UNIVERSITAT DE BARCELONA

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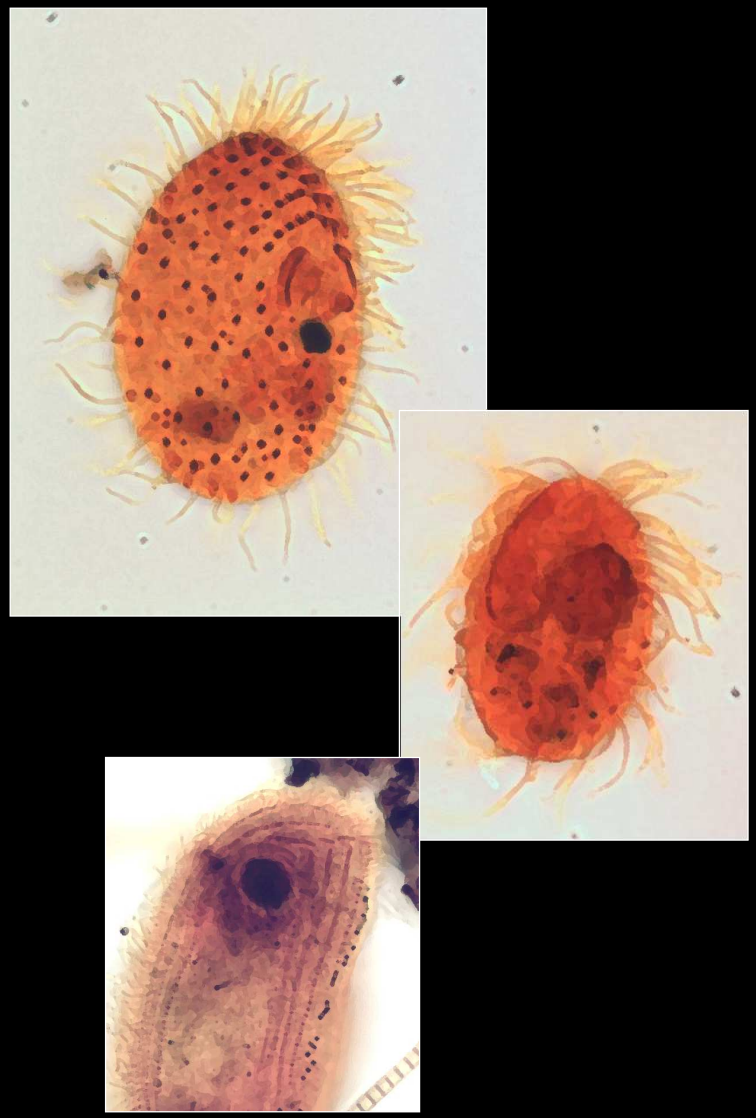

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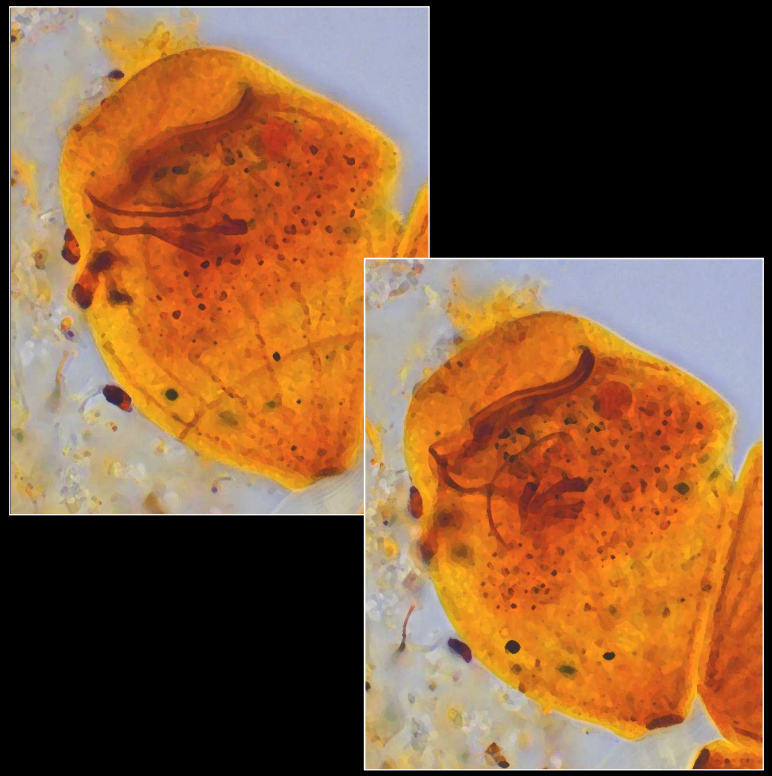

ORIOL CANALS DELGADO

2017

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PROTOZOUS EN SISTEMES AVANÇATS DE TRACTAMENT D'AIGÜES RESIDUALS: CARACTERITZACIÓ DE LES POBLACIONS I CERCA DE LES ESPÈCIES BIOINDICADORES.

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Tesi Doctoral

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Facultat de Biologia

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Programa de doctorat en Biodiversitat

PROTOZOUS EN SISTEMES AVANÇATS DE TRACTAMENT D'AIGÜES RESIDUALS: CARACTERITZACIÓ DE LES POBLACIONS I CERCA DE LES ESPÈCIES BIOINDICADORES.

Memòria presentada per

ORIOL CANALS DELGADO

per optar al grau de Doctor per la Universitat de Barcelona

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Oriol Canals Delgado

Director i tutor de la tesi

Dr. Humbert Salvadó Cabré

Universitat de Barcelona

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Les birres queden pendents, me les demaneu i celebrem. No crec que pagui jo, per això...

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ABREVIACIONS

EDAR – Estació depuradora d'aigües residuals.

BF – Biofilm.

ML – Licor mescla.

DQO – Demanda química d'oxigen.

sDQO – Demanda química d'oxigen en fracció soluble.

DBO_5 – Demanda biològica d'oxigen en 5 dies.

TAN – Nitrogen amoniacal total.

MBR – *Membrane biological reactor*.

RBC – *Rotating biological contactor*.

BAF – *Biological aerated filter*.

MBBR – *Moving-bed biofilm reactor*.

IFAS - *Integrated fixed-film activated sludge*.

BNR – *Biological nitrogen removal*.

cBNR – *Conventional biological nitrogen removal*.

SBNR – *Shortcut biological nitrogen removal*.

PN – *Partial nitriation*.

Anammox – *Anaerobic ammonium oxidation*.

NGS – *Next generation sequencing*.

1. INTRODUCCIÓ

1. INTRODUCCIÓ

Aquesta memòria recull els principals resultats de l'estudi de la comunitat microeucariota en dos sistemes avançats de tractament d'aigües residuals amb alta càrrega amoniacal. La present introducció, dividia en dos blocs, pretén donar el context necessari que justifiqui l'interès i importància dels estudis realitzats.

1.1. Aigües residuals i sistemes de tractament d'aigües residuals

L'ésser humà, com la resta d'éssers vius, necessita l'aigua per la seva supervivència. Aquesta necessitat d'aigua no es concep només a nivell fisiològic, sinó que també abasta les activitats diàries d'àmbit domèstic, de regadiu, d'activitat industrial, obtenció d'energia i d'oci, entre altres. Aquest ús de l'aigua comporta en la gran majoria de casos el seu deteriorament, principalment relacionada amb càrrega de compostos contaminants molt diversos, generant el que s'anomenen les aigües residuals. Abans de retornar les aigües utilitzades al medi, ja sigui a la llera pública o a la mar, la càrrega contaminant ha de ser eliminada fins a uns valors mínims, per tal d'evitar els efectes nocius que aquesta contaminació pot exercir sobre el medi ambient i ecosistemes. A la Unió Europea, aquests valors mínims de qualitat de l'aigua ja depurada són normalment establerts per la legislació de cada estat, tot i que la Unió Europea estableix unes directives que, tot i no regular de forma directa, marca el camí a seguir en termes de qualitat i protecció dels medis aquàtics dels seus estats membres.

1.1.1. Aigües residuals

Es defineix com a aigua residual aquella aigua que ha patit un deteriorament de la seva qualitat degut a l'ús humà. Aquest empitjorament de la qualitat, que depèn directament de l'ús a la que l'aigua ha estat sotmesa, consisteix en una modificació de les seves característiques físiques així com l'adquisició (i transport) de contaminants (Mackenzie 2010). Els contaminants poden trobar-se suspesos en l'aigua, en forma col·loïdal o en forma soluble. Independentment de la forma en la que es trobin, els contaminants de les aigües residuals es classifiquen en diferents categories, que poden variar entre els diferents països i estats, en funció de les respectives normatives. A l'estat espanyol, en el marc de la Unió Europea, els contaminants es classifiquen en tres categories: contaminants convencionals, contaminants prioritaris i contaminants emergents.

Es consideren contaminants convencionals la gran majoria de contaminants habituals en aigües residuals domèstiques. Els contaminants prioritaris es defineixen com aquells que presenten un risc significatiu pel medi aquàtic o la salut humana per la seva toxicitat, persistència o per ser bioacumulables (Annex II de la Directiva 2008/105/CE). Per últim, els contaminants emergents son aquells dels que es desconeix en gran mesura la seva presència localitzada, concentració i impacte en el medi aquàtic, estant normalment sota estudi i no recollits a la majoria de normatives vigents sobre aigües.

Tot i que en la present memòria els contaminants emergents no son objecte d'estudi, crec imperatiu fer un incís per remarcar la necessitat de controlar i monitoritzar la presència d'aquest grup difós de contaminants tant en les estacions depuradores con en els entorns naturals, així com la necessitat d'avançar en el coneixement dels possibles efectes nocius que puguin tenir sobre el medi ambient a tots els nivells. És com a mínim inquietant, sinó preocupant, l'increment que s'ha observat pel que fa la detecció d'aquests contaminants en la gran majoria, per no dir totes, les aigües residuals (tant domèstiques com urbanes) a l'estat espanyol, així com en aigües que ja han passat pel procés de depuració i que poden dispersar aquests contaminants al medi (Marín et al. 2009).

Les aigües residuals es classifiquen, a *grosso modo*, en aigües residuals domèstiques (o urbanes) i aigües residuals industrials. Les aigües residuals urbanes son aquelles que provenen dels habitatges, comerços, edificis institucionals i construccions similars destinades a les activitats diàries i quotidianes de les persones. Les aigües residuals industrials, com el seu nom indica, són les que provenen de l'activitat industrial (indústries i polígons industrials). Degut a la presència habitual d'aigües residuals formades per una barreja entre aigües d'origen domèstic i industrial, la normativa de l'estat espanyol contempla, a efectes de cànon, la classificació de les aigües residuals en urbanes o assimilables (màxim del 30% de fracció industrial), industrial de classe I (entre el 30 i el 70% de fracció industrial) i industrial (més del 70% de fracció industrial) (del Río 2016).

A part de les aigües residuals domèstiques i industrials, en aquells territoris que presenten clavegueram unitari, les aigües residuals també contemplen les possibles aportacions a la xarxa de les aigües pluvials (provinents de l'escorrentia superficial i desglaç de neu) i les aportacions incontrolades (aigües pluvials que s'incorporen a la xarxa de clavegueram mitjançant les xarxes de pluvials, els baixants d'edificis, tapes de pous de registre, etc.). Per últim, degut a un mal manteniment de la xarxa, les aigües residuals també poden contenir entrades d'aigües d'infiltració (aigua que entra a la xarxa a través de fractures, esquerdes i canonades en mal estat, i que principalment prové de pous subterranis) (Metcalf i Eddy 2003).

Característiques de les aigües residuals

Les aigües residuals es caracteritzen en funció de les seves característiques físiques, químiques i biològiques.

Les característiques físiques de l'aigua residual urbana varien en funció de si aquesta és recentment generada o si es tracta d'una aigua residual més vella i/o sèptica. L'aigua residual urbana recentment generada es diu que presenta una olor de querosè o terra recent remogut i de color normalment gris. L'aigua residual sèptica, per la seva part, presenta una olor més desagradable degut majoritàriament a la formació d'àcid sulfhídric i de tiols (indicadors d'un ambient sèptic) i color negre. La temperatura de l'aigua residual acostuma a ser sempre lleugerament superior a la de l'aigua potable, i fluctua en funció de l'època de l'any. Les aigües residuals urbanes presenten una elevada terbolesa després de deixar-la decantar.

Les aigües residuals domèstiques presenten un número molt divers de compostos químics, que s'agrupen en uns pocs grups generals per a facilitar la seva caracterització. Cada un dels grups inclou tot el conjunt de components químics que són mesurats utilitzant una mateixa anàlisi fisicoquímica, anàlisi de la que el grup en pren el nom (Mackenzie 2010). Així doncs, el valor de DQO (demanda química d'oxigen) s'utilitza per conèixer la quantitat de substàncies que conté una aigua residual que són susceptibles de ser oxidades químicament independentment de la forma química en la que aquesta matèria orgànica es trobi. La DQO, tot i ser una mesura de matèria orgànica, pot patir interferències en cas de presència de substàncies inorgàniques susceptibles de ser oxidades com sulfurs, sulfits o iodurs, que també es reflecteixen en la mesura. La DBO_5 (demanda bioquímica d'oxigen en 5 dies) representa la matèria orgànica susceptible de ser oxidada biològicament en un temps determinat, i el seu valor és per definició inferior al valor de DQO. El NKT (Nitrogen Kjeldahl total) és una mesura del total de nitrogen orgànic o en forma amoniacal. Per últim, el paràmetre fòsfor total dona una mesura de la concentració del fòsfor que conté l'aigua residual. En base a la concentració que presenten aquests paràmetres en l'aigua residuals domèstica, aquesta es pot classificar en dèbil, mitja i forta (Metcalf i Eddy 2003, Taula 1.1).

Taula 1.1. Composició habitual de les aigües residuals domèstiques dèbil, mitja i forta (font: Mackenzie 2010).

Constituent	Weak	Medium	Strong
	(all mg/L except settleable solids)		
Alkalinity (as $CaCO_3$)[a]	50	100	200
Ammonia (free)	10	25	50
BOD_5 (as O_2)[b]	100	200	300
Chloride[a]	30	50	100
COD (as O_2)	250	500	1,000
Total suspended solids (TSS)	120	210	400
Volatile (VSS)	95	160	315
Fixed	25	50	85
Settleable solids, mL/L	5	10	20
Sulfates[a]	20	30	50
Total dissolved solids (TDS)	200	500	1,000
Total Kjeldahl nitrogen (TKN) (as N)	20	40	80
Total organic carbon (TOC) (as C)	75	150	300
Total phosphorus (as P)	5	10	20

[a]To be added to amount in domestic water supply. Chloride is exclusive of contribution from water-softener backwash.
[b]For newer, tighter collection systems, or where water conservation is practiced, these numbers may be considerably higher.

Com es mostra a la taula1.2, aquests paràmetres principals es poden subdividir en categories depenent de la forma en la que es trobin. La forma que presentin els diferents paràmetres a l'aigua residual molt sovint determina la facilitat d'assimilació dels compostos per part dels microorganismes. Per exemple, el nitrogen en forma amoniacal serà més fàcilment assimilable

per la microbiota nitrificant que el nitrogen orgànic, que requereix un procés previ d'hidròlisis per ser alliberat al medi.

Taula 1.2. Principals paràmetres fisicoquímics de l'aigua residual i llistat de la categoria en la que aquests es poden trobar (font: Mackenzie 2010).

Constituent[a, b]	Definition
BOD	
BOD or BOD_5	Total 5-d biochemical oxygen demand
sBOD	Soluble 5-d biochemical oxygen demand
UBOD or BOD_u	Ultimate biochemical oxygen demand
COD	
COD	Total chemical oxygen demand
bCOD	Biodegradable chemical oxygen demand
pCOD	Particulate chemical oxygen demand
sCOD	Soluble chemical oxygen demand
nbCOD	Nonbiodegradable chemical oxygen demand
rbCOD	Readily biodegradable chemical oxygen demand
rbsCOD	Readily biodegradable soluble chemical oxygen demand
sbCOD	Slowly biodegradable chemical oxygen demand
bpCOD	Biodegradable particulate chemical oxygen demand
nbpCOD	Nonbiodegradable particulate chemical oxygen demand
nbsCOD	Nonbiodegradable soluble chemical oxygen demand
Nitrogen	
TKN	Total Kjeldahl nitrogen
bTKN	Biodegradable total Kjeldahl nitrogen
sTKN	Soluble (filtered) total Kjeldahl nitrogen
ON	Organic nitrogen
bON	Biodegradable organic nitrogen
nbON	Nonbiodegradable organic nitrogen
pON	Particulate organic nitrogen
nbpON	Nonbiodegradable particulate organic nitrogen
sON	Soluble organic nitrogen
nbsON	Nonbiodegradable soluble organic nitrogen
Suspended Solids	
TSS	Total suspended solids
VSS	Volatile suspended solids
nbVSS	Nonbiodegradable volatile suspended solids
iTSS	Inert total suspended solids

[a]*Note:* b = biodegradable; i = inert; n = non; p = particulate; s = soluble.
[b]Measured constituent values, based on the terminology given in this table, will vary depending on the technique used to fractionate a particular constituent.

Tot i que aquests paràmetres són els principals i més utilitzats per determinar la qualitat d'un efluent de depuradora, existeixen altres paràmetres de mesura de qualitat d'efluents com la conductivitat, el pH, l'alcalinitat o els clorurs, entre d'altres (von Sperling 2007).

Les característiques biològiques de les aigües residuals domèstiques fan referència a l'elevat número de microorganismes que aquestes presenten, alguns d'ells patògens o paràsits per als

humans com algunes soques del coliforme fecal *Escherichia coli*, virus, helmints i protists (von Sperling 2007). Així doncs, un dels motius principals pel tractament de les aigües residuals és l'eliminació d'aquests agents patògens d'origen humà.

Les aigües residuals industrials també es caracteritzen física i químicament mitjançant els paràmetres mencionats abans per les aigües residuals domèstiques. A diferència d'aquestes últimes, els processos industrials generen una diversitat molt elevada de compostos contaminants, que poden variar enormement d'una indústria a una altra en funció de l'activitat que aquestes desenvolupen. L'enorme diversitat i heterogeneïtat de contaminants de les aigües residuals industrials molt sovint fa que aquesta sigui pràcticament impossible de classificar (Mackenzie 2010).

Independentment del tipus de indústria, les principals diferències entre les aigües residuals industrials i les domèstiques són: a) que les aigües residuals d'origen industrial poden presentar una molt major concentració dels contaminants convencionals i prioritaris (DQO, DBO_5, nitrogen total, fòsfor total...) que sovint dificulten l'operació òptima dels processos de depuració; b) que les aigües residuals industrials poden presentar una enorme diversitat de contaminants prioritaris i contaminants emergents que han de ser retirats de l'aigua de forma específica abans de retornar-la al medi; i c) que poden presentar una molt elevada varietat de característiques físiques, sobretot relacionades amb la olor, el color i la temperatura (Mackenzie 2010).

Aigües residuals amb alta càrrega amoniacal

Les aigües residuals amb alta càrrega amoniacal són aquelles que presenten una concentració molt elevada de nitrogen en forma d'amoni. L'origen d'aquest tipus d'aigua residual és força divers, i degut a aquesta diversitat d'origen, les diferents aigües residuals amb alta càrrega amoniacal poden presentar diferències en relació a la resta de paràmetres fisicoquímics. Alguns exemples d'aigües residuals amb alta càrrega amoniacal són les generades per indústries alimentàries (gelatina, llet, càrniques) i/o instal·lacions relacionades amb el processament de la carn de bestiar (escorxadors), els residus que es generen en granges d'animals, especialment en granges porcines (purins), els residus generats en abocadors (lixiviats) i els efluents de processos de tractament anaerobis de fangs de depuradores urbanes, entre d'altres.

En alguns casos, aquesta càrrega nitrogenada no es genera directament en forma amoniacal, ja que en les aigües residuals provinents d'indústries alimentàries (càrniques, lleteres o de gelatina), dels escorxadors o els residus de les granges de porcs, la fracció nitrogenada es troba inicialment en forma de nitrogen orgànic, principalment en forma de proteïnes. Les condicions anaeròbies de la mateixa aigua residual durant l'emmagatzematge o durant el recorregut a través dels col·lectors fins la depuradora faciliten la transformació d'aquest nitrogen orgànic a amoni, la seva forma més reduïda. En altres casos, les condicions d'anaerobiosi dels reactors anaerobis o dels abocadors,generen *per se* residus amb elevada càrrega amoniacal.

Les aigües residuals amb alta càrrega amoniacal poden presentar importants diferències entre elles, tant pel que fa a la concentració d'amoni (Taula 1.3) com dels altres paràmetres fisicoquímics. En el context de la present memòria convé destacar les aigües residuals amb alta càrrega amoniacal resultants del centrifugat dels efluents dels digestors anaerobis de fangs, aigües que es caracteritzen per la relació particularment baixa de carboni/nitrogen, resultat de les elevades concentracions d'amoni (en ocasions superiors als 1000 mg/l) i dels valors baixos de matèria orgànica carbonosa biodegradable (Galí et al. 2007; Kampschreur et al. 2008).

Taula 1.3. Concentració de nitrogen amoniacal en diferents tipus d'aigua residual amb alta càrrega amoniacal (font: Ren-Cun et al. 2012).

Wastewater / industry	Ammonium nitrogen concentration(mg L^{-1})
Monosodium glutamate wastewater	15,000–25,000
Sludge digestion liquid	1200–4000
Landfill leachate	1400–2800
	0.2–13,000
Coke-ovens wastewaters	330–5100
Digester liquor of swine wastewater	3000–5000
Livestock: cattle or swine	500–2300
Fertilizer	200–940
Oil Refinery	23.8–865

1.1.2. Estacions depuradores d'aigües residuals

El procés de depuració de les aigües residual es du a terme en unes instal·lacions dissenyades específicament per acomplir aquest objectiu, anomenades estacions depuradores d'aigües residuals (EDARs). L'objectiu principal de les EDARs és eliminar la càrrega contaminant en suspensió i soluble, incloent-hi els nutrients, que l'aigua arrossega després d'haver estat utilitzada per l'ésser humà. Com ja s'ha comentat anteriorment, aquesta depuració es realitza fins a uns límits o valors mínims establerts per la legislació vigent de cada estat o territori. A part de les variacions entre estats, aquests límits també varien en funció de la zona on l'aigua ja depurada serà retornada al medi i en funció del possible ús que se li donarà.

El procés de depuració de les aigües residuals en les estacions depuradores consta habitualment d'un pretractament i tres fases de tractament anomenades tractament primari, secundari i terciari (figura 1.1).

Durant la fase de pretractament es retira de l'aigua residual els objectes i partícules de mida més gran (plàstics, troncs, etc.) fent passar l'aigua a través del procés de desbast per evitar possibles obturacions posteriors i possibles desperfectes que aquests objectes puguin provocar sobre les bombes i vàlvules al llarg del procés. A continuació l'aigua residual és enviada a un desarenador-desgreixador on es retiren, mitjançant decantació, els sòlids no solubles de mida més petita com les graves o sorres que no han estat retirats en el procés de desbast, així com els greixos, olis i altres compostos hidrofòbics, que queden surant sobre la massa d'aigua.

Durant el tractament primari l'aigua es deixa reposar durant unes hores en un tanc de decantació anomenat decantador primari, amb la finalitat de retirar la matèria orgànica sedimentable i altres partícules inorgàniques que no han estat retingudes en el pretractament mitjançant un procés de decantació. L'objectiu del tractament primari és retirar un mínim del 50% dels sòlids i del 20% de la DBO_5 de l'aigua residual. Si la càrrega de l'aigua residual és molt elevada, el tractament primari contempla l'addició de compostos químics per millorar la seva floculació i/o coagulació.

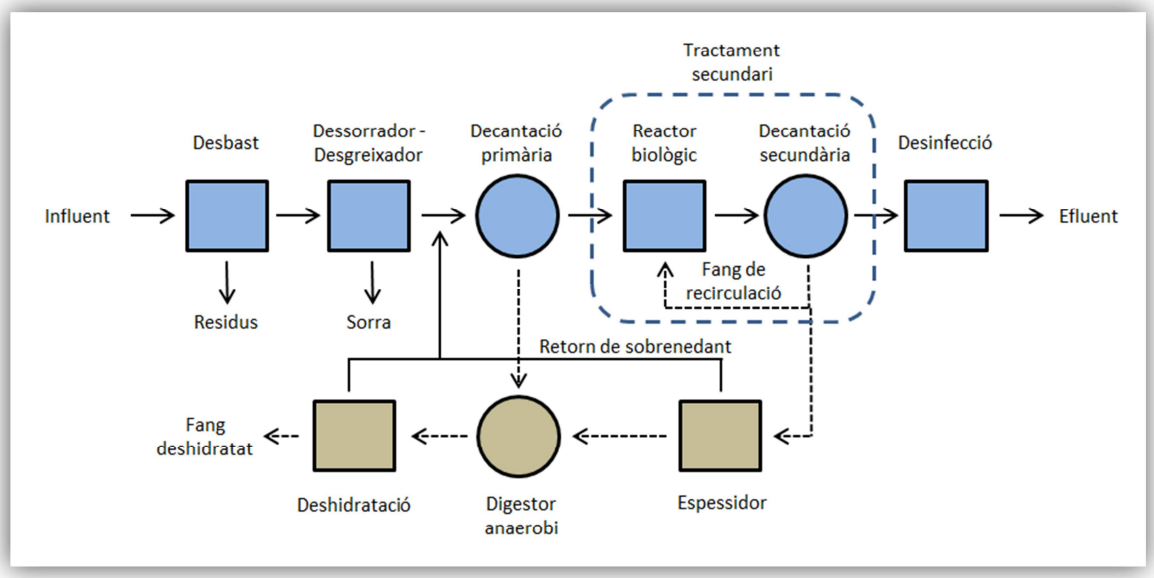


Figura 1.1. Esquema habitual d'una estació depuradora de tractament d'aigües residuals (modificat de Metcalf i Eddy 2003).

El tractament secundari pretén la degradació de la matèria orgànica dissolta mitjançant l'acció dels microorganismes. Aquest procés es realitza en un dipòsit anomenat reactor biològic, on l'aigua residual entra en contacte amb la biomassa bacteriana. El reactor biològic pot operar en condicions aeròbies, anòxiques, anaeròbies o en combinació d'aquestes, i és el lloc on es desenvolupa la microbiota associada als processos de tractament d'aigües residuals.

Per últim, el tractament terciari consisteix en tota una sèrie de processos biològics, físics i/o químics, o modificacions afegides al procés convencional de les EDARs per tal d'incrementar la qualitat de l'efluent eliminant de l'aigua residual compostos específics. Un dels tractaments terciaris més àmpliament utilitzats actualment és el procés avançat d'eliminació de nutrients, mitjançant el qual s'incrementa l'eliminació el nitrogen i el fòsfor de l'aigua residual per part dels microorganismes. L'eliminació incrementada de nitrogen es du a terme mitjançant el procés biològic de nitrificació-desnitrificació. El fòsfor, tot i l'existència de processos biològics dissenyats per la seva eliminació incrementada, s'acostuma a eliminar mitjançant l'addició de compostos químics (per exemple clorur fèrric).

Existeixen una gran varietat de sistemes biològics de tractament d'aigües residuals, en funció de les particularitats de la seva configuració, dels processos biològics que utilitzen per la depuració de l'aigua residual i de com es desenvolupa la biomassa en el sistema. El tipus de sistema a implementar a cada EDAR es determina durant el disseny de la EDAR en qüestió, en funció del tipus d'aigua residuala tractar, de la seva càrrega contaminant i de l'exigència sobre la qualitat de l'aigua depurada. De totes maneres, el disseny del sistema de tractament pot ser modificat posteriorment si aquest queda obsolet o per variacions significatives en les característiques de l'aigua residual a tractar. Per les característiques dels sistemes estudiats, en la present memòria no es desenvoluparan ni els sistemes de tractament tous (llacunatges i llits de canyes) ni els sistemes anaerobis.

La configuració del sistema fa referència al disseny de la pròpia planta de tractament, i determina el nombre de reactors biològics del sistema, la forma i característiques dels reactors, la continuïtat o discontinuïtat del caudal d'entrada (reactors seqüencials o de flux continu), el volum i velocitat del caudal de recirculació, la presència de recirculació interna o la presència de decantador secundari, entre d'altres. Les modificacions en la configuració del sistema acostumen a requerir la realització d'obres a la EDAR.

1.1.2.1. Biomassa en els sistemes de tractament

En relació a com es desenvolupa la biomassa en el reactor biològic, els sistemes es poden classificar en tres tipus: a) els sistemes que operen amb la biomassa en suspensió (fangs actius, reactor biològic de membranes, fang granular); b) els sistemes que operen amb la biomassa adherida a un suport, ja sigui fix (sistemes de biodiscs, filtres percoladors, BAF) o mòbil (*moving bed biofilm reactor*); i c) els sistemes híbrids (*integrated fixed and activated sludge*).

Sistemes de biomassa en suspensió. Els sistemes que operen amb la biomassa en suspensió són aquells en els que la biomassa és barrejada amb l'aigua residual a depurar, donant com a resultat una matriu líquida d'aspecte fangós que rep el nom de fangs activats (o fangs actius). La tecnologia o sistema de fangs actius es va desenvolupar l'any 1914 (Ardren i Lockett 1914) i, en l'actualitat, més de 100 anys després, continuen essent el sistema de tractament d'aigües residuals que més s'utilitza a tot el planeta. Per definició, el sistema de fangs actius s'utilitza en condicions aeròbies o en combinació d'etapes aeròbies, anòxiques i anaeròbies. Precisament les condicions aeròbies a les que operen els sistemes de fangs actius generen una elevada producció de biomassa en forma de fangs que han de ser retirats regularment del sistema. Aquests fangs es retiren mitjançant un sistema de purga, generant la necessitat d'un tractament adequat d'aquests excedents (línia de fangs). Una de les opcions més emprades pel seu tractament són els processos de digestió anaeròbia, processos que generen aigua residual amb alta càrrega amoniacal i una baixa concentració de matèria orgànica biodegradable.

Els sistemes de fangs actius, en operar amb els microorganismes en suspensió barrejats amb l'aigua, requereixen una etapa de separació de la fracció sòlida (biomassa) i la fracció líquida posterior al reactor biològic. En la majoria de casos, aquesta separació es dóna per decantació

dels fangs en un tanc de decantació (decantador secundari). La biomassa o fracció sòlida és enviada de nou cap al reactor biològic o eliminada del sistema, i la fracció líquida o aigua ja depurada serà retornada al medi, reutilitzada o enviada a plantes potabilitzadores, entre altres destins.

En aquest context es situa la principal problemàtica associada als sistemes de fangs actius: els episodis d'esponjament del fang (*bulking*) i/o de formació d'escumes (*foaming*) causats per proliferacions massives de microorganismes filamentosos, que poden comprometre de forma important la qualitat del'aigua de sortida. L'esponjament del fang és una alteració en la decantabilitat dels fangs degut a un creixement massiu de microorganismes filamentosos, en la que l'elevada abundància de filaments dificulta la decantació del mateix fang, evitant la seva compactació al decantador secundari. Per la seva part, els episodisd'escumes es tradueixen en la formació d'una capa d'aspecte esponjós surant a la superfície del reactor biològic i decantador secundari, resultat de l'acumulació de microorganismes filamentosos. En ambdós casos es corre el risc que l'aigua de sortida no compleixi els paràmetres d'abocament, principalment per sortida de sòlids amb l'efluent.

El sistema de fangs actius presenta també limitacions associades a l'espai, com succeeix en situacions en les que l'aigua residual a tractar presenta una càrrega de contaminació orgànica o una concentració de matèria orgànica lentament biodegradable més elevada del que és habitual. El sistema de fangs actius, per tal d'eliminar aquest excésde matèria orgànica entrant, requereix d'un major temps de contacte entre l'aigua residual i la microbiota (major temps de retenció hidràulic), solució que en moltes EDARs només es pot aconseguir augmentant el volum del reactor biològic; o una concentració més elevada de microorganismes que permetil'eliminació de la pol·lució entrant. Un augment pertinent de la concentració de fangs és molt probable que acabi traduint-se en problemes de decantació del fang al decantador secundari. A més a més, aquesta segona opció és directament inviable en aquelles EDARs que ja operen amb una concentració de fangs elevada o una capacitat de oxigenació limitada.

En aquest context es van desenvolupar altres sistemes com els MBR (*Membrane Biological Reactor*) i els sistemes de fang granular. El procés MBR es diferencia del sistema de fangs activats per la presència d'un sistema de ultrafiltració (diàmetre efectiu del porus <0.1µm; Judd 2008) mitjançant membranes com a mètode de separació de la biomassa i de l'aigua ja tractada en substitució del tanc de decantació típicament associat al sistema de fangs actius. El sistema de ultrafiltració assegura una bona clarificació de l'efluent (separació de les fraccions líquida i sòlida del licor mescla) independentment de la concentració de fangs a la que operi el reactor i de l'abundància de microorganismes filamentosos, fet que permet al sistema MBR operar amb elevades concentracions de fangs i, en conseqüència, el tractament d'influents amb una major càrrega orgànica (Judd 2006). Les elevades concentracions de sòlids a les que opera el sistema MBR (de 8 a 10 g/l; Ekama 2015) comporta també una reducció de la mida del reactor biològic i promou el desenvolupament de les poblacions nitrificants, incrementant l'eliminació d'amoni. Com a contrapunt, el sistema de membranes requereix d'una inversió energètica molt major a la requerida per un sistema convencional de fangs actius. Els sistemes de fang granular es caracteritzen per presentar la biomassa formant conglomerats d'una estructura densa i aparença ben definida, anomenats grànuls (Schmidt i Ahring 1995). Els

sistemes de fang granular presenten, en comparació amb els sistemes de fangs actius, una major facilitat de separació sòlid-líquid a causa de la compactació de la biomassa i la seva menor barreja amb el líquid circumdant que permet operar en un sol reactor, sense necessitat de decantador secundari ni bombes de recirculació (Ekama 2015), una millor retenció dels microorganismes al sistema, major capacitat de suportar elevades càrregues orgàniques (Adav et al. 2008) i capacitat d'operar a una concentració de sòlids també més elevada (de 8 a 10 g/l) que a la que poden operar els fangs actius (3 a 6 g/l) (Ekama 2015).

Sistemes de biomassa adherida o biofilm. En els sistemes de biofilm, la biomassa es troba adherida a una superfície. Comparats amb els processos de fangs actius, els processos de biofilm tenen una majorcapacitat de tractament del volum de càrrega, una menor necessitat d'espai degut a la major concentració de biomassa amb la que operen i una menor relació entre la qualitat de l'efluent i l'efectivitat del procés de decantació dels fangs (Metcalf i Eddy 2003).

Els sistemes de tractament d'aigües residuals que operen amb la biomassa adherida a un suport es classifiquen en funció de si el suport és fix (sistema de biodiscs o *Rotating Biological Contactors* o RBC, filtres percoladors, filtres biològics airejats o *Biological Aerated Filters* o BAF) o mòbil (reactors de llit mòbil o *Moving Bed Biological Reactor* o MBBR). En aquests sistemes l'aigua residual fa un recorregut a través del sistema de biofilms durant el transcurs del qual és depurada, donant lloc a una clara zonació pel que fa a la microbiota. En el cas dels sistemes de filtres percoladors l'aigua residual és introduïda per la part superior dels filtres i es va escolant fins arribar a la base, mentre els microorganismes que conformen el biofilm oxiden la càrrega orgànica de l'influent. A la zona superior, doncs, la microbiota rep la major quantitat de càrrega contaminant, pel que serà colonitzada per espècies altament tolerants a la contaminació. A mesura que anem descendint pels filtres, la composició específica de la comunitat dels biofilms anirà variant cap a espècies cada cop més exigents pel que fa a la qualitat de l'aigua. El mateix succeeix en un sistema RBC, però en aquest cas l'aigua es desplaça en sentit horitzontal.

En la majoria de sistemes de biofilm fix, l'aportació d'oxigen no es du a terme de forma activa, sinó que normalment és producte del mateix moviment rotatori dels biofilms com en el cas dels RBC o per simple contacte amb l'aire en el cas dels filtres percoladors. Els BAF son els únics que requereixen oxigenació activa així com neteges periòdiques de la matriu on es desenvolupen els microorganismes i queden retinguts els sòlids en suspensió.

El sistema MBBR, o de biofilm mòbil, va ser desenvolupat a finals dels anys 1980 per HallvardØdegaard (Ødegaard et al. 1994). En aquests sistemes la biomassa s'adhereix a unes estructures anomenades *carriers*, d'una densitat molt propera a la de l'aigua (1 g/cm^3), que es mantenen en suspensió al reactor barrejats juntament amb el líquid mescla, normalment gràcies al mateix sistema d'oxigenació del reactor, garantint *a priori* un millor contacte dels components de l'aigua residual amb la biomassa que es desenvolupa als *carriers*. Per evitar que els *carriers* escapin del procés simplement s'afegeix una xarxa o estructura porosa que impedeixi la sortida d'aquests amb l'efluent. Una de les principals avantatges del sistema MBBR és el disseny dels *carriers* com a estructures de mida petita i independents del conjunt

d'instal·lacions de la EDAR, que permet la seva implementació com a modificacions dels tancs de fangs activats ja existents adaptant el grau d'ompliment del reactor en funció dels requeriments concrets que necessiti el sistema en cada moment, augmentat així la capacitat de tractament del sistema evitant la necessitat d'augmentar la mida del reactor obsolet i/o la construcció de nous tancs. Els sistemes que operen amb tecnologia MBBR no requereixen línia de recirculació de fangs, ja que la biomassa creix adherida als suports plàstics, fent innecessària la presència d'un decantador secundari (Ødegaard 1999). Altres avantatges del procés MBBR són un major temps de retenció de la biomassa, fet que afavoreix l'establiment de la biomassa responsable dels processos de nitrificació (Wang et al. 2006), una major resposta a fluctuacions de càrrega, una menor producció de fangs i una major resiliència a entrades de tòxics (Barwal i Chaudhary 2014).

Sistemes híbrids. Per últim, els processos anomenats híbrids combinen la tecnologia de biomassa en suspensió juntament amb la tecnologia de biofilm. En la majoria de casos els sistemes híbrids operen amb tecnologia MBBR, tot i que també es pot aplicar amb tecnologia de biofilm en suport fix. Aquests sistemes acostumen a estar enfocats a modificacions de plantes depuradores ja existents i que han quedat obsoletes. Un exemple és el sistema anomenat *Integrated Fixed-Film ActivatedSludge* (IFAS). La principal diferència entre els sistemes híbrids i els sistemes de biofilm és la presència d'una línia de recirculació de fangs, necessària per mantenir una suficient concentració de biomassa en suspensió, així com la presència d'un decantador secundari.

1.1.2.2. Processos biològics de depuració

Els processos biològics de depuració de les aigües residuals són tot el conjunt de processos metabòlics mitjançant els quals els microorganismeseliminen els contaminantsde l'aigua. Els diferents processos de depuració venen determinats pel tipus d'acceptor d'electrons disponible per la realització de les vies catabòliques (transformació de les molècules orgàniques o biomolècules complexes en molècules senzilles i en l'emmagatzematge de l'energia química despresa en forma d'enllaços fosfat de molècules d'ATP) per part dels microorganismes, i es classifiquen en tres tipus: processos aerobis, anòxics i anaerobis, segons el metabolisme predominant (Mackenzie 2010).

Processos de depuració aerobis

Els processos de depuració aerobis (o oxidació aeròbia) són aquells en els que l'acceptor final d'electrons és l'oxigen enforma molecular (O_2), oxigen que sovint ha de ser dosificat activament al sistema. Dins els processos de depuració aerobis podem diferenciar entre el procés d'oxidació heteròtrofa de la matèria orgànica i la nitrificació (Henze et al. 2008).

- **Oxidació de la matèria orgànica**

El procés d'oxidació de la matèria orgànica és aquell mitjançant el qual els microorganismes eliminen de l'aigua la matèria orgànica carbonatada (en base carboni), mesurada normalment en forma de DBO_5 i/o DQO. En comparació amb els processos anòxics i anaerobis, els processos de depuració aerobis són els que poden degradarun espectre més ampli de matèria orgànica (Mackenzie 2010). Com a productes finals del metabolisme, l'oxidació aeròbia genera principalment diòxid de carboni, aigua i nou material cel·lular, tots ells productes estables, és a dir, que no suposen un perill pel medi ambient ni suposen molèsties com males olors. Els organismes que utilitzen els processos de depuració aerobis presenten normalment taxes de creixement més elevades que les dels organismes que utilitzen processos de depuració anòxics o anaeròbics. Aquest fet implica que ens els processos aerobis la degradació de la matèria orgànica és més ràpida i eficient però, com a contrapartida, genera una major producció de biomassa (fangs). Degut precisament a aquesta rapidesa i eficiència de la depuració aeròbia, aquesta és especialment utilitzada per tractar grans quantitats d'aigües residuals amb concentracions de DBO_5 inferiors als 500 mg/l.

Els processos aerobis no es consideren generalment apropiats per tractar aigües residuals amb molta càrrega de matèria orgànica (DBO_5> 1000 mg/l) degut a la dificultat i a l'alt cost de proveir l'oxigen suficient per la seva oxidació. Existeixen, però, alguns casos en els que aquesta premissa no es compleix i la depuració aeròbia és capaç de tractar satisfactòriament aigües residuals amb concentracions de DBO_5 superiors a 3000 mg/l, com és el cas de l'aplicació de llacunes airejades per tractar comunitats petites o algunes indústries concretes, on el caudal d'entrada d'aigua residual al sistema és reduït i el temps de retenció de l'aigua és molt elevat (Mackenzie 2010).

- **Nitrificació**

Durant el procés de nitrificació la fracció nitrogenada de l'aigua residual que es troba en forma amoniacal és oxidada en un primer pas fins a nitrit i, posteriorment, fins a nitrat. Els microorganismes responsables de la nitrificació reben el nom de microorganismes nitrificants, i es classifiquen en dues grans comunitats en funció de la fase oxidativa en la que actuen, els microorganismes amonioxidants i els nitritoxidants. Els processos de nitrificació s'expliquen amb més profunditat posteriorment en la mateixa memòria.

Desnitrificació

En absència d'oxigen molecular, alguns organismes poden utilitzar el nitrat (NO_3^-) o el nitrit (NO_2^-) com a acceptor final d'electrons en els seus processos catabòlics. Aquesta ruta metabòlica rep el nom de desnitrificació i genera, com a productes finals, nitrogen gas, diòxid de carboni, aigua i nou material cel·lular (biomassa). La taxa de reproducció dels organismes que utilitzen aquesta via catabòlica també és força elevada, tot i que no tant com la que permet la oxidació aeròbia. En els últims anys, el procés de desnitrificació ha adquirit una gran importància en els sistemes de tractament d'aigües residuals per la necessitat d'incrementar l'eliminació de la càrrega nitrogenada de l'aigua residual abans de retornar-la al medi. Aquesta eliminació de la càrrega nitrogenada es du a terme mitjançant processos d'eliminació biològica de nutrients o BNR (*Biological Nitrogen Removal*), que combina tancs o fases oxigenades (per

oxidar la matèria orgànica, tant la carbonatada com la nitrogenada) amb fases anòxiques on els microorganismes porten a terme la desnitrificació.

Processos de depuració anaerobis

Els processos de depuració anaerobis o de fermentació de la matèria orgànica es donen a terme quan el sistema no presenta ni oxigen molecular ni nitrats ni nitrits. En aquestes condicions, els microorganismes amb rutes catabòliques anaeròbies degraden la matèria orgànica utilitzant molècules de diòxid de carboni, sulfats (SO_4^{2-}) o compostos orgànics susceptibles de ser reduïts com a acceptors finals d'electrons. Els principals productes finals d'aquest procés son diòxid de carboni, metà i aigua; tot i que també es genera amoni, sulfur d'hidrogen (H_2S) i tot un conjunt de compostos orgànics sulfurosos anomenats tiols, compostos responsables de la desagradable olor que caracteritza aquests processos. La depuració anaeròbia presenta una producció molt baixa de fangs en comparació amb els altres dos tipus de processos, pel que és àmpliament utilitzat per a estabilitzar i reduir el volum de fangs que es produeixen precisament durant els processos de depuració aerobi i anòxic, així com per tractar aigües residuals amb una càrrega contaminant molt elevada (DBO_5> 1000 mg/l), entre d'altres aplicacions (Mackenzie 2010).

1.1.2.3. Sistemes d'eliminació biològica incrementada de nitrogen

Donat que la present memòria té com a objectiu l'estudi de sistemes dissenyats per depurar aigües residuals amb grans concentracions de nitrogen (fins a 1000 mg de nitrogen amoniacal per litre), a continuació s'expliquen amb més profunditat els principals sistemes d'eliminació biològica incrementada de nitrogen.

A Europa, la Directiva 91/271/CEE determina que l'eliminació biològica incrementada de nitrogen de l'aigua residual és d'obligat requeriment en aquelles EDARs que aboquen a zones declarades sensibles, és a dir, zones que compleixen amb els criteris establerts en l'apartat I de l'annex II del RD 509/1996 de 15 de març. A grans trets, es considera zona sensible aquells cossos d'aigua (llacs, llacunes, rius i aigües marítimes) que presenten risc d'eutrofització o que ja són eutròfics i aquelles zones d'aigües continentals destinades a l'obtenció d'aigua potable amb concentracions de nitrats superiors a 50 mg/l. Aquestes EDARs, doncs, han d'eliminar tant la matèria orgànica carbonosa de l'aigua com els nutrients (nitrogen i fòsfor).

Pràcticament la totalitat de EDARs (tant industrials com urbanes) eliminen el nitrogen mitjançant el procés convencional d'eliminació biològica de nitrogen o *Biological Nitrogen Removal* (en endavant a la present memòria cBNR). Però durant els últims anys s'han desenvolupat i implementat a gran escala dos processos innovadors d'eliminació biològica incrementada de nitrogen: el *Shortcut Biological Nitrogen Removal* (SBNR) i el procés combinat *Partial Nitritation – Anaerobic Ammonium Oxidation* (PN-Anammox), aquest últim arran del descobriment del procés Anammox (Mulder et al. 1995; de Graaf et al. 1995). Les diferents rutes metabòliques d'aquests processos d'eliminació biològica de nitrogen es mostren de forma esquemàtica a la figura 1.2.

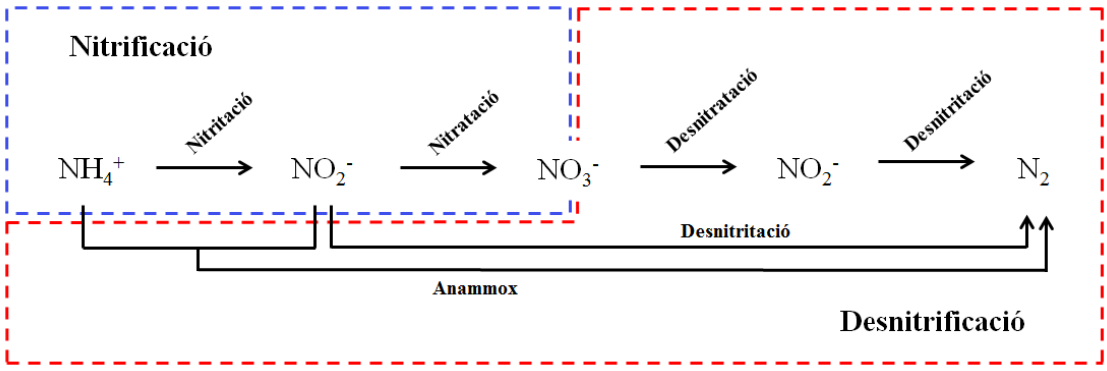


Figura 1.2. Principals rutes metabòliques dels processos d'eliminació biològica de nitrogen.

De forma global, en aquests processos el nitrogen amoniacal és oxidat total o parcialment fins a nitrit o nitrat pels microorganismes nitrificants en condicions aeròbies (nitrificació) i, en segon terme, és alliberat a l'atmosfera en forma de nitrogen gas (N_2) en condicions anòxiques o anaeròbies. La conversió de les formes oxidades de nitrogen (nitrit i nitrat) a nitrogen gas la duen a terme els microorganismes desnitrificants mitjançant el procés de desnitrificació. Els bacteris Anammox alliberen el nitrogen a l'atmosfera utilitzant conjuntament molècules de nitrit i amoni en el que es coneix com a oxidació anaeròbia de l'amoni.

Procés convencional d'eliminació de nitrogen (cBNR)

El procés convencional d'eliminació biològica de nitrogen és el procés mitjançant el qual el nitrogen en forma amoniacal present a l'aigua residual és eliminat i alliberat a l'atmosfera en forma de nitrogen gas (N_2) via nitrat. És el procés més comú i emprat en les EDAR pel tractament de les aigües residuals urbanes o que presenten una relació C/N similar a la de l'aigua residual domèstica.

Durant la nitrificació del procés cBNR, el nitrogen en forma amoniacal és oxidat completament fins a nitrat (equació 1.1). Aquesta conversió la duen a terme dos conjunts de microorganismes procariotes quimioautotròfs: els amonioxidants, que obtenen energia de la oxidació de l'amoni fins a nitrit (equació 1.2, nitritació); i els nitritoxidants, encarregats de la transformació dels nitrits a nitrats (equació 1.3, nitratació).

Equació 1.1 (nitrificació). $NH_4^+ + 2O_2 \rightarrow NO_3^- + 2H^+ + H_2O$

Equació 1.2 (nitritació). $NH_4^+ + 1.5O_2 \rightarrow NO_2^- + H_2O + 2H^+$

Equació 1.3 (nitratació). $NO_2^- + 0.5O_2 \rightarrow NO_3^-$

Durant la desnitrificació les molècules de nitrat son reduïdes fins a nitrogen gas (N_2), que és alliberat a l'atmosfera. Aquesta etapa la duen a terme un conjunt molt variat i ampli de microorganismes heteròtrofs que reben el nom de microorganismes desnitrificants. Els

microorganismes desnitrificants son organismes facultatius, essent capaços de realitzar el seu metabolisme tant en condicions aeròbies com anòxiques. Tot i preferir l'oxigen molecular dosificat en condicions d'aeració, poden utilitzar el nitrat com a acceptor d'electrons enlloc de l'oxigen dissolt per al seu metabolisme (equació 1.4, on CH_2O representa el donant orgànic d'electrons) quan la concentració d'oxigen dissolt al medi és inferior a 0,3-0,5 mg/l (Daigger et al. 1988).

Equació 1.4 (desnitrificació via nitrat). $NO_3^- + 1.25CH_2O \rightarrow 0.5N_2 + 1.25HCO_3^- + 1.25H^+$

A diferència dels sistemes convencionals de fangs actius sense eliminació incrementada de nitrogen, els sistemes amb cBNR requereixen operar amb un major temps de retenció cel·lular (major edat del fang), a causa de la menor velocitat de creixement que presenten les espècies nitrificants (Seviour i Nielsen 2010).

Shortcut Biological Nitrogen Removal (SBNR)

El procés SBNR, així com el procés combinat PN-Anammox (que es detalla a continuació), van ser dissenyats per tractar aigües residuals amb alta càrrega amoniacal i baixa relació carboni/nitrogen, com per exemple l'aigua de sortida de digestors anaerobis, per disminuir el cost energètic i econòmic que comporta el seu tractament mitjançant el procés cBNR.

El sistema d'eliminació incrementada de nitrogen SBNR està basat en l'eliminació del nitrogen amoniacal de l'aigua residual via nitrit, sense necessitat d'oxidar les formes nitrogenades fins a nitrat. En el procés SBNR la nitrificació es refereix únicament al pas de nitritació (equació 1.2), portada a terme pels microorganismes amonioxidants.

L'etapa de desnitrificació en el procés SBNR es mostra a l'equació 1.5 (on CH_2O representa el donant orgànic d'electrons).

Equació 1.5 (desnitrificació via nitrit). $NO_2^- + 0.75CH_2O \rightarrow 0.5N_2 + 0.75HCO_3^- + 0.75H^+$

Comparant l'estequiometria del procés SBNR i del cBNR, es pot observar com el procés SBNR comporta l'estalvi de fins a un 25% de la demanda d'oxigen en la fase aeròbia i fins a un 40% de matèria orgànica requerida per la desnitrificació, fent-lo un mètode de tractament efectivament més econòmic que el procés cBNR per tractar aigües residuals amb alta càrrega amoniacal i/o una baixa relació C:N. Tot i els beneficis que comporta el procés SBNR, alguns autors (Fux i Siegrist 2004; Hwang et al. 2006) han comprovat que les elevades concentracions de nitrit al reactor poden implicar una producció significativa d'òxid nitrós (N_2O), un dels principals gasos d'efecte hivernacle, amb el conseqüent impacte sobre el medi ambient.

La inhibició del desenvolupament i l'activitat dels bacteris nitritoxidants es controla principalment mitjançant elevats valors de temperatura (vora els 35ºC), valors de pH entre 7 i 8 (com més elevats major concentració d'amoníac lliure) i/o baixes concentracions d'oxigen dissolt (Hellinga et al. 1998; Chung et al. 2007).

Partial Nitritation – Anaerobic Ammonium Oxidation (PN-Anammox)

Per últim, el procés combinat PN-Anammox està basat en l'acció d'un grup de bacteris quimiolitoautòtrofs anomenats anammox, tots ells pertanyents al phylum Planctomycetes (Strous et al. 1999; Jetten et al. 2009), que són capaços d'oxidar l'amoni en condicions anòxiques utilitzant el nitrit com a acceptor d'electrons i eliminant el nitrogen del sistema en forma de nitrogen gas (equació 1.6, Strous et al. 1998).

Equació 1.6 (anammox). $1NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O0.5N0.15 + 2.03H_2O$

Com es pot observar a l'equació 6, mitjançant aquesta ruta metabòlica la obtenció de nou material cel·lular és molt escassa, és a dir, la velocitat de creixement de les poblacions de bacteris anammox és molt lenta. Aquest fet implica un inconvenient a l'hora de posar en marxa aquest tipus de sistemes, però l'avantatge, al mateix temps, que aquests sistemes generen un volum de fangs fins a un 90% menor que l'associat al procés cBNR. Existeixen varis factors que poden inhibir l'activitat anammox: la presència d'oxigen dissolt (Strous et al. 1997), temperatures superiors a 45ºC (Dosta et al. 2008), alguns compostos de carboni orgànic com alcohols, valors de pH fora del rang de 6,5 a 8,8 (valor òptim de 8) i els mateixos substrats que els bacteris anammox utilitzen per dur a terme els seus processos metàbolics (carboni inorgànic, nitrit, amoni i la forma no ionitzada de l'amoni, l'amoníac lliure) (Ren-Cun et al. 2012; Magrí et al. 2013), tot i que per alguns d'aquests factors encara no hi ha consens a la literatura sobre quins són els valors límit d'inhibició.

El principal requeriment del procés anammox és l'entrada d'un efluent adequat per a l'acció dels bacteris anammox. Aquest efluent ha de presentar una ratio nitrit:amoni de 1.32 (equació 1.6), ratio que s'obté gràcies al procés PN (nitritació parcial) previ a la fase anammox. Durant la fase de PN únicament la meitat aproximada del nitrogen amoniacal és oxidat a nitrit en condicions aeròbies (equació 1.7), permetent un estalvi d'oxigen durant la fase d'oxidació d'entre el 50 i el 60% que el que és requerit en un procés cBNR.

Equació 1.7 (nitritació parcial). $1NH_4^+ + 0.75O_2 \rightarrow 0.5NO_2^- + 0.5NH_4^+ + 0.5H_2O + H^+$

1.2. Comunitats microeucariotes dels sistemes de tractament d'aigües residuals

1.2.1. Organismes microeucariotes

El terme microeucariota és un terme que inclou a tots aquells organismes eucariotes (és a dir, que no són bacteris ni arqueus) de mida inferior a 1 mm i que majoritàriament requereixen de l'ús d'un microscopi per la seva detecció, observació i estudi. El terme compren des d'organismes unicel·lulars "senzills" fins a organismes pluricel·lulars més complexes com els micrometazous, amb diferenciació cel·lular i presència de teixits i òrgans.

La comunitat microeucariota associada als processos de tractament biològic d'aigües residuals està formada per protists, metazous i fongs. D'aquests, els protists i els micrometazous (aquests últims en menor mesura) són els que presenten un paper més rellevant i una major incidència sobre l'eficiència del tractament biològic d'aigües residuals[1] i, en conseqüència, han estat els més estudiats.

1.2.1.1. Protists

El terme Protist va ser emprat per primer cop l'any 1866 per Ernst Haeckel. Es consideren protists tots aquells organismes eucariotes microscòpics, unicel·lulars o pluricel·lulars sense teixits especialitzats, que no pertanyen a cap dels tres grans grups d'eucariotes pluricel·lulars: els fongs, animals i plantes. Els organismes classificats com a protists presenten una enorme heterogeneïtat, i comprenen una gran diversitat de característiques morfològiques, estructurals, d'hàbitat, nutricionals, reproductives i de desenvolupament (Vargas i Zardoya 2012).

Des del seu descobriment en els temps de van Leeuwenhoek fins pràcticament a finals del segle XX, els protists (anomenats també Protozoa o Protoctista) s'han classificat seguint únicament o en gran mesura criteris morfològics, com la classificació de Levine et al. (1980), una de les últimes classificacions anteriors a la filogènia molecular. Aquesta classificació, basada en semblances morfològiques i caràcters d'ultraestructura, ordena els protists en set fílums, dels quals cinc estan formats majoritàriament per organismes paràsits (Apicomplexa, Microspora, Labyrinthomorpha, Ascetospora i Myxozoa), mentre que els altres dos (Sarcomastigophora i Ciliophora) comprenen la gran majoria de protists de vida lliure. El fílum Ciliophora comprèn exclusivament els ciliats, organismes que es reconeixen fàcilment per presentar el cos (cèl·lula) recobert de cilis, mentre que el fílum Sarcomastigophora comprèn als organismes que presenten flagells (subfílum Mastigophora) i als que es desplacen mitjançant pseudopodis (subfílum Sarcodina).

Aquestes classificacions van començar a quedar obsoletes amb l'aparició dels primers estudis de filogènia molecular a finals de la dècada dels 80 i principis dels 90, basats en l'anàlisi molecular de la subunitat petita del ribosoma. Les noves dades moleculars, proveïdes per una tecnologia molecular incipient, van posar a debat la classificació tradicional del moment, com

[1] Recordem que, en la present memòria, en el concepte de 'sistemes de tractament biològics d'aigües residuals' no s'inclouen ni els sistemes de tractament tous (llacunatges i llits de canyes) ni els sistemes de tractament anaerobis.

va recollir Cavalier-Smith (1995), i van suposar el punt de partida de la reestructuració taxonòmica dels protists. Durant els últims anys del segle XX i la primera dècada del segle XXI l'ús de tècniques moleculars i anàlisis filogenètiques es va multiplicar, tant per protists com per la resta d'organismes eucariotes, gràcies a les cada vegada més potents, estandarditzades i accessibles econòmicament tecnologies moleculars. En aquest context de descobriment i reestructuració de la classificació dels protists (i de tot el conjunt d'organismes eucariotes) es va publicar la classificació d'Adl et al. (2005), posteriorment revisada i actualitzada (Adl et al. 2012).

L'arbre evolutiu dels eucariotes proposat per Adl et al. (2012) classifica a aquests organismes en 5 grans grups: Opisthokonta, Amoebozoa, Excavata, Archaeplastida i SAR, grups que segueixen essent força acceptats actualment, i altres taxons sense ubicació clara (Figura 1.3). Els organismes tradicionalment classificats com a protists componen la gran majoria de grups de l'arbre eucariota, alguns d'ells estretament emparentats amb eucariotes multicel·lulars com els coanoflagel·lats (emparentats amb els Metazoa), els nucleàrids (amb els Fungi) i els glaucòfits (amb els Plantae), i d'altres, la gran majoria, formant grups independents. De fet, 3 dels 5 supergrups d'eucariotes el componen exclusivament organismes protists: Amoebozoa, SAR i Excavata, fet que serveix per fer-se una idea de l'enorme diversitat eucariota unicel·lular existent.

L'impacte de la reorganització dels protists en l'arbre evolutiu dels eucariotes s'exemplifica en la posició dels organismes flagel·lats i ameboides del grup Sarcomastigophora de Levine et al. (1980). Els flagel·lats (antics Mastigophora) estan presents en tots els grans grups de l'arbre, i la forma ameboide (Sarcodina) únicament no es troba representada en el grup dels Archaeplastida. En aquesta nova classificació, doncs, la presència de flagells i pseudopodis sembla ser un caràcter força basal del conjunt dels eucariotes. Aquesta important reorganització dels organismes flagel·lats i ameboides dóna també una idea de la dificultat que presenta la sistemàtica d'aquests organismes mitjançant microscòpia, molts d'ells de mida inferior a 20 µm i de caràcters difícilment observables. El grup dels Ciliophora, en canvi, ha estat dels pocs als que la filogènia molecular ha reconegut com a grup monofilètic i, juntament amb els Apicomplexa i Dinoflagellata, conformen els Alveolata.

Cal tenir en compte que la classificació dels protists (i dels eucariotes en el seu conjunt) és un camp viu, que està en constant procés de reestructuració i que encara presenta nombroses qüestions no resoltes que generen debat en la comunitat científica. Algunes d'aquestes qüestions són la monofília del grup Excavata (Rodríguez-Ezpeleta et al. 2007; Cavalier-Smith 2016), o el suport a l'existència com a supergrup dels SAR, que alguns autors (Pawlowski 2013) prefereixen mantenir de forma separada, atorgant una major categoria taxonòmica als tres grups que el componen: Stramenopiles, Alveolata i Rhizaria. Per últim, les noves classificacions moleculars mostren encara un gran nombre de grups *Incertae sedis*, la posició dels quals queda sense resoldre en l'arbre filogenètic, com s'observa en la classificació de Adl et al. (2012)i, tot i que en menor mesura, en la de Pawlowski (2013).

Els protists associats habitualment als sistemes biològics de tractament d'aigües residuals pertanyen a tots els grans grups d'eucariotes: Opisthokonta, Amoebozoa, Excavata, Stramenopiles, Rhizaria, Alveolata i, de forma molt més ocasional, Archaeplastida. D'aquests,

els ciliats (Ciliophora, dins els Alveolata) són els que presenten una major rellevància per al correcte funcionament dels processos i, al mateix temps, són els que tenen un major valor com a bioindicadors de les condicions del sistema, motiu pel qual s'expliquen més detalladament a la present memòria. Per qüestions pràctiques, les formes flagel·lades i ameboides s'explicaran de forma agrupada sota els termes tradicionals flagel·lats i amebes.

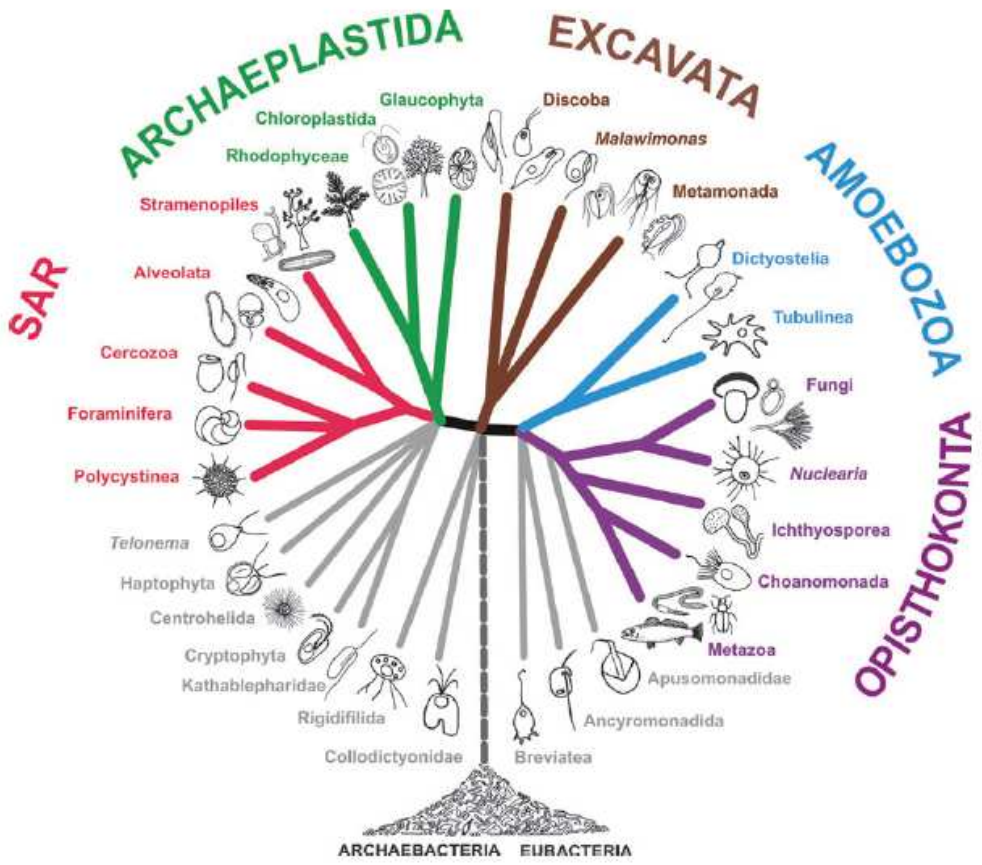


Figura 1.3. Classificació filogenètica dels eucariotes (font: Adl et al. 2012).

1.2.1.1.1. Flagel·lats

El terme flagel·lat (terme sense implicacions taxonòmiques) engloba a tots aquells organismes que presenten un o més flagells, que són utilitzats per a la locomoció, adhesió a un substrat i/o alimentació. El nombre i longitud dels flagells i la posició d'aquests al cos són caràcters que s'utilitzen per la seva identificació microscòpica, així com el tipus de moviment del flagel·lat, les característiques morfomètriques i la flexibilitat del cos.

Els sistemes de tractament d'aigües residuals poden ser colonitzats per una gran diversitat de flagel·lats, essent habitualment els organismes dominants a nivell d'abundància poblacional, tot i que degut a la seva petita mida tenen un impacte força menor en termes de biomassa. A causa de la seva petita mida i la conseqüent dificultat d'identificació, els flagel·lats de mida

inferior a 20 µm sovint s'agrupen conjuntament com a "nanoflagel·lats". Entre els nanoflagel·lats més comuns destaquen els pertanyents als grups Kinetoplastea i Metamonada (Excavata) i Chrysophyceae (Stramenopiles). Els flagel·lats de mida més gran, la gran majoria pertanyents al grup Euglenida (Excavata), i aquells amb característiques molt concretes i fàcilment identificables com els coanoflagel·lats (Choanomonada, Opisthokonta), s'acostumen a identificar a nivell de gènere o espècie (Hawkes 1960; Curds i Cockburn 1970a).

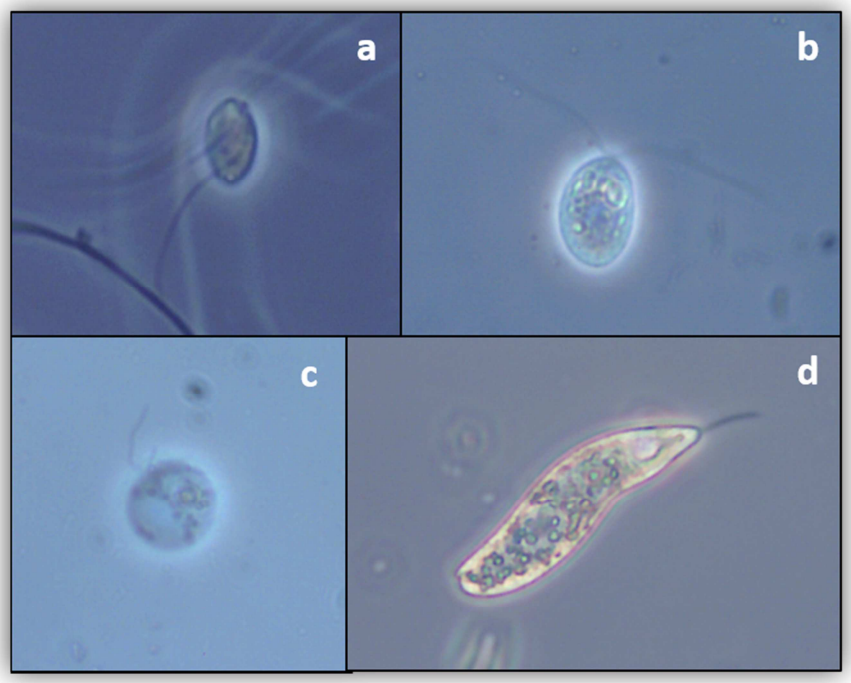


Figura 1.4. Imatges de flagel·lats de sistemes de tractament d'aigües residuals. a. *Bodo* sp. (Kinetoplastea, Excavata); b. *Polytoma* sp. (Chlorophyceae, Archaeaplastida); c. Ochromonadal (Chrysophyceae, Stramenopiles); d. *Peranema* sp. (Euglenida, Excavata). Autor: Oriol Canals.

1.2.1.1.2. Amebes

Igual que succeeix amb els flagel·lats, el terme ameba és un terme que fa referència a la presència d'un caràcter morfològic compartit, i no implica proximitat filogenètica entre els seus membres. El fet característic de les amebes és la presència de pseudopodis per al seu desplaçament i alimentació. Existeixen una considerable varietat de tipus de pseudopodis, com els lobopodis, actinopodis, filopodis i reticulopodis.

De forma pràctica, a nivell microscòpic les amebes es divideixen en dos grans grups, les amebes nues o gimnamebes i les amebes testàcies o amebes amb teca. Les gimnamebes són probablement els organismes que presenten major dificultat d'identificació, ja que no presenten una forma ni estructures externes més o menys fixes com els flagel·lats. En el context dels sistemes de tractament d'aigües residuals, les gimnamebes es poden agrupar en base a la seva mida en gimnamebes petites (< 20 µm), mitjanes (entre 20 i 50 µm) i grans (> 50 µm) (Salvadó 1994; Pérez-Uz et al. 2010), i pertanyen principalment al grup Amoebozoa i

Heterolobosea (Excavata). De forma més ocasional les amebes poden pertànyer al grups dels Nucleariida (Opisthokonta) i Actinophryidae (Stramenopiles), entre d'altres.

Les amebes testàcies, en canvi, s'acostumen a identificar fins a nivell de gènere o espècie (Curds i Cockburn 1970a; Salvadó 1994). La seva identificació es basa principalment en la forma, mida i altres característiques de la teca, com la presència d'estructures similars a espines o la coloració. La gran majoria d'amebes testàcies pertanyen als grups Arcellinida (Amoebozoa) i Euglyphida (Rhizaria).

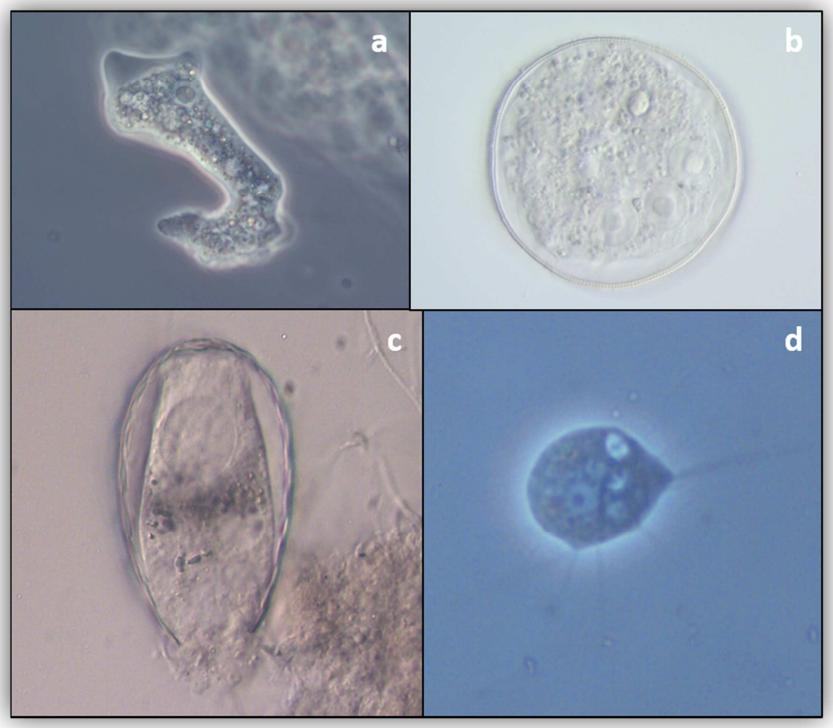


Figura 1.5. Imatges de gimnamebes i d'amebes testàcies de sistemes de tractament d'aigües residuals. a. Gimnameba (Amoebozoa); b. *Arcella* sp. (Arcellinida, Amoebozoa); c. *Euglypha* sp. (Euglyhida, Rhizaria); d. Nucelariida (Opisthokonta). Autor: Oriol Canals.

1.2.1.1.3. Ciliats (Ciliophora Doflein, 1901)

Tot i que a primer cop d'ull la característica més òbvia que defineix als ciliats és la presència de nombrosos cilis recobrint en menor o major mesura el seu cos, el tret distintiu que defineix als ciliats com a grup monofilètic és la combinació de tres caràcters: la construcció del còrtex o capa cortical, la presència de dualisme nuclear i el fenomen de la conjugació durant la fase de reproducció sexual.

El còrtex és el responsable de la forma de les diferents espècies de ciliats, i el que determina la rigidesa o flexibilitat d'aquestes. Breument, el còrtex està format principalment per dos components, la pel·lícula i les cinètides (cinetosomes i estructures associades), tot i que també s'hi poden trobar estructures addicionals com fibres contràctils i altres orgànuls (Hausman et

al. 2003). La pel·lícula la formen la membrana cel·lular i, en ocasions, una capa addicional anomenada *perilemma* com a component extracel·lular. Just per sota la membrana cel·lular s'hi troba un sistema d'alvèols disposat en forma de mosaic que normalment segueix un patró específic de cada espècie. En ocasions, els alvèols contenen plaques proteiques (gènere *Euplotes*) o plaques calcificades (gènere *Coleps*, figura 1.9, imatge b) que donen encara més estabilitat a la capa cortical. En el seu sentit més ampli, la pel·lícula també inclou una capa anomenada epiplasma, subjacent a les vacuoles alveolars, i bandes longitudinals de microtúbuls paral·leles a l'epiplasma, que probablement tenen una funció estabilitzadora del còrtex així com un rol en el procés de morfogènesis d'aquest (figura1.6).

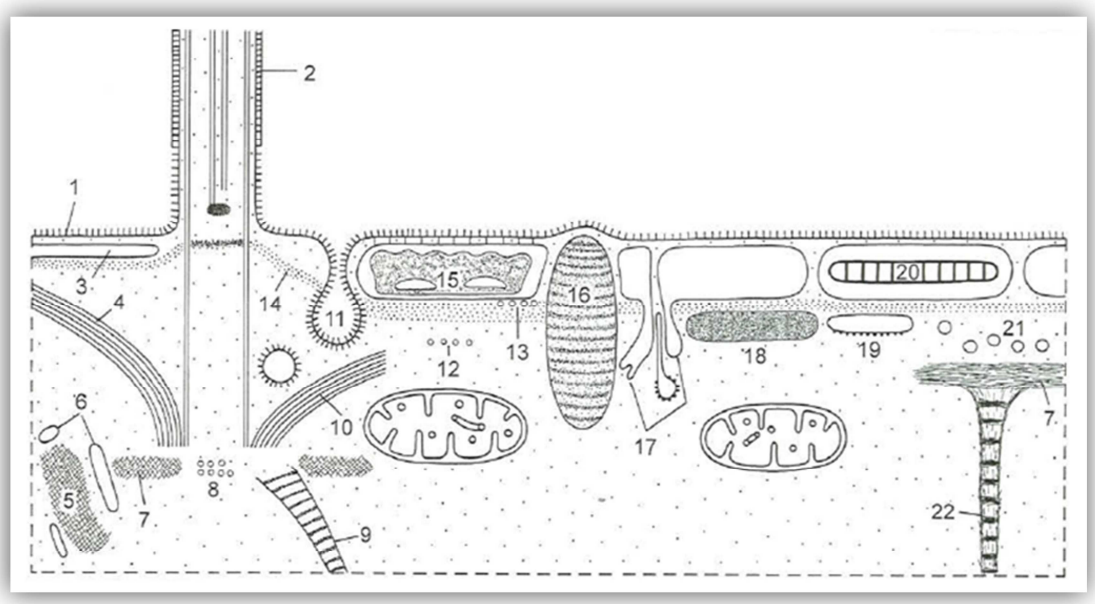


Figura 1.6. Representació de l'esquema del còrtex dels ciliats. 1- membrana cel·lular, 2- *perilemma*, 3- alvèols, 4- microtúbuls postciliars, 5- mionema, 6- cisterna de reticle endoplasmàtic, 7- capa de filaments, 8- microtúbuls subcinetials, 9- fibra cinetodesmal, 10- microtúbuls transversals, 11- sac parasomal, 12 i 13- microtúbuls longitudinals, 14- epiplasma, 15- placa alveolar calcificada, 16- extrusoma, 17- alveolocist, 18- placa polisacàrida, 19- cisterna de reticle endoplasmàtic rugós, 20- placa alveolar proteica, 21- vesícules, 22- feix de filaments transversals estriats (font: Hausman et al. 2003).

Les cinètides són els components fonamentals del sistema infraciliar (o infraciliació) dels ciliats, un sistema únic d'aquests organismes i responsable del moviment coordinat dels cilis (figura1.7). Cada cinètida està formada per la combinació de un, dos o múltiples cinetosomes (cossos basals dels cilis) associats a vàries estructures connectades a un complex estructural superior. Les estructures associades als cinetosomes comprenen una sèrie de fibres i bandes de microtúbuls que connecten i orienten els diferents cinetosomes i cilis al llarg del cos del ciliat, permetent la organització dels cilis somàtics i orals. Els cilis somàtics, implicats en la locomoció, s'organitzen en files que segueixen l'eix longitudinal del cos, anomenades cinèties, i que poden ser monocinèties o dicinèties en funció del nombre de cinetosomes que presenti cada cinètida. Aquest patró únicament s'interromp en la regió oral, on les cinèties passen a

anomenar-se cinèties periorals. Les cinèties associades al citostoma presenten una major complexitat d'organització i reben el nom de policinèties orals. Les cinètides que conformen les policinèties orals estan formades per varis cinetosomes.

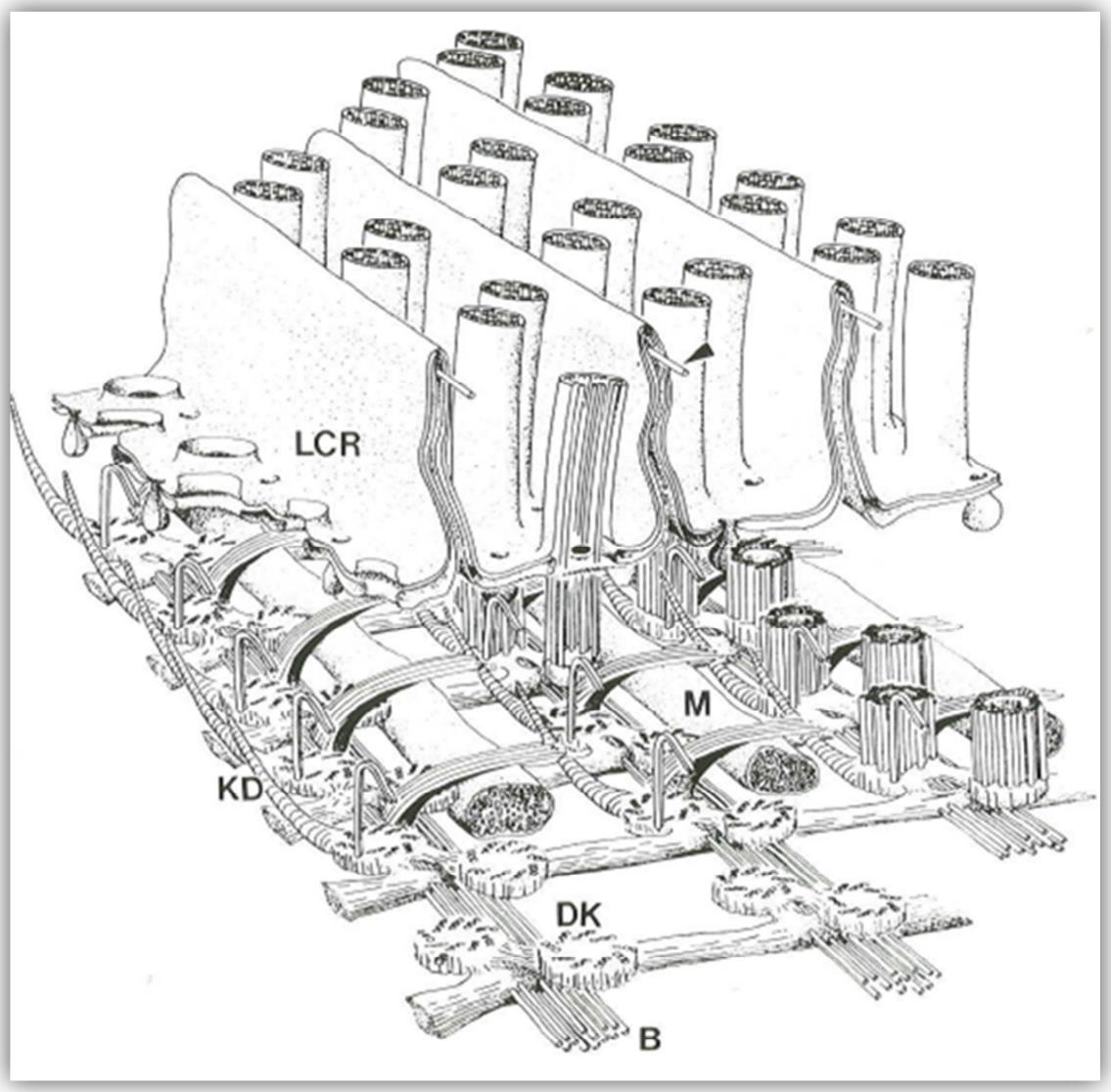


Figura 1.7. Exemple d'un esquema del sistema infraciliar en ciliats, en aquest cas en *Conchophthirus curtus* (Hymenostomatia). B- feix de microtúbuls basals, DK- dicinètides, LCR- cresta cortical longitudinal, KD- fibra cinetodesmal, M- mitocondris (font: Hausman i Bradbury 1996).

Els diferents patrons i característiques de la infraciliació dels ciliats, tant somàtica com oral, aporten informació rellevant per la classificació i identificació d'aquests organismes (Hausman et al. 2003; Lynn 2008).

En certa manera, els cilis poden ser definits com la part externa i visible del sistema infraciliar. Són estructures similar a petits pèls que aquests protists utilitzen per la locomoció i alimentació. Els cilis poden cobrir uniformement tota la cèl·lula del ciliat, cobrir només una

part del cos (per exemple la zona ventral o dorsal) o trobar-se exclusivament en algunes zones concretes (per exemple ala regió oral en el cas de la majoria de perítrics). En ocasions els cilis somàtics poden aparèixer agrupats donant lloc a unes estructures locomotores anomenades cirrus (figura 1.9, imatge e), o, en el cas dels cilis orals, formant estructures aplanades anomenades membranel·les (figura 1.9, imatge d). Els ciliats que es desplacen activament per la columna d'aigua també poden presentar un o més cilis de major longitud a l'extrem caudal del cos, probablement implicats en tasques de moviment i direcció.

El dualisme nuclear és potser la característica més àmpliament reconeguda com a caràcter unitari dels ciliats. Cada individu ciliat presenta, normalment, un o més nuclis diploides de mida generalment petita i forma esfèrica anomenats micronuclis, i un o més nuclis de mida més gran i forma variable anomenats macronuclis, amb un contingut de DNA significativament major que el que es pot trobar al micronucli. Als macronuclis es duen a terme tots els processos de síntesis de RNA, essent els responsables del metabolisme bàsic de la cèl·lula. Per la seva part, als micronuclis s'hi dona la recombinació genètica. Durant la fase de reproducció sexual el macronucli normalment degenera, essent posteriorment re-establert a partir del nou micronucli. En els ciliats, la reproducció sexual es dóna mitjançant el procés de conjugació, en el que dos individus de la mateixa espècie es fusionen de forma parcial. Durant aquest procés, els dos individus s'intercanvien un nucli haploide derivat del micronucli. Un cop finalitzat l'intercanvi genètic, en la majoria de grups els dos individus es tornen a separar.

Identificació dels ciliats

La identificació microscòpica dels ciliats, tot i no ser fàcil per a un observador no habituat a treballar amb el microscopi, és generalment molt més senzilla que la identificació de la resta de protists com ara les formes flagel·lades i formes ameboides (amb l'excepció de les amebes testàcies). Gràcies a la presència del còrtex, els ciliats presenten una forma més o menys constant i estable dins una mateixa espècie que fa més fàcil el seu reconeixement, i normalment presenten una mida suficientment gran per poder diferenciar les seves característiques mitjançant les diferents tècniques de microscòpia òptica.

Els caràcters que s'utilitzen per a la identificació microscòpica dels ciliats són la mida del cos, el número i la posició al cos de vacuoles contràctils, la presència o absència d'estructures externes com lorica o peduncle, el tipus de moviment, la posició del citostoma (boca), la organització de les estructures orals (en ocasions formant membranel·les), el grau de cobertura ciliar del cos, el patró de les cinèties somàtiques i la presència de cirrus, entre d'altres.

La majoria d'aquestes característiques requereixen l'observació d'individus vius mitjançant microscòpia òptica de camp clar o contrast de fases, amb l'excepció del sistema infraciliar, que requereix l'observació d'individus tenyits amb tècniques específiques com la impregnació argèntica (Chatton-Lwoff 1930; Klein 1958; Fernández-Galiano 1994) (figura 1.8) o el flutax, basada en microscòpia d'epifluorescència (Arregui et al. 2003).

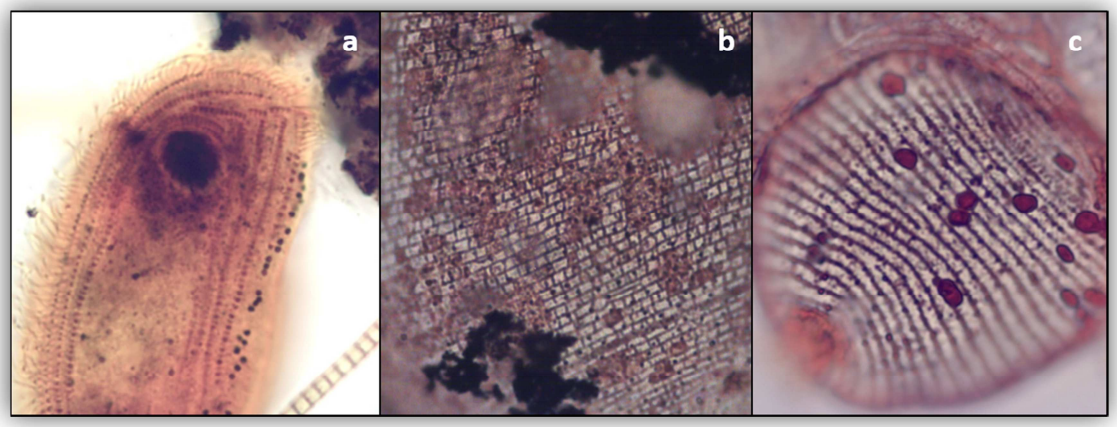


Figura 1.8. Imatges de ciliats després de impregnació argèntica. a. *Pseudochilodonopsis fluviatilis* (Phyllopharyngea) tenyit amb la tècnica d'impregnació argèntica de Galiano; b. Individu del gènere *Pseudovorticella* (Peritrichea, Oligohymenophorea) tenyit amb la tècnica d'impregnació argèntica de Klein; c. *Vorticellides aquadulcis* (Peritrichea, Oligohymenophorea) tenyida amb impregnació argèntica de Klein. Autor: Oriol Canals.

Altra informació addicional útil per la identificació dels ciliats és la posició que ocupen a la xarxa tròfica (bacterívors o depredadors) i trets característics de la seva ecologia, com l'hàbitat on es desenvolupa o el seu índex de saprobietat.

En els sistemes de tractament d'aigües residuals els ciliats s'acostumen a ordenar en grups funcionals segons el seu grau d'afinitat amb la biomassa (Curds 1965), separant entre aquells que es desplacen lliurement per l'aigua (lliure nedadors), els que estan fixats de forma més o menys permanent a un substrat (sèssils) i els que es desplacen activament per sobre el substrat (reptants). Aquesta agrupació funcional també contempla com a grup els ciliats depredadors, independentment de si aquests són de vida lliure o es troben fixats a un substrat com és el cas del grup dels ciliats suctors.

En els últims anys, el ràpid avenç que ha experimentat el camp de tecnologiesde seqüenciació de DNA està atorgant un paper cada cop més protagonista a les tècniques moleculars com a sistema de caracterització de les comunitats de ciliats (i del conjunt de protists) tant d'entorns naturals com de sistemes de tractament d'aigües residuals (Matsunaga et al. 2014; Medinger et al. 2010; Stoeck et al. 2014). En aquest context, les tècniques que més interès poden tenir per ser aplicades en estudis de biodiversitat són les que s'engloben sota el nom de *Next Generation Sequencing* (NGS) o *High-Throughput Sequencing* (HTS), tècniques enfocades a obtenir la màxima diversitat genètica d'un únic gen o regió d'un gen present a una mostra. Alguns exemples d'aquestes tècniques són la piroseqüenciació 454 o Illumina.

Figura 1.9. Imatges d'alguns ciliats habituals de sistemes de tractament d'aigües residuals. a. *Litonotus lamella* (Litostomatea); b. *Coleps hirtus* (Prostomatea); c. *Dexiotricha* sp. (Oligohymenophorea); d. *Spirostomum* sp. (Heterotrichea), la fletxa indica les membranel·les; e. *Aspidisca lynceus* (Spirotrichea), la fletxa indica els cirrus; f. *Epicarchesium* sp. (Olighymenophorea); g. *Holoprhyadiscolor* (Prostomatea); h. *Acineta tuberosa* (Phyllopharyngea). Autor: Oriol Canals.

Classificació dels ciliats

La classificació dels ciliats ha sofert grans canvis des de l'època de Haeckel, a mesura que s'hananat descobrint les característiques d'aquests organismes i, en paral·lel, s'han anat desenvolupant les metodologies per al seu estudi. Alguns autors, com Lynn (2008), classifiquen les diferents etapes de coneixement i estudi dels ciliats en 4 grans èpoques.

a. Època del descobriment (1880-1930) i expansió (1930-1950), representada principalment per Bütschli i Kahl, on les classificacions de la qual es centraven en les característiques de la ciliació oral i somàtica observables mitjançant microscòpia òptica. Aquestes primeres classificacions es basaven en la premissa que en l'evolució les formes més simples van precedir a les formes més complexes.

b. Època de la infraciliació (1950-1970), directament relacionada amb la normalització de l'ús de les tècniques de impregnació argèntiques, i durant la qual es van proposar classificacions que integraven les noves dades referents al patró del sistema infraciliar dels ciliats i a la morfogènesis de la zona oral durant la divisió cel·lular. Les estructures orals van continuar mantenint un rol important en la determinació dels clades. Alguns dels autors més destacats d'aquesta època van ser Fauré-Fremiet i Corliss.

c. Època de la ultraestructura (1970-1990). Durant aquest període les tècniques d'impregnació argèntica es van continuar utilitzant, però va ser el microscopi electrònic el que va anar guanyat preferència alhora de resoldre qüestions de sistemàtica de ciliats. Les classificacions van assimilar els patrons de complexitat del còrtex dels ciliats, principalment relacionats amb l'estructura de les cinètides somàtiques, ja que presentaven una molt menor variació entre grups que les que mostraven les cinètides orals, fet normalment associat a una senyal filogenètica més profunda. Les cinètides somàtiques es van utilitzar, doncs, per assignar la monofília dels diferents clades. Lynn, de Puytorac i el mateix Corliss van ser alguns dels autors amb un major protagonisme en aquesta època de la ultraestructura.

d. Època del refinament (1990-actualitat), en la que les classificacions incorporen les dades moleculars resultants de les anàlisis de seqüenciació de gens, principalment del gen 18S rDNA. Les últimes classificacions i estudis filogenètics de ciliats, com les de Adl et al. (2012) i Gao et al. (2016), sembla que recolzen clarament l'existència de 2 subfílums: Postciliodesmatophora i Intramacronucleata. El subfílum Postciliodesmatophora està compost per les classes Karyorelictea i Heterotrichea, mentre que el subfílum Intramacronucleata engloba les 9 classes de ciliats restants acceptades actualment (Colpodea, Oligohymenophorea, Nassophorea, Plagiopylea, Prostomatea, Phyllopharyngea, Litostomatea, Armorphorea i Spirotrichea) (figura1.10). Aquests resultats impliquen una clara relació entre les posicions basals de l'arbre i els ciliats amb un menor dimorfisme nuclear i amb la presència de dicinètides somàtiques amb postciliodesmata (bandes de microtúbuls postciliars lateralment superposades, estructura que dóna nom al subfílum Postciliodesmatophora).

Bona part de la sistemàtica de ciliats, sobretot a nivell de grans grups, es basa en gran mesura en les característiques estructurals de les cinètides somàtiques (observables mitjançant microscòpia electrònica), com per exemple el nombre de bandes de microtúbuls que presenten, la seva orientació, el seu punt d'ancoratge als cinetosomes, etc. (figura 1.11). En la present memòria no s'aprofundirà en les característiques estructurals de les cinètides en els diferents grups, però es vol deixar constància de la seva importància en la classificació filogenètica dels ciliats.

La classificació filogenètica del fílum Ciliophora, com succeeix amb l'arbre dels eucariotes, encara no es pot considerar resolta, i continuen existint dubtes sobre les relacions entre els clades que el constitueixen (Gao et al. 2016). Alguns autors, com Adl et al. (2012), utilitzen el grup CONthreeP per referir-se al conjunt de les classes Colpodea, Oligohymenophorea, Nassophorea, Plagiopylea, Prostomatea i Phyllopharyngea, tot i l'absència de sinapomorfies que el justifiquin. El mateix succeeix amb l'agrupació de les classes Spirotrichea, Armorphorea i Litostomatea en el grup SAL, tot i que aquest terme és molt menys estès. També és destacable l'existència d'alguns grups de ciliats que van canviant la seva posició dins l'arbre filogenètic

com *Protocruzia*, considerat com a Spirotrichea per Adl et al. (2012), posició poc suportada en els resultats obtinguts per Gao et al. (2016), autors que el situen a la base de la branca del subfílum Intramacronucleata. Gao et al. (2016) també contempla establir els organismes de l'ordre Mesodiniida (del gènere *Mesodinium*) com un dels grups més basals de l'arbre dels ciliats.

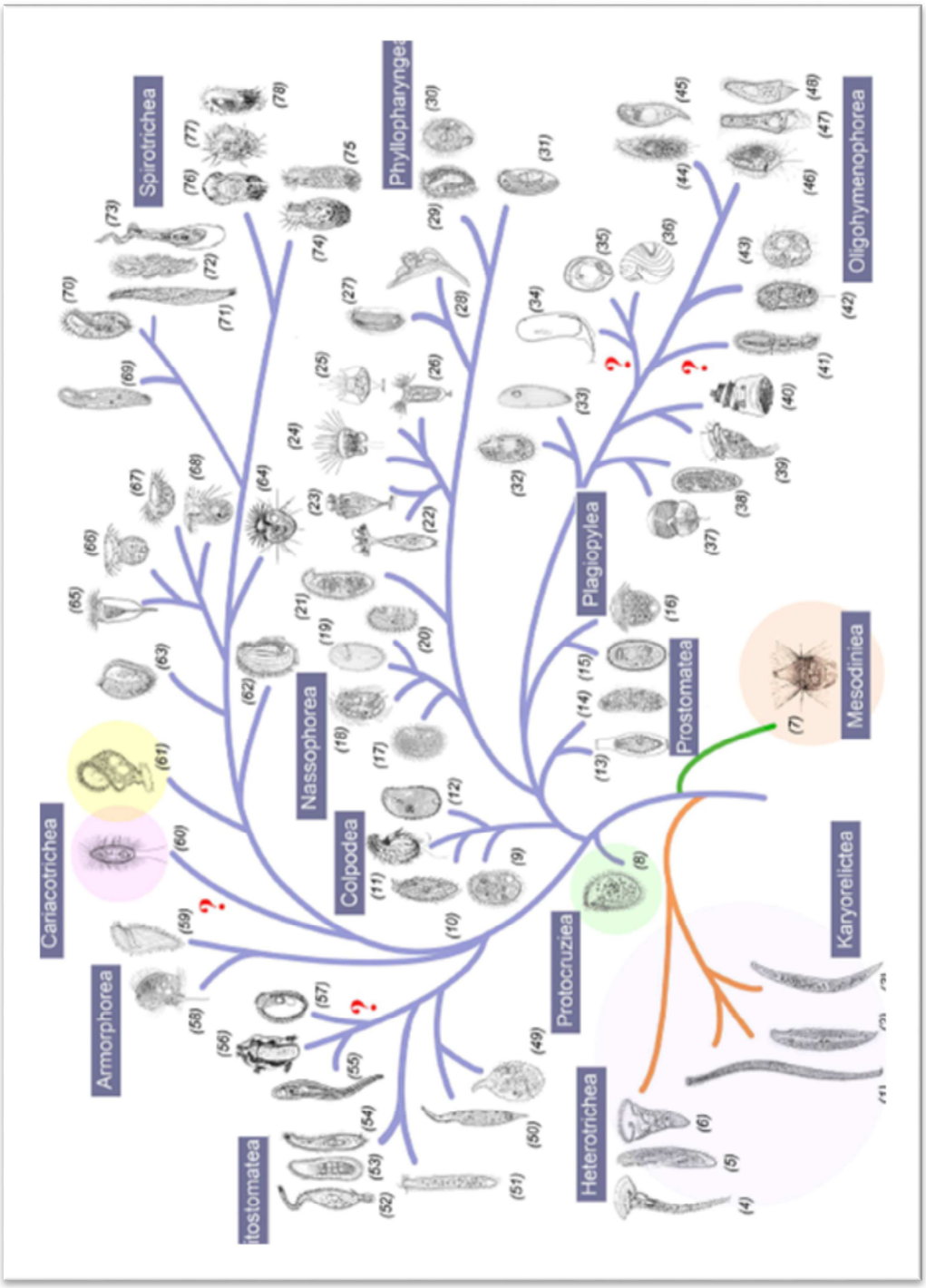


Figura 1.10. Evolució hipotètica dels ciliats, basada en dades morfològiques i moleculars, mostrant la relació i les posicions dels taxons a nivell d'ordre (font: Gao et al. 2016).

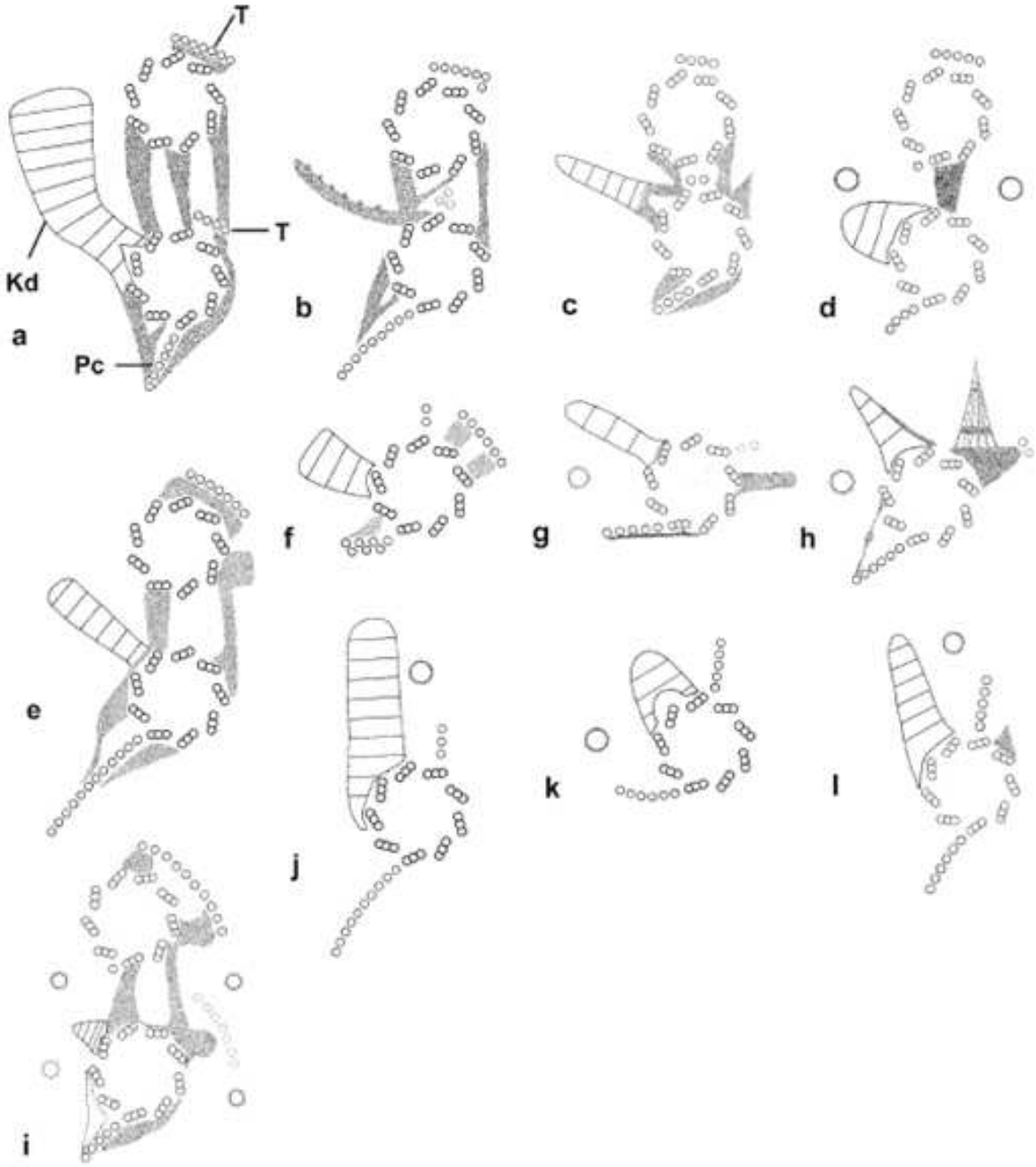


Figura 1.11. Esquema de les cinètides somàtiques de diferents gèneres representant les diferents classes del fílum Ciliophora. a- *Loxodes*, classe Karyorelictea; b- *Blepharisma*, classe Heterotrichea; c- *Protocruzia*; d- *Euplotes*, classe Spirotrichea; e- *Metopus*, classe Armophorea; f- *Balantidium*, classe *Litostomatea*; g- *Chilodonella*, classe Phyllopharyngea; h- *Obertrumia*, classe Nassophorea; i- *Colpoda*, classe Colpodea; j- *Plagiopyla*, classe Plagiopylea; k- *Holophrya*, classe Prostomatea; l- *Tetrahymena*, classe Oligohymenophorea. Kd- fibra cinetodesmal, Pc- cinta de microtúbuls postciliar, T- cinta transversal de microtúbuls (font: Lynn 2008).

De les 11 classes acceptades actualment, en el present apartat es tractaran amb major profunditat la classe Colpodea i les subclasses Scuticociliatia i Peritrichia de la classe

Oligohymenophorea, tots ells del subfílum Intramacronucleata, per ser els grups als quals pertanyen les espècies de ciliats més rellevants en els estudis que aquí es presenten.

Classe Colpodea Small & Lynn, 1981

Els Colpodea van ser elevats al rang de classe per Small i Lynn l'any 1981 basant-se bàsicament en les característiques estructurals de les cinètides somàtiques. Les espècies de la classe Colpodea viuen majoritàriament en hàbitats terrestres o d'aigua dolça amb una alta probabilitat de dessecació com sòls, molses o fullaraca, i es consideren els organismes amb una major habilitat en la formació de cists (estructures de resistència), principalment els pertanyents al gènere *Colpoda*. Fins avui dia, es creu que totes les espècies conegudes de Colpodea són capaces d'encistar-se en resposta a situacions de dessecació (Lynn 2008). Respecte l'alimentació, els membres de la classe presenten una gran varietat de preses en funció de la mida de cada espècie, que pot variar des de 10µm fins a més de 1 mm.

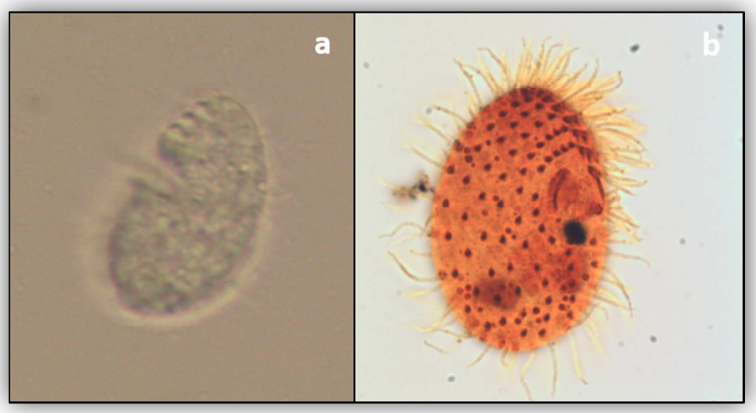


Figura 1.12. Imatges de *Colpoda aspera* (Colpodea). a. *In vivo*; b. tenyit amb impregnació argèntica de Galiano. Autor: Oriol Canals.

Els Colpodea presenten una gran varietat de preses en funció de la seva mida. Els més petits s'alimenten de bacteris, mentre que els de major mida com *Bursaria* depreden sobre altres ciliats de mida gran com *Paramecium*. Presenten una variabilitat molt elevada pel que fa a l'aparell oral, fet que provoca que alguns gèneres presentin fortes semblances amb altres tipus de ciliats i siguin fàcils de confondre. La conjugació en la classe Colpodea ha estat observada molt poques vegades, i sovint s'assumeix que aquests ciliats són bàsicament asexuals. Els membres de la família Colpididae, entre els que es troba el gènere *Colpoda*, presenten de manera molt habitual el cos amb forma de ronyó, fet característic del grup.

Els últims estudis moleculars recolzen l'existència de 4 clades dins la classe Colpodea (Foissner et al. 2011): Colpodida, Cyrtolophosidida, Bursariomorphida i Platyophryida.

Classe Oligohymenophorea de Puytoreac et al., 1974

Els individus de la classe Oligohymenophorea reben aquest nom per presentar a la regió oral un màxim de tres policinèties orals i una policinètia paroral (en ocasions molt prominent). A part d'aquest fet, però, no existeixen pràcticament evidències morfològiques o clares sinapomorfies entre els ciliats que conformen la classe Oligohymenophorea. De fet, la classe comprèn una enorme diversitat morfològica i una àmplia varietat d'estratègies, des d'espècies de vida lliure (nedadores i sèssils) fins a simbionts i paràsits d'altres organismes, majoritàriament metazous. Els Oligohymenophorea són comuns tant en ambients marins com d'aigua dolça, i es considera la classe de ciliats més àmpliament distribuïda.

La classe inclou sis subclasses: Peniculia, Scuticociliatia, Hymenostomatia, Peritrichia, Apostomatia i Astomatia. Membres de les subclasses Scuticociliatia, Hymenostomatia i Peritrichia es troben entre els organismes més habituals dels sistemes de tractament d'aigües residuals (Curds 1975).

Subclasse Scuticociliatia Small, 1967

La principal sinapomorfia de la subclasse Scuticociliatia és la presència de *scutica* (figura 1.13, imatge c). La *scutica* és una estructura en forma de ganxo situat en la zona posterior de la futura membrana paroral durant la estomatogènesis, tant del ciliat nou com del que es divideix. La *scutica* pot ser transitòria o trobar-se de forma permanent en el ciliat. Quan aquesta és permanent, el segment posterior de la policinètia paroral sovint rep el seu nom.

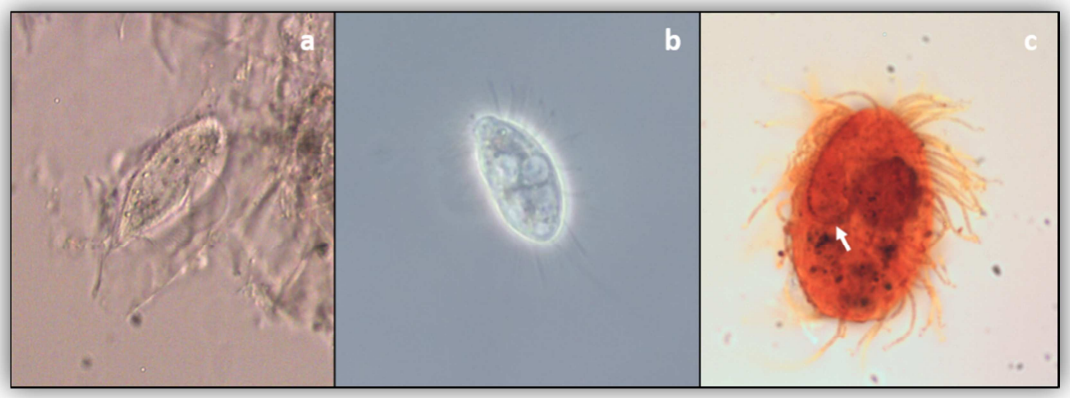


Figura 1.13. Imatges de scuticociliats presents en sistemes de tractament d'aigües residuals. a. *Calyptotricha lanuginosa*; b. *Cyclidium glaucoma*; c. *Cyclidium glaucoma* tenyit amb impregnació argèntica de Galiano. La fletxa indica la *scutica*. Autor: Oriol Canals.

És característic dels Scuticociliatia la presència d'una membrana ondulant com a policinètia paroral. Aquesta membrana ondulant sovint és molt prominent, en alguns casos podent arribar a ser pràcticament tant llarga com la longitud del cos del ciliat (gèneres *Pseudocohnilembus* o *Calyptotricha*, figura 1.13, imatge a). Els Scuticociliatia són típicament

bacterívors micròfags, essent més abundants en hàbitats eutrofitzats. En llacs i zones de costa es troben de forma força habitual en les aigües més profundes, sovint en condicions microaeròfiles (Lynn 2008).

La subclasse ha tingut fins ara un fort suport en els arbres filogenètics basats en seqüències de la subunitat petita del ribosoma, però existeixen dubtes sobre la monofília d'algunes famílies i la validesa d'alguns gèneres (Gao et al. 2014; Pan et al. 2015), entre elles la família Cyclidiidae. Els arbres filogenètics actuals sembla que recolzen la classificació dels Scuticociliatia en dos ordres: Philasterida i Pleuronematida, a l'espera de resoldre la posició de la família Cyclidiidae i dels membres de l'antic ordre Thigmotrichida, ambdós grups estretament relacionats i situats com a grup basal de l'ordre Pleuronematida.

Subclasse Peritrichia Stein, 1859

Els grup dels perítrics és, sinó el més important, un dels grups de ciliats de major rellevància en els sistemes biològics aerobis de tractament d'aigües residuals amb presència d'algun substrat de fixació (flòculs o biofilm). Els ciliats perítrics es caracteritzen per presentar una ciliació gairebé exclusivament oral, en forma d'un anell ciliar en posició adoral format per una policinètia oral i la policinètia paroral (o haplokinètia), que gira en el sentit contrari a les agulles del rellotge des del peristoma (regió oral) fins al citostoma. La policinètia oral es divideix en tres membranel·les a l'interior de la cavitat oral. La majoria de perítrics no presenten pràcticament cilis somàtics, amb l'excepció d'una banda aboral ciliada en les espècies no sèssils (gèneres *Astylozoon*, *Opisthonecta*) i en els telotrocs, formes lliure nedadores implicades en la dispersió i reproducció sexual.

Els perítrics es classifiquen en dos ordres, l'ordre Mobilida (Kahl, 1933) i l'ordre Sessilida (Kahl, 1933). Els ciliats de l'ordre Mobilida no són gens habituals en sistemes de tractament d'aigües residuals. Es caracteritzen per presentar una corona ciliar basal connectada a un aparell adhesiu que els permet fixar-se de forma temporal sobre la superfície epitelial d'un hoste, sobre el que es pot desplaçar i al que pot produir patologies epitelials greus. Els seus hostes poden ser cnidaris, briozous, larves d'amfibis i peixos.

La monofília de la subclasse Peritrichia està actualment sota qüestió a causa de la ubicació del grup dels Mobilida en nombrosos arbres filogenètics (Sun et al. 2011), motiu pel qual alguns autors proposen l'establiment de la nova subclasse Mobilida. De totes maneres, per altres autors (Gao et al. 2016) les evidències de la no monofília de la subclasse Peritrichia no són prou concloents i aposten per mantenir, a l'espera de més resultats, els Mobilida dins la subclasse Peritrichia.

La gran majoria d'espècies de perítrics que colonitzen i es desenvolupen de forma habitual en sistemes biològics de tractament d'aigües residuals pertanyen a l'ordre Sessilida.

Ordre Sessilida Kahl, 1933

Els ciliats de l'ordre Sessilida es caracteritzen per presentar, normalment, una forma sèssil com a forma implicada en l'alimentació. Aquesta forma sèssil rep el nom de trofont, i es pot trobar adherida a un substrat o com a organisme epizoic d'alguns metazous aquàtics com copèpodes o gasteròpodes.

Un fet característic de molts gèneres de sessílids és la presència de peduncle, estructura mitjançant la qual s'adhereixen al substrat. Aquest peduncle és generat pel mateix ciliat a partir d'un orgànul especial anomenat *scopula*. El peduncle pot ser contràctil, que permet als individus retraure's sobtadament en situacions desfavorables, com és el cas dels gèneres *Vorticella*, *Vorticellides*, *Carchesium*, *Epicarchesium* i *Pseudovorticella*; o rígid, organismes que només poden contraure el cos, com els gèneres *Opercularia* i *Epistylis*. Altres gèneres viuen a l'interior d'una lorica (*Vaginicola*, *Thuricola* o *Pyxicola*) o dins una matriu gelatinosa (*Ophrydium*). És habitual en els sessílids la formació de colònies formades per molts individus compartint un únic peduncle ramificat.

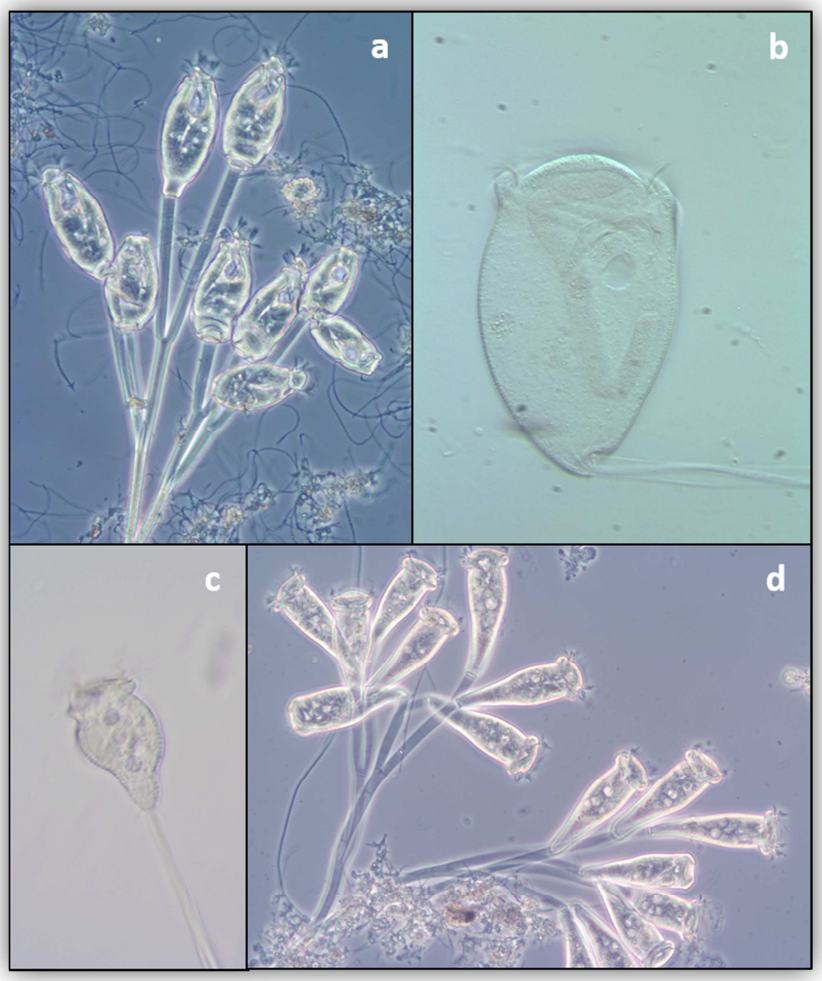


Figura 1.14. Imatges d'algunes espècies de sessílids habituals en sistemes de tractament d'aigües residuals. a. *Opercularia articulata* (Opercolariidae); b. *Vorticella convallaria* (Vorticellidae); c. *Vorticellides aquadulcis* (Vorticellidae); d. *Epistylis plicatilis* (Epistylididae). Autor: Oriol Canals.

Els sessílids són ciliats amb sistema de filtració molt eficient que, juntament amb la seva capacitat per desenvolupar-se de forma massiva i/o formant grans agregats d'individus en colònies, fa d'aquests perítrics un grup molt important per ala obtenció d'un efluent de qualitat en els processos de tractament d'aigües residuals (Fried et al. 2000).

L'ordre Sessilida és reconegut com a un grup monofilètic en la gran majoria (per no dir tots) d'estudis filogenètics (Miao et al. 2004). Els resultats moleculars, però, qüestionen la validesa d'algunes de les 14 famílies que proposa Lynn (2008) com les famílies Astylozoidae i Opisthonectidae, així com la monofília d'alguns gèneres (*Vorticella*, *Opisthonecta*), aquests últims resolts amb la definició dels nous gèneres *Vorticellides* (Foissner et al. 2010) i *Telotrochidium* (Martín-Cereceda et al. 2007).

Les espècies més importants de sessílids dels sistemes de tractament d'aigües residuals pertanyen majoritàriament a les famílies Epistylididae, Vorticellidae i Operculariidae.

1.2.1.2. Micrometazous

Els micrometazous que més habitualment s'associen als sistemes biològics aerobis de tractament d'aigües residuals són els grups dels rotífers, nematodes, gastròtrics, anèl·lids i tardígrads, tot i que de forma molt ocasional es poden observar també algunes espècies de platihelmints turbel·laris (principalment de la família Catenulida) i àcars.

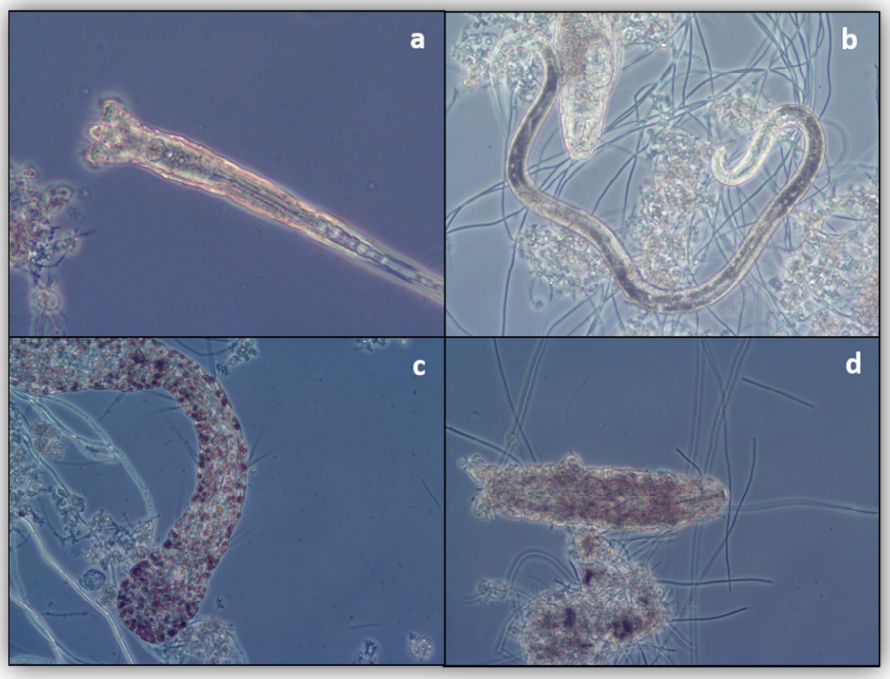


Figura 1.15. Imatges d'alguns metazous de sistemes de tractament d'aigües residuals. a. Rotífer de la família Philodinidae; b. Nematode; c. Anèl·lid del gènere *Aelosoma*; d. Tardígrad. Autor: Oriol Canals.

D'aquests, els que presenten una major rellevància són els rotífers. Aquests micrometazous es troben de forma molt habitual tant en sistemes amb la biomassa en suspensió (com els fangs actius i els MBR) com en sistemes de biofilm (filtres percoladors i sistemes de biodiscs), mostrant sovint abundàncies elevades i essent en algunes ocasions l'organisme majoritari del sistema. Els rotífers estan ben integrats en la xarxa tròfica dels reactors biològics dels sistemes de tractament d'aigües residuals, essent depredats per algunes espècies de protozous ciliats (gènere *Holophrya*) i desenvolupant tasques de predació per filtració sobre bacteris dispersos (família Philodinidae) i de "brosteig" sobre flòculs i predació sobre bacteris filamentosos (família Lecanidae) (Fialkowska i Pajdak-Stós 2008).

Els nematodes i, en menor mesura, algunes espècies d'anèl·lids, poden arribar a ser força abundants en els sistemes de biofilm, on mantenen un paper important en el sistema gràcies al seu desplaçament a través de la biopel·lícula, obrint canals que faciliten la difusió dels nutrients a les zones més internes del biofilm (Salvadó et al. 2004).

1.2.2. Importància de la comunitatmicroeucariota en elssistemes de tractament d'aigües residuals

Tot i que els organismes principalment encarregats de la depuració de l'aigua residual són els procariotes, la comunitat microeucariota és una part implicada activament en els sistemes biològics de tractament d'aigües residuals, on pot arribar a representar fins el10 % del total de la biomassa en sistemes de fangs actius i fins el 20% en sistemes RBC (Madoni 1994a).

Els microeucariotes han colonitzat des d'un bon principi els sistemes biològics de tractament d'aigües residuals, tot i que, inicialment, la concepció que es tenia sobre aquests organismes era que la seva presència podia tenir conseqüències negatives per al bon funcionament del procés (Fairbrother & Renshaw 1922). Actualment, però, és àmpliament reconegut que els microeucariotes, especialment els protists, tenen un paper determinant per la obtenció de bons rendiments de depuració i de efluents de millor qualitat.

Les principals funcions atribuïdes a la comunitat microeucariota són:

1. Millora de la qualitat i clarificació de l'efluent per depredació sobre bacteris dispersos, principalment per l'activitat filtradora dels microeucariotes, com per exemple els sessílids (Curds et al. 1968; Curds 1973; Madoni 1994b).

2. Mineralització del fang. Ratsak et al. (1996) recullen, en aquesta revisió, les principals causes que expliquen com l'activitat depredadora dels microeucariotes sobre la comunitat microbiana té un efecte estimulador sobre la descomposició biològica del carboni. Algunes d'aquestes causes són l'excreció per part dels microeucariotes de compostos necessaris pel desenvolupament de les comunitats bacterianes a les que aquestes no tenen fàcil accés en els sistemes de tractament d'aigües residuals i que permeten als bacteris una major velocitat de creixement poblacional, i l'alliberament al medi de compostos derivats de la lisis cel·lular dels microeucariotes, compostos que

poden ser utilitzats pels bacteris. L'activitat de la comunitat microeucariota també implica una reducció de la producció de fangs, amb el conseqüent estalvi energètic que comporta una menor gestió d'excedents de fangs (Lee i Welander 1996; Ghyoot i Verstraete 1999).

3. Incrementar la diversitat bacteriana present al reactor biològic. Curds (1974) i Jost et al. (1973) van observar que l'efecte depredador dels protists impedeix que el reactor biològic sigui colonitzat de forma massiva per un nombre baix de bacteris, permetent la supervivència de grups bacterians que en absència de protists es veurien desplaçades del sistema. Aquest fet podria implicar, alhora, una millora de la qualitat del procés degut precisament a la major diversitat bacteriana, explicada per una major diversitat de metabolismes, capaços de degradar un major nombre de substrats (Ratsak et al. 1996).

4. Millora de la clarificació de l'efluent en sistemes de fangs actius per selecció de bacteris floculants. L'efecte depredador dels microeucariotes sobre els bacteris dispersos afavoreix el desenvolupament de bacteris formadors de flòcul i, en conseqüència, la formació d'aquests grans agregats bacterians. La formació de flòculs de gran mida (i pes) són claus per obtenir una bona velocitat de sedimentació dels fangs i, en conseqüència, un efluent correctament clarificat (Curds 1982).

5. Millora de la clarificació de l'efluent en sistemes de fangs actius per la seva influència sobre els processos de biofloculació. L'estímul de l'activitat bacteriana per part dels microeucariotes incrementa la secreció de substàncies polimèriques extracel·lulars (Curds 1977; Arregui et al. 2007, 2008) que fan funció de "mesella" dels flòculs, afavorint la ràpida sedimentació d'aquests.

6. Eliminació de bacteris patògens del medi. S'ha demostrat que els protists també depreden i redueixen la població de bacteris patògens de l'aigua residual (Curds & Fey 1969; Bitton 2002).

7. En sistemes de biofilm, s'ha demostrat que els fluxos d'aigua generats per l'acció filtradora de perítrics del gènere *Epistylis* afavoreix el moviment i la renovació de l'aigua (i els nutrients) a les zones més internes del biofilm (Fried i Lemmer 2003), implicant un millor contacte dels microorganismes del biofilm amb els substrats a depurar.

8. Per últim, alguns investigadors afirmen que l'activitat predadora dels protists també incrementa la taxa de nitrificació en els sistemes dissenyats per a l'eliminació avançada de nitrogen (Petropoulos i Gilbride 2005; Pogue i Gilbride 2007) o que, com a mínim, no té efectes negatius sobre la taxa d'oxidació de l'amoni (Padjak-Stós et al. 2010; Yu et al. 2011), tot i que sí pot modificar la mida i forma de les colònies de bacteris nitrificants.

1.2.3. La comunitat microeucariota com a bioindicadora dels processos de tractament d'aigües residuals

La utilitat de les espècies microeucariotes (principalment els ciliats) com a bioindicadores dels processos de tractament d'aigües residuals és molt probablement el principal motiu dels nombrosos estudis que s'han realitzat sobre aquests organismes microscòpics durant les últimes dècades.

Segons Hawkes (1960), el primer en reportar la presència de protists en sistemes de fangs actius va ser Johnson (1914), el mateix any en el que Ardren i Lockett publicaven el sistema de tractament biològic de fangs actius. De totes maneres és esperable que la comunitat eucariota, i més específicament els protists, hagin estat associats als sistemes de tractament d'aigües residuals des de la implementació del primer reactor biològic. Uns anys més tard, altres autors van confirmar la presència d'organismes microeucariotes com a part activa dels fangs actius (Richards i Sawyer 1922; Buswell i Long 1923; Kolkwitz 1926). Ardren i Lockett (1936) probablement van ser els primers autors que van relacionar directament la presència de certs tipus de protists amb l'eficiència del procés, donant valor i utilitat a les anàlisis microscòpiques com a forma de control del procés de fangs actius. A aquest primer estudi el van seguir Lackey et al. (1938) i Baines et al. (1953), entre molts altres. Probablement, el punt d'inflexió en el coneixement i aplicació dels microeucariotes com a bioindicadors van ser els treballs de Curds, principalment el publicat l'any 1970 en col·laboració amb Cockburn titulat *Protozoa in biological sewage-treatment processes II. Protozoa as indicators in the activated-sludge process.* En aquesta publicació, Curds i Cockburn van demostrar que es podia predir el rang de concentració de DBO_5 de l'efluent a partir de la identificació a nivell d'espècie dels ciliats del fang actiu amb el 83% d'efectivitat.

Posteriorment, Poole (1984) també va observar l'existència de relació entre diferents espècies de protists i certs paràmetres fisicoquímics com la concentració de DBO_5 i la concentració de sòlids en suspensió a l'efluent, i va reportar les primeres espècies de protists associades a plantes de tractament amb eliminació biològica de nitrogen, relacionant la concentració de nitrogen amoniacal de l'efluent amb la presència de certes espècies microeucariotes. Relacions entre la DBO_5 i espècies de ciliats també van ser obtingudes per Luna-Pabello et al. (1990) i Salvadó et al. (1995). Al-Shahwani i Horan (1991) van observar, mitjançant anàlisis estadístiques de regressió múltiple entre els valors d'abundància de les diferents espècies i els paràmetres fisicoquímics i operacionals de la planta, la relació entre alguns ciliats i l'edat del fang (o temps de retenció cel·lular de la biomassa) del sistema, relació també observada per Salvadó (1994). Altres publicacions referents en el camp de la bioindicació de protists en sistemes de tractament d'aigües residuals són els publicats per Madoni (1991), Esteban et al. (1990 i 1991), Salvadó i Gracia (1993), Martín-Cereceda et al. (1996), Nicolau et al. (2001), entre d'altres.

Entre la comunitat de microeucariotes, els ciliats han acaparat la major part de publicacions com a organismes bioindicadors del funcionament dels processos de tractament d'aigües residuals, a causa de la seva elevada freqüència d'aparició als sistemes, la seva elevada abundància i la seva fàcil identificació. Tot i això, la presència d'altres organismes microeucariotes als fangs actius, com rotífers i nematodes, ja va ser posada de manifest per

Hawkes (1960), i Poole i Fry (1980)van observar que rotífers de la família Bdelloidea, nematodes, oligoquets de la família Aelosomatidae i, de forma més esporàdica, tardígrads, apareixien al licor mescla de sistemes amb processos de nitrificació que operaven amb elevada concentració de sòlids al reactor i elevada edat dels fangs.

Paral·lelament als estudis de fangs actius, alguns autors van analitzar la comunitat microeucariota associada a sistemes de tractament amb la biomassa adherida a un suport (sistemes de biofilm). Els mateixos Curds i Cockburn (1970a) van estudiar i llistar les espècies presents a l'efluent de vàries plantes de tractament de filtres percoladors, confirmant en bona part les troballes realitzades prèviament per Barker (1943) i Clay (1964). Uns anys més tard, Madoni i Ghetti (1981) van caracteritzar l'estructura de les comunitats de ciliats en diferents sistemes de biodiscs o RBC. Estudis sobre la diversitat microeucariota en sistemes RBC també van ser duts a terme per Chung i Strom (1991), Hul (1992), Luna-Pabello (1992) i Pérez-Uz et al. (1998), entre d'altres. La capacitat de bioindicació de la comunitat microeucariota en sistemes RBC va ser estudiada per Kinner (1984),Martín-Cereceda et al. (2001 i 2002) i Salvadó et al. (2004). Aquests autors van observar com la comunitat de ciliats variava en funció de la càrrega orgànica (DBO_5) (Martín-Cereceda et al. 2001), van determinar aquelles espècies associades a un millor rendiment del procés (Martín-Cereceda et al. 2002) i van observar que la presència i l'acció de micrometazous nematodes estimulava el desenvolupament del biofilm (Salvadó et al. 2004).

Tot i que la capacitat bioindicadora de la comunitat microeucariota en els sistemes convencionals (principalment de fangs actius) continua essent objecte d'estudi per part dels investigadors (Dubber i Gray 2011a; Hu et al. 2013), durant la última dècada bona part de l'atenció de la comunitat científica s'ha dirigit a l'estudi dels nous sistemes i tecnologies de tractament que han anat sorgint com a resposta a l'exigència d'efluents de més qualitat per part de les administracions, fruit de la situació socioeconòmicade creixement demogràfic, increment de la industrialització i de major conscienciació social de la necessitat d'una major sostenibilitat mediambiental. Un clar exemple d'aquesta situació és l'extensa aplicació de tractaments terciaris per l'eliminació avançada de nutrients.

En aquest context, Liu et al. (2008) va observar com la comunitat microeucariota variava en relació al sistema de tractament, obtenint unes comunitats més similars en aquelles depuradores que empraven una mateixa configuració. Posteriorment, Dubber i Gray (2011b), analitzant la comunitat microeucariota d'un sistema amb eliminació de nutrients que alternava fases aeròbies i anòxiques, van observar que el temps de exposició a condicions d'anòxia tenia influència sobre la diversitat i abundància de la comunitat de ciliats, i que afavoria o perjudicava el desenvolupament d'algunes espècies enfront d'altres. Durant aquest mateix període, Pérez-Uz et al. (2010) van observar que la comunitat microeucariota associada a sistemes amb eliminació avançada de nutrients era diferent a la que es desenvolupa en sistemes convencionals, i van determinar les espècies amb capacitat bioindicadora per aquests tipus de processos. Aquests estudis semblen demostrar, doncs, que els diferents processos de tractament, així com les diferents configuracions o tecnologies que presenten les EDAR, afecten i condicionen la comunitat microeucariota, fent poc extrapolable la capacitat bioindicadora de les mateixes espècies en sistemes diferents i al mateix temps necessària la

realització d'anàlisis microscòpiques per a la caracterització de les comunitats de microeucariotes associades als nous sistemes.

El nitrogen, en les seves formes amoniacal, nitrit o nitrat, és un dels paràmetres de gestió diària d'una planta de tractament d'aigües residuals amb una major capacitat d'afectació al medi, ja que pot desestabilitzar l'ecosistema receptor ocasionant, en els casos més extrems, episodis d'eutrofització. Alguns estudis han demostrat que les comunitats microeucariotes presenten canvis estructurals com a resposta a la toxicitat de l'amoni (Puigagut et al. 2005), podent esser indicadores de la qualitat de l'efluent en sistemes de tractament de purins (Puigagut et al. 2009). La toxicitat del nitrogen amoniacal, el nitrit i l'amoníac lliure sobre la comunitat microeucariota també ha estat objecte d'estudi per part de Klimek et al. (2012), Sobczyk et al. (2015) i Xu et al. (2004, 2005). Aquests estudis confirmen que, de forma general, elevades concentracions d'aquests compostos afecten negativament a les espècies microeucariotes, tot i que la intensitat de l'efecte nociu d'aquests paràmetres difereix per les diferents espècies estudiades.

2. OBJECTIUS II

2. OBJECTIUS

Els sistemes de tractament d'aigües residuals s'han erigit com un dels grans baluards d'estudi dels protists. Nombrosos treballs han estat publicats a l'empara dels tractaments biològics d'aigües residuals, contribuint enormement: i) al coneixement de la biodiversitat eucariota amb la descripció de noves espècies, ii) al coneixement de l'ecologia d'aquestes espècies i del seu potencial com a bioindicadores en sistemes aquàtics continentals, d'especial interès en la millora de la gestió i optimització dels sistemes de tractament d'aigües residuals, iii) a un major coneixement del rol d'aquests organismes en els ecosistemes i les relacions tròfiques que estableixen.

En aquest context, la present tesi doctoral té com a **objectiu principal** l'exploració i caracterització de les comunitats microeucariotes que colonitzen i es desenvolupen en dos sistemes avançats de tractament d'aigües residuals amb alta càrrega amoniacal, les comunitats dels quals no han estat estudiades fins al moment.

Aquest objectiu principal planteja el desenvolupament d'un protocol d'extracció del biofilm adequat per al propòsit de la tesi, ja que ambdós sistemes operen amb tecnologia de biofilm *Moving Bed Biofilm Reactor* (MBBR). Aquest protocol ha de permetre l'estudi independent de la comunitat microeucariota en suspensió i la associada al biofilm.

Com a **segon objectiu principal**, i supeditat als resultats obtinguts en el primer objectiu, la present memòria pretén la determinació d'aquelles espècies amb potencial com a bioindicadores del funcionament dels processos, per tal de contribuir al desenvolupament de les eines de gestió complementàries als paràmetres fisicoquímics i operacionals.

La present memòria contempla també els següents **objectius secundaris**:

- **i)** La descripció morfològica, ecològica i molecular d'aquelles espècies no descrites a la bibliografia.
- **ii)** Contribuir al coneixement actual sobre l'ecologia de les diferents espècies microeucariotes, principalment en relació a la seva tolerància a l'amoni i al nitrit.
- **iii)** Valorar la viabilitat d'aplicació dels mètodes moleculars de seqüenciació massiva (*Next Generation Sequencing*) en els sistemes de tractament d'aigües residuals estudiats.

Els objectius assolits es mostren en els tres capítols de resultats sota els títols "*Microfauna communities as performance indicators for an A/O Shortcut Biological Nitrogen Removal moving-bed biofilm reactor*" (capítol 1), "*Description of Epistylis camprubii n. sp., a species highly tolerant to ammonium and nitrite*" (capítol 2) i "*Microeukaryote community in a partial nitritation reactor prior to anammox and an insight into the potential of ciliates as performance bioindicators*" (capítol 3).

3. RESULTATS III

3.1. INFORME DEL FACTOR D'IMPACTE DE LES PUBLICACIONS

El doctorand Oriol Canals Delgado presenta a la present tesi doctoral un total de 3 articles signats com a primer autor. Aquests articles han estat publicats en revistes científiques internacionals, de les quals una d'elles pertany al primer quartil (*Water Research*; posició 1/81 a la categoria Water Resources), una al segon quartil (*NewBiotechnology;* posició 45/161 a la categoria Biotechnology and Applied Microbiology) i la restant al quart quartil (*Acta Protozoologica*; posició 99/123 a la categoria Microbiology).

A continuació es detalla la informació dels 3 articles que conformen pròpiament la tesi amb el factor d'impacte de les revistes en els anys de publicació segons el *Science Citation Index*, així com la contribució científica del doctorand i dels coautors en cadascun dels articles.

Canals O.; Salvado H.; Auset M.; Hernández C.; Malfeito J.J. 2013. Microfauna communities as performance indicators for an A/O Shortcut Biological Nitrogen Removal moving-bed biofilm reactor. Water Research 47(9): 3141-50.

Journal Impact Factor (2013): 5.323

Disseny del treball: **OC**, HS, JJM

Mostreig i anàlisi de les mostres: **OC**, CH, MA

Anàlisi de resultats: **OC**, HS

Redacció del manuscrit: **OC**, HS, MA

Canals O.; Salvadó H. 2016. Description of *Epistylis camprubii* n. sp., a Species Highly Tolerant to Ammonium and Nitrite. Acta Protozoologica 55(1): 7-18.

Journal Impact Factor (2015): 1.491

Disseny del treball: **OC**, HS

Mostreig i anàlisi de les mostres: **OC**

Anàlisi de resultats: **OC**, HS

Redacció del manuscrit: **OC**, HS

Canals O.; Massana R.; Riera J.L.; Balagué V., Salvadó H. 2017. Microeukaryote community in a partial nitritation reactor prior to anammox and an insight into the potential of ciliates as performance bioindicators. New Biotechnology. In Press.

Journal Impact Factor (2015): 3.199

Disseny del treball: **OC**, HS

Mostreig i anàlisi de les mostres: **OC**, VB

Anàlisi de resultats: **OC**, RM, JLR

Redacció del manuscrit: **OC**, RM, HS

Cap d'aquests 3 articles ha estat utilitzat prèviament, ni pretenen ser utilitzats en un futur, en cap altra tesi doctoral.

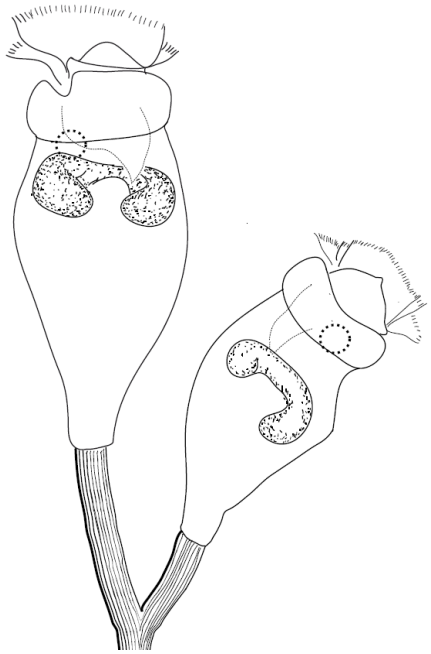
Com a annex de la present tesi es mostren 4 publicacions més en les quals ha signat el doctorand Oriol Canals Delgado, una d'elles també com a primer autor. Aquestes publicacions també han estat publicades en revistes científiques internacionals: *Water Research* (primer quartil), *Environmental Science and Pollution Research* (primer quartil) i *Journal of Applied Microbiology* (segon quartil).

A Barcelona, 23 de maig de 2017,

Dr. Humbert Salvadó i Cabré

3.2. CAPÍTOL 1

MICROFAUNA COMMUNITIES AS PERFORMANCE INDICATORS FOR AN A/O SHORTCUT BIOLOGICAL NITROGEN REMOVAL MOVING-BED BIOFILM REACTOR



Resum

S'ha caracteritzat la comunitat de microfauna present al licor mescla i al biofilm d'un procés Anòxic/Òxic *Shortcut Biological Nitrogen Removal* amb tecnologia *moving-bed biofilm reactor* (MBBR) amb l'objectiu d'optimitzar el control del procés mitjançant l'ús de bioindicadors. El sistema va operar a elevades concentracions d'amoni, amb una mitjana de 588 ± 220 mg N-NH_4^+ L^{-1} a l'influent, 161 ± 80 mg L^{-1} al reactor anòxic i 74 ± 71.2 mg L^{-1} al reactor aerobi. Es van identificar un total de 20 taxons diferents, incloent ciliats (4), flagel·lats (11), amebes (4) i nematodes (1). Comparat amb processos de tractament d'aigües residuals convencionals, aquest procés es pot definir com a un sistema dominat per flagel·lats i amb una baixa diversitat de ciliats. Els flagel·lats van dominar principalment al licor mescla, demostrant una elevada tolerància a l'amoni i la capacitat de sobreviure llargs períodes de temps en condicions anòxiques. Les dades obtingudes aporten dades interessants sobre els valors màxims i mínims de tolerància a l'amoni, nitrat i nitrit de les espècies *Cyclidium glaucoma*, *Colpoda ecaudata*, *Vorticellamicrostoma*-complex i *Epistylis* cf. *rotans*. Aquesta última espècie va ser l'únic ciliat que va presentar una població constant i abundant, gairebé de forma exclusiva al biofilm aerobi. La dinàmica poblacional de *Epistylis* cf. *rotans* va mostrar una forta correlació negativa amb les variacions d'amoni i una correlació positiva amb l'eficiència d'eliminació d'amoni. Els resultats indiquen, doncs, que *Epistylis* cf. *rotans* és un bon bioindicador del procés de nitrificació en aquest sistema. L'estudi de les comunitats de protozous en sistemes de tractament d'aigües residuals inexplorats aporta noves sobre l'ecologia de les espècies i del seu rol en aquests sistemes en condicions fisicoquímiques poc habituals, podent oferir noves eines biològiques de gestió del procés.

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Microfauna communities as performance indicators for an A/O Shortcut Biological Nitrogen Removal moving-bed biofilm reactor

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ABSTRACT

The microfauna communities present in the mixed liquor and biofilm of an Anoxic/Oxic Shortcut Biofilm Nitrogen Removal moving-bed biofilm process were characterised in order to optimise process control through the use of bioindicators. The system operated at high ammonium concentrations, with an average of 588 ± 220 mg N–NH_4^+ L^{-1} in the influent, 161 ± 80 mg L^{-1} in the anoxic reactor and 74 ± 71.2 mg L^{-1} in the aerobic reactor. Up to 20 different taxa were identified, including ciliates (4), flagellates (11), amoebae (4) and nematodes (1). Compared to conventional wastewater treatment processes (WWTPs), this process can be defined as a flagellates-predominant system with a low diversity of ciliates. Flagellates were mainly dominant in the mixed liquor, demonstrating high tolerance to ammonium and the capacity for survival over a long time under anoxic conditions. The data obtained provide interesting values of maximum and minimum tolerance ranges to ammonium, nitrates and nitrites for the ciliate species *Cyclidium glaucoma*, *Colpoda ecaudata*, *Vorticella microstoma*-complex and *Epistylis* cf. *rotans*. The last of these was the only ciliate species that presented a constant and abundant population, almost exclusively in the aerobic biofilm. *Epistylis* cf. *rotans* dynamics showed a high negative correlation with ammonium variations and a positive correlation with ammonium removal efficiency. Hence, the results indicate that *Epistylis* cf. *rotans* is a good bioindicator of the nitrification process in this system. The study of protozoan communities in unexplored WWTPs sheds light on species ecology and their role under conditions that have been little studied in WWTPs, and could offer new biological management tools.

1. Introduction

Over the last decade, new wastewater treatment processes and technologies have been developed and implemented for nitrogen removal of high ammonium loaded wastewater in response to the need to reduce energy costs and improve treatment efficiency. For example, **A**noxic/**O**xic **S**hortcut **B**iological **N**itrogen **R**emoval (A/O SBNR) processes, which focus on nitrogen removal via nitrite, can reduce oxygen demand and organic source demand due to nitrification by up to 25% and 40%, respectively (Chung et al., 2007). In addition, moving-bed biofilm technology theoretically avoids bulking problems

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Nomenclature

A/O SBNR	Anoxic/Oxic Shortcut Biological Nitrogen Removal
WWTP	Wastewater Treatment Plant
TSS	Total Suspended Solids
VSS	Volatile Suspended Solids
tCOD	Total Chemical Oxygen Demand
fCOD	Filtered Chemical Oxygen Demand
DO	Dissolved Oxygen
$N–NH_4^+$	Nitrogen Ammonia
$N–NO_2^-$	Nitrogen Nitrite
$N–NO_3^-$	Nitrogen Nitrate
FISH	Fluorescence In Situ Hybridisation
AOB	Ammonium Oxidising Bacteria
NOB	Nitrite Oxidising Bacteria
OXI-ML	Aerobic Mixed Liquor
OXI-BF	Aerobic Biofilm
ANX-ML	Anoxic Mixed Liquor
ANX-BF	Anoxic Biofilm
SD	Standard Deviation
Max	Maximum value
Min	Minimum value

and can operate with a more specific biomass for each treatment step. Moreover, no liquid–solid separation is needed (Andreottola et al., 2000).

As regards the microorganisms involved, microfauna communities in activated sludge processes have been widely studied in recent decades due to their importance for the correct performance of treatment systems and their role as bioindicators (Curds, 1982; Madoni, 1994). Protozoan species, and in particular ciliates, have been related to nitrification processes (Poole, 1984; Pérez-Uz et al., 2010), effluent quality (Madoni and Ghetti, 1981; Luna-Pabello et al., 1990; Salvadó et al., 1995; Zhou et al., 2008), organic loading ratio (Salvadó and Gracia, 1993), cellular retention time (Salvadó, 1994), dissolved oxygen (Esteban et al., 1990) and presence of toxic compounds such as heavy metals (Martín-González et al., 2005) and ammonium (Puigagut et al., 2005, 2009).

However, the knowledge of microfauna communities and the development of new wastewater treatment systems have not evolved at the same pace. No studies have been found concerning the microfauna inhabiting an A/O SBNR moving bed biofilm system. Although Van Dongen et al. (2001) referred to the presence of protozoa in the high ammonium loaded process SHARON (**S**ingle reactor system for **H**igh activity **A**mmonium **R**emoval **O**ver **N**itrite), no protozoan species were identified.

Only a few studies, such as that by Fried et al. (2000), have been found regarding microfauna inhabiting the biofilm of a moving-bed biofilm system. However, microfauna communities in other biofilm technologies have been more extensively studied, such as those in fixed bed reactors (Lorda-de-los-Ríos et al., 2002), aerated biofilters (García-Santana and González-Martínez, 2002) or rotating biological contactors (Pérez-Uz et al., 1998; Martín-Cereceda et al., 2002). Therefore, the aim of this study was to explore and characterise the microfauna communities, and in particular the ciliate communities, present in an A/O SBNR moving bed biofilm system treating high ammonium loaded wastewater, and to determine which species are useful as process performance indicators.

2. Material and methods

2.1. Experimental set-up

The study was carried out using a pilot-scale A/O SBNR reactor system (Fig. 1). The system consisted of two consecutive reactors of 2 m^3 volume. The first was operated as anoxic while the second was operated as aerobic. Water remained in the system for an average of 4.6 ± 2 days. To understand the conditions under which the microfauna developed, the effect of the recirculation flow on hydraulic residence time should be considered. Thus, the uninterrupted hydraulic residence time for each tank (7.6 ± 1.3 h) was calculated using *Volume reactor/(Influent Flow + Recirculation Flow)*. Both reactors presented 1 m^3 of carriers (specific surface of 500 $m^2\ m^{-3}$) held in suspension by stirrers (400 mm diameter; velocity of stirrers was 50rpm in both anoxic and oxic reactors). Aeration was provided by a blower connected to a perforated tube and DO (dissolved oxygen) concentration was controlled with an on-line sensor (LDO HACH LANGE). Both reactors also presented on-line temperature, pH and $N–NO_2^-$ analysers, signals from which were sent to a SCADA system. Influent and recirculation flows were controlled by cavity pumps. The carriers from both reactors were kept separate by using a perforated pipe with a pore diameter smaller than the carrier diameter.

2.2. Wastewater

The system was fed with centrifuged leachates from the anaerobic digester at the wastewater treatment plant (WWTP) in Rubí, Barcelona, with an average of 483 ± 270 mg fCOD L^{-1} and 588 ± 220 mg $N–NH_4^+$ L^{-1} (wastewater characteristics in Table 1). The chemical oxygen demand to total nitrogen ratio (COD/TN) of wastewater was about 0.77, and the extra carbon source needed for the denitrification process was provided by addition of methanol by using a control system depending on the $N–NO_2^-$ concentration in the anoxic tank (average of methanol solution COD: 18.4 ± 7.6 g L^{-1}; average of methanol flow input: 2.3 ± 1.6 L h^{-1}). The wastewater was heated in a settler before feeding the anoxic reactor.

2.3. Physicochemical parameters

Samples were collected daily from the feed, settler and both the anoxic and aerobic reactors. Total and filtered chemical oxygen demand (tCOD and fCOD), ammonium ($N–NH_4^+$), nitrite ($N–NO_2^-$) and nitrate ($N–NO_3^-$) were analysed according to Hach Lange. DO, pH and temperature were measured on-line. The alkalinity, total suspended solids (TSS) and volatile suspended solids (VSS) of biofilm and mixed liquor were measured according to standard methods (APHA, 1998). TSS and VSS values were expressed in g L^{-1}.

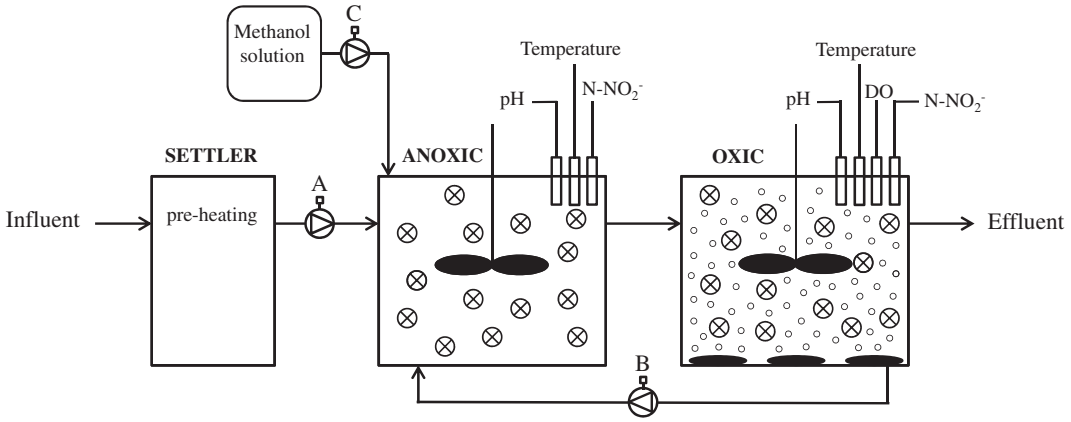

Fig. 1 – Diagram of the pilot-scale A/O SBNR system. A: Influent flow (average: 47.5 ± 15.3; min: 20; max: 60 L h^{-1}); B: Internal recirculation flow (mean: 228.8 ± 24.5; min: 130; max: 249 L h^{-1}); C: Methanol flow (mean: 2.3 ± 1.6; min: 0.3; max: 8.6 L h^{-1}).

2.4. *Sampling and sample treatment*

Microscopic analyses were carried out every two weeks over a period of 7 months. Four samples were analysed for each sampling: aerobic mixed liquor (OXI-ML), aerobic biofilm (OXI-BF), anoxic mixed liquor (ANX-ML) and anoxic biofilm (ANX-BF). To study the microfauna of both anoxic and aerobic biofilm, biofilm was detached from the carriers using a modified method of the Andreottola et al. (2000) method: an average of 8 carriers were collected with tweezers from each reactor and suspended in 80 mL of filtered mixed liquor using a 0.45 μm membrane. The biofilm was then carefully scraped off the carriers with a 4–5 mm diameter laboratory brush. This method resulted appropriate to analyze both solids attached to the carriers and the colonizing microfauna.

2.5. *Fluorescence In Situ Hybridisation*

Nitrifying bacteria were detected by Fluorescence In Situ Hybridisation (FISH) probes using the NitriVit kit (Vermicon, Munich, Germany). Presence of ammonium oxidising bacteria (AOB) and nitrite oxidising bacteria (NOB) in OXI-BF and ANX-BF was studied throughout the last 6 months of sampling.

2.6. *Counts of filamentous bacteria and microfauna (protozoa and metazoa)*

Microscopic analyses were performed using a Zeiss microscope *Axioskop 40*. Abundance of filamentous bacteria was measured using the counting technique described by Salvadó (1990), and expressed as metres per millilitre (m mL^{-1}). Filamentous bacteria counts were carried out at 400× magnification by phase contrast. Ciliates, amoebae > 20 μm and metazoa were counted by analysing two 25 μL replicates *in vivo* at 100× magnification. Small flagellates (<20 μm) and amoebae (<20 μm) were counted at 400× magnification and, for each replicate, 30 microscopic fields on the coverslip (20 × 20 mm) were analysed. The counts were carried out using well-homogenised samples.

Table 1 – Mean, standard deviation (SD), minimum (Min) and maximum (Max) values and number of analysis performed (N) of the physicochemical parameters measured in the influent, anoxic reactor and aerobic reactor. *COD values of influent do not include methanol COD input.

Physicochemical parameters		Influent				Anoxic reactor				Aerobic reactor			
		Mean ± SD	Max	Min	N	Mean ± SD	Max	Min	N	Mean ± SD	Max	Min	N
Total chemical oxygen demand (mg L^{-1})*		483.6 ± 270.5	818	243	151	579 ± 156.3	728	359	151	617.4 ± 129.9	743	414	151
Filtered chemical oxygen demand (mg L^{-1})*		228.7 ± 94.5	358	23	151	202.1 ± 50.8	278	99	151	218.5 ± 72.4	362	131	151
Nitrogen ammonia (mg L^{-1})		588.2 ± 220	1050	303	204	161.8 ± 80	312	34	204	74 ± 71.2	231	0.5	204
Nitrogen nitrite (mg L^{-1})	High DO period	<0.6			151	59.1 ± 50.5	231	0	64	134.2 ± 94.3	360	4.7	64
	Low DO period					66.1 ± 56.7	279	0	140	123.6 ± 74.4	426	0.7	140
Nitrogen nitrate (mg L^{-1})	High DO period	<0.23			151	120.3 ± 129.5	284	0	64	145 ± 149	321	0	64
	Low DO period					1.8 ± 2.9	6.9	0	140	0.3 ± 0.6	1.6	0	140
Alcalinity		2085 ± 661	3209	1090	182	1438 ± 448.6	2150	599	182	1242 ± 548	1998	422	182
Temperature (°C)		37.8 ± 5.3	44.3	29.1	204	28.7 ± 3.1	32.8	22.6	204	29.2 ± 3.1	33.6	24.3	204
pH		7.7 ± 0.1	8.1	7.3	204	8.3 ± 0.2	8.7	8	204	7.7 ± 0.4	8.1	6.9	204
Dissolved oxygen (mg L^{-1})	High DO period		–			<0.1				2.9 ± 0.3	3.5	2.5	64
	Low DO period									1.1 ± 0.3	1.6	0.6	140
Ammonium removal (%)			–			70.6 ± 18.6	90	17.5	204	84 ± 20.8	99.8	23.8	204

The frequency of detection of the species was calculated by dividing the number of samples in which the species appeared by the total number of samples. Its value was expressed as a percentage.

Identification of protozoan species was conducted at different levels: ciliates and flagellates were determined to species level and amoebae were grouped according to their size and morphology. It should be emphasised that the groups of amoebae cannot be considered as taxa since they might be composed of several amoebae species. Several keys were used during the study. Lee et al. (2000) was employed to identify flagellates. Gymnamoebae were determined according to Page (1988). Stiller (1971), Foissner (1993), Foissner et al. (1991, 1992 and 1994) and Curds et al. (2008) were used for specific identification of ciliates.

Abundance values were expressed as individuals per millilitre (ind mL^{-1}). In the case of biofilm, abundances were expressed as individuals per square centimetre (ind cm^{-2}) and transformed to ind mL^{-1} using the specific surface of carriers (500 m^2 m^{-3}).

2.7. Statistical analysis

Data were analysed statistically using the Statgraphics Centurion XVI software package. The normality of variables was determined by using Shapiro–Wilks test. Variance analysis tests (Student *t*-test for parametric variables or Mann–Whitney *U*-test for nonparametric data) and correlation tests (Pearson correlation for parametric variables or Spearman correlation for nonparametric data) were carried out depending on the normality of variables. Data were transformed applying logarithmic or square root transformation wherever possible.

3. Results

3.1. Pilot plant performance

Operational parameters, percentage of ammonium removal, nitrite and nitrate accumulation and total nitrogen removal were measured throughout the experimental period (Table 1). The A/O SBNR moving bed biofilm process treating high ammonium loaded wastewater presented an average ammonium removal of 84.0 ± 20.8%, with a maximum value of 99.8%. An average of 84.5 ± 23% of oxidised N-forms was accumulated as nitrites, while 15.5 ± 23% completed the nitrification process to nitrates. DO was increased up to 3.5 mg L^{-1} in the aerobic reactor from day 60 to day 105 (Supplementary Data Fig. 1), promoting the activity of NOB, which explains the low nitrite accumulation in this period. Excluding this period of high DO, nitrite accumulation in the aerobic reactor reached up to 93.6 ± 14%.

3.2. Biofilm and mixed liquor colonisation

ANX-BF presented the highest values of both TSS and VSS within the system (Table 2), although only 18.6% corresponded to organic compounds, in contrast to the percentage observed in the other subsystems (from 81.6% to 89.8%). The remaining 81.4% of TSS from ANX-BF corresponded mostly to calcium carbonate (formed in the anoxic reactor due to denitrification processes). The Student's *T*-test did not show statistically significant differences between suspended solids in OXI-BF, ANX-ML and OXI-ML.

Table 2 – Mean, standard deviation (SD) and number of analysis performed of the total suspended solids (g L^{-1}) and percentage of volatile suspended solids in anoxic mixed liquor, anoxic biofilm, aerobic mixed liquor and aerobic biofilm.

	Total suspended solids (g L^{-1})		VSS/TSS (%)
	Mean ± SD	*N*	Mean ± SD
Influent	0.079 ± 0.059	13	94.6 ± 6.7
Anoxic mixed liquor	0.4 ± 0.13	15	81.6 ± 14.1
Anoxic biofilm	6.05 ± 1.94	15	18.6 ± 7.7
Aerobic mixed liquor	0.42 ± 0.13	15	84.6 ± 11.4
Aerobic biofilm	0.42 ± 0.3	15	89.8 ± 8.9

FISH analyses of nitrifying bacteria were carried out to determine the distribution of AOB and NOB in both aerobic and anoxic biofilms. In OXI-BF, AOB were observed to form monocolonies continuously over the duration of the study, normally distributed throughout the biofilm. In ANX-BF, solitary AOB were found occasionally, probably due to OXI-BF detachment processes. NOB were only observed during the period of high aeration in the OXI-BF, although no correlation could be established between the two parameters.

As expected in a moving bed biofilm process, filamentous bacteria showed very low abundances in both mixed liquor samples (Tables 3a and 3b). No differences were observed between abundance of filamentous bacteria in anoxic and oxic mixed liquor. Moreover, filamentous bacteria populations in anoxic and oxic mixed liquor showed a statistically significant relationship (Pearson correlation coefficient = 0.8126; *p*-value = 0.0008). Student's *T*-test demonstrated that filamentous bacteria were statistically more abundant in the ANX-BF (28.5 ± 15.3 m mL^{-1}) than in the OXI-BF (8.3 ± 10.6 m mL^{-1}) (*p*-value = 0.0002). Results confirmed that the abundance of filamentous bacteria in an A/O SBNR moving bed biofilm system presented lower values than in a conventional activated sludge process. Nevertheless, the filamentous bacteria population should not be discounted, since it contributes to the macro-structure of floc and biofilm.

3.3. Microfauna

The microfauna associated with the mixed liquor and biofilm of an A/O SBNR moving bed reactor process treating high ammonium loaded wastewater were made up of ciliates, flagellates, amoebae and nematodes. Up to 20 different taxa were identified (Tables 3a and 3b). Flagellates presented the largest number of taxa (up to 11), followed by ciliates (4) and amoebae groups (4). Nematodes were observed once. The greatest diversity was found in OXI-ML, with 85% of total taxa, followed by ANX-ML (75%), OXI-BF (65%) and ANX-BF (40%).

Considering the system as a whole, an average of 5.2 ± 2.2 taxa per sampling (maximum of 11 and a minimum of 2 taxa)

Table 3a – Frequencies (%) and average abundances of microfauna taxa (ind mL^{-1}) and filamentous bacteria (m mL^{-1}) in anoxic mixed liquor and anoxic biofilm during the study period.

	Anoxic mixed liquor				Anoxic biofilm			
	Mean ± SD	Max	Min	Freq (%)	Mean ± SD	Max	Min	Freq (%)
Total flagellates <20 μm	62,939 ± 101,936	347,112	0	87	4585 ± 7571	24,598	0	53
Order Kinetoplastea								
Fam. Bodonidae	15,641 ± 42,213	158,557	0	47	2030 ± 6423	24,598	0	13
Order Diplomonadida								
Fam. Hexamitidae	714 ± 2242	8571	0	13	0	0	0	0
Order Volvocidae								
Polytoma sp	180 ± 561	2143	0	13	0	0	0	0
Class Chrysomonada								
Spumella guttula	22,141 ± 83,989	325,685	0	13	1318 ± 5104	19,766	0	7
Class Heterolobosea								
Paratretamitus sp-like	12,070 ± 29,491	94,277	0	27	586 ± 1546	4392	0	13
Tetramitus rostratus	1928 ± 3393	10,713	0	33	73 ± 284	1098	0	7
Unidentified Heterolobosea	0	0	0	0	0	0	0	0
Other heterotrophic flagellates								
Cercomonas sp-like	0	0	0	0	0	0	0	0
Unidentified sp1	1714 ± 6071	23,569	0	13	0	0	0	0
Unidentified sp2	7142 ± 27,662	107,133	0	7	73 ± 284	1098	0	7
Unidentified sp3	643 ± 2210	8571	0	13	505 ± 1232	4392	0	20
Gymnamoebae <20 μm	2571 ± 3334	12,856	0	73	2619 ± 8475	32,944	0	20
Filopodia amoebae <20 μm	143 ± 553	2143	0	7	0	0	0	0
Gymnamoebae >20 μm	15 ± 57	220	0	7	0	0	0	0
Testate amoebae >20 μm	0	0	0	0	0	0	0	0
Total ciliates	80 ± 89	240	0	73	3.7 ± 8	21	0	20
Colpoda ecaudata	1.3 ± 5	20	0	7	0	0	0	0
Cyclidium glaucoma	0	0	0	0	0	0	0	0
Epistylis cf. *rotans*	53 ± 66	200	0	73	2.5 ± 7	21	0	13
Vorticella microstoma-complex	4 ± 15	60	0	7	0	0	0	0
Telotroch	21 ± 58	220	0	20	1.2 ± 5	18	0	7
Nematoda	0	0	0	0	0	0	0	0
Filamentous bacteria (m mL^{-1})	13.3 ± 9.3	30.3	2.4	100	14.2 ± 7.7	29.9	3.9	100

were observed. Flagellates comprised the most abundant and diverse group (up to 7 species per sampling), regardless of sample type, and were clearly dominant in the mixed liquor. Ciliates developed mainly in OXI-BF and also inhabited both anoxic and aerobic mixed liquor, albeit at low abundances (frequency of 73% and 87%, respectively). In ANX-BF, ciliates appeared sporadically. Amoebae were observed in both anoxic and aerobic systems and were less frequent in biofilm samples, specifically in ANX-BF.

Most of the taxa were observed in 30% or less of the samples, and the only ones with frequencies higher than 70% were the ciliate *Epistylis* cf. *rotans* and the gymnamoebae <20 μm.

As expected, the ANX-BF presented the most unfavourable conditions for colonisation by the microfauna. Although flagellates, ciliates and amoebae were occasionally observed, no protozoan species or group truly colonised the anoxic biofilm, as demonstrated by the low frequencies that protozoan taxa reached (Table 3a) and the low number of taxa per sampling observed (average of 1.1 ± 1.0, maximum of 3).

Mixed liquor samples (aerobic and anoxic) presented the highest diversity of protozoan species within the system, with 3.9 ± 2.1 taxa per sampling in OXI-ML and 3.5 ± 1.6 in ANX-ML. In terms of abundance, mixed liquor samples exhibited the largest population of total flagellates <20 μm, which were slightly more abundant in ANX-ML than in OXI-ML. The abundance and diversity of flagellates decreased during the high DO period, although no statistically significant results were obtained. The flagellate species colonising the mixed liquor varied considerably throughout the study, as indicated by the low frequencies shown in Tables 3a and 3b. Bodonidae were the most frequently occurring taxa, with frequencies of 40% and 47% in OXI-ML and ANX-ML, respectively. Ciliates (specifically *Epistylis* cf. *rotans*) and amoebae showed low abundance values, often near the limit of detection, although they were observed with frequencies higher than 70%.

A statistical analysis of microfauna abundance demonstrated the existence of a single mixed liquor system instead of the operational two independent subsystems (oxic and anoxic): a statistically significant Spearman correlation was observed between the abundance of most taxa, and the abundances of the most frequently occurring groups in ANX-ML and OXI-ML did not show statistically significant differences (Mann–Whitney *U*-test, 95% confidence).

The OXI-BF presented an average of 2.7 ± 1.5 taxa per sampling. The ciliates *Vorticella microstoma*-complex, *Colpoda ecaudata* and *Epistylis* cf. *rotans* were detected. Only *Epistylis* cf. *rotans* was continuously observed throughout the study and maintained a permanent and abundant population. *V. microstoma*-complex presented a frequency of 27%, while *Colpoda ecaudata* was observed once. The flagellate taxa corresponded to those colonising the mixed liquor, the surrounding medium.

Table 3b – Frequencies (%) and average abundances of microfauna taxa (ind mL^{-1}) and filamentous bacteria (m mL^{-1}) in aerobic mixed liquor and aerobic biofilm during the study period.

	Aerobic mixed liquor				Aerobic biofilm			
	Mean ± SD	Max	Min	Freq %	Mean ± SD	Max	Min	Freq %
Total flagellates <20 μm	49,979 ± 90,489	327,828	0	67	18,956 ± 50,116	180,970	0	47
Order Kinetoplastea								
Fam. Bodonidae	12,142 ± 34,660	134988	0	40	11,564 ± 42,991	166,914	0	27
Order Diplomonadida								
Fam. Hexamitidae	429 ± 1660	6428	0	7	0	0	0	0
Order Volvocidae								
Polytoma sp	0	0	0	0	0	0	0	0
Class Chrysomonada								
Spumella guttula	20,570 ± 77,905	302116	0	13	4978 ± 19,280	74,672	0	7
Class Heterolobosea								
Paratretamitus sp-like	10,428 ± 22,804	72,851	0	20	439 ± 1159	3294	0	13
Tetramitus rostratus	1037 ± 2750	10,715	0	33	878 ± 2851	10,981	0	13
Unidentified Heterolobosea	37 ± 143	553	0	7	0	0	0	0
Other heterotrophic flagellates								
Cercomonas sp-like	857 ± 2782	10,693	0	13	0	0	0	0
Unidentified sp1	2608 ± 9949	38,568	0	13	1010 ± 3620	14,056	0	13
Unidentified sp2	357 ± 1121	4285	0	13	146 ± 567	2196	0	7
Unidentified sp3	243 ± 600	2143	0	20	0	0	0	0
Gymnamoebae <20 μm	4214 ± 4782	17,141	0	80	1550 ± 2140	6589	0	53
Filopodia amoebae <20 μm	214 ± 444	1071	0	20	73 ± 284	1098	0	7
Gymnamoebae >20 μm	4 ± 15	60	0	7	0	0	0	0
Testate amoebae >20 μm	0	0	0	0	1.1 ± 4	16	0	7
Total ciliates	191 ± 259	980	0	87	6165 ± 3941	14,063	72	100
Colpoda ecaudata	0	0	0	0	0.2 ± 0.8	3.3	0	7
Cyclidium glaucoma	1.3 ± 5.2	20	0	7	0	0	0	0
Epistylis cf. *rotans*	115 ± 132	460	0	80	6079 ± 3891	14,043	66	100
Vorticella microstoma-complex	1.3 ± 5.2	20	0	7	8 ± 17	55	0	27
Telotroch	73 ± 208	820	0	53	78 ± 215	849	0	60
Nematoda	1.3 ± 5.2	20	0	7	0	0	0	0
Filamentous bacteria (m mL^{-1})	17.3 ± 11.5	41.7	4.1	100	4.1 ± 5.3	20.6	0.1	100

3.4. *Bioindication analyses*

Data showed that the population dynamics of *Epistylis* cf. *rotans* in OXI-BF responded directly to variations in the ammonium concentration in the aerobic tank (Fig. 2), with a statistically significant negative correlation (Pearson correlation coefficient = −0.6524; *p*-value = 0.0156). *Epistylis* cf. *rotans* also presented a statistically significant positive correlation with ammonium removal efficiency (Pearson correlation coefficient = 0.7363, *p*-value = 0.0063).

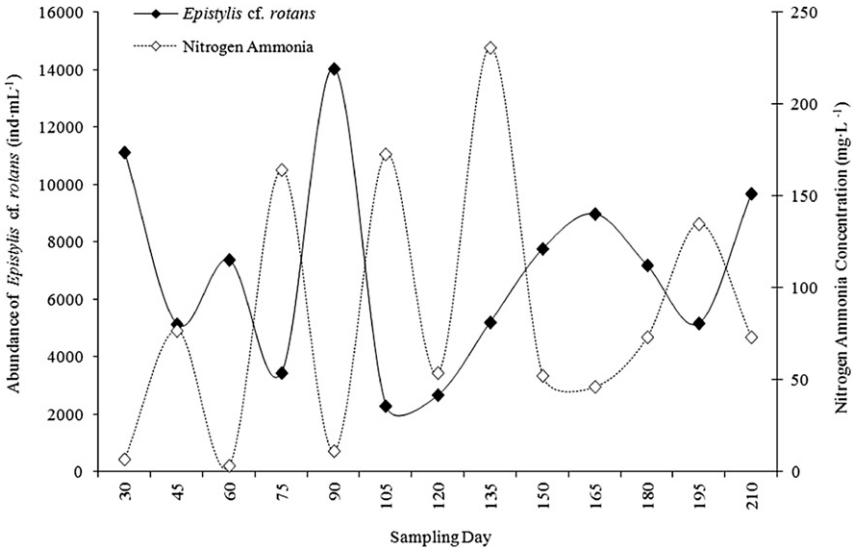

Fig. 2 – Changes of ammonium concentration (mg L^{-1}) and abundance of *Epistylis* cf. *rotans* (ind mL^{-1}) in the aerobic reactor.

3.5. *Tolerance ranges of ciliates*

The characteristics of an A/O SBNR system treating high ammonium loaded wastewater, a process which focuses on nitrite accumulation, provided new data on tolerance ranges of ciliate species to ammonium, nitrites, nitrates, temperature and pH, shown in Table 4.

4. Discussion

The protozoan community in an A/O SBNR moving bed biofilm system treating high ammonium loaded wastewater is different from the protozoan community in conventional WWTPs. In conventional processes, flagellates are rarely predominant except for episodes of low dissolved oxygen and/or high organic pollution (Madoni, 1994), whereas ciliate communities habitually consist of 15–20 species (Dubber and Gray, 2009), sometimes reaching up to more than 30 species (Pérez-Uz et al., 2010). Furthermore, our results are also different compared to those for other biofilm processes, such as rotating biological contactors (Pérez-Uz et al., 1998; Martín-Cereceda et al., 2002; Salvadó et al., 2004), where the number of ciliate species ranged from 28 to 67, and fixed bed biofilm systems (Lorda-de-los-Ríos et al., 2002) and aerated biofilters (García-Santana and González-Martínez, 2002), where ciliate species ranged from 10 to 15, in contrast to the 4 ciliate species detected in the A/O SBNR system. Therefore an A/O SBNR moving bed biofilm system treating high ammonium loaded wastewater could be defined as a flagellates-predominant system with a low diversity of ciliates.

4.1. *Biofilm*

The ANX-BF operated continuously under anoxic conditions and received a high ammonium loaded influent. Few protozoan species have demonstrated tolerance to low dissolved oxygen and some have been positively related to short anoxic/aerobic cycles of anoxia (Dubber and Gray, 2011). Nevertheless, low levels or absence of DO are known to be unfavourable for most free-living protozoan species in WWTPs, as confirmed by Maurines-Carboneill et al. (1998), who reported that protozoa and metazoa disappear after three days of anaerobiosis. Moreover, Puigagut et al. (2005, 2009) showed that ammonium adversely affects protozoan communities, diminishing their abundance and community complexity. Thus, the lack of DO coupled with the toxicity of high ammonium concentrations might lead to stressful conditions for microfauna, explaining the residual colonisation of the anoxic biofilm by protozoan species. The presence of protozoa in the ANX-BF was most probably caused by dispersion processes from the mixed liquor, the surrounding medium for the carriers. This was confirmed by the fact that the protozoan species observed in ANX-BF were also detected in the mixed liquor in most samplings.

In contrast to ANX-BF, the lower ammonium concentration and the aerobic conditions under which the OXI-BF operated favoured colonisation by ciliates, specifically by sessile species of the subclass Peritrichia, such as *Epistylis* cf. *rotans* and *V. microstoma*-complex. During the study, up to 3 ciliate species were observed in the aerobic biofilm, although in most cases *Epistylis* cf. *rotans* was the only ciliate species detected. These data reveal a low level of ciliate diversity compared to the results reported by Fried et al. (2000), who observed from 6 to 12 ciliate species inhabiting the biofilm of plastic particles (carriers). These researchers also observed high abundances of nematodes and rotifers, which were not observed in the aerobic biofilm in the present study (Table 4). Hence, the results obtained confirm that ammonium concentrations adversely affect ciliate diversity, and also suggest that ammonium hinders biofilm colonisation by micrometazoa such as nematodes and rotifers.

The capacity to adhere to the substrate seemed to be essential for the development of ciliate species in the A/O SBNR moving bed biofilm system. Sessile ciliates such as *Epistylis* cf. *rotans* could avoid the anoxic conditions of an A/O process due to their close relationship with the biofilm, whereas the free-swimming ciliates *Colpoda ecaudata* and *Cyclidium glaucoma* probably could not, although they were occasionally observed in the system.

As for the flagellates, the data showed that the species in OXI-BF were related to those in the mixed liquor, so their

Table 4 – Tolerance ranges of *Colpoda ecaudata*, *Cyclidium glaucoma*, *Epistylis* cf. *rotans* and *Vorticella microstoma*-complex to nitrogen ammonia, nitrogen nitrite, temperature and pH. Obs: Observed data; Rep: Reported data from several authors, published in Foissner et al. (1994).

	Ranges	*Colpoda ecaudata*		*Cyclidium glaucoma*		*Epistylis* cf. *rotans*		*Vorticella microstoma*-complex	
		Min	Max	Min	Max	Min	Max	Min	Max
Nitrogen ammonia (mg L^{-1})	Obs.	0.54	207	73.2	83.7	0.54	267	0.5	164.4
	Rep	–		0	85	–		–	
Nitrogen nitrite (mg L^{-1})	Obs.	28.8	194.2	27.4	60.5	0	360	23	194.2
	Rep.	–		0	2	–		–	
Nitrogen nitrate (mg L^{-1})	Obs.	0.8	3.4	28.7	45.1	0	321	0	45.3
	Rep.	–		0	2	–		–	
Temperature (°C)	Obs.	25.4	28.8	26.2	33.6	22.6	33.6	28.8	33.1
	Rep.	–		0	51	–		–	
pH	Obs.	7.8	8.01	7.09	7.78	6.91	8.66	7.72	8.36
	Rep.	–		5.7	9.8	–		–	

presence in OXI-BF should be considered a result of dispersion processes from the mixed liquor. The mixed liquor might be a dispersion vector for flagellate species that colonised both aerobic and anoxic biofilm, as well as the means by which *Epistylis* cf. *rotans* sporadically appeared in the ANX-BF.

4.2. *Mixed liquor*

In the statistical analysis of microfauna distribution, the initial four compartments were reduced to 3 subsystems (mixed liquor, OXI-BF and ANX-BF), since despite the physicochemical differences between OXI-ML and ANX-ML, the effect of the high recirculation flow was that both OXI-ML and ANX-ML subsystems were considered the same fluid for microfauna.

The mixed liquor circulated continuously between the anoxic and aerobic reactors. According to Van Dongen et al. (2001), intermittent aeration in a SHARON reactor does not permit the development of protozoan community. Moreover, Dubber and Gray (2011) demonstrated that the longer the anoxia, the more protozoan community complexity is reduced (by up to 50%–73% in 3.3 h anoxic cycles). Considering these earlier conclusions, the uninterrupted hydraulic residence time in each reactor was calculated (7.6 ± 1.3 h; maximum: 13.3 h, minimum: 6.5 h) in order to determine the time that the mixed liquor remained in anoxic or aerobic conditions before returning to the other reactor. Results demonstrated that flagellates were able to develop in the mixed liquor in spite of the 6.5–13 h anoxic/aerobic cycles and the high ammonium concentration. The opportunistic role of flagellates, usually more tolerant of organic pollution and lack of DO than ciliates (Madoni, 1994), allowed them to rapidly increase their population when system conditions became sufficiently favourable.

The low abundance of ciliates in the mixed liquor suggests that 7.6 h anoxic/aerobic cycles may be sufficient to prevent stable colonisation by ciliates of the mixed liquor over time. The presence of *Epistylis* cf. *rotans* in the mixed liquor should be considered as the result of dispersion or detachment processes from OXI-BF, the only subsystem where *Epistylis* cf. *rotans* maintained its presence throughout the entire sampling period. Alternating anoxic/aerobic conditions seems to enhance the development of flagellates rather than ciliates, in agreement with several studies in which flagellates were reported at high abundances in conventional nutrient removal processes with at least one anoxic reactor (Pérez-Uz et al., 2010).

4.3. *Protozoa as bioindicators*

As shown in Fig. 2, a lower ammonium concentration was related to higher abundances of *Epistylis* cf. *rotans*, whereas high ammonium concentrations hindered ciliate development. Consequently, the ciliate *Epistylis* cf. *rotans* could be a useful bioindicator of ammonium removal efficiency in an A/O SBNR moving bed biofilm system treating high ammonium loaded wastewater.

The strong negative correlation between the *Epistylis* cf. *rotans* population in the OXI-BF and the ammonium concentration in the aerobic reactor could be explained by the lack of competition from other ciliates. With no competition, only the effect of toxic or adverse parameters, such as ammonium, could influence the *Epistylis* cf. *rotans* population dynamics.

Epistylis cf. *rotans* was also positively related to ammonium removal efficiency. These results agree with those obtained by Pajdak-Stós et al. (2010) and Yu et al. (2011), who demonstrated that predation pressure exerted by protozoa on nitrifying bacteria colonies does not negatively affect ammonium removal effectiveness. Moreover, Fried and Lemmer (2003) reported that filtering species such as *Epistylis* are able to produce intense water currents due to their grazing activity, bringing nutrient-rich water to the inner parts of the biofilm and consequently improving ammonium removal efficiency. Therefore, our results support the hypothesis that the presence of protozoa is positive for optimal performance of an SBNR process.

Given that all protozoa species thrive within their specific parameter ranges, our study provides information about the ecology of the ciliate species observed (Table 4). Results confirm the tolerance ranges of *Cyclidium glaucoma* to ammonium concentrations established by Bick and Kunze (reported in Foissner et al., 1994) and greatly expand this species' limits of tolerance to nitrite and nitrate concentrations (60.5 mg L^{-1} and 45.1 mg L^{-1}, respectively). Furthermore, our study establishes broad limits of tolerance for *Colpoda ecaudata*, *V. microstoma*-complex and *Epistylis* cf. *rotans* to nitrite, nitrate and ammonium levels.

The study of protozoan communities in new wastewater treatment processes sheds light on species ecology and their role under conditions that have been little studied to date in wastewater treatment processes, and could offer new biological management tools.

5. Conclusions

The A/O SBNR moving bed biofilm system treating high ammonium loaded wastewater can be defined as a flagellates-predominant system with a low diversity of ciliates compared to conventional WWTPs.

The anoxic conditions of ANX-BF and 7.3 h anoxic/aerobic cycles in mixed liquor impeded colonisation by ciliates. Moreover, ammonium has also been confirmed as a negative factor for diversity of ciliate community. Only four ciliate species were detected: *Colpoda ecauta*, *Cyclidium glaucoma*, *Epistylis* cf. *rotans* and *V. microstoma*-complex, but *Epistylis* cf. *rotans* was the only species that presented a constant and abundant population, almost exclusively in the OXI-BF. The ability to adhere to the substrate has proved to be essential for the development of ciliate species in an A/O SBNR moving bed biofilm system. Flagellates abundantly colonised the mixed liquor, results that confirm that flagellates are more tolerant than ciliates to stressful conditions such as anoxic/aerobic cycles and high ammonium concentrations.

Protozoa, and more specifically ciliates, do not adversely affect the efficiency of nitrification, as suggested in previous studies. Moreover, *Epistylis* cf. *rotans* was positively related to ammonium removal efficiency. Therefore, *Epistylis* cf. *rotans* is a useful bioindicator species of the ammonium removal efficiency of an A/O SBNR process.

The results obtained demonstrate that ciliates are useful as bioindicators in an A/O SBNR moving bed biofilm system. Moreover, they encourage us to study new and unexplored treatment systems and determine their bioindicator species.

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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.watres.2013.03.017.

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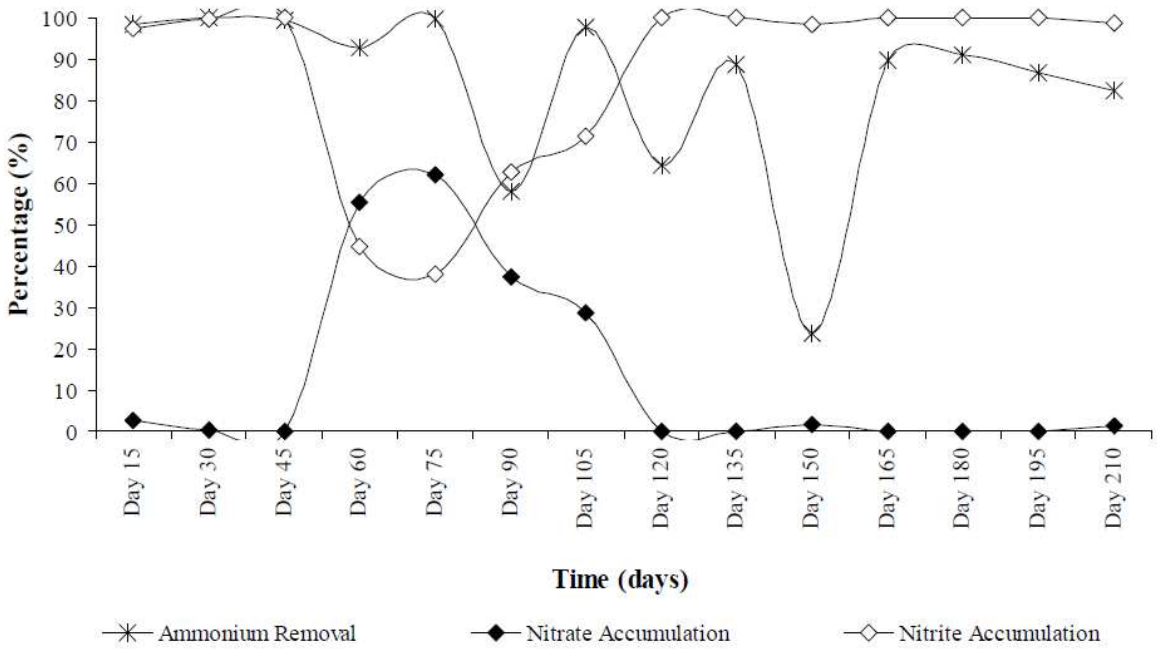


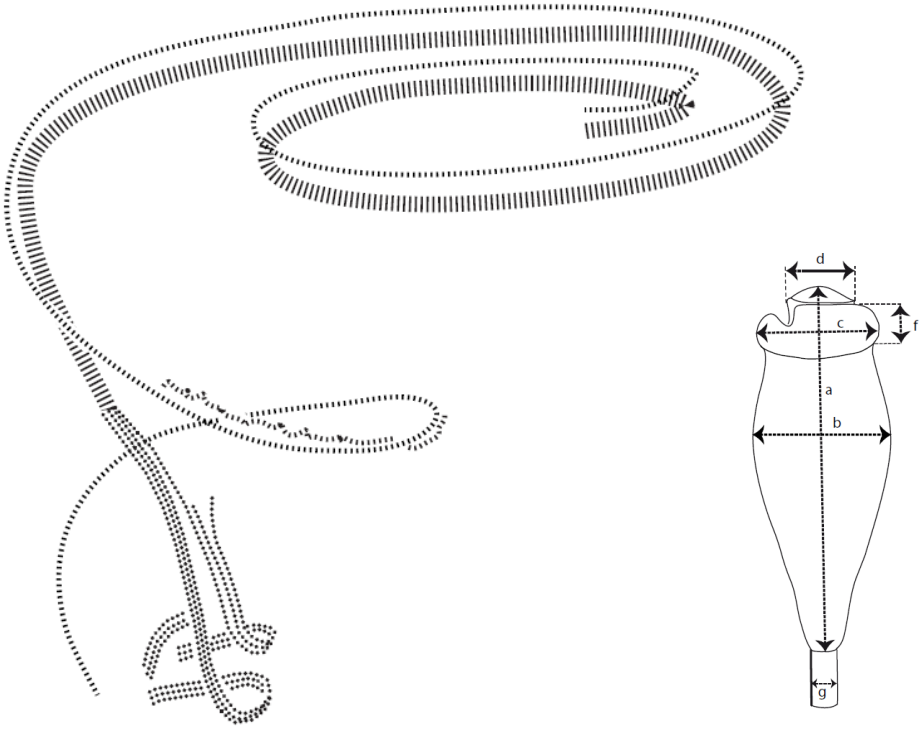
Supplementary Data Fig. 1. Evolution of nitrite and nitrate accumulation percentages and ammonium removal efficiency in the aerobic reactor.

3.3. CAPÍTOL 2

DESCRIPTION OF *EPISTYLIS CAMPRUBII* n. sp., A SPECIES HIGHLY TOLERANT TO AMMONIUM AND NITRITE.

"*Una descripció moderna d'una nova espècie de ciliat hauria d'incloure, si és possible, dades obtingudes mitjançant la observació de individus vius, individus tenyits i dades de seqüenciació de gens*"

(Lynn, 2008)



Resum

Una nova espècie de perítric altament tolerant a l'amoni i al nitrit, *Epistylis camprubii* n. sp., es va trobar adherit al biofilm de dos plantes de tractament avançat d'aigües residuals tractant aigua residual amb alta càrrega amoniacal a Rubí, Spain. S'ha estudiat la seva morfologia, infraciliació oral i posició filogenètica en el clade de la subclasse Peritrichia. La nova espècie és un perítric amb forma de gerro, amb constricció sota el llavi peristomial, una longitud mitjana in vivo de 58.7 ± 10.1 µm, una amplada mitjana de 32.0 ± 5.4 µm, i un peduncle compacte i longitudinalment estriat que ocasionalment mostra un gruix desigual i de forma poc habitual segments transversals. El disc peristomial és normalment arrodonit o lleugerament punxegut, estranyament umbilicat. El nucli en forma de C es situa a la meitat adoral del cos, i la única vacuola contràctil es troba al terç adoral del zooid. L'anàlisi filogenètic de la seqüència del gen 18S situa *Epistylis camprubii* n. sp. juntament amb les altres espècies de *Epistylis*, amb l'excepció de *Epistylis galea*.

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Description of *Epistylis camprubii* n. sp., a Species Highly Tolerant to Ammonium and Nitrite

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Abstract. A new peritrich species highly tolerant to ammonium and nitrite, *Epistylis camprubii* n. sp., was found adhered to the biofilm of two advanced wastewater treatment plants treating high ammonium-loaded wastewater in Rubí, Spain. Its morphology, oral infraciliature and phylogenetic position in the peritrich clade were studied. The new species is a vase-shaped peritrich, constricted below the peristomial lip, with an *in vivo* average length of 58.7 ± 10.1 μm, average width of 32.0 ± 5.4 μm, and a longitudinally striated, compact stalk that occasionally exhibits uneven thickness and rarely shows transverse segments. The peristomial disc is commonly rounded or pointed, and rarely umbilicated. The C-shaped macronucleus is located in the adoral half of the body, and the only contractile vacuole lies in the adoral third of the zooid. The molecular analysis of the 18s gene sequence clustered *E. camprubii* n. sp. together with the other *Epistylis*, with the exception of *Epistylis galea*.

Key words: Peritrichia, wastewater treatment, ciliates, 18s rRNA, ammonium, nitrite.

Abbreviations: A/O SBNR – Anoxic/Oxic Shortcut Biological Nitrogen Removal; PN – Partial Nitrification; MBBR – Moving Bed Biofilm Reactor.

INTRODUCTION

The genus *Epistylis* Ehrenberg, 1830 comprises a great number of species from aquatic environments including both freshwater and marine ecosystems. The *Epistylis* species are colonial and normally attached to a non-living substrate, although several species are known to be capable of living as epibiont on aquatic invertebrates (Stiller 1971). Just a few species have been described as free-living. *Epistylis* zooids are usually elongated, showing one micronucleus and a macronucleus, an eversible peristomial lip, and a non-contractile stalk without a spasmoneme. *Epistylis* are very common in wastewater treatment systems, and play an important role in effluent clarification. In addition, *Epistylis* species of wastewater treatment plants can be used as performance bioindicators of a great variety of parameters and processes (Curds 1982, Salvadó *et al*. 1995, Berger and Foissner 2003, Canals *et al*. 2013).

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Epistylis camprubii n. sp. was found and collected from two advanced treatment plants treating high ammonium wastewater. Firstly identified as *Epistylis* cf. *rotans*, a deeper study provided enough evidences to consider this organism, which is highly tolerant to ammonium and nitrite, as a new *Epistylis* species. It was named *Epistylis camprubii* n. sp. The present study describes the morphologic and molecular characteristics of the new *Epistylis* species, and provides detailed images and drawings of its oral infraciliature.

MATERIAL AND METHODS

1. Sampling. Samples containing *Epistylis camprubii* n. sp. were obtained by analysing the microfauna of an *Anoxic/Oxic Shortcut Biological Nitrogen Removal Moving Bed Biofilm Reactor* (A/O SBNR-MBBR) and a *Partial Nitritation Moving Bed Biofilm Reactor* (PN-MBBR). Both pilot wastewater treatment plants were located in Rubí, Spain, and operated during different periods. A *Moving Bed Biofilm Reactor* (MBBR) process is characterised by the presence of plastic supports held in suspension (named 'carriers'), on which the biofilm develops. *Epistylis camprubii* n. sp. was mainly observed attached to the biofilm of carriers. Consequently, the biofilm needed to be detached from the carriers to undertake the study. The method of detachment is detailed in Canals *et al*. (2013).

2. Morphological analysis. The length and width of the zooid, the peristomial disc diameter, the height and width of peristomal lip, and the stalk width (see Fig. 1d) were measured using a Zeiss Axioskop 40 microscope. Measurements were always made on *in vivo* specimens, using a calibrated ocular micrometer. The ammoniacal silver carbonate method (Fernández-Galiano 1994) was carried out to study the oral infraciliature of the stained specimens. Ammoniacal silver carbonate and Klein's silver impregation (Klein 1958) methods were used to determine the number of transverse silverlines of the stained zooids. Pictures were taken on a ProgResC3 camera (Jenoptik, Germany) and Zeiss Axioskop 40 and Leitz DMRB microscopes for *in vivo* and stained specimens respectively. Drawings were made using Illustrator software based on *in vivo* and after silver staining method specimens.

3. Single Cell PCR, sequencing and data analysis. Isolated cells of *Epistylis camprubii* n. sp. were collected in a 0.2-mL PCR tube and frozen at –80°C until PCR was performed. The cells were isolated using an inverted microscope (Meiji TC5000). To perform Single Cell amplifications, the following primer combinations were used: 528F (5'-GCGGTAATTCCAGCTCCAA-3', Elwood *et al*. 1985) and EukR (5'-TGATCCTTCTGCAGGTTCACCTAC-3', EUK B, Medlin *et al*. 1988). The PCR mixtures (100 µL of volume) contained: 20 µL approximately of the culture medium from which each cell (DNA template) was isolated, 2 µL of deoxynucleoside triphosphate mix at a concentration of 10 mM each, 4 µL of $MgCl_2$ (50 mM solution), 10 µL of PCR buffer (Invitrogen; USA), 8 µL of each primer at a concentration of 10 µM, 0.75 µL of Taq DNA polymerase (Invitrogen) and 47.25 µL of 0.2 µm-filtered sterilised water (up to 100 µL). The PCR program included an initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, extension at 72°C for 3 min, and a final extension cycle at 72°C for 10 min. An aliquot of the PCR product was run in a 1% agarose gel by electrophoresis, stained with SYBR SAFE (0.5 × final concentration; Invitrogen), and checked by comparison to a standard (Low DNA Mass Ladder; Invitrogen).

PCR products were sent to Genoscreen (Lille, France) to be purified and subsequently sequenced with the primers 528F, EukR (both sequences detailed previously), 1209F (5'-CAGGTCTGTGATGCCC-3', Littlewood and Olson 2001) and 1209R (5'-GGGCATCACAGACCTG-3', Giovannoni *et al*. 1988). The sequences obtained for each sample were assembled using Geneious software and the resulting consensus sequence was screened and then compared with public DNA databases using BLAST (Altschul *et al*. 1997). The sequence can be found in GenBank (accession number KP713786).

To study the phylogenetic position of *Epistylis cambrubii* n. sp. in the Peritrichia phylogenetic tree, as well as its position within the *Epistylis* clade, some previously published sequences from GenBank were used in addition to the sequence obtained for *E. camprubii*: *Campanella umbellaria* AF401524; *Carchesium polypinum* GU187053; *Cyclidium glaucoma* EU032356; *Epicarchesium abrae* DQ190462; *Epistylis chlorelligerum* KM096375; *Epistylis chrysemydis* AF335514; *Epistylis galea* AF401527; *Epistylis hentscheli* AF335513; *Epistylis plicatilis* AF335517; *Epistylis riograndensis* KM594566; *Epistylis urceolata* AF335516; *Epistylis wenrichi* AF335515; *Opercularia microdiscum* AF401525; *Pseudovorticella punctata* DQ190466; *Pseudovorticella sinensis* DQ845295; *Tetrahymena pyriformis* EF070255; *Vaginicola crystallina* AF401521; *Vorticella aequilata* JN120215; *Vorticella convallaria* JN120220; *Vorticellides aquadulcis* JQ723990; *Vorticellides infusionum* JN120203; *Vorticellides microstoma* JN120206; *Zoothamnium alternans* DQ662855 and *Zoothamnium pluma* DQ662854.

The 18s rRNA sequences were aligned with MAFFT V.6 online software (Katoh and Toh 2008), using the Q-INS-i approach with default settings (gap opening penalty GOP = 1.53 and offset value set to 0.0). Gaps were recoded as presence/absence data following the simple method of Simmons and Ochotorena (2000), as implemented in the program SeqState v1.4.1 (Müller 2005). The final matrix had 1819 nucleotide position and 90 characters corresponding to the gaps scored as absence/presence. The best-fitting evolutionary model of the alignment was assessed using Partitionfinder software (Lanfear *et al*. 2012). The suggested model (GTR+G+I) was used for subsequent analysis.

Phylogenetic trees were inferred using three methods: maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI). MP analysis was carried out using TNT software (Goloboff *et al*. 2008) with gaps scored as absence/presence. The search consisted of 1000 replicates of random sequence addition, followed by TBR branch swapping and holding five trees per iteration. Clade support was assessed with 1000 bootstrap replicates. Maximum likelihood inference was conducted using RAxML v.8.1.16 program (Stamatakis 2014) run under the RAxML-GUI software (Silvestro and Michalak 2012). Two independent models were defined, a GRT+G+I for the nucleotide and a binary model for the gap partition. The best tree was selected from 100 iterations and support assessed with 1000 replicates of bootstrap resampling. Bayesian Inference was conducted using MrBayes 3.2 (Ronquist and Huelsenbeck 2003). Two independent runs of 5×10^7 genera-

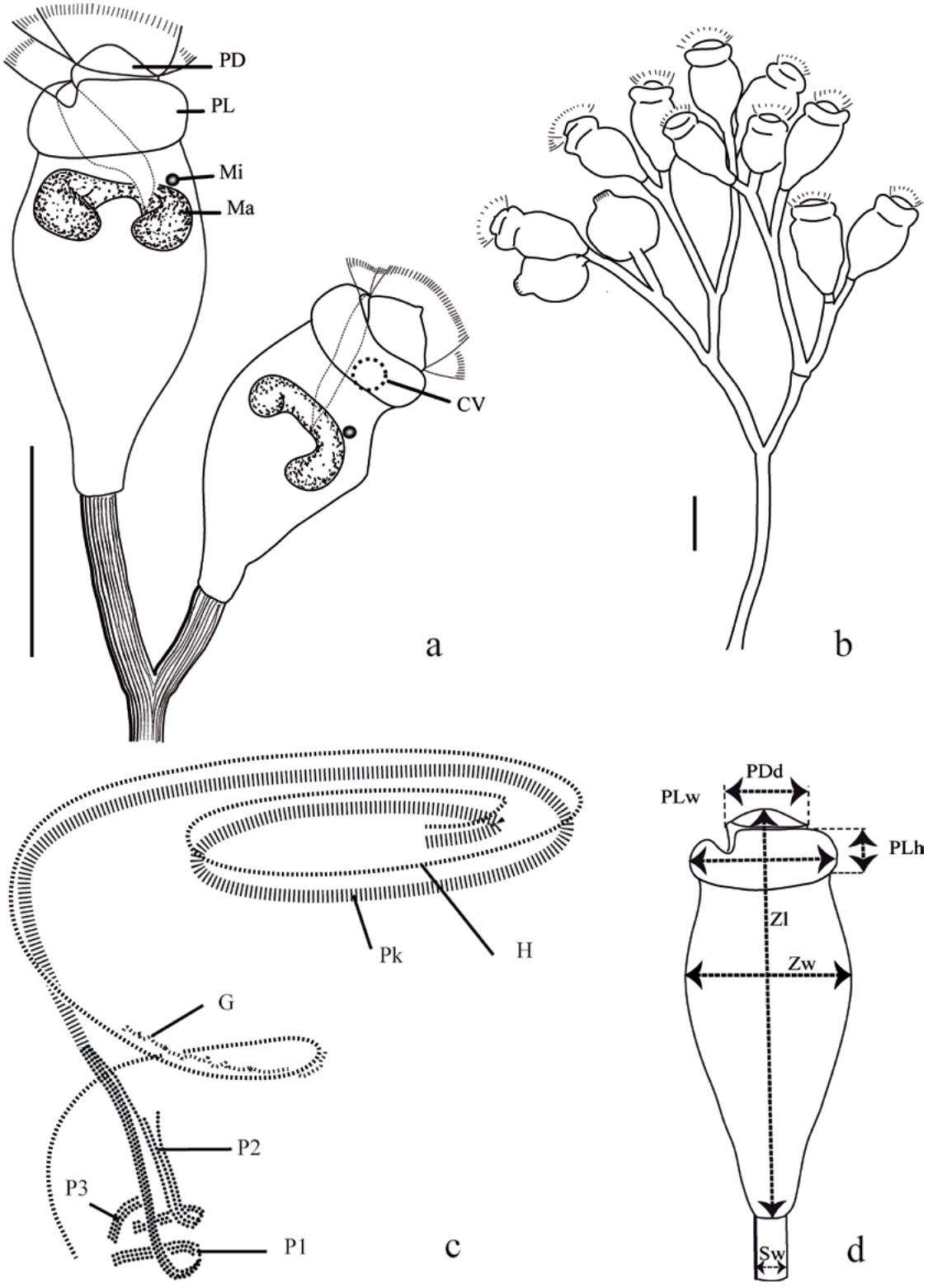


Fig. 1a–d. Morphological and oral infraciliature details of *Epistylis camprubii*. **a** – detail of the zooid. PD – peristomial disk; PL – peristomial lip; CV – contractile vacuole; Ma – macronucleus; Mi – micronucleus; **b** – scheme of a colony; **c** – oral infraciliature. Pk – polykinety; H – haplokinety; G – germinal kinety; P1 – polykinety 1; P2 – polykinety 2; P3 – polykinety 3; **d** – morphological characteristics measured. PDd – peristomial disk diameter; PLw – peristomial lip width; PLh – peristomial lip height; Zl – zooid lenght; Zw – zooid width; Sw – stalk width. Scale bars: 25 μm.

tions with 4 MCMC (Markov Chain Monte Carlo) chains per run, starting from random trees and resampling each 1000 generations were run simultaneously. The first 25% generations of each run were discarded as a burn-in for the analyses. Run convergence was assessed by means of the average standard deviation of split frequencies (ASDSF < 0.01).

RESULTS

Epistylis camprubii n. sp.

1. Diagnosis. Freshwater bacterivorous *Epistylis*, measuring on average 58.7 µm in length and 32.0 µm in width. The zooids are vase-shaped and constricted below the thick peristomial lip. Persitomial disc commonly rounded or pointed, very rarely umbilicated. C-shaped macronucleus located in the adoral half of the zooid and transversely oriented. Contractile vacuole placed in the adoral third of the body, on dorsal wall of infundibulum. Row 3 of P2 slightly divergent to the other rows at their abstomal ends, extending, together with row 2, approximately 2/3 of the length of row 1. Transverse silverlines numbering 106 to 136 from peristome to aboral trochal band and 33 to 48 from aboral trochal band to scopula.

2. Description. *Epistylis camprubii* n. sp. is a vase-shaped peritrich, constricted below the peristomial lip. The zooid length ranges from 35.3 to 98.1 µm (average of 58.7 ± 10.1 µm) and the width from 18.0 to 65.2 µm (average of 32.0 ± 5.4 µm). The peristomial disc is commonly rounded or pointed, and very rarely umbilicated. It is possible, but not common, to observe zooids of the same colony showing different peristomial disc shapes. The diameter of the peristomial disc ranges from 11.2 to 21.3 µm (average of 15.5 ± 1.9 µm). *E. camprubii* shows a thick peristomial lip with a height ranging from 5.0 to 10.6 µm (average of 7.8 ± 1.2 µm) and a width from 16.2 to 31.7 µm (average of 24.2 ± 2.9 µm). The width of the stalk varies from 3.1 to 8.4 µm (average of 5.3 ± 0.9 µm). The stalk is longitudinally striated and compact, occasionally exhibits uneven thickness, and rarely shows transverse segments. Stalk thickening is often associated with separation nodes, but they can also be seen in the internodal part of the stalk (Fig. 2h–j). Thicker stalks or branches, as well as more frequent stalk thickening, are related to shorter stalks. *E. camprubii* presents a colourless cytoplasm and a transversely oriented C-shaped macronucleus located in the adoral half of the zooid. The only contractile vacuole lies in the adoral third of the body, on dorsal wall of infundibulum.

In the wastewater treatment systems where *E. camprubii* was found, 88.2% of the colonies showed a number of zooids between 2 and 20 (Fig. 4), with a maximum value of 124. The ramification is initially dichotomic, but becomes irregular after the second level of ramification. Sometimes the ramification is totally irregular.

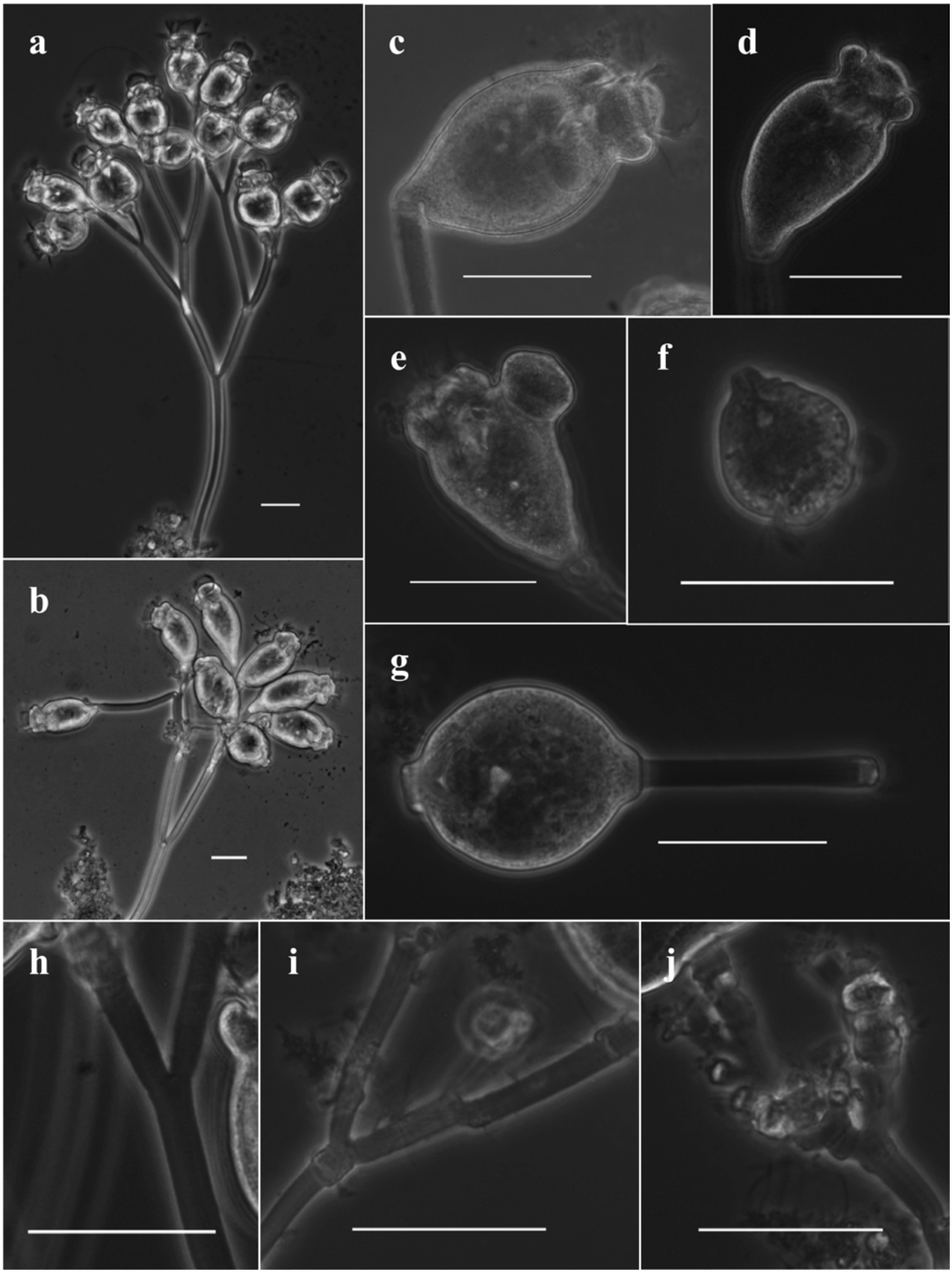
E. camprubii presents the typical oral infraciliature of the genus *Epistylis*. Specifically, it has very similar oral infraciliature to *Epistylis chrysemydis* (detailed by Foissner *et al*. 1992). The haplokinety and the polykinety make between one and one and a half circuits before entering the infundibulum, where they make a turn again. In the infundibulum, the haplokinety is initially accompanied by a germinal kinety. The germinal kinety is no longer observed when the haplokinety turns and follows the longitudinal axis of the zooid. The polykinety becomes three infundibular polykineties (named P1, P2, P3), each consisting of 3 rows of kinetosomes. The abstomal end of P1 comes directly from the original polykinety and its three rows are equal in length. The abstomal end of row 1 of P2 extends the entire distance to P1 and merges with it near the oral opening. Rows 2 and 3 of P2 are much shorter than row 1, extending only ~ 2/3 of the length of row 1 at their abstomal ends. Row 3 of P2 is slightly divergent from the other two rows at its abstomal end. The adstomal ends of all three rows of P2 end a short distance before the adstomal ends of P1 and P3. P3 is the shortest polykinety and presents the three rows equal in length. P3 appears after the last turn of P1 and P2 and its adstomal end is very close to the adstomal end of P1 (Figs 1c and 3d–e).

Epistylis camprubii presents 106 to 136 transverse silverlines from peristome to aboral trochal band and 33 to 48 from aboral trochal band to the scopula.

3. Etymology. The name *camprubii* refers to the surname of the Catalan biologist Jordi Camprubí Capella, in personal recognition of a life dedicated to teaching and sharing his love for biology, especially for ciliate protozoa.

▸▸

Fig. 2a–j. Images of *Epistylis camprubii, in vivo*. **a–b –** example of colonies; **c–d** – two examples of extended zooids; **e** – zooid during conjugation; **f** – conjugation; **g** – contracted zooid; **h–j** – images of the stalk and branches, from smoother and larger to shorter and thicker. Scale bars: 25 µm.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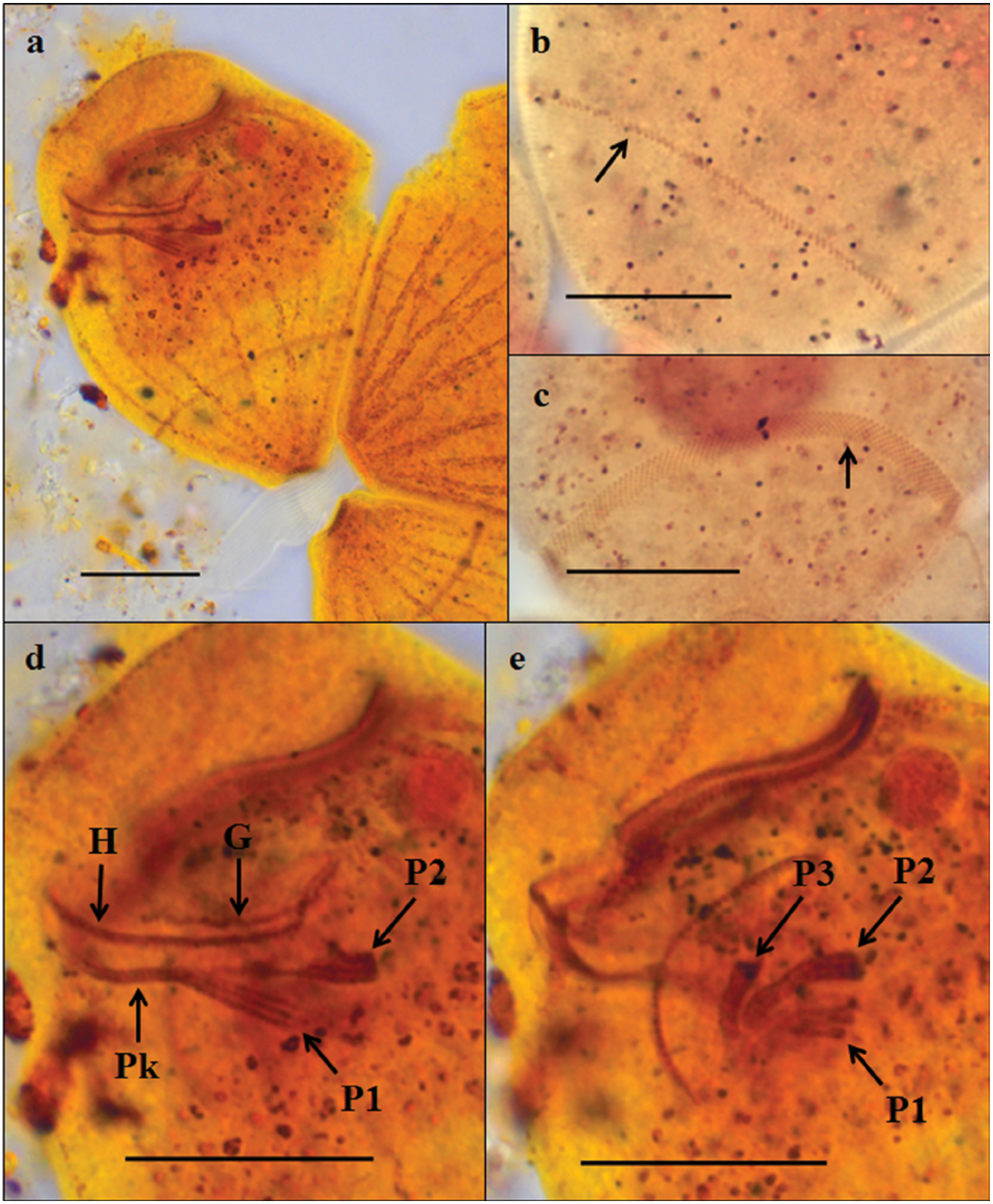

Fig. 3a–e. Images of *Epistylis camprubii*, after silver staining method. **a** – view of the longitudinal fibers and the oral infraciliature; **b** – detail of the aboral trochal band of a feeding zooid; **c** – aboral trochal band of a zooid during swimmer formation; **d–e** – oral infraciliature details. H – haplokinety; G – germinal kinety; Pk – polykinety; P1 – polykinety 1; P2 – polykinety 2; P3 – polykinety 3. Scale bars: 15 µm.

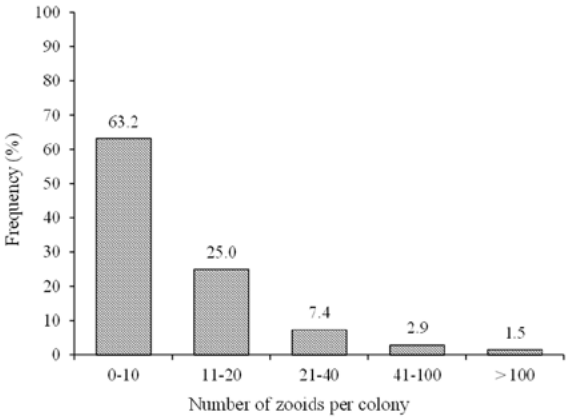

Fig. 4. Frequency of the number of zooids per colony observed in *Epistylis camprubii* colonies (number of analyzed colonies = 71).

4. Ecology. *Epistylis camprubii* is a bacterivorous peritrich. It was first observed in the aerobic reactor of an A/O SBNR-MBBR process and was initially identified as *Epistylis* cf. *rotans* (Canals *et al*. 2013). It was observed mainly inhabiting the biofilm adhered to carriers (the plastic support for the biofilm attachment), but was also found colonising the liquid phase, adhered to flocs. It was observed again attached to the biofilm and colonising the liquid phase of a PN-MBBR and PN-granular processes, both previous to an Anammox reactor.

A/O SBNR and PN processes are designed to treat high ammonium-loaded wastewater (up to over 10^3 $N\text{-}NH_4^+$ $mg \cdot L^{-1}$). Specifically, the aerobic reactor of an A/O SBNR process is designed to oxidise the ammonium in the influent and accumulate the oxidised nitrogen form as nitrite, while the PN process is focused on achieving an adequate effluent for the posterior Anammox reactor, that is, a nitrite: ammonium ratio close to 1.2. The range of ammonium and nitrite values in which *Epistylis camprubii* was observed are shown in Table 2.

Some organisms were identified cohabiting with *E. camprubii*: the ciliates *Colpoda* spp., *Cyclidium glaucoma*, *Opercularia coarctata*, *Telotrochidium matiense*, *Vorticellides microstoma*-complex and two unidentified Hypotrichia; *Tetramitus rostratus*, *Polytoma* sp., *Anthophysa* sp. and *Trimastix* sp. among other unidentified flagellates; nematoda; and unidentified gymnamoeba smaller than 20 μm. Most of these species, specifically the ciliates, are usually observed in conventional activated sludge wastewater treatment plants. It would not be surprising if *Epistylis camprubii* had been misidentified in this kind of treatment systems.

5. Type Sequence. GenBank accession number KP713786.

6. Type material. An holotype slide with 36 specimens (registration number CRBA-28006) and a paratype slide with 52 specimens (registration number CRBA-28007) after ammoniacal silver carbonate method were deposited in the Centre de Recursos de Biodiversitat Animal (CRBA), Facultat de Biologia, Universitat de Barcelona, Barcelona (Spain). Another paratype slide with 35 specimens was deposited in the Natural History Museum of London, UK (registration number NHMUK 2015.4.20.1).

7. Phylogenetic position. The molecular analysis of the 18s rRNA gene sequences supported the inclusion of *E. camprubii* within the Peritrichia, regardless of the inference method implemented. All analyses agreed in supporting the monophyly of *E. camprubii* with the other *Epistylis* sequences used in the present study, with the single exception of *E. galea* (Fig. 5).

8. Comparative diagnosis. A large number of *Epistylis* species have been described. Among them, just a few showed morphological similarities to *E. camprubii* and/or are typical species of wastewater treatment plants.

E. camprubii was formerly reported as *Epistylis* cf. *rotans* (Canals *et al*. 2013), since *E. rotans* is a vase-shaped peritrich that is often observed in wastewater treatment plants, and presents a longitudinally striated and transversally segmented stalk, as *E. camprubii* does. But further observations revealed that transverse segments are not always present in *E. camprubii*, in contrast to *E. rotans*. Moreover, *E. rotans* is longer (70 to 100 μm) and was described as an oligosaprobic species by Curds (1969), while *E. camprubii* is shorter (Table 1), inhabits more polluted environments, and shows a high tolerance to ammonium and nitrite compounds (Table 2). It must be noted that Foissner *et al*. (1992) considers *E. rotans* a synonym of *E. procumbens*, a funnel-shaped peritrich with a zooid length from 60 to 140 μm, often bent and with transverse segments before the stalk division, whose characteristics clearly do not match those of *E. camprubii*.

A comparison of *E. camprubii* and other *Epistylis* species of wastewater treatment plants showed that *E. camprubii* is shorter than *E. chrysemydis* (120 to 220 μm), *E. coronata* (70 to 120 μm), *E. entzii* (125 to 190 μm), *E. hentscheli* (110 to 170 μm), *E. balatonica* (90 to 100 μm) and *E. plicatilis* (90 to 160 μm). In addition, most of these species have a wider stalk, ranging from 7 to 20 μm width (Foissner *et al*. 1992), while the stalk of *E. camprubii* ranges from 3.1 to a maxi-

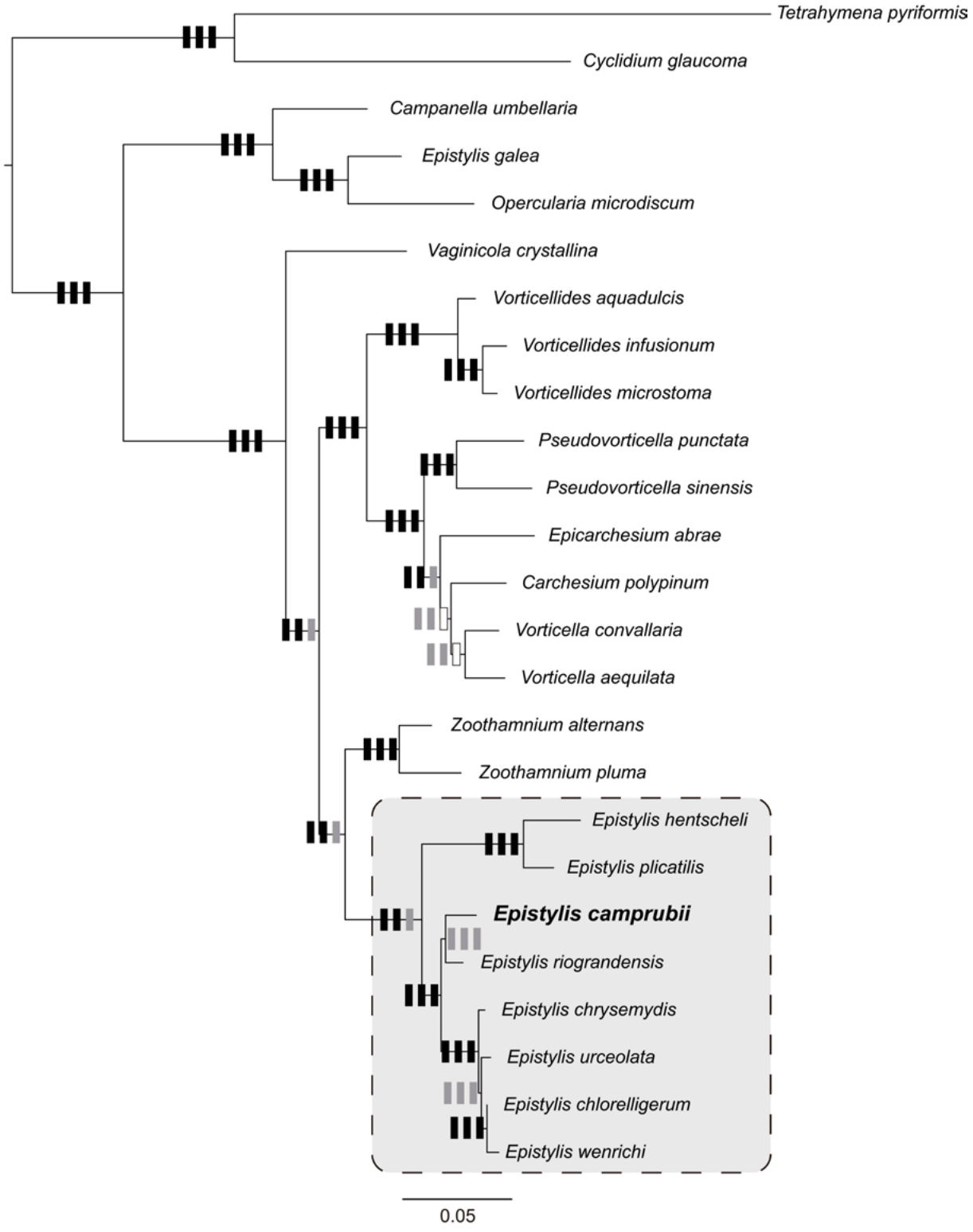

Fig. 5. The preferred 18s rRNA tree under maximum likelihood (ML). Rectangles on branches denote the support recovered in analyses under alternative inference methods. Left rectangle refers to maximum likelihood (ML), the middle one to Bayesian inference (BI) and the right one to maximum parsimony (MP). Black coloured rectangle indicates bootstrap support > 80 or posterior probability > 0.95, grey rectangle indicates clade recovered but with lower support than the former values, and white rectangle indicates the clade was not recovered. Main *Epistylis* clade boxed.

mum of 8.4 µm when stalk thickening occurs (Table 1). Other clearly different traits are that *E. chrysemydis* and *E. balatonica* have two lips in the peristome, in contrast to the one lip of *E. camprubii*. *E. coronata* always shows an umbilicated peristomial disc and *E. plicatilis* and *E. hentscheli* are clearly funnel-shaped.

Outside the field of wastewater treatment systems, three *Epistylis* species presented similar characteristics to *E. camprubii*: *E. epistyliformis*, *E. thienemanni* and *E. variabilis* (Stiller 1971). *E. epistyliformis*, the most similar species, is a vase-shaped peritrich with a clearly pointed and rarely umbilicated peristomial disc, a zooid length from 43 to 62 µm, and clearly constricted below the peristomial lip. Nevertheless, the C-shaped macronucleus of *E. epistyliformis* is flattened, located in half of the body, and follows the longitudinal axis of the zooid, in contrast to the transverse position of the macronucleus of *E. camprubii*. In addition, Stiller's guide (1971) does not specify in the text or in the drawings whether the stalk of *E. epistyliformis* is longitudinally striated or not. *E. variabilis* shows variability in the stalk and branches that strongly resembles that of *E. camprubii*. Colonies of *E. variabilis* inhabiting calm waters present long and smooth branches, while the branches of colonies from vigorous water flows are shorter, articulated and thicker or show uneven thickness. Nevertheless, there is no information on the specific width of the stalk in *E. variabilis*, and the main stalk of the colony is always short, a feature that does not always occur in *E. camprubii*. Moreover, *E. variabilis* zooids are funnel-shaped, in contrast to the vase-shaped zooids of *E. camprubii*. Finally, *E. thienemanni*

Table 1. Mean, standard deviation (SD), maximum values (Max), minimum values (Min), coefficient of variation (CV) and number of measurements realized (N) of morphological characteristics of *Epistylis camprubii*.

	Mean	SD	Max	Min	CV	N
Zooid length, *in vivo* (µm)	58.7	10.1	98.1	35.3	17.2	343
Zooid width, *in vivo* (µm)	32.0	5.4	65.2	18.0	16.8	342
Peristomial disc diameter, *in vivo* (µm)	15.5	1.9	21.3	11.2	12.1	94
Peristomial lip height, *in vivo* (µm)	7.8	1.2	10.6	5.0	15.9	149
Peristomial lip width, *in vivo* (µm)	24.2	2.9	31.7	16.2	11.8	110
Stalk width, *in vivo* (µm)	5.3	0.9	8.4	3.1	16.9	152
Number of silverlines from peristome to aboral trochal band	120.8	10.1	136.0	106.0	8.4	11
Number of silverlines from aboral trochal band to scopula	40.7	5.1	48.0	33.0	12.5	11
Macronucleous characteristics	C-shaped, transversely oriented, in the adoral half of the cell					
Number of contractile vacuoles, position	One, in the adoral third of the body, on dorsal wall of vestibulum					

Table 2. Minimum (Min) and maximum values (Max) of the main physicochemical parameters in which *Epistylis camprubii* was observed, and comparison with available ecological data of other *Epistylis* species.

	Epistylis camprubii	*E. chrysemydis*	*E. coronata*	*E. hentscheli*	*E. plicatilis*	*E. rotans/ E. procumbens*
	Min–Max	Min–Max	Min–Max	Min–Max	Min–Max	Min–Max
Soluble Chemical Oxygen Demand ($mg \cdot L^{-1}$)	53.8–415.3	–	–	–	–	–
Ammonium ($N\text{-}NH_4^+$, $mg \cdot L^{-1}$)	0.54–491	2.1–5.9	0–1.6	0–1.9	0–27	0–0.018
Free ammonia ($N\text{-}NH_3$, $mg \cdot L^{-1}$)	0.024–18.01	–	–	–	–	–
Nitrites ($N\text{-}NO_2^-$, $mg \cdot L^{-1}$)	0–572.1	0.1–0.8	–	–	0–61	–
Free nitrous acid ($N\text{-}HNO_2$, $mg \cdot L^{-1}$)	0.00013–0.47	–	–	–	–	–
Nitrates ($N\text{-}NO_3^-$, $mg \cdot L^{-1}$)	0–321	1.5–4.2	–	–	0.14–52	–
Temperature (°C)	22.6–33.6	10–35	2–12	2–32	6.6–32	6–23
pH	6.3–8.66	7.0–8.6	7.2–7.6	7.0–8.6	4.7–8.5	7.2–7.8

Table 3a. Comparison between *Epistylis camprubii* and the other *Epistylis* species mentioned in the manuscript: characteristics of zooid and persitome.

Species	Body lenght *in vivo* (µm)	Body width *in vivo* (µm)	Zooid shape	Peristomial disc diameter *in vivo* (µm)	Peristomial disc shape	Peristomial lip height *in vivo* (µm)	Peristomial lip width *in vivo* (µm)	Number of peristomial lips	Data source
Epistylis camprubii	35.3–98.1	18.0–65.2	Vase-shaped	11.2–21.3	Rounded, pointed, rarely umbilicated	5.0–10.6	16.2–31.7	1	Present manuscript
E. balatonica	90–100	45–55	Vase-shaped	–	–	–	–	2	Stiller 1971
E. chrysemydis	120–220	60–110	Vase-shaped	–	Umbilicated	–	50–80	2	Foissner *et al*. 1992
E. coronata	70–120	–	Vase-shaped	–	Slightly umbilicated and oblique	–	32–65	1	Foissner *et al*. 1992
E. entzii	125–190	80	Cylindrical	–	Convex, slightly oblique	–	–	1	Foissner *et al*. 1992
E. epistyliformis	43–62	20–27	Vase-shaped	–	Convex, sometimes conical	–	–	1	Stiller 1971
E. hentscheli	110–170	38–60	Asymmetric and bell-shaped, narrowed down to the stem	–	Convex, slightly oblique	–	–	1	Foissner *et al*. 1992
E. plicatilis	90–160	25–50	Funnel-shaped	–	Not umbilicated	–	36–60	1	Foissner *et al*. 1992
E. rotans/E. procumbens	60–140	2–2½ times as long as wide	Irregular (sigmoidal shape, bent at right angles, slightly tilted backwards)	–	Flat or slightly convex, slightly oblique	–	–	1	Foissner *et al*. 1999
E. rotans	70–100	–	Vase-shaped	–	Arched	–	–	1	Curds 1969
E. thienemanni	67–120	–	Vase-shaped	–	Conical	–	–	1	Stiller 1971
E. variabilis	50–100	–	Funnel-shaped	–	Slightly convex and obliquely protuberant	–	–	1	Stiller 1971

Table 3b. Comparison between *Epistylis camprubii* and the other *Epistylis* species mentioned in the manuscript: characteristics of stalk, macronucleus and contractile vacuole.

Species	Stalk width *in vivo* (µm)	Stalk striation / segmentation	Macronucleus	Contractile vacuole	Data source
Epistylis camprubii	3.1–8.4	Longitudinally striated, occasionally transeverse segmentation	C-shaped, transversely oriented; adoral half of the body	1, adoral third of the body, on dorsal wall of vestibulum	Present manuscript
E. balatonica	–	Longitudinally finely striated	Horinzotal horsehoe-shaped in the middle of the body	1, in the heigh of the peristomial lip	Stiller 1971
E. chrysemydis	13–25	Longitudinally striated, occasionally transeverse segmentation	C-shaped, transversely oriented; adoral half of the body	1, close or in the heigh of the peristomial lips, on ventral wall of vestibulum	Foissner *et al*. 1992
E. coronata	11–18	–	Semicircular, adoral half of the body	1, in the height of the peristomial lips, on dorsal wall of vestibulum	Foissner *et al*. 1992
E. entzii	18	–	3/4 circular, adoral half of the body	1, in the heigh of the peristomial lip, on dorsal wall of vestibulum	Foissner *et al*. 1992
E. epistyliformis	–	Transverse segmentation	Intenselly flattened, horsehoe-shaped	1, adoral third of the body	Stiller 1971
E. hentscheli	12–20, sometimes 25	Occasionally finely annulated	Semicircular, adoral half of the body	1, in the heigh of the peristomial lip, on ventral wall of vestibulum	Foissner *et al*. 1992
E. plicatilis	7–18	Longitudinally finely striated	Semicircular, adoral half of the body	1, in the heigh of the peristomial lip, on dorsal wall of vestibulum	Foissner *et al*. 1992
E. rotans/E. procumbens	–	Fine longitudinally striated, transverse segmentation	Reniform to semicircular, in transverse axis and adoral half of zooid	1, at level of peristomial lip, on dorsal wall of vestibulum	Foissner *et al*. 1999
E. rotans	–	Longitudinally striated, transeverse segmentation	C-shaped, transversely oriented, adoral third of the body	1, located in the adoral third of the body	Curds 1969
E. thienemanni	–	–	Flattened ribbon-like and horsehoe-shaped, adoral third of the body	1, located in the peristomial disc, on dorsal wall of vestibulum	Stiller 1971
E. variabilis	Variable	–	–	1, at the level of the peristomial lip	Stiller 1971

(Stiller 1971, first reported as *Rhabdostyla thienemanni* by Nenninger 1948) is an epibiont of leeches, and is larger (67 to 120 µm), but with a shorter stalk than *E. camprubii*. It has the contractile vacuole located in the peristomial disc. A summary of the comparison between *Epistylis camprubii* and the other *Epistylis* species can be seen in Tables 3a and 3b.

9. Comments. The great variability observed in the appearance of the stalk of *Epistylis camprubii* could be related to water hydrodynamics, as Stiller (1971) suggested regarding the stalk variability of *E. variabilis*. The authors also consider that the low number of zooids observed per colony (63% of colonies showed between 2 and 10 zooids, Fig. 4) may also be influenced or limited by the flow, agitation speed or other hydrodynamic parameters of water, in addition to the availability of food (bacteria) in the reactor. Further research is needed in order to shed light on these unanswered questions.

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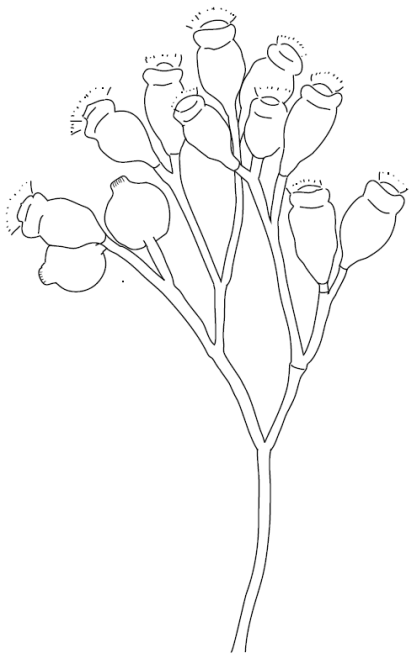
3.4. CAPÍTOL 3

MICROEUKARYOTE COMMUNITY IN A PARTIAL NITRITATION REACTOR PRIOR TO ANAMMOX AND AN INSIGHT INTO THE POTENTIAL OF CILIATES AS PERFORMANCE BIOINDICATORS



Resum

S'ha dut a terme un estudi en profunditat, a llarg termini i multidisciplinar amb l'objectiu d'estudiar la comunitat microeucariota en un reactor de nitritació parcial (PN) anterior a un reactor anammox. El reactor PN va operar amb tecnologia moving bed biofilm reactor (MBBR), utilitzant suports plàstics (*carriers*) pel desenvolupament del biofilm. La comunitat microeucariota del biofilm (BF) i del líquid que l'envolta (licor mescla o ML) va ser analitzat de forma independent. Tot i les condicions fisicoquímiques a les que operava el reactor PN (mitjana de 305.9 ± 117 mg TAN l^{-1} i 328.4 ± 131.9 mg $N\text{-}NO_2^-$ l^{-1}), es van observar fins a 24 taxons microeucariotes mitjançant anàlisis microscòpiques. Les espècies microeucariotes van mostrar una distribució desigual en el reactor PN, fet que suggereix l'existència de dos hàbitats clarament diferenciats: el BF, preferit per espècies amb estructures específiques d'adherència al substrat, com els perítrics pedunculats, i el ML, preferit per espècies lliure nedadores o que no depenen de substrat. Els resultats indiquen que les dinàmiques poblacionals de la gran majoria de ciliats va respondre a variacions en la concentració d'àcid nitrós i amoníac lliure i, en menor mesura, a la concentració de sDQO. Al BF, les variacions poblacionals de *Epistylis camprubii* i *Opercularia coarctata* suggereixen l'existència de competència a causa de solapament de nínxols ecològics. S'ha realitzat un estudi molecular (Ilumina) de la regió V4 del gen 18S rDNA d'algunes mostres amb l'objectiu de detectar el màxim nombre d'espècies eucariotes detectades microscòpicament durant el període d'estudi. Les dades de diversitat i abundància obtingudes mitjançant els dos mètodes han estat comparades. L'estudi ha ajudat a determinar rangs de tolerància més amplis dels taxons microeucariotes als paràmetres fisicoquímics analitzats.

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Microeukaryote community in a partial nitritation reactor prior to anammox and an insight into the potential of ciliates as performance bioindicators

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ABSTRACT

An in-depth, long-term, multidisciplinary study was conducted in order to study the microeukaryote community in a partial nitritation (PN) reactor prior to anammox. The PN reactor operated with moving bed biofilm reactor (MBBR) technology, using plastic supports (carriers) for biofilm development. The microeukaryote community from the biofilm (BF) and the surrounding media (mixed liquor or ML) were analysed separately. Despite the physicochemical conditions under which the PN-MBBR operated (an average of 305.9 ± 117 mg TAN l^{-1} and 328.4 ± 131.9 mg N-NO_2^- l^{-1}), up to 24 microeukaryotic taxa were observed by microscope. Microeukaryote species showed an uneven distribution in the PN-MBBR, thus suggesting the existence of two habitats: the BF, preferred by species with specific structures for adhering to a substrate, such as the stalked Peritrichia, and the ML, preferred by free-swimming or non-substrate dependent species. The results indicated that most ciliate population dynamics mainly responded to the nitrous acid and free ammonia concentrations and, to a lesser extent, to sCOD values. In the BF, variations in the population of *Epistylis camprubii* and *Opercularia coarctata* suggest the existence of competition between these species due to niche overlap. A V4 18S rDNA molecular survey (Illumina) was carried out for some samples with the aim of obtaining maximum coverage of the main eukaryote species that were microscopically detected throughout the study. The diversity and abundance data provided by both detection methods were compared. The study helped identify broader tolerance ranges of the microeukaryote taxa to the physicochemical parameters analysed.

Introduction

One of the main goals in wastewater treatment research is to reduce energy consumption. In this contextthe combined partial nitritation (PN) – anaerobic ammonium oxidation (anammox) process is one of the most promising technologies for treating high ammonium-loaded wastewatersuch as sludge digester liquor livestock wastewater and landfill leachates. Compared with traditional nitrification-denitrification processes the PN-anammox process implies a 50%–60% reduction in oxygen demand does not require an external organic carbon source for denitrification and reduces sludge production by about 90% [1–3]. Over the last decade a variety of PN-anammox configurations have been developed and full-scale implemented. These configurations include the moving bed biofilm reactor (MBBR) process granular sludge processes SBR RBC and activated sludge systems [2]. However the successful application of the two-stage PN-anammox process relies on how effectively the ammonium is partially oxidised into nitrite thus providing an adequate influent to the anammox reactor *i.e.* an influent with a nitrite:ammonium ratio of 1.32 [1]. In addition the anammox bacteria are sensitive to a great variety of abiotic parameters such as dissolved oxygen biodegradable organic carbon and ammonium and nitrite concentrations [4] which can inhibit the anammox activity. Therefore optimising the PN step could also serve as a way to control these inhibitor parameters and ensure the success of the whole PN-anammox process.

Extensive studies have been conducted on the prokaryote community that develops in the innovative PN-anammox process [5–7], but no detailed information regarding the microeukaryote community of a PN reactor has yet been published. Microeukaryotes have been reported in a wide variety of wastewater

Abbreviations: Anammox, anaerobic ammonium oxidation; BF, biofilm; MBBR, moving bed biofilm reactor; ML, mixed liquor; OTU, operational taxonomic unit; PN, partial nitritation; sCOD, soluble chemical oxygen demand; TAN, total ammonia nitrogen; TBFS, total biofilm solids; TMLS, total mixed liquor solids.

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treatment processes and technologies [8–10], where they play an important role in ensuring the correct performance of treatment processes and are very useful bioindicators of process performance [8]. Protists and metazoa have also been described in processes to treat high ammonium-loaded wastewater, such as the single reactor system for high activity ammonium removal over nitrite (SHARON) [3] and the anoxic/oxic shortcut biological nitrogen removal moving bed biofilm reactor (A/O SBNR-MBBR) [11], both processes that focus on limiting the oxidation of ammonia to nitrate and accumulating the oxidised N-forms as nitrite.

Traditionally the study of eukaryote communities in wastewater treatment plants has been conducted by microscope but recent advances in molecular technology have led to the increased use and implementation of next-generation sequencing methods such as 454 pyrosequencing and Illumina as the main tools for assessing the diversity and abundance of microeukaryote organisms in the aquatic environment [12,13]. In wastewater treatment systems next-generation sequencing technology has been applied primarily to the study of the prokaryote community and very few authors [14] have applied these technologies for the study of the eukaryotic microorganisms that inhabit the biological reactors in wastewater treatment plants.

The aim of the present research was to conduct the first in-depth long-term multidisciplinary study of the microeukaryote community inhabiting a PN reactor with MBBR technology and to assess the potential of microeukaryote species as performance bioindicators.

Material and methods

Pilot plant configuration

The study was carried out in a 2 m^3 volume reactor containing 1 m^3 of carriers (K1 AnoxKaldnes, with a specific surface of 500 $m^2\,m^{-3}$) held in suspension by stirrers (diameter of 400 mm, velocity of stirrers 50 rpm). The carriers were maintained in the tank by means of a perforated tube with a pore diameter smaller than the carrier diameter, which prevented the loss of carriers with the effluent. Tank aeration was provided by a blower connected to a perforated tube, and the dissolved oxygen concentration was controlled with an online sensor (LDO Hach Lange). The system also incorporated online temperature, pH and nitrite analysers, and the signals from these were sent to a SCADA system. Influent flow was controlled by cavity pumps.

The system was fed with centrifuged leachates from the anaerobic digester at the wastewater treatment plant in Rubí, Barcelona. The wastewater was heated in a settler before being fed into the PN reactor. The average influent flow was $59.8 \pm 21.2\,L\,h^{-1}$, with a maximum value of 100 and a minimum value of 15. Water remained in the PN reactor for an average of 1.7 ± 0.9 days.

Physicochemical parameters

Physicochemical data from the settler (influent) and PN reactor were analysed. Total and soluble chemical oxygen demand (tCOD and sCOD), total ammonia nitrogen (TAN), nitrites and nitrates were analysed according to Hach Lange test kits. Dissolved oxygen, pH and temperature were measured online. Alkalinity, total suspended solids (TSS) and volatile suspended solids (VSS) were measured according to standard methods [15]. Analyses of total and volatile suspended solids were conducted independently for biofilm and mixed liquor samples, which allowed the total biofilm solids (TBFS), volatile biofilm solids (VBFS), total mixed liquor solids (TMLS) and volatile mixed liquor solids (VMLS) to be obtained. The fractions of uncharged ammonia (free ammonia or N-NH_3) and nitrous acid (N-HNO_2) were calculated as follows:

$$\mathrm{N-NH_3}\left(mgl^{-1}\right) = \frac{Total\ ammonia\ as\ N\ \left(mgl^{-1}\right)}{1 + \left(10^{-pH}\right)/\left(e^{-6344/(temperature^{\circ}C+273)}\right)}$$

$$\mathrm{N-HNO_2}\left(mgl^{-1}\right) = \frac{Total\ nitrite\ as\ N\ \left(mgl^{-1}\right)}{1 + \left(e^{-2300/(temperature\,^{\circ}C+273)}\right)/\left(10^{-pH}\right)}$$

Sampling and sample treatment

Microscopic analyses were carried out weekly over a period of 15 months. Two samples were analysed for each sampling procedure: mixed liquor (ML) and biofilm (BF). Biofilm was detached from the carriers as described in Canals et al. [11]. Briefly, 80 mL of mixed liquor was filtered using a 0.45 μm membrane, then the carriers were collected with tweezers and suspended in the previously filtered mixed liquor. The biofilm was then carefully scraped off the carriers with a 4–5 mm diameter laboratory brush. The mixed liquor samples were collected directly from the reactor.

Counts of filamentous bacteria and eukaryote community (protists and metazoa)

Microscopic analyses were performed using a Zeiss Axioskop 40 microscope (Carl Zeiss, Germany). The abundance of filamentous bacteria was measured according to Salvadó [16] and expressed as metres per millilitre ($m\,ml^{-1}$). Filamentous bacteria counts were carried out by analysing two 25 μL replicates *in vivo* at 400 × magnification by phase contrast (20 × 20 mm coverslip). Although these organisms are prokaryotes rather than eukaryotes, their abundance was measured due to the serious negative effect they can have on the performance of most conventional wastewater treatment systems and their involvement in the macrostructure of the mixed liquor.

Ciliates, metazoa and amoeboid and flagellated organisms larger than 20 μm were counted according to Madoni [17] by analysing two 25 μL replicates *in vivo* at 100 × magnification using a 20 × 20 mm coverslip. The abundance of amoeboid and flagellated organisms smaller than 20 μm was approximated *in vivo* at 400 × magnification by counting 30 microscopic fields on the coverslip (20 × 20 mm) for each replicate. The counts were carried out using well-homogenised samples. Abundance values were expressed as individuals per millilitre ($ind\,ml^{-1}$). In the case of biofilm, abundances were obtained as individuals per square centimetre ($ind\,cm^{-2}$) and transformed to $ind\,ml^{-1}$ using the specific surface of carriers ($500\,m^2\,m^{-3}$).

The identification of protist species was conducted at different levels: ciliates were determined to species level according to Foissner [18], Foissner et al. [19–21] and Canals and Salvadó [22], while flagellated and amoeboid protists were determined according to Jeuck and Arndt [23], Lee et al. [24] and Page [25]. The frequency of species detection was calculated by comparing the number of samples in which the species appeared with the total number of samples, and was expressed as a percentage. The results of the eukaryotic taxa are expressed in accordance with the taxonomic classification proposed by Adl et al. [26].

Illumina analysis: sampling and DNA extraction

Illumina analysis was carried out as a complement to the microscopic results in order to verify the feasibility of the microscopic and molecular analyses. Two BF and two ML samples,

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stored at −80 °C, were chosen in order to obtain maximum coverage of the main eukaryote species that were microscopically detected throughout the sampling process. The abundance and diversity results of the microscopic analysis of these four samples are shown in Supplementary Table S1.

Nucleic acids from these four samples were extracted separately using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA), in accordance with the manufacturer's instructions. The nucleic acid extracts were quantified and quality checked with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and stored at −80 °C. Similar DNA amounts of each of the four samples were mixed (500 ng of DNA in total) and sent to the Research & Testing Laboratory (Texas, USA) for Illumina sequencing.

Illumina analysis: sequencing analysis

The mixed DNA sample was used as a template for amplifying the V4 region of the 18S rRNA gene using the universal eukaryotic primers TAReuk454FWD1 and TAReukREV3 [27], and the PCR product was sequenced in a MiSeq Illumina sequencer. Paired-end reads from each amplicon were merged by FLASH and analysed for quality and chimera detection using mothur v.1.33.1 [28]. Reads with an average Phred score lower than 25 in sliding windows of 50 base pairs were discarded, and chimera detection was performed using ChimeraSlayer [29] based on the alignment file released by SILVA 108 (http://www.arb-silva.de). Individual Illumina reads were clustered by Swarm [30], with only one base-pair difference considered between nodes and the sequence from the most abundant and connected node considered as representative of the operational taxonomic unit (OTU). Representative sequences were then taxonomically assigned using BLAST [31] against the SILVA 115 reference database (http://www.arb-silva.de).

Statistical analysis

Relationships between ciliates and selected environmental variables were analysed and visualised in reduced dimensions using distance-based redundancy analysis (dbRDA). RDA is a multivariate technique that provides ordination of a species matrix where the axes are constrained to be the best linear combinations of environmental variables. It implicitly uses the Euclidean distance, so the technique is akin to multivariate multiple regression. However, the Euclidean distance often proves to be inadequate for species abundance data because it is overly sensitive to abundant species, so the Bray-Curtis dissimilarity is often preferred. Distance-based RDA extends RDA to other dissimilarities [32]. We applied dbRDA to a Bray-Curtis distance matrix calculated on log-transformed abundance data to produce separate ordination diagrams for the mixed liquor and the biofilm. The significance of the first two axes was checked using permutation tests. Analyses were carried out with the "capscale" function in the vegan package [33] using R version 3.3.1 [34]. As a measure of the association between individual species and environmental variables, the nonparametric Spearman's rank correlation coefficient was used (Statgraphics Centurion XVI software package).

Results

Pilot plant performance and carrier colonisation

The physicochemical and operational parameter values of the influent and PN reactor are shown in Table 1. Throughout the whole study period, the average nitrite:ammonium ratio in the PN reactor was 1.4 ± 1.5, and 90% of the oxidised N-forms were accumulated as nitrites.

As expected in a MBBR system, the biomass within the PN reactor mainly adhered to the carriers instead of remaining in suspension. Specifically, the average TBFS percentage represented 81.2 ± 8.2% of the total solids in the system, with a maximum percentage of 92.5% and a minimum of 49.1%. The evolution of BF colonisation is shown in Supplementary Fig. S1c. During the first four months of the study, carrier colonisation gradually increased until it reached stable values of between 1 and 1.3 g TBFS l^{-1}. In April, the system became destabilised due to unexpected and repeated outages in the pilot plant facility that in some cases lasted longer than 48 h, and the TBFS dropped to below 1 g l^{-1}. These outages affected influent pumping, oxygenation and other system performance mechanisms. The TBFS recovered to stable values when the outages ended. Supplementary Fig. S1b shows the

Table 1
Mean, standard deviation (SD), minimum (Min) and maximum values (Max) and number of analyses performed (n) on the physicochemical and operational parameters in the influent and PN reactor.

	Influent				Partial Nitritation Reactor			
	Mean ± SD	Max	Min	n	Mean ± SD	Max	Min	n
TMLS (mg l^{-1})	–				216.7 ± 103.4	427.5	37.5	56
VMLS (mg l^{-1})	–				172.2 ± 93.7	375.0	17.5	55
TBFS (mg l^{-1})	–				1,015.9 ± 232.2	1299.7	393.7	56
VBFS (mg l^{-1})	–				829.3 ± 210.0	1220.2	313.6	55
Soluble COD (mg l^{-1})	526.2 ± 378.2	2600	108	274	216.1 ± 95.6	459.7	27.8	275
Soluble COD removal (%)	–				53.2 ± 15.6	88.8	5.7	272
Total COD (mg l^{-1})	773.4 ± 326.7	1587	216	45	473.9 ± 170.6	794.3	159.3	45
Total COD removal (%)	–				35.9 ± 13.8	65.8	1.8	45
Alkalinity	2,481.5 ± 525.8	3598	1102	287	499.7 ± 242.0	1552	160	286
pH	7.7 ± 0.1	8.4	7.3	297	6.7 ± 0.4	7.9	6.1	297
Temperature (°C)	37.9 ± 5.2	50	28.9	297	30.6 ± 1.5	34.8	24.2	296
TAN (mg l^{-1})	683.8 ± 192.1	1110	277	297	305.9 ± 117.0	586	34.4	296
Ratio sCOD:TAN	0.79 ± 0.54	2.7	0.22	274	0.22 ± 0.19	2.06	0.06	272
TAN removal (%)	–				54.7 ± 14.0	92.7	18.0	296
Free ammonia (mg l^{-1})	–				1.66 ± 1.68	11.17	0.12	296
Nitrites (mg l^{-1})	–				328.4 ± 131.9	628.6	15.8	296
Nitrous acid (mg l^{-1})	–				0.21 ± 0.18	0.71	0.003	296
Ratio nitrite:ammonium	–				1.4 ± 1.5	12.11	0.07	296
Nitrates (mg l^{-1})	–				27.3 ± 33.3	251	0	296
Dissolved Oxygen (mg l^{-1})	–				1.8 ± 0.6	3.0	1.0	295
Hydraulic retention time (days)	–				1.7 ± 0.9	5.6	0.8	296

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evolution of nitrogen forms and sCOD concentrations during the study period. Finally, the evolution of free ammonia and nitrous acid is shown in Supplementary Fig. S1a.

The macrostructure of the mixed liquor was fairly similar to that of the activated sludge, but showed much lower concentration of flocs and filamentous bacteria. The majority of flocs measured between 100 μm and 300 μm and presented a compact structure.

Microeukaryote community in the PN-MBBR

Up to 24 eukaryotic taxa were observed inhabiting the PN-MBBR during the study period (Table 2). Of these 24 taxa, nine belonged to the Alveolata species, four to Excavata, two each to Amoebozoa, Opisthokonta and Stramenopiles, and one each to Archaeplastida and Rhizaria. The three remaining undetermined taxa were classified as *Incertae sedis* and named depending on their morphology, *i.e.* flagellated or amoeboid organisms. Twenty-three taxa colonised the ML and 20 colonised the BF.

Despite the large number of species that colonised the PN-MBBR reactor, the average number of taxa detected per sampling procedure was much lower: 7.6 ± 1.7, with a maximum of 11 and a minimum of 4. The average number of taxa per sampling procedure was even lower when the ML and BF were analysed separately: an average of 6.6 ± 1.8 taxa per sampling procedure in the ML (maximum of 10 and minimum of 3) and 5.3 ± 1.7 in the BF (maximum of 8 and a minimum of 1).

Table 2
Mean, standard deviation (SD), minimum value (Min), maximum value (Max) and frequency (Freq; %) of eukaryotic taxa and filamentous bacteria in the ML and BF of the PN reactor. The symbol '*' alongside taxa indicates that counting was performed at 400 x magnification. A: amoeboid organism, C: ciliate, F: flagellate, M: metazoa, T: testate amoebae.

		Mixed Liquor				Biofilm			
		Mean ± SD	Max	Min	Freq	Mean ± SD	Max	Min	Freq
Filamentous Bacteria (m ml^{-1})		10.19 ± 17.82	86.20	0.47	100	9.77 ± 4.13	20.51	2.61	100
Alveolata (ind ml^{-1})									
Ciliophora	C	3140 ± 4915	26,700	0	96.1	1910 ± 1812	9055	0	98.0
Colpoda aspera	C	2532 ± 4915	26,620	0	68.6	56 ± 116	441	0	41.2
Cyclidium glaucoma	C	299 ± 1001	5220	0	19.6	4.1 ± 17	118	0	11.8
Cyrtolophosis acuta	C	0.4 ± 2.8	20	0	2.0	0	0	0	0.0
Epistylis camprubii	C	95 ± 168	760	0	52.9	1055 ± 1855	8996	0	60.8
Hypotrichia (not identified)	C	6.3 ± 26	160	0	9.8	0.4 ± 2.8	20	0	2.0
Opercularia coarctata	C	124 ± 314	1500	0	45.1	535 ± 854	3609	0	76.5
Vorticellides microstoma-complex	C	19 ± 68	440	0	15.7	95 ± 237	1142	0	29.4
Non-attached peritrichia	C	61 ± 99	520	0	62.7	148 ± 166	886	0	86.3
Other Ciliophora	C	2.7 ± 14	100	0	5.9	1.2 ± 8.8	63	0	2.0
Amoebozoa (ind ml^{-1})									
Cochliopodium sp. <20 μm *	T	23 ± 150	1071	0	3.9	0.8 ± 5.9	42	0	2.0
Other Discosea	T	0	0	0	0.0	0.4 ± 2.8	20	0	2.0
Archaeplastida (ind ml^{-1})									
Polytoma sp.	F	394 ± 960	5000	0	41.2	78 ± 227	1440	0	37.3
Excavata (ind ml^{-1})									
Kinetoplastea *	F	16,490 ± 53,590	250,692	0	31.4	2102 ± 6233	32,920	0	21.6
Euglenida <20 μm *	F	1415 ± 4831	23,569	0	13.7	153 ± 894	6327	0	9.8
Tetramitus rostratus	F	5678 ± 9257	37,300	0	76.5	104 ± 358	2520	0	39.2
Trimastix sp	F	7 ± 45	320	0	3.9	0	0	0	0.0
Opisthokonta (ind ml^{-1})									
Nematoda	M	0.8 ± 3.9	20	0	4	0	0	0	0
Nucleariids <20 μm *	A	7546 ± 16,981	113,561	0	64.7	458 ± 1120	6172	0	19.6
Rhizaria (ind ml^{-1})									
Cercomonas sp.-like *	F	42 ± 300	2143	0	2.0	0	0	0	0.0
Stramenopiles (ind ml^{-1})									
Anthophysa sp. *	F	1116 ± 5000	32,340	0	21.6	2.7 ± 19	138	0	2.0
Other Ochromonadales*	F	42,097 ± 179,935	1,133,471	0	17.6	4019 ± 14,593	73,812	0	11.8
Incertae sedis (ind ml^{-1})									
Amoeboid organisms <20 μm *	A	7462 ± 9579	51,424	0	88.2	4393 ± 4499	16,871	0	70.6
Flagellated sp1	F	1823 ± 10,358	71,400	0	9.8	48 ± 295	2087	0	5.9
Flagellated sp2	F	75 ± 538	3840	0	2.0	0.4 ± 2.8	20	0	2.0

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The most diverse phylogenetic group was the Ciliophora, with at least nine different taxa colonising the PN reactor during the study period. The frequency with which Ciliophora occurred was 96.1% and 98% in the ML and BF, respectively, thereby presenting an average of 2.3 ± 1.2 (maximum of 6 and minimum of 0) and 2.2 ± 0.9 (maximum of 4 and minimum of 0) taxa per sampling procedure in the ML and BF, respectively. Compared to the total taxa observed per sampling procedure, Ciliophora represented an average of 42.7 ± 18.4 and 62.5 ± 22.4% of the taxa present in the ML and BF, respectively, but these values occasionally reached 83.3% in the ML and the entire diversity in the BF. *Colpoda aspera*, *Cyclidium glaucoma*, *Epistylis camprubii*, *Opercularia coarctata* and *Vorticellides microstoma*-complex were the most abundant and/or frequent Ciliophora species.

Although all Ciliophora species (with the exception of *Cyrtolophosis acuta*, which was observed only in the ML) were detected in both the ML and BF, the microscopic results presented an uneven distribution of the microeukaryote taxa within the PN-MBBR reactor, thus suggesting the existence of two clearly differentiated habitats, *i.e.* ML and BF, and consequently an ecological distribution of ciliate species (Fig. 1). As expected, the ML was colonised mainly by free-swimming species, organisms that remained in suspension in the liquid phase, whilst the BF was dominated by sessile species, which can adhere to a fixed substrate using specific structures, *e.g.* Peritrichia stalks. As an example, 96.6% of the total abundance (total abundance understood as the sum of ML and BF abundance) of the free-swimming *Colpoda aspera* was detected in the ML samples, while 82.1% of the total abundance of the sessile *Epistylis camprubii* was observed in the BF samples. Surprisingly, non-attached Peritrichia were the only free-swimming organisms that were more abundant in the BF than in the ML.

The ciliate community composition varied throughout the whole study period in both the ML and BF habitats (See Supplementary Fig. S2). In the ML, the data (Table 3, Fig. 2a) suggest a negative relationship between the concentrations of *Cyclidium glaucoma* and sCOD (Spearman's correlation coefficient = −0.6512; p-value < 0.001) and nitrous acid (Spearman's correlation coefficient = −0.5777; p-value < 0.001). Meanwhile, it seems likely that *C. aspera* developed mainly at higher TAN concen-

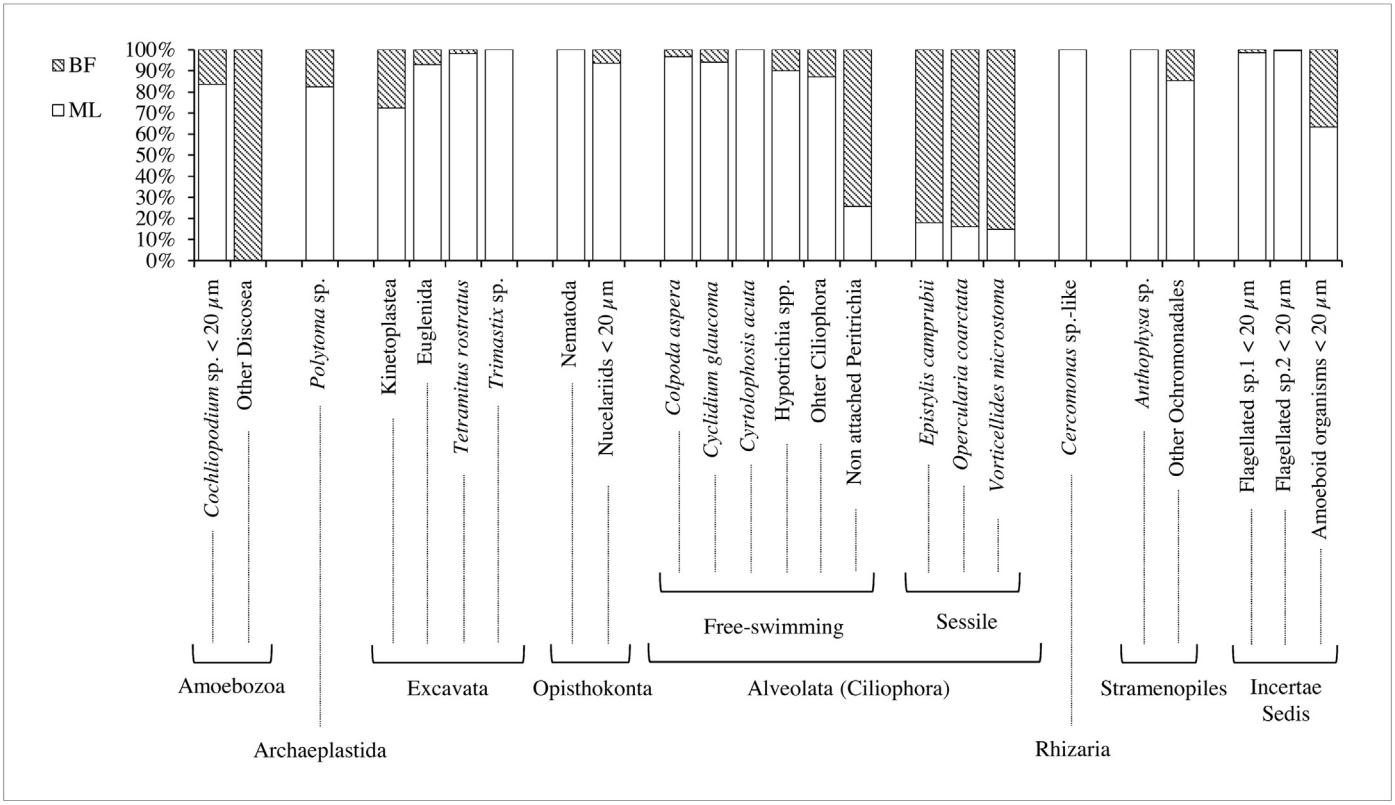

Fig. 1. Distribution of population percentages of each taxon in the PN reactor. The percentage of the biofilm population is shown in grey, while the percentage of the mixed liquor population is shown in white.

Table 3
Spearman's correlation coefficients between abundance variations of the main ciliate species and soluble chemical oxygen demand (sCOD), total ammonia nitrogen (TAN), free ammonia, nitrite ($N—NO_2^-$) and nitrous acid. '*' indicates a p-value ≤ 0.001.

Taxa	Partial Nitritation Reactor				
	sCOD	TAN	Free ammonia	$N\text{-}NO_2^-$	Nitrous acid
Colpoda aspera		0.5936*			
Cyclidium glaucoma	−0.6512*			−0.5486*	−0.5777*
Epistylis camprubii	−0.528*		0.4774*		−0.4655*
Opercularia assymetrica	0.6132*			0.4919*	0.6087*
Vorticellides microstoma-komplex					−0.5414*

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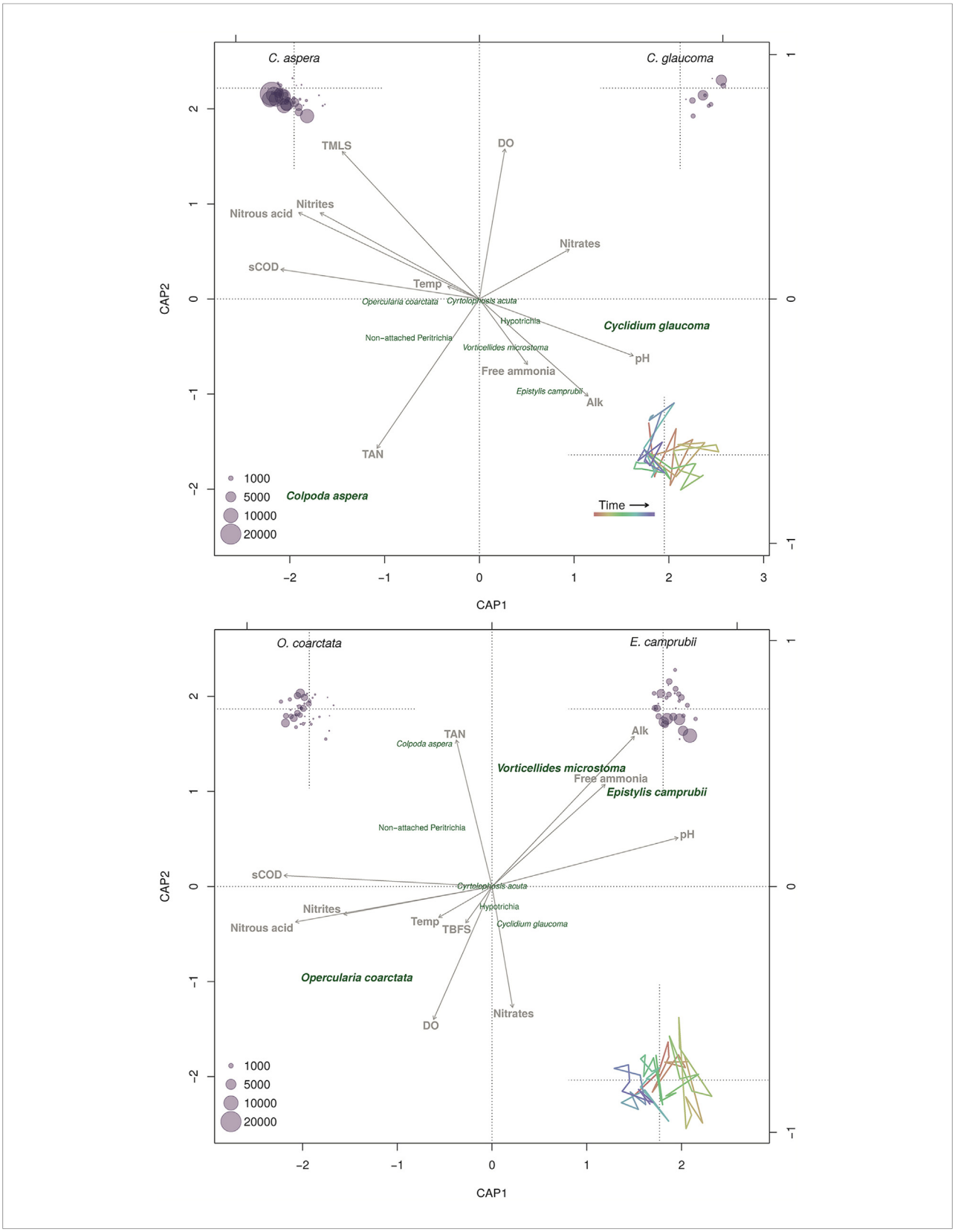

Fig. 2. a) Direct ordination diagrams of ciliate assemblage against selected environmental variables using distance-based redundancy analyses for mixed liquor (ML). Abundance plots for the focal species of this study are shown in insets as bubble plots, with abundance proportional to symbol size according to the legend at the bottom left of the graph. The temporal sequence of observations is also shown as a colour-coded line at the bottom right of each graph. In both cases, the first two axes were significant at $p < 0.001$. **b**) Direct ordination diagrams of ciliate assemblage against selected environmental variables using distance-based redundancy analyses for biofilm (BF). Abundance plots for the focal species of this study are shown in insets as bubble plots, with abundance proportional to symbol size according to the legend at the bottom left of the graph. The temporal sequence of observations is also shown as a colour-coded line at the bottom right of each graph. In both cases, the first two axes were significant at $p < 0.001$.

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trations (Spearman's correlation coefficient = 0.5936; p-value < 0.001) and, to a lesser extent, at high sCOD values (not a statistically supported correlation). In the BF, statistical analyses (Table 3, Fig. 2b) indicate that *Epistylis camprubii* positively responded to the free ammonia concentration (Spearman's correlation coefficient = 0.4774; p-value < 0.001), and negatively correlated with sCOD (Spearman's correlation coefficient = -0.5280; p-value = 0.001) and nitrous acid (Spearman's correlation coefficient = -0.4655; p-value < 0.001), among others. *Opercularia coarctata* seemed to positively respond to sCOD (Spearman's correlation coefficient = 0.6132; p-value < 0.001) and nitrous acid (Spearman's correlation coefficient = 0.6087; p-value < 0.001). Lastly, *Vorticellides microstoma*-complex dynamics seemed to respond positively to free ammonia (not a statistically supported correlation) and negatively to nitrous acid (Spearman's correlation coefficient = -0.5414; p-value < 0.001).

Besides the Ciliophora, most non-ciliated taxa showed a clear preference for the ML over the BF (see Fig. 1). The Excavata *Tetramitus rostratus*, the Archaeplastida *Polytoma* sp., nucleariids <20 μm and the *Incertae sedis* amoeboid organisms <20 μm were the most relevant non-ciliated species in the PN reactor. Nevertheless, the maximum abundance values were obtained for the small flagellated organisms belonging to the Ochromonadales (Stramenopiles), with a maximum value of over a million ind ml^{-1}. Nucleariids were mainly observed attached to the surface of dead peritrich individuals, thus suggesting they may play a carrion feeder or predator role in the PN-MBBR process.

Filamentous bacteria did not reach high abundance in either the ML or the BF.

Table 4 shows the maximum and minimum values of abiotic parameters at which the species identified were observed in the PN-MBBR during the study period. Most of these data greatly increase the previously reported tolerance ranges [11].

Illumina results

A total of 26,718 reads of the V4 region in the 18S rDNA were obtained and clustered using Swarm into 2664 operational taxonomic units (OTUs). OTUs with fewer than five reads were discarded, and the remaining 34 OTUs were classified using the basic local alignment sequence tool (BLAST) against NCBI-nr. Some of these 34 OTUs were very similar to another OTU with greater amplicon representation and were discarded after alignment visualisation, as recommended by Decelle et al. [35]. In fact, the variability seen in these discarded OTUs was often found in highly conserved domains of the V4 18S rDNA region, which would suggest that they likely derived from sequencing errors. Finally, 16 OTUs, representing 88.1% of the initial reads, were obtained (Table 5). Seven OTUs corresponded to Alveolata (specifically to Ciliophora), four to Opisthokonta, two to Archaeplastida and one each to Amoebozoa, Rhizaria and Stramenopiles. No Excavata sequences were obtained.

Eleven out of these 16 OTUs showed a sequence similarity >95% to their closest BLAST hit. The OTUs with most reads corresponded to *Opisthonecta henneguyi* (42.66%) and *Epistylis camprubii* (28.07%). The OTUs that corresponded to *Colpoda aspera*, *Telotrochidium matiense* and *Spumella elongata* accounted for 1%–10%, whereas the remaining OTUs represented less than 1% of the reads.

Discussion

The eukaryote community in the PN-MBBR process showed strong similarities to that of an A/O SBNR-MBBR process [11]. Specifically, 14 of the 24 taxa observed in the PN-MBBR have also been reported to inhabit the A/O SBNR-MBBR process. This correspondence in terms of taxa diversity can be explained by the similar abiotic characteristics of the two processes. Both PN and SBNR processes are wastewater treatment technologies that are designed to treat high ammonium-loaded wastewater and focus on limiting the complete oxidation of ammonia to nitrate, thus accumulating the oxidised N-forms as nitrite.

Despite the fact that the PN reactor operates at high ammonium and nitrite concentrations, both compounds that are *a priori* adverse for eukaryotic microorganisms [36–38], the microscopic analysis revealed that a notable number of microeukaryote species colonised the PN-MBBR. Indeed, several taxa were observed in the reactor for long and continuous periods of time, and in some cases presented frequency values of between 60% and 80% (Table 2). Surprisingly, the greatest number of these species belonged to ciliates instead of flagellated organisms; the latter are commonly used as bioindicators of polluted and stressful conditions in other wastewater treatment processes [17,39].

In terms of nitrogen compounds, the PN-MBBR showed two clearly differentiated phases during the study period (See Supplementary Fig. S1a): the free ammonia phase, which corresponded to the first seven months of the study and was characterised by high free ammonia concentrations (mean of 2.63 ± 1.87 mg l^{-1}) and low nitrous acid values (mean of 0.05 ± 0.03 mg l^{-1}); and the nitrous acid phase, which was characterised by an increase in nitrous acid (mean of 0.33 ± 0.10 mg l^{-1}) and a reduction in unionised ammonia (mean of 0.7 ± 0.44 mg l^{-1}). The nitrous acid phase continued up to the end of the analyses. This shift responded mainly to a reduction in pH (mean of 7.03 ± 0.18 and 6.39 ± 0.17 during the free ammonia and nitrous acid phases, respectively). The dynamics of the ciliate species, with the exception of *Colpoda aspera*, seemed to respond to this toxicity scenario and, to a lesser extent, to variations in sCOD.

In the ML, *Cyclidium glaucoma* appeared during the declining sCOD period of the free ammonia phase (See Supplementary Figs. S1 and S2), whilst *Colpoda aspera* showed an irregular pattern of occurrence and was the only ciliate species that abundantly colonised the PN reactor, even in the free ammonia and nitrous acid phases (See Supplementary Figs. S1 and S2). In the BF, *Epistylis camprubii* developed mainly in the free ammonia phase and reached greater abundance when sCOD presented lower

Table 4
Minimum and maximum values of soluble chemical oxygen demand (sCOD), total ammonia nitrogen (TAN), free ammonia, nitrite ($N\text{-}NO_2^-$), nitrous acid, nitrate ($N\text{-}NO_3^-$), temperature and pH at which the most relevant eukaryotic species were observed.

	sCOD	TAN	Free ammonia	$N\text{-}NO_2^-$	Nitrous acid	$N\text{-}NO_3^-$	Temperature	pH
Colpoda aspera	54–415	216–491	0.38–6.26	117.3–511.7	0.015–0.47	0–58.1	28.2–32.5	6.3–7.37
Cyclidium glaucoma	54–127	154–386	1.28–2.75	28.2–341.7	0.006–0.065	0–221.2	28.5–32.2	7–7.14
Epistylis camprubii	53.8–415.3	65.5–491	0.4–6.26	28.2–572.1	0.006–0.47	0–221.2	28.2–32.2	6.3–7.37
Opercularia coarctata	53.8–415.3	42.4–491	0.15–8.78	28.2–572.1	0.006–0.598	0–221.2	28.2–34	6.24–7.45
Vorticellides microstoma-complex	53.8–406.6	42.4–364	0.4–8.78	37.5–572.1	0.007–0.112	0–115.3	29–34	6.45–7.45
Tetramitus rostratus	53.8–415.3	42.4–491	0.15–8.78	28.2–572.1	0.006–0.598	0–221.2	28.2–34	6.24–7.45
Polytoma sp.	83.5–415.3	65.5–400	0.38–8.78	37–572.1	0.007–0.421	0–115.3	29.1–34.0	6.3–7.45

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Table 5
List of the 16 main OTUs obtained by Illumina. The symbol '‡' indicates that the microscopic correspondence of the OTU was not observed in the samples used for Illumina analysis, but was detected in other samples throughout the study.

OTU	Phylogenetic group	Closest Cultured Match	Number of reads (%)	Similarity (%)	Closest Match	Similarity (%)	Microscopic concordance	Microscopic abundance ind ml^{-1} (%)
1	Amoebozoa	Amoebozoa sp.	11 (0.04)	336/395 (85.1)	SIBO1176_N12D1_18S_E	273/298 (91.6)	Amoeboid organisms <20 μm	7251 (59.62)
2	Archaeaplastida, Chlorophyceae	*Polytoma uvella*	68 (0.26)	378/378 (100)			*Polytoma* sp.	54 (0.45)
3	Archaeaplastida, Chlorophyceae	*Pyrobotrys stellata*	21 (0.08)	373/378 (98.7)	SB12_2010	378/378 (100)	–	–
4	Opisthokonta, Holozoa, Metazoa	*Rhabditella axei*	29 (0.11)	369/369 (100)			Nematoda ‡	0 (0)
5	Opisthokonta, Nucletmycea, Fungi	*Trichosporon montevideense*	14 (0.05)	380/380 (100)			–	–
6	Opisthokonta, Nucletmycea, Fungi	*Leucogyrophana mollusca*	12 (0.05)	197/222 (88.7)	D53	340/344 (98.8)	–	–
7	Opisthokonta, Nucletmycea, Fungi	*Glomus mosseae*	10 (0.04)	195/221 (88.2)	Re-St.7-47	338/344 (98.3)	–	–
8	Alveolata, Ciliophora, Colpodea	*Colpoda aspera*	1787 (6.75)	371/375 (98.9)	Colpoda sp.	373/374 (99.7)	*Colpoda aspera*	2515 (20.68)
9	Alveolata, Ciliophora, Peritrichia	*Epistylis camprubii*	7426 (28.07)	369/369 (100)			*Epistylis camprubii*	909 (7.48)
10	Alveolata, Ciliophora, Peritrichia	*Opisthonecta henneguyi*	11,288 (42.66)	368/368 (100)			Non-attached peritrichia	267 (2.20)
11	Alveolata, Ciliophora, Peritrichia	*Telotrochidium matiense*	2391 (9.04)	365/369 (98.9)			Non-attached peritrichia	267 (2.20)
12	Alveolata, Ciliophora, Scuticociliata	*Cyclidium glaucoma*	17 (0.06)	374/374 (100)			*Cyclidium glaucoma*	395 (3.25)
13	Alveolata, Ciliophora, Spirotricheae	*Paraparentocirrus sibillinensis*	86 (0.33)	374/374 (100)			Hypotrichia spp. ‡	0 (0)
14	Alveolata, Ciliophora, Spirotricheae	*Gonostomum strenuum*	18 (0.07)	375/376 (99.7)	Stichotrichia sp. St05	375/376 (99.7)	Hypotrichia spp. ‡	0 (0)
15	Rhizaria, Cercozoa	*Allantion* sp.	7 (0.03)	114/130 (87.7)			*Cercomonas* sp.-like ‡	0 (0)
16	Stramenopiles, Chrysophyceae	*Spumella elongata*	345 (1.30)	354/383 (92.4)	RW2_2011	367/380 (96.6)	*Anthophysa* sp.	485 (3.99)

concentrations, while *Opercularia coarctata* showed greater abundance during the nitrous acid phase (See Supplementary Figs. S1 and S2). The opposite abiotic preferences of both sessile species, together with the fact that they coexisted largely in the PN-MBBR, suggest the existence of competition between them, most probably due to an ecological niche overlap. In this scenario, the toxicity conditions of the PN reactor, depending on the free ammonia and nitrous acid values, together with the sCOD concentration, would determine the colonisation success of both stalked ciliates in the BF.

The high tolerance of microeukaryote species to nitrogen forms in the PN reactor may be explained by the protozoan species' capacity to acclimatise. The acclimatisation of ciliates and gymnamoebae to ammonia nitrogen was observed by Puigagut et al. [36] in microeukaryote communities in activated sludge, although at lower ammonia concentrations than those referred to in the present manuscript. Some studies concerning the toxicity of nitrogen forms (mainly ammonia and nitrites) to microeukaryotes have been conducted [37,38,40,41], but no reports regarding the acclimatisation of microeukaryote taxa to nitrites or to the unionised nitrous acid and free ammonia forms have been found. The fact that free ammonia values were almost permanently higher than those of nitrous acid (even in the nitrous acid phase) may suggest that nitrous acid is more harmful than free ammonia to ciliates.

The aim of the PN process is to provide an influent with a nitrite: ammonium ratio of 1.32 for the subsequent anammox process. From our point of view, bioindication relies on the principle that each species has a specific tolerance range to each abiotic parameter. In this context, the search of bioindicator species for the nitrite:ammonium ratio value makes little sense, since the same ratio can be obtained at very different nitrite and ammonium concentrations, with different toxic effects for the microeukaryote community. Moreover, as shown in the present study, two samples showing the same nitrite:ammonium ratio at the same concentration can have different toxic effects on the microeukaryote community due to the influence of the pH on the nitrite:nitrous acid and ammonium:unionised ammonia ratios.

It is interesting to highlight the importance of long-term studies of strictly controlled and physicochemically monitored ecosystems, especially if they are colonised by a very low number of taxa. The lower food-web complexity in such ecosystems makes it possible to obtain fairly clear results regarding the interspecific relationships and the relationship between these species and the environmental factors that hinder or enhance their development. In addition, it should be noted that the findings of the present study concerning the relationship between microeukaryote taxa and abiotic parameters apply only to the abiotic conditions and concentration ranges under which the PN-MBBR operated.

Comparing microscopic and illumina results

The Illumina analysis provided 16 main OTUs (Table 5), only 13 of which were comparable by microscopic observation, since the remaining three OTUs belonged to Fungi, organisms that were not considered in the microscope analysis. A total of 12 eukaryotic taxa were obtained microscopically (See Supplementary Table S1), and four of these were consistent with the molecular results: the green algae *Polytoma* sp. and the ciliates *Epistylis camprubii*, *Colpoda aspera* and *Cyclidium glaucoma*. Five taxa were not detected in the molecular survey: nucleariids <20 μm, the ciliates *Opercularia coarctata* and *Vorticellides microstoma*-complex, and the Excavata *Tetramitus rostratus* and some kinetoplastids. Stoeck et al. [13] proposed some reasons to explain the amplification or sequencing

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failure of some ciliate species, including the presence of longer inserts in the V4 region and mismatches in the used primers. The absence of Excavata sequences in the Illumina results was somewhat expected, since the V4 region of the 18S gene for this group is known to be longer and, therefore, is amplified inefficiently [42].

The taxon known as 'amoeboid organisms <20m' was assigned to OTU 1, which was affiliated within Amoebozoa, while the stramenopile *Anthophysa* sp. was assigned to OTU 16. Both sequences showed a low percentage of similarity to their closest cultured matches (85.1% and 92.4%, respectively), which indicates that the corresponding organism has not been cultured and sequenced at the 18S rDNA region. These two cases exemplify the need for comprehensive taxonomic studies in order to provide morphological and molecular data on more eukaryote species, since, as stated by Stoeck et al. [13], the value of a molecular sequence data set can only be as strong as the underlying reference database.

With respect to the molecular results, five of the 16 OTUs obtained were not detected by microscopy in the samples analysed, although the majority were observed in other samples throughout the study period (Table 2). This suggests that these taxa were indeed present in the analysed samples, but at abundances lower than the detection limit of the counting methodology or as resting stages. Both possibilities have previously been considered by [12,13]. Another putative explanation concerns the amplification of non-living DNA. Lastly, the free-swimming peritrich OTUs *Opisthonecta henneguyi* and *Telotrochidium matiense* were assigned to the taxa termed 'non-attached Peritrichia'.

As shown in Table 5, substantial differences were observed between the abundance values obtained by microscopy and those yielded by the molecular survey. In accordance with the observations of Medinger et al. [12] and Stoeck et al. [13], our results also yielded an overestimation of ciliates. This could be explained by the high number of copies of the rDNA operon in ciliates compared with other eukaryotic groups [43]. The results obtained do not support the Illumina analysis as feasible for determining the abundance of eukaryote taxa in these samples, which are dominated by relatively large protist species.

Conclusions

A microeukaryote community inhabiting the PN-MBBR, a wastewater treatment system designed to operate at high ammonia and nitrite concentrations, has been described here. The taxa that form part of the microeukaryote community should be considered highly tolerant to ammonia, nitrite and at least one of the unionised forms of these nitrogen compounds, *i.e.* free ammonia and nitrous acid. As a consequence of the high nitrogen compound concentrations at which the PN-MBBR operates, broader tolerance ranges of the microeukaryote taxa to the abiotic parameters were reported.

The use of MBBR technology implied an uneven distribution of the microeukaryote community in two habitats, the mixed liquor and the biofilm. As expected, the mixed liquor was predominantly colonised by free-swimming or non-substrate dependent organisms, while the biofilm was colonised mainly by sessile species. Free ammonia, nitrous acid and, to a lesser extent, sCOD concentrations were the physicochemical parameters that best explained the colonisation success of ciliate species in both BF and ML habitats. Temporal segregation between *Epistylis camprubii* and *Opercularia coarctata* was observed in the BF, most probably due to differential competitive abilities in response to variations in the aforementioned abiotic parameters. In addition, the results obtained suggest that nitrous acid has a higher toxicity effect for ciliates than the toxicity presented by free ammonia.

Lastly, it is important to point out the need for exhaustive taxonomic studies in order to provide further morphological and molecular data on microeukaryote species.

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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.nbt.2017.05.003

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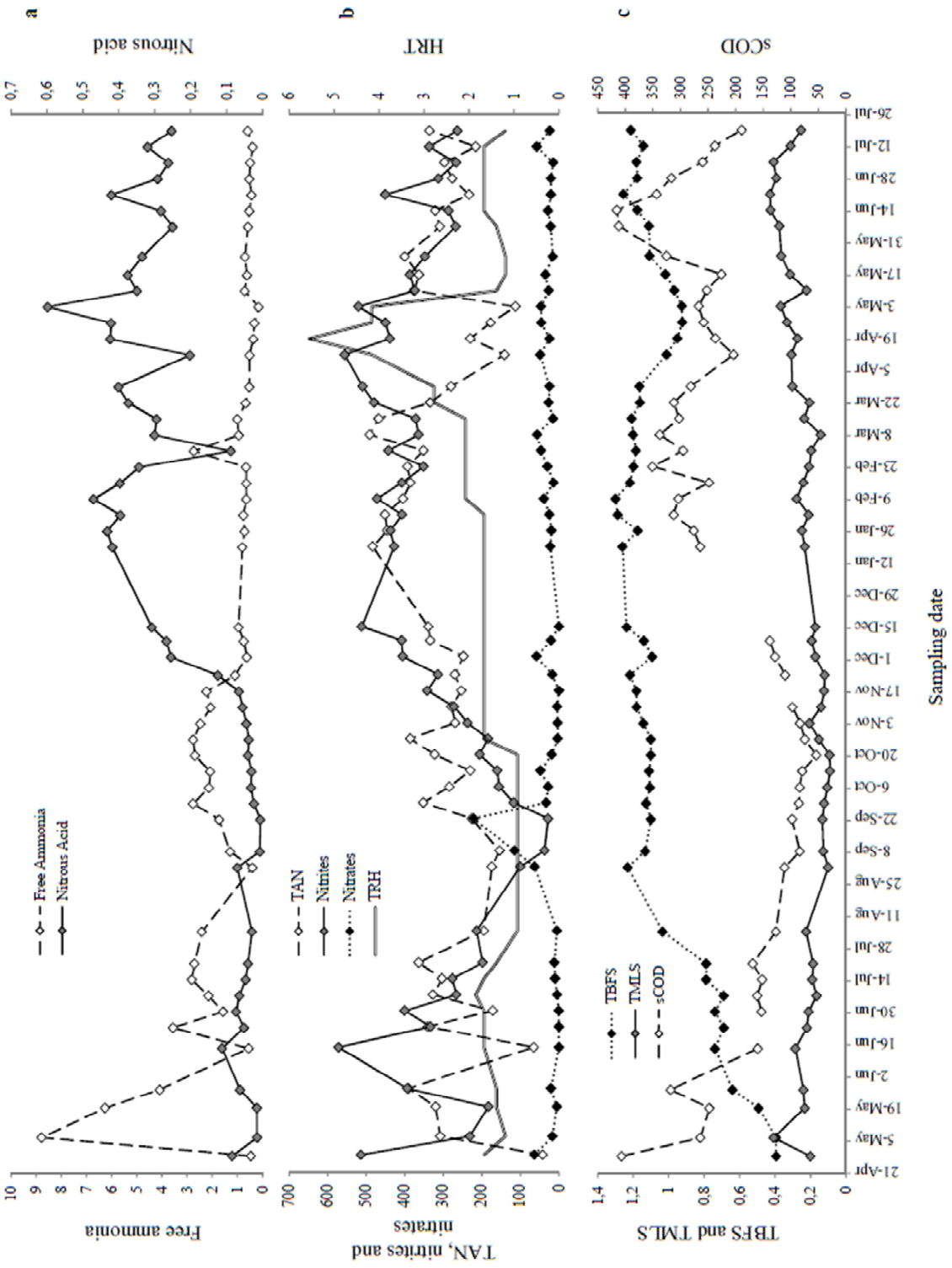
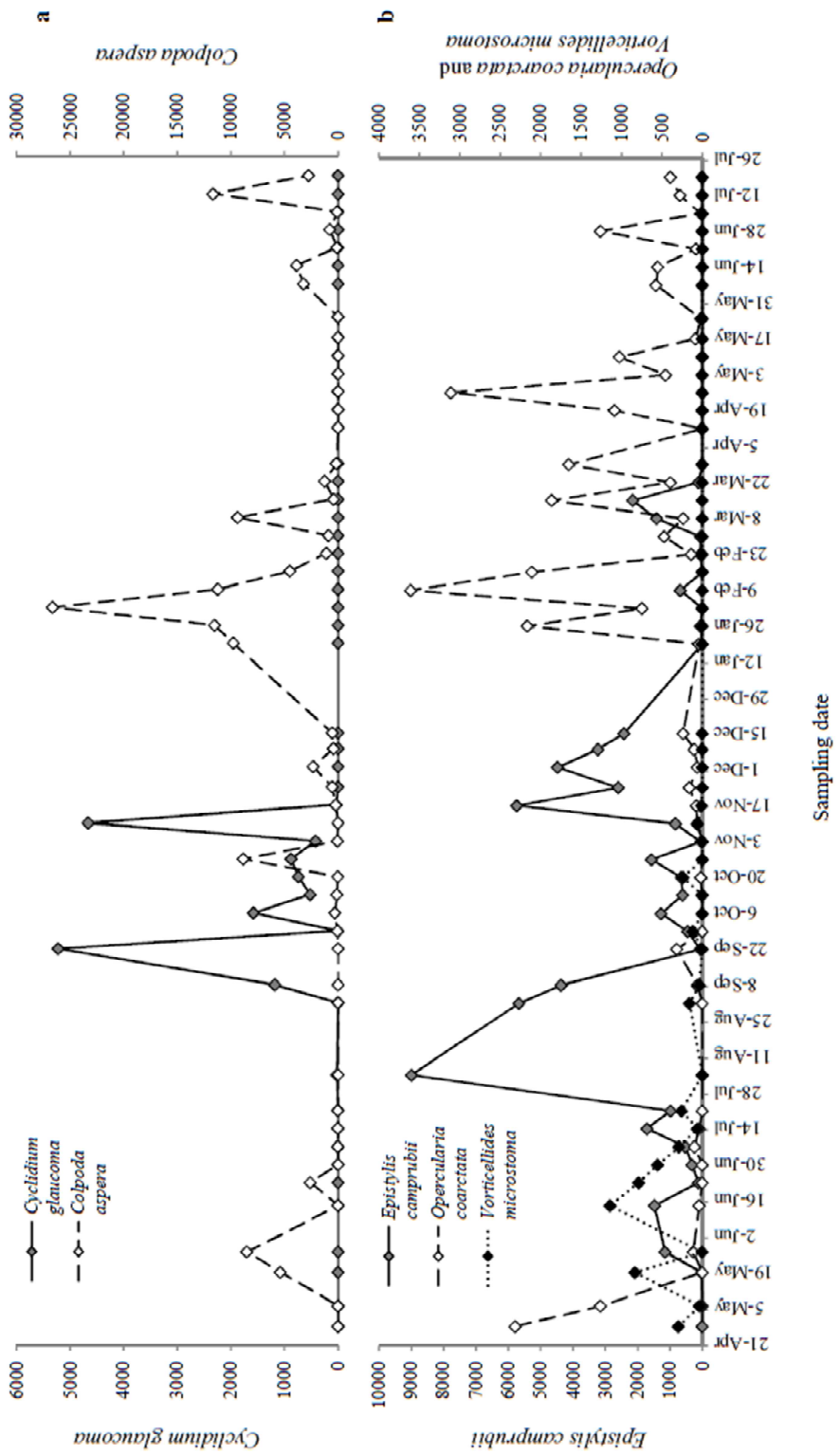


Supplementary Figure S1. Variation of free ammonia and nitrous acid (a); nitrite, nitrate, total ammonia nitrogen (TAN) and hydraulic retention time (HRT) (b); and total biofilm solids (TBFS), total mixed liquor solids (TMLS) and soluble chemical oxygen demand (sCOD) concentrations (c). Values are expressed in mg l^{-1}, with the exception of TBFS and TMLS, which are expressed in g l^{-1}, and HRT, which is expressed in days.



Supplementary Figure S2. Evolution of the abundance of *Cyclidium glaucoma* and *Colpoda aspera* in the ML (a) and *Epistylis camprubii*, *Opercularia coarctata* and *Vorticellides microstoma*-complex in the BF (b). Values are expressed in individuals ml^{-1}.

Supplementary Table S1. List of the taxa and abundance values of eukaryote taxa in the samples chosen for Illumina analysis. The symbol '*' alongside taxa indicates that counting was performed at 400x magnification. A: amoeboid organism, C: ciliate, F: flagellate, M: metazoa.

Taxa		Mixed Liquor samples		Biofilm samples		Average Abundance
		ML 1	ML 2	BF 1	BF 2	
Archaeplastida (ind ml^{-1})						
Polytoma sp.	F	20	0	197	0	54
Excavata (ind ml^{-1})						
Kinetoplastea *	F	0	4.285	4.218	0	2.126
Tetramitus rostratus	F	1.260	300	118	0	420
Opisthokonta (ind ml^{-1})						
Nucleariids < 20 µm *	A	1.286	0	0	0	321
Alveolata (ind ml^{-1})						
Ciliophora						
Colpoda aspera	C	280	9.780	0	0	2.515
Cyclidium glaucoma	C	1.580	0	0	0	395
Epistylis camprubii	C	10	0	1.476	2.151	909
Opercularia coarctata	C	0	0	39	1.863	476
Vorticellides microstoma -complex	C	0	0	1.142	0	285
Non-attached peritrichia	C	100	520	315	134	267
Stramenopiles (ind ml^{-1})						
Anthophysa sp. *	F	1.940	0	0	0	485
Incertae sedis (ind ml^{-1})						
Amoeboid organisms < 20 µm*	A	1.286	19.284	8.436	0	7.251

4. DISCUSSIÓ GENERAL IV

4.DISCUSSIÓ GENERAL

La present memòria s'ha centrat en la caracterització de la comunitat microeucariota que es desenvolupa en dos sistemes avançats de tractament d'aigües residuals amb alta càrrega amoniacal operant amb la biomassa adherida a un substrat mòbil (MBBR) i la determinació d'aquelles espècies amb possible potencial com a bioindicadores del seu funcionament. Els resultats obtinguts han permès aportar noves dades sobre l'ecologia de les espècies que han colonitzat els dos sistemes, establint (o eixamplant) els rangs de tolerància d'aquests organismes al nitrogen amoniacal, nitrit, nitrat, amoníac lliure i àcid nitrós, entre altres paràmetres abiòtics. Per últim, s'ha valorat el potencial de la tècnica de *Next Generation Sequencing* Ilumina, comparant els resultats de biodiversitat obtinguts mitjançant l'anàlisi molecular amb els resultats obtinguts mitjançant microscòpia òptica.

L'estudi del biofilm en sistemes MBBR

La comunitat microeucariota associada als sistemes de tractament d'aigües residuals de biofilm ha estat objecte d'estudi per part de la comunitat científica probablement des de la implementació de cada un dels sistemes (Madoni 2011). Tot i això, l'estudi dels sistemes de biofilm sovint ha estat acompanyat de certa dificultat a l'hora de realitzar el mostreig. Curds i Cockburn (1970a) van estudiar la comunitat microeucariota d'un sistema de filtres percoladors mitjançant la observació de l'efluent, donada la dificultat d'obtenir una mostra representativa de biofilm del sistema. En sistemes RBC, Madoni i Ghetti (1981) van observar una mostra de biofilm diluïda en aigua però sense mesurar la superfície de volum que raspaven. Posteriorment, Kinner i Curds (1987) van intentar estandarditzar el mètode de mostreig en sistemes RBC, mesurant la superfície de biofilm a extreure i afegint el biofilm a un volum conegut d'aigua destil·lada. Martín-Cereceda et al. (2001), enlloc d'afegir aigua destil·lada al biofilm, van afegir aigua del mateix punt del mostreig prèviament filtrada fins a un volum de 20ml, fent possible així la obtenció de dades d'abundància comparables entre mostres.

Andreottola et al. (2000), comparant l'activitat de la biomassa d'un sistema MBBR i un sistema de fangs activats, van extreure la biomassa dels *carriers* mitjançant un raspall de laboratori de petit diàmetre, diluint posteriorment el raspat en un volum determinat i conegut d'aigua desmineralitzada. Coneixent la superfície específica dels *carriers*(superfície/volum) i el percentatge del reactor omplert amb aquests suports plàstics, van poder comparar els valors de sòlids en suspensió i adherits als suports plàstics. En el context dels estudis de la comunitat microeucariota, a causa de la importància dels processos de osmosi per a gran part d'aquests microorganismes, vam optar per emprar aigua prèviament filtrada del mateix reactor d'on es recollien els *carriers*, com anteriorment havien fet Martín-Cereceda et al. (2001) en sistemes RBC, i així mantenir el mínim alterades possibles les condicions fisicoquímiques originals. Per intentar evitar al màxim el pas d'individus del licor mescla cap al biofilm i viceversa, els *carriers* van ser transportats cap al laboratori suspesos en licor mescla prèviament filtrat en el mateix moment del mostreig. També es va eliminar l'aigua que quedava als espais buits dels *carriers* al treure'ls del reactor, per evitar al màxim possible contaminació d'organismes i sòlids del licor mescla.

El sistema d'extracció del biofilm proposat a la present memòria s'ha demostrat també útil per a l'estudi de la comunitat bacteriana de sistemes MBBR (Abzazou et al. 2015).

L'estudi de la comunitat microeucariota en sistemes MBBR és, fins al moment, molt escassa. De fet, la cerca d'articles ha estat pràcticament infructuosa, havent trobat només dos articles relacionats (Fried et al. 2000; Fried i Lemmer 2003) enfocats a l'estudi de microeucariotes en dos sistemes de biofilms (un amb *carriers* i l'altre amb *expanded shale*) operant de forma seqüencial. En aquests articles no es detalla amb suficient precisió el mètode d'anàlisi de les comunitats de microeucariotes, tot i que sembla que només es van realitzar anàlisis microscòpiques de les mostres de licor mescla (obviant la possible biomassa eucariota allotjada al biofilm), ja que els valors d'abundància dels diferents grups de microeucariotes es donen en individus per mil·lilitre. En aquests estudis, Fried i Lemmer (2003) conclouen que el sistema amb *expanded shale* presenta sempre una major abundància de ciliats que el sistema de *carriers*. Al mateix temps, però, observen també una quantitat molt elevada de biofilm a les parts internes dels *carriers*.

En els sistemes MBBR estudiats a la present memòria s'ha observat que l'abundància de les espècies amb capacitat per adherir-se de forma fixe a un substrat ha estat sempre molt major al biofilm que al licor mescla. En concret, de mitjana, el 93,2 i 82,1% de l'abundància total dels individus del ciliat sèssil *Epistylis camprubii* es van trobar al biofilm del sistema SBNR i el reactor PN, respectivament. Per la seva banda, les poblacions associades al biofilm de *Opercularia coarctata* i *Vorticellides microstoma*-complex va representar el 83,8 i 85% de l'abundància total d'aquestes espècies al conjunt del reactor PN. Els nostres resultats, doncs, indiquen la possibilitat que els estudis realitzats en sistemes MBBR, com el de Fried i Lemmer (2003),que no contemplen l'anàlisi independent de les comunitats de biofilm i de licor mescla probablement estaran subestimant la població de microeucariotes del sistema, principalment de la població de sessílids, amb més del 80% dels seus individus adherits al biofilm.

En base als nostres resultats, doncs, seria interessant que futurs estudis en sistemes MBBR contemplessin la realització d'anàlisis independents dels dos hàbitats (biofilm i licor mescla) per tenir una idea més aproximada a la realitat de l'abundància de les diferents espècies, així com garantir o apostar per mètodes d'extracció de la biomassa que permetin comparacions entre les poblacions dels diferents hàbitats, per tal de conèixer la importància relativa de cada població en el conjunt del procés.

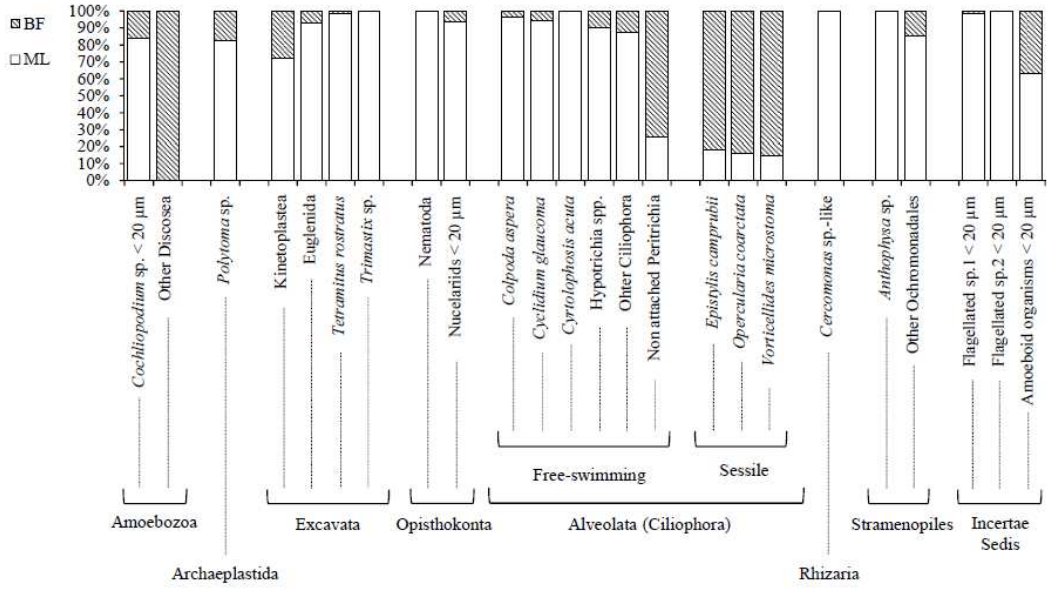
Diferenciació de comunitats en sistemes MBBR

Les anàlisis microscòpiques del licor mescla i del biofilm al sistema SBNR i al reactor PN han mostrat que la comunitat microeucariota no es presenta de forma homogènia en els dos hàbitats (licor mescla i biofilm) d'un sistema MBBR, sinó que en els dos processos existeix una diferenciació evident entre una comunitat més íntimament associada al licor mescla i una altra comunitat que colonitza majoritàriament el biofilm dels *carriers*. Aquesta diferenciació de comunitats respon al grau d'afinitat amb el substrat de les diferents espècies, com ja va observar anteriorment Curds 1965. Aquest autor va classificar les diferents espècies de ciliats dels sistemes de fangs actius en lliure-nedadors, que es desplacen activament per la columna

d'aigua, reptants, que es desplacen activament sobre el substrat però sense estar-hi adherit de manera fixe, i sèssils, que es troben íntimament associats al substrat, sovint mitjançant estructures d'adhesió com peduncles.

En els sistemes MBBR l'existència de dos hàbitats físicament diferenciats fa molt més evident la segregació de comunitats que la que es dóna en els sistemes de fangs actius. Aquesta diferenciació de comunitats es reflecteix de forma clara en l'abundància de les espècies en els dos hàbitats (figura 4.1), però té una incidència menor pel que fa a la biodiversitat. En aquest tipus de sistemes, les espècies adherides al biofilm i les associades al licor mescla no es poden mantenir aïllades, sinó que existeix un flux d'individus entre hàbitats, fet pel qual la gran majoria d'espècies van ser observades en ambdós subsistemes.

Figura 4.1. Distribució (en percentatge) de les poblacions del licor mescla (ML) i del biofilm (BF) de cada taxó. El percentatge de la població del biofilm es mostra en gris, mentre que el percentatge de la població del licor mescla es mostra en blanc.



Els taxons que van ser observats només en un hàbitat van aparèixer de forma més aviat esporàdica al sistema (majoritàriament amb valors de freqüència entre el 2 i el 4% al reactor PN i entre el 7 i el 13% al reactor aerobi del sistema SBNR, valors més elevats per haver realitzat un nombre menor de mostrejos) i amb abundàncies molt baixes, en la majoria de casos properes al límit de detecció del mètode d'anàlisi microscòpica. És més probable que l'absència d'aquests organismes a un dels subsistemes respongui a qüestions d'esforç de mostreig i de límit de detecció del mètode de recompte, i no a absència d'aquests taxons a un dels hàbitats.

La diferenciació de les comunitats té incidència també en l'estudi de la capacitat bioindicadora de les espècies. Com es pot observar en la taula 4.1, la població més representativa de cada espècie (és a dir, la població associada al licor mescla d'aquelles espècies lliure nedadores o la

població associada al biofilm de les espècies sèssils) és la que presenta relacions significatives entre variacions poblacions i els paràmetres fisicoquímics dels processos. Així, tot i que individus sèssils poden trobar-se al licor mescla dels sistemes MBBR, la seva presència i variacions poblacionals sembla que no venen explicades per les condicions ambientals (paràmetres fisicoquímics) sinó per la presència d'una població "mare" al biofilm que fa funció de reservori i punt de partida de processos d'expansió, les variacions poblacionals de la qual sí que es veuen estretament relacionades amb les condicions ambientals. El mateix fenomen succeeix amb les poblacions dels ciliats lliure nedadors, on la població més representativa, és a dir, l'associada al licor mescla, és la que presenta una major correlació amb els paràmetres abiòtics en termes de bioindicació.

Els resultats obtinguts confirmen de nou la importància de caracteritzar, de forma independent, les comunitats del biofilm i del licor mescla en sistemes MBBR (i probablement en el conjunt de sistemes de tractament que operen amb la biomassa adherida a un substrat), per poder determinar aquelles espècies del biofilm amb potencial bioindicador.

Espècie	Hàbitat	PN Reactor				
		sDQO	TAN	Amoníac lliure	$N\text{-}NO_2^-$	Àcid nitrós
Colpoda aspera (LN)	**ML**		**0,5936**			
	BF					
Cyclidium glaucoma (LN)	**ML**	**-0,6512**			**-0,5486**	**-0,5777**
	BF	-0,4890				
Epistylis camprubii (S)	ML					
	BF	**-0,5280**		**0,4774**		**-0,4655**
Opercularia coarctata (S)	ML					
	BF	**0,6132**			**0,4919**	**0,6087**
Vorticellides microstoma-komplex (S)	ML					
	BF					**-0,5414**

Taula 4.1. Coeficients de correlació de Spearman entre les variacions poblacionals del licor mescla (ML) i del biofilm (BF) de les principals espècies de ciliats i la DQO soluble (sDQO), el nitrogen amoniacal total (TAN), l'amoníac lliure, el nitrit ($N\text{-}NO_2^-$) i l'àcid nitrós al reactor PN. L'abreviació LN indica que l'espècie és lliure nedadora, mentre que la lletra S indica que l'espècie és sèssil. En negreta es marca la població majoritària de cada taxó.

Efectes de l'aigua residual amb alta càrrega amoniacal sobre la comunitat microeucariota

Hi ha pocs estudis a la bibliografia que contemplin l'efecte dels compostos inorgànics de nitrogen amoni, amoníac lliure, nitrit i àcid nitrós, sobre la comunitat microeucariota. Entre aquests destaquen els treballs realitzats per Puigagut et al. (2005, 2009), centrats en la toxicitat del nitrogen amoniacal. Aquests autors van observar com un increment puntual de la concentració de nitrogen amoniacal al medi (fins a valors de 80 mg/l; Puigagut et al. 2005)

tenia un efecte immediat negatiu sobre la diversitat de ciliats i sobre la seva abundància total. Tot i que en la majoria de casos (amb l'excepció dels experiments amb major concentració d'amoni) la població total acabava per recuperar-se, aquesta recuperació era protagonitzada únicament per una o dues espècies de ciliats, que representaven fins el 70% de l'abundància total. Posteriorment, Puigagut et al. (2009) van estudiar la comunitat microeucariota en un reactor seqüencial (SBR) tractant un influent amb una concentració mitjana d'amoni de 316 ± 63 mg/l, provinent d'una granja de porcs. En aquest estudi, Puigagut i col·laboradors van observar com concentracions de nitrogen amoniacal majors a 100 mg/l a l'efluent es relacionava amb una reducció de l'abundància de ciliats al reactor.

Com s'ha mostrat als capítols 1 i 3 de la present memòria, la comunitat microeucariota associada al sistema SBNR i al reactor PN, processos dissenyats pel tractament d'aigua residual amb alta càrrega amoniacal, ha presentat una diversitat de ciliats molt menor que les observades en sistemes de tractament d'aigües residuals més convencionals, tant en sistemes de biomassa en suspensió (Al-Shahwani i Noran 1991; Arévalo et al. 2009; Pérez-Uz et al. 2010) com en sistemes de biofilm (Martín-Cereceda et al. 2001, 2002; Fried i Lemmer 2003, Salvadó et al. 2004). Concretament, als processos SBNR i PN es van identificar microscòpicament un total de 7 espècies de ciliats (4 al sistema SBNR i 7 al reactor PN) durant tot el període de mostreig, de les que només 5 (*Colpoda* spp. i *Cyclidium glaucoma* al licor mescla; *Epistylis camprubii*, *Opercularia coarctata* i *Vorticellides microstoma*-complex al biofilm) van presentar abundàncies i freqüències elevades.

Aquests resultats, doncs, semblen confirmar l'efecte advers de concentracions elevades de nitrogen amoniacal sobre la diversitat de ciliats en sistemes de tractament d'aigües residuals, observada prèviament per Puigagut et al. (2005, 2009).

D'altra banda, és interessant comentar que precisament el baix nombre d'espècies de ciliats associats al sistema SBNR i al reactor PN ha permès comprendre de forma molt més precisa i profunda les dinàmiques poblacionals d'aquestes espècies. Degut al baix grau d'interaccions interespecífiques per absència de depredadors, molt freqüents en altres sistemes, i de competència per part d'altres espècies bacterívores, les variacions poblacionals de les principals espècies de ciliats han respost de forma quasi exclusiva a les variacions abiòtiques del medi (paràmetres fisicoquímics), permetent una descripció molt més completa d'aquelles espècies que no havien estat descrites (basada no només en els caràcters morfològics de les mateixes, sinó també en les seves característiques ecològiques), i l'establiment i/o eixamplament de les característiques ecològiques d'aquelles espècies ja descrites (Taula 4.2).

Taula 4.2. Valors mínim i màxim dels paràmetres fisicoquímics sDQO, TAN, amoníac lliure, nitrit, àcid nitrós, nitrat, temperatura i pH als quals van ser observats els diferents taxons. Aquests valors integren tant els resultats obtinguts al sistema SBNR com els obtinguts al reactor PN. (*Pàgina següent*). →

	sDQO	TAN	Amoníac lliure	$N\text{-}NO_2^-$	Àcid nitrós	$N\text{-}NO_3^-$	Temperatura	pH
Colpoda aspera	54-415	216-491	0,38-6,26	117,3-511,7	0,015-0,47	0-58,1	28,2-32,5	6,3-7,37
Colpoda ecaudata	180,0	0,54-207	0,02-11,56	28,8-194,2	<0,001-0,006	0,78-0,8	25,4-28,8	7,8-8,01
Cyclidium glaucoma	54-127	73,2-386	1,28-2,75	27,4-341,7	0,006-0,065	0-221,2	26,2-33,6	7-7,78
Epistylis camprubii	53,8-415,3	0,54-491	0,4-6,26	0-572,1	0,006-0,47	0-321	22,6-33,6	6,3-8,66
Opercularia coarctata	53,8-415,3	42,4-491	0,15-8,78	28,2-572,1	0,006-0,598	0-221,2	28,2-34	6,24-7,45
Vorticellides microstoma-complex	53,8-406,6	0,5-364	0,4-8,78	23-572,1	0,007-0,112	0-115,3	28,8-34	6,45-8,36
Tetramitus rostratus	53,8-415,3	42,4-491	0,15-8,78	28,2-572,1	0,006-0,598	0-221,2	28,2-34	6,24-7,45
Polytoma sp.	83,5-415,3	65,5-400	0,38-8,78	37-572,1	0,007-0,421	0-115,3	29,1-34	6,3-7,45

Toxicitat de l'amoníac lliure sobre els ciliats lliure nedadors *Colpoda* spp. i *Cyclidium glaucoma*

Tot i la presència ocasional de *Colpoda* spp. i de *Cyclidium glaucoma*, el licor mescla del sistema SBNR es va caracteritzar per presentar una població molt poc abundant i d'aparició esporàdica de ciliats lliure nedadors. En el capítol 1 de la present memòria es va atribuir aquesta incapacitat de colonització, per part dels ciliats, del licor mescla del sistema SBNR a la combinació de dos factors: toxicitat produïda per les elevades concentracions de nitrogen amoniacal (161,8 ± 80 mg/l, min: 34, max:312) (Puigagut et al. 2005, 2009; Xu et al. 2004, 2005); i exposició prolongada a condicions anòxiques (Dubber i Gray, 2011) a causa de l'elevat caudal de recirculació (de més del 400% del caudal d'entrada) entre els reactors anòxic i aerobi, que es traduïa en un temps de retenció hidràulic continuat a cada reactor de 7,6 ± 1,3 hores (min: 6,5; max: 13,3).

Durant l'estudi del reactor PN (capítol 3 de la present memòria), però, es va observar que tant *Colpoda* spp. com *Cyclidium glaucoma* eren capaços de colonitzar el licor mescla durant llargs períodes de temps (fins a 4 mesos *Colpoda* spp. i més de 2 mesos *Cyclidium glaucoma*) i amb abundàncies considerablement elevades, tot i que la concentració de nitrogen amoniacal en aquest reactor era significativament superior (305,9 ± 117 mg/l, min: 34,4, max: 586) a la que presentava el reactor anòxic del sistema SBNR. Aquests resultats semblaven invalidar, doncs, la hipòtesis de la toxicitat del nitrogen amoniacal sobre els ciliats *Colpoda* spp. i *Cyclidium glaucoma* com a possible causa de la seva incapacitat per colonitzar el licor mescla del sistema SBNR.

Arrel de la gran influència que les variacions d'amoníac lliure i àcid nitrós exerceixen sobre la comunitat de ciliats al reactor PN, es va calcular la concentració d'aquests compostos al

sistema SBNR, compostos que no havien estat contemplats durant el període d'estudi i de publicació dels resultats. Al reactor anòxic la concentració d'amoníac lliure va resultar ser sorprenentment elevada (23,9 ± 20,4 mg/l, min: 2,52, max: 86,3) en comparació amb els reactors aerobis SBNR (3,32 ± 5 mg/l, min: 0,02, max: 18,01) i PN (1,66 ± 1,68 mg/l, min: 0,12, max: 11,17), indicant que molt probablement sigui l'exposició prolongada de forma cíclica a l'elevada concentració de la fracció no ionitzada del nitrogen amoniacal (i no la fracció ionitzada o el valor de nitrogen amoniacal total), en combinació amb les condicions anòxiques, el que impedeix la colonització del licor mescla del sistema SBNR per part dels ciliats *Colpoda* spp. i *Cyclidium glaucoma*.

Aquests resultats sembla que fan vàlida per als ciliats l'escrit per Sobczyk et al. (2015): "*la toxicitat de la solució es correlaciona positivament amb la quantitat de amoni no ionitzat (...). L'amoni ionitzat només és moderadament tòxic per als animals aquàtics, mentre que l'amoni no ionitzat és molt tòxic i els seus efectes negatius són àmpliament descrits en la literatura*".

Efectivament, existeixen bastantes referències sobre l'efecte de l'amoni no ionitzat sobre animals aquàtics, tant vertebrats com invertebrats (Snell i Janssen 1995; Arauzo i Valladolid 2003; Sobczyk et al. 2015), però el seu efecte sobre protists s'ha estudiat en comptades ocasions (Xu et al. 2005). La toxicitat del nitrogen amoniacal sobre protists, en canvi, sí ha captat més l'atenció dels investigadors, principalment en el context del tractament d'aigües residuals (Puigagut et al. 2005; Xu et al. 2004, 2005; Klimek et al. 2012).

Seria convenient que en futurs estudis sobre la capacitat bioindicadora de les espècies microeucariotes en sistemes de tractament d'aigües residuals, especialment en aquells sistemes que tracten aigües residuals amb alta càrrega amoniacal, es contemplin les formes no ionitzades de l'amoni i del nitrit com a paràmetres independents, així com la realització d'estudis de toxicitat d'aquests compostos sobre les espècies de protists.

Integrant resultats del sistema SBNR i PN per a una millor comprensió de l'ecologia de *E. camprubii* i *Opercularia coarctata*

Els resultats de la present memòria recolzen el potencial de *Epistylis camprubii* com a espècie bioindicadora tant en el sistema SBNR com al reactor PN. De totes maneres, crida l'atenció que la seva dinàmica poblacional es correlacioni amb paràmetres fisicoquímics diferents ens els dos sistemes estudiats. En concret, les variacions poblacionals de *Epistylis camprubii* es correlaciona amb la concentració de nitrogen amoniacal total o TAN (coeficient de correlació de Spearman = -0.6524; p-valor = 0.0156) al sistema SBNR; i amb la concentració de sDQO (coeficient de correlació de Spearman = -0.5280; p-valor ≤ 0.001), àcid nitrós (coeficient de correlació de Spearman = -0.4655; p-valor ≤ 0.001) i amoníac lliure (coeficient de correlació de Spearman = 0.4774, p-valor ≤ 0.001) al reactor PN.

Aquest fet, per si sol, es pot considerar normal si els rangs de valors dels paràmetres en els dos processos són suficientment diferents, com de fet succeeix amb les concentracions d'àcid nitrós i de TAN. Com es pot observar a les figures 4.2 i 4.3, els rangs de TAN i àcid nitrós als quals van operar el sistema SBNR i el reactor PN presenten poca superposició. En aquests dos

casos, l'anàlisi integrada de les dades, amb la conseqüent ampliació del rang de valors dels paràmetres (un rang de 0 a 500 mg/l en el cas del TAN), permet obtenir un coneixement més profund de l'efecte d'aquests paràmetres abiòtics sobre *Epistylis camprubii*. Els resultats de l'anàlisi integrada semblen confirmar que, efectivament, la dinàmica poblacional de *Epistylis camprubii* es veu afectada negativament pel TAN (coeficient de correlació de Spearman = -0.5326; p-valor ≤ 0.001) i per l'àcid nitrós (coeficient de correlació de Spearman = -0.7316; p-valor ≤ 0.001).

Figura 4.2. Gràfic de dispersió de l'abundància de *Epistylis camprubii* al biofilm i la concentració de nitrogen amoniacal total (TAN). Els rombes blancs corresponen als resultats obtinguts al sistema SBNR, mentre que els rombes negres fan corresponen als resultats del reactor PN.

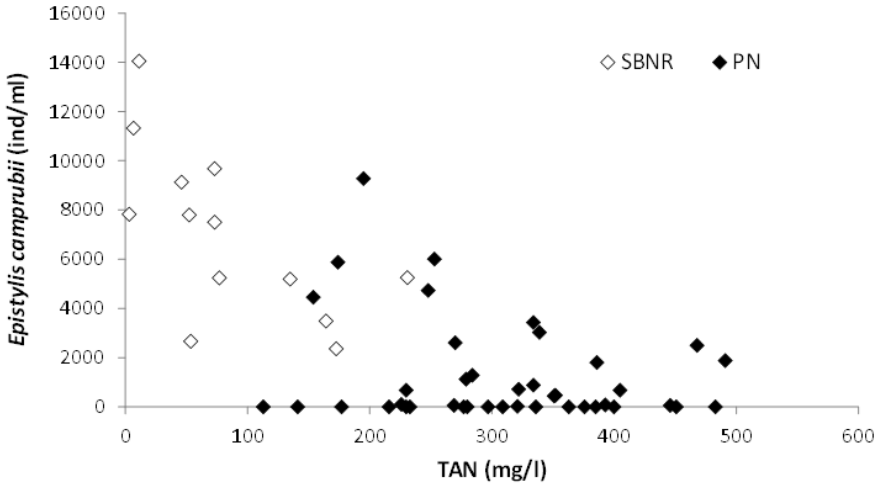
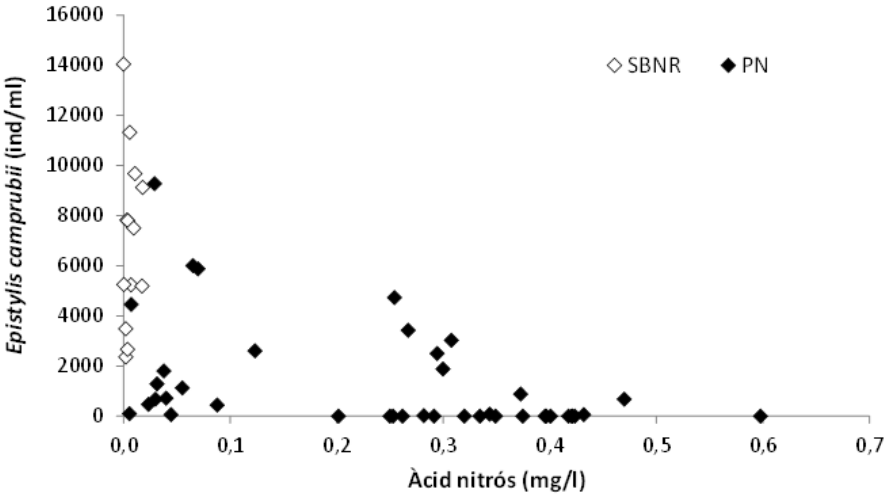
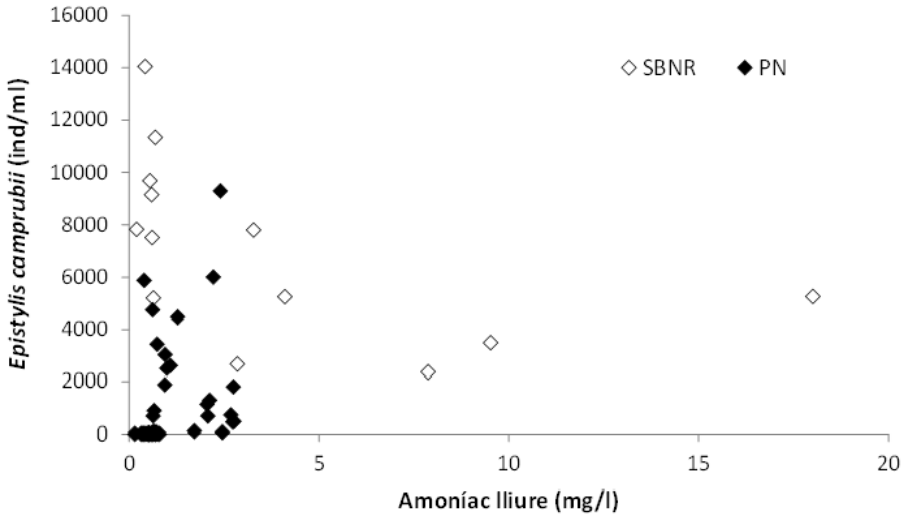


Figura 4.3. Gràfic de dispersió de l'abundància de *Epistylis camprubii* al biofilm i la concentració d'àcid nitrós. Els rombes blancs corresponen als resultats obtinguts al sistema SBNR, mentre que els rombes negres fan corresponen als resultats del reactor PN.



Sí que existeix, en canvi, solapament de rangs dels valors d'amoníac lliure i de sDQO entre el sistema SBNR i el reactor PN. En relació a l'amoníac lliure (figura 4.4), l'anàlisi integrada dels resultats no recolza la correlació positiva obtinguda al reactor PN entre aquest paràmetre i la dinàmica poblacional de *Epistylis camprubii*, tot i que tampoc indica que la concentració d'aquest compost en el rang de valors estudiat (de 0 a 18 mg/l) afecti negativament la dinàmica poblacional d'aquesta espècie de sessílid, evidenciant l'elevada capacitat de resistència o tolerància d'aquest perítric a la toxicitat produïda per l'amoníac lliure en el rang de concentracions estudiat.

Figura 4.4. Gràfic de dispersió de l'abundància de *Epistylis camprubii* al biofilm i la concentració d'amoníac lliure. Els rombes blancs corresponen als resultats obtinguts al sistema SBNR, mentre que els rombes negres fan corresponen als resultats del reactor PN.



L'anàlisi integrada de les dades tampoc recolza la correlació negativa obtinguda al reactor PN entre la dinàmica poblacional de *Epistylis camprubii* i la concentració de sDQO. A la figura 4.5 es pot observar clarament que els dos processos (SBNR i PN) van operar a valors de concentració de sDQO força similars, tot i que l'abundància de *Epistylis camprubii* al sistema SBNR és pràcticament sempre major a la que presenta al reactor PN, principalment en el rang de valors de 250 a 350 mg/l. L'explicació d'aquesta menor abundància de *Epistylis camprubii* al reactor PN en el rang de concentració indicat probablement s'expliqui per la relació de competència que s'estableix al biofilm del reactor PN entre *Epistylis camprubii* i *Opercularia coarctata*. Com es mostra al capítol 3 de la present memòria, *Opercularia coarctata* es correlaciona positivament amb la sDQO (coeficient de correlació de Spearman = 0.6132; p-valor ≤ 0.001), i presenta els seus màxims valors d'abundància justament en el rang de valors comprès entre els 250 i 350 mg/l (figura 4.6).

Figura 4.5. Gràfic de dispersió de l'abundància de *Epistylis camprubii* al biofilm i la concentració de sDQO. Els rombes blancs corresponen als resultats obtinguts al sistema SBNR, mentre que els rombes negres fan corresponen als resultats del reactor PN.

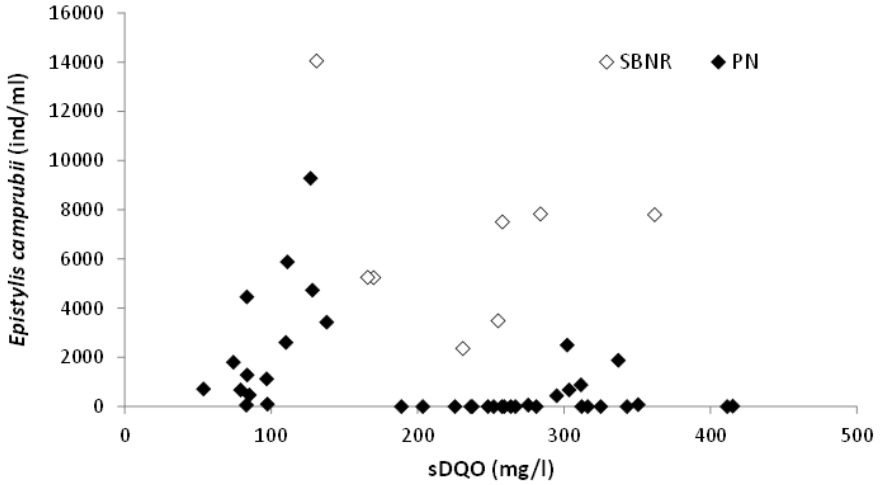
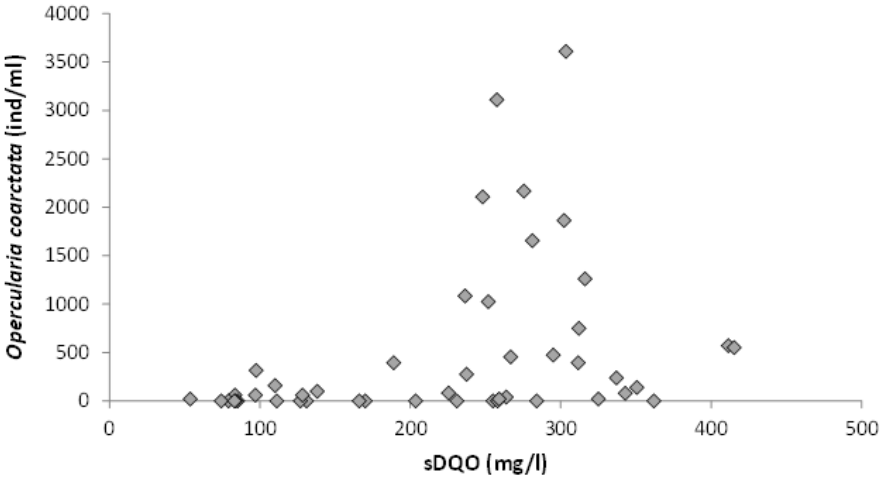


Figura 4.**6.** Gràfic de dispersió de l'abundància de *Opercularia coarctata* al biofilm i la concentració de DQO soluble (sDQO) al reactor PN.

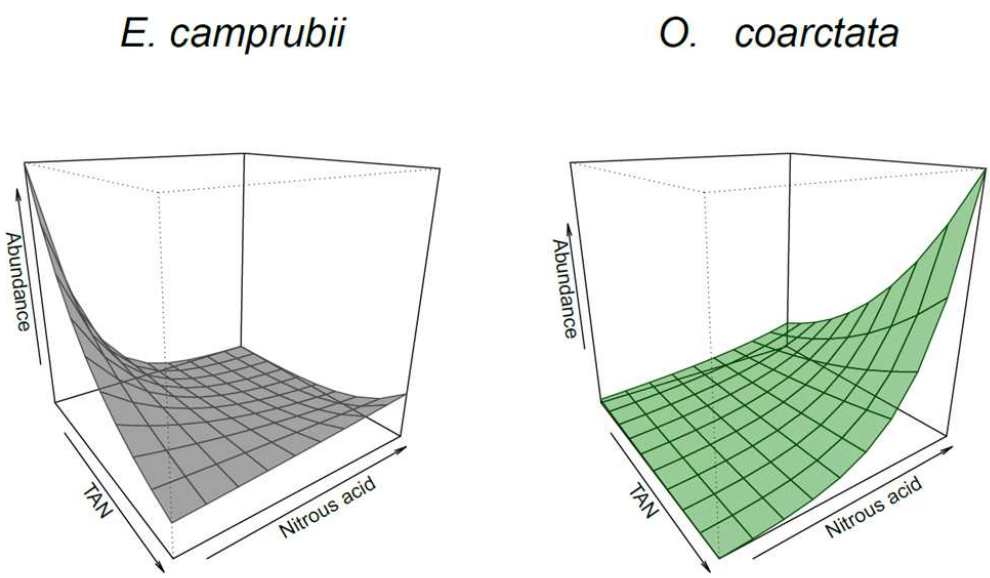


L'anàlisi integrada dels resultats semblen indicar, doncs, que les correlacions obtingudes al reactor PN entre la concentració de sDQO i el ciliat *Epistylis camprubii* probablement siguin producte de la relació de competència existent entre els dos sessílids i no causats per un efecte directe de la sDQO sobre aquestes espècies de ciliats.

Per acabar de tancar el cercle, s'ha volgut incidir en quins són o poden ser els paràmetres que regulen o que determinen la relació de competència entre *Epistylis camprubii* i *Opercularia coarctata* en els biofilms del sistema SBNR i reactor PN. Analitzant les dades de forma integrada, els resultats estadístics semblen apuntar que les dinàmiques poblacionals dels ciliats *Epistylis camprubii* i *Opercularia coarctata* responen a la interacció dels paràmetres àcid nitrós i nitrogen amoniacal total (figura 4.7), descartant la sDQO com a paràmetre implicat en aquest escenari de competència.

Entre aquests dos paràmetres, àcid nitrós i TAN, són especialment sorprenents els valors de correlació entre l'àcid nitrós i les dues espècies de sessílids si s'analitzen els resultats del sistema SBNR i el reactor PN de forma integrada. En concret, *Opercularia coractata* va presentar un coeficient de correlació de Spearman de 0.7915 (p-valor ≤ 0.001), relació pràcticament inversa a la obtinguda per *Epistylis camprubii* (coeficient de correlació de Spearman = -0.7316; p-valor ≤ 0.001). Aquests resultats suggereixen, doncs, que l'àcid nitrós és el principal paràmetre regulador de les poblacions de *Epistylis camprubii* i *Opercularia coarctata* als biofilms del sistema SBNR i del reactor PN, i molt probablement expliqui l'absència d'*Opercularia coarctata* al biofilm del reactor aerobi del sistema SBNR, on l'àcid nitrós va presentar permanentment concentracions molt més baixes que les obtingudes al reactor PN (figura 4.3).

Figura 4.7. Superfície de resposta modelada de l'abundància de *Epistylis camprubii* (esquerra) i *Opercularia coarctata* (dreta) en funció de les concentracions de nitrogen amoniacal total (TAN) i àcid nitrós.



Tot i que la toxicitat de l'àcid nitrós és coneguda i ha estat descrita sobre organismes procariotes, principalment sobre els bacteris nitrit oxidants (Anthonisen et al. 1976; Svehla et al. 2014), no s'han trobat treballs que estudiïn l'efecte d'aquest compost sobre la comunitat microeucariota, tant en sistemes naturals com en sistemes de tractament d'aigües residuals, probablement a causa de que el nitrit estranyament s'acumula en grans concentracions en els sistemes aquàtics naturals i que la gran majoria de sistemes de tractament d'aigües residuals dissenyats per a l'eliminació incrementada del nitrogen realitzen la desnitrificació mitjançant el procés convencional (via nitrat), sense acumulació de nitrits al reactor.

Els resultats de la present memòria posen de manifest la importància de considerar l'àcid nitrós com a paràmetre clau i d'especial rellevància sobre la microbiota (com a mínim sobre la

comunitat microeucariota) en els sistemes avançats d'eliminació de nitrogen dissenyats per desnitrificar via nitrit, especialment en aquells que tracten aigües residual amb alta càrrega amoniacal.

Epistylis camprubii en el context dels tractaments d'aigües residuals i el seu potencial com a bioindicador

Epistylis camprubii probablement sigui l'espècie més representativa de la present memòria, havent colonitzat de forma abundant i força constant els biofilms dels sistemes innovadors de tractament d'aigües residuals amb alta càrrega amoniacal. Des de la seva publicació, però, *Epistylis camprubii* s'ha anat observant de forma força constant també en sistemes de fangs activats (dades no publicades, experiència personal i comunicacions personals de Meritxell Mas de Hydrolab Microbiologica, Eva Rodrígez del Grupo de Bioindicación de Sevilla, i Andrés Zornoza de la Universitat Politècnica de València), on es podria catalogar com a espècie "de estranya aparició", seguint la terminologia de Madoni (2011).

És molt probable que, anteriorment a la seva descripció, *Epistylis camprubii* ja hagués estat observat però no identificat o determinat com a *Epistylis* sp. en sistemes de fangs actius. Les possibles causes que aquesta espècie de sessílid no hagi estat descrit abans probablement es deguin a la combinació de varis factors: i) la seva menor freqüència d'aparició en comparació a altres espècies de *Epistylis*; ii) la manca de característiques morfològiques clarament distintives microscòpicament (per exemple, el gruix del llavi peristomial no és un caràcter habitual per a identificar espècies de *Epistylis* en la majoria de guies de identificació); iii) el limitat esforç taxonòmic per part de la comunitat de protistòlegs que treballem en sistemes de tractament d'aigües residuals (Foissner 2016).

Tot i que no hi ha dades al respecte, sembla força evident que la presència de *Epistylis camprubii* als fangs actius de les EDARs (urbanes i industrials) s'associa a l'entrada d'elevades càrregues de nitrogen (principalment nitrogen amoniacal), tot i que, donada l'elevada tolerància de *Epistylis camprubii* a l'amoníac lliure, també podria ser indicador de toxicitat per part d'aquest compost. És necessària la realització de més estudis per tal de conèixer amb major exactitud la seva utilitat com a organisme bioindicador. El que sí que sembla evident és que el valor de *Epistylis camprubii* com a espècie bioindicadora no només es restringeix als sistemes SBNR i PN, sinó que *Epistylis camprubii* es pot considerar un ciliat amb potencial bioindicador transversal en els sistemes de tractament d'aigües residuals.

Aplicació de tècniques moleculars *Next Generation Sequencing* per l'anàlisi de la biodiversitat microeucariota

Durant els últims anys hi ha hagut un fort increment d'estudis utilitzant tècniques moleculars com a mètode per avaluar la biodiversitat de la comunitat microeucariota dels ecosistemes aquàtics del planeta, tant en l'àmbit dels ecosistemes aquàtics continentals (Medinger et al. 2010; Charvet et al. 2012), marins (Decelle et al. 2014; Logares et al. 2012, 2014) com en els sistemes de tractament d'aigües residuals (Matsunaga et al. 2014; Chouari et al. 2017). Entre

aquestes tècniques moleculars destaquen sobretot les tècniques anomenades *Next Generation Sequencing* (NGS) o *High-Throughput Sequencing* (HTS), per la seva gran capacitat de detecció d'espècies amb baixos valors d'abundància (Massana et al. 2015). De totes maneres, en aquells estudis en que s'ha comparat els resultats moleculars amb els resultats mitjançant microscòpia s'ha vist que existeixen diferències en els resultats obtinguts mitjançant els dos mètodes d'estudi de la biodiversitat (Medninger et al. 2010; Stoeck et al. 2014).

Com es mostra al capítol 3 de la present memòria, mitjançant l'ús de Ilumina es van detectar alguns taxons no observats prèviament mitjançant microscòpia durant la realització dels recomptes. És interessant comentar que la gran majoria d'aquests taxons, però, sí que havien estat observats en altres mostrejos realitzats al reactor PN durant el període d'estudi, pel que és força probable que aquests organismes mantinguessin una població constant al reactor amb valors d'abundància difícilment detectables mitjançant mètode de recompte utilitzat, recolzant la major capacitat de detecció d'organismes molt poc abundants de les tècniques moleculars en comparació amb les anàlisis microscòpiques.

Els resultats obtinguts, però, també evidencien certes limitacions de les tècniques NGS en la detecció de la biodiversitat eucariota, fins i tot en un sistema amb baixa biodiversitat com el reactor PN. Deixant de banda els Excavata, que presenten una regió V4 més llarga que la resta d'eucariotes i que dificulta la seva amplificació mitjançant tècniques NGS (Pawlowski et al. 2011), l'anàlisi de seqüenciació massiva Ilumina no va detectar nucleàrids ni les espècies de ciliats *Opercularia coarctata* i *Vorticellides microstoma*-complex, observades microscòpicament a les mostres.

Entre aquests organismes, la no detecció de *Vorticellides microstoma*-complex és potser el cas més inesperat, doncs existeixen força seqüències a les bases de dades d'espècies del clade format pels gèneres *Vorticellides* (degut al recent canvi de nom del gènere algunes espècies apareixen encara com a *Vorticella*), *Astylozoon* i *Opisthonecta* (Miao et al. 2004), fet que no suggereix dificultats d'amplificació o seqüenciació de les seves espècies. De fet, l'anàlisi molecular del reactor PN sí que va detectar *Opisthonecta henneguyi*, un perítric lliure nedador (sense fase sèssil) ubicat al mateix clade que *Vorticellides* (Williams i Clamp 2007). El gènere *Opercularia*, en canvi, sí presenta un escàs nombre de seqüències del gen 18S rDNA a les bases de dades (8 resultats per la cerca de '*Opercularia* 18S' a dia 09/05/2017; https://www.ncbi.nlm.nih.gov/). La falta de seqüències de *Opercularia* podria indicar l'existència de dificultats d'amplificació o seqüenciació de les seves espècies, justificant la seva no detecció mitjançant Ilumina.

Els principals motius pel qual alguns taxons no són amplificats o seqüenciats mitjançant tècniques moleculars de *Next Generation Sequencing* són la presència de grans insercions en la regió amplificada que impedirien la hibridació de les seqüències *forward* i *reverse* (com succeeix amb el grup dels Excavata), l'existència d'estructures secundàries addicionals producte d'aquestes insercions i la presència de diferències en la seqüència nucleotídica dels encebadors i la regió d'hibridació d'aquests en les diferents espècies (Stoeck et al. 2014). Totes aquestes possibilitats s'han descartat tant per *Opercularia* com per *Vorticellides* després de realitzar una comprovació visual dels alineaments.

Les incongruències entre Ilumina i els taxons observats microscòpicament també poden ser degudes a la no amplificació d'aquests últims per una qüestió d'atzar. En aquest cas, com opina de forma Prosser (2010) en el seu article de títol contundent "*Replicate or lie*", seria molt convenient incloure sempre la realització de rèpliques en els dissenys de futurs estudis moleculars (tot i que l'encara elevat cost d'aquestes tècniques fan de les rèpliques un objectiu poc assumible per alguns grups d'investigació), així com la realització d'estudis que investiguin el grau de variabilitat d'aquestes tècniques moleculars.

A nivell personal, l'aplicació de les tècniques moleculars de NGS m'han servit per descobrir el gran potencial d'aquestes com a eina d'estudi de la biodiversitat en medis aquàtics. De totes maneres, els resultats obtinguts han mostrat certes limitacions de l'estat actual de la tècnica Ilumina, que s'han traduït en importants diferències en comparació amb els resultats microscòpics. Entre aquestes limitacions destaquem la no detecció d'alguns taxons majoritaris microscòpicament i la impossibilitat de relacionar l'abundància de les seqüències amplificades amb l'abundància d'individus de les diferents espècies a causa de l'elevat nombre de còpies del gen 18S rDNA que presenten alguns grups com els ciliats, en comparació amb el nombre de còpies reduït d'alguns organismes flagel·lats (Gong et al. 2013; Zhu et al. 2005).

Actualment, doncs, les tècniques moleculars de NGS s'haurien de considerar com a un mètode complementari a les anàlisis microscòpiques, especialment útils per detectar aquells taxons que presenten baixes abundàncies i/o organismes difícilment identificables mitjançant microscòpia òptica.

Al contrari del que es podria pensar, però, la irrupció de les tècniques moleculars com a eina d'anàlisi de la diversitat fa més necessària que mai la realització d'estudis taxonòmics profunds (sobretot en protists) que contemplin tant els caràcters morfològics com els moleculars, en vista de proveir una bona base de dades on comparar les seqüències obtingudes mitjançant tècniques de NGS.

Sobre el gen 18S rDNA i la regió V4

El gen més utilitzat en els estudis moleculars de biodiversitat microeucariota és el gen 18S rDNA, i concretament les regions hipervariables V4 (Massana et al. 2015; Stock et al. 2013, Stoeck et al. 2014), V9 (Amaral-Zettler, 2009; Pawloswki et al. 2011) i, en menor mesura, la regió V2 (Hadziavdic et al. 2014). És important tenir en compte que cap de les regions del gen 18S rDNA és capaç de detectar tots els organismes eucariotes. La regió V4, per exemple, no detecta organismes del grup dels Excavata, mentre que la regió V9 no és útil per a la detecció de les espècies del supergrup Amoebozoa (Pawloswki et al. 2012). De les diferents regions, la V4 és la que presenta una major correlació de variació amb la variació del total del gen 18S rDNA (Dunthorn et al. 2012), motiu pel qual alguns autors (Pawlowski et al. 2012) recomanen utilitzar-la com a primer pas per als estudis moleculars de biodiversitat eucariota. Potser el més recomanable és, en cas de tenir el suport econòmic suficient, utilitzar com a mínim les dues regions del 18S rDNA més informatives (V4 i V9).

Les regions del gen 18S rDNA són molt útils per diferenciar les seqüències a nivell de grans grups, però sovint no serveix per assignar les seqüències a una espècie (Pawlowski et al. 2012), ja que el grau de variabilitat o de conservació de la seqüència del gen 18S rDNA difereix entre els diferents grups taxonòmics. Per exemple, el gen 18S rDNA serveix per diferenciar espècies en foraminífers i algunes diatomees, però no és capaç de resoldre les relacions interespecífiques en la gran majoria de grups.

Aquesta "limitació" de l'ús del gen 18S rDNA l'hem observat en els resultats moleculars obtinguts en l'anàlisi del reactor PN, en els que la seqüència d'un ciliat de la classe Spirotrichea va presentar el 100% de coincidències amb les seqüències de fins a 4 espècies de 3 gèneres diferents: *Paraparentocirrus sibillinensis* (número d'accés KF184655), *Pattersoniella vitiphila* (AJ310495), *Sterkiella histriomuscorum* (HQ615720) i *Sterkiella cavicola* (GU942565). Els tres gèneres pertanyen a un mateix grup de la família Oxytrichidae anomenat "Stylonychine Oxytrichidae" (Kumara et al. 2014), i serveix com a exemple per evidenciar la poca capacitat de discriminació d'espècies que ofereix la seqüenciació massiva del gen 18S rDNA, en aquest cas a causa de poca variabilitat de la regió V4 del gen 18S rDNA en aquest grup de la classe Spirotrichea.

En la mateixa línia, la seqüència obtinguda corresponent a *Opisthonecta henneguyi*va mostrar una coincidència del 100% amb la seqüència de *Opisthonecta minima* (EF417834) i de fins el 99% amb altres espècies de l'actual gènere *Vorticellides* (anteriorment *Vorticella*) com *Vorticellides microstoma* (DQ868347), *Vorticellides infusionum* (JN120203) i *Vorticellides astyliformis* (JN120204), fet que efectivament sembla demostrar l'existència d'una baixa variabilitat de la regió V4 dins el clade *Vorticellides*. En aquest context de baixa variabilitat de la regió V4, i recordant que les espècies del clade *Vorticellides* no presenten dificultats d'amplificació i seqüenciació, ens vam arribar a plantejar si aquesta seqüència podria pertànyer a *Vorticellides microstoma*-complex, espècie observada microscòpicament però no detectada mitjançant Ilumina, i no a *Opisthonecta henneguyi*. Aquesta hipòtesis, però, es va descartar al realitzar la visualització dels alineaments entre la seqüència obtinguda assignada a *Opisthonecta henneguyi* i les seqüències pertanyents a *Vorticellides microstoma* (DQ868347), *Vorticellides infusionum* (JN120203) i *Vorticellides astyliformis* (JN120204). En aquests alineaments es va observar, de forma repetida, la presència d'un *gap* en la mateixa posició en totes les seqüències del gènere *Vorticellides*, *gap* no existent en les seqüències de *Opisthonecta henneguyi* ni *Opisthonecta minima*. La presència d'aquest *gap* pot ser útil per corroborar la correcta determinació dels gèneres *Vorticellides* i *Opisthonecta* en anàlisis NGScentrats en la regió V4 del gen 18S rDNA.

Per realitzar estudis més profunds i específics sobre la comunitat de ciliats seria recomanable l'ús d'altres gens amb major variabilitat intraclade com el 28S rDNA, ITS rDNA, la subunitat I del citocrom oxidasa c (COI) i el gen citocrom b (*cyt b*) (Chantangsi et al. 2007; Gentekaki i Lynn 2009; Strüder-Kypke i Lynn 2010; Pawloswki et al. 2012), gens que permeten discriminar els ciliats a nivell d'espècie. Com a contrapartida, però, cal tenir en compte que les bases de dades com GenBank presentin un número molt menor de seqüències d'aquests gens en comparació amb el 18S rDNA.

5. CONCLUSIONS V

5. CONCLUSIONS

1. S'ha establert un protocol que permet l'estudi de la comunitat microeucariota en sistemes MBBR, mantenint separades les poblacions que es desenvolupen en suspensió al licor mescla i les que es desenvolupen adherides de forma fixe al biofilm dels *carriers*. El protocol també permet comparar les abundàncies de les espècies als dos hàbitats a l'utilitzar la mateixa unitat de mesura (ind/ml).

2. S'ha caracteritzat les comunitats microeucariotes associades al sistema SBNR i al reactor PN. El sistema SBNR ha estat colonitzat per un total de 20 taxons, dels quals 11 pertanyen a flagel·lats, 4 a ciliats, 4 a amebes i 1 a metazous nematodes. Per la seva banda, al reactor PN s'han detectat 24 taxons, 10 pertanyents a flagel·lats (4 Excavata, 2 Stramenopiles, 1 Archaeplastida, 1 Rhizaria i 2 *Incertae sedis*), 9 a ciliats (Alveolata), 4 a amebes (2 Amoebozoa, 1 Opisthokonta i 1 *Incertae sedis*) i 1 a metazous nematodes (Opisthokonta).

3. L'anàlisi independent del licor mescla i el biofilm ha mostrat que en els sistemes MBBR existeix una diferenciació entre les comunitats de microeucariotes que colonitzen els dos hàbitats. La gran majoria de la població de les espècies lliure nedadores es va observar al licor mescla, mentre que les espècies sèssils van presentar la major part de la seva població total colonitzant el biofilm dels *carriers*.

4. Arrel de les anàlisis microscòpiques del biofilm dels sistemes estudiats, s'ha descrit una nova espècie de ciliat perítric sessílid, *Epistylis camprubii*, altament tolerant a l'amoni i al nitrit. La seva descripció aporta els principals caràcters morfològicsi ecològics, així com la seqüència molecular del gen 18S rDNA. *Epistylis camprubii* és una espècie també present en sistemes de tractament d'aigües residuals urbanes i industrials operant amb tecnologia de fangs actius, pel que la seva descripció ajudarà a la realització de diagnòstics més precisos en les anàlisis rutinàries de bioindicació d'aquests sistemes.

5. Els sistemes estudiats de tractament d'aigües residuals amb alta càrrega amoniacal s'han caracteritzat per presentar un número baix d'espècies de ciliats en comparació amb sistemes dissenyats per al tractament d'aigües residuals amb càrrega amoniacal més habituals (entre 10 i 50 mg/l de mitjana). El baix nombre d'espècies de ciliats al sistema SBNR (amb un màxim de 2 i 3 espèciesde ciliats observades en un mateix mostreig al licor mescla i al biofilm, respectivament) i al reactor PN (màxim de 6 i 4), sembla confirmar l'efecte negatiu de les concentracions elevades de nitrogen amoniacal sobre la diversitat de ciliats.

6. S'han determinat les principals espècies amb potencial com a bioindicadores del sistema SBNR i del reactor PN.
   6.1. En el sistema SBNR, *Epistylis camprubii* (prèviament anomenat *Epistylis* cf. *rotans*) ha estat l'espècie amb major rellevància com a bioindicador del procés, essent l'únic eucariota capaç de colonitzar de forma permanent el reactor aerobi del sistema. En el context abiòtic i biòtic del reactor aerobi del sistema SBNR, la dinàmica poblacional

d'*Epistylis camprubii* s'ha correlacionat negativament amb la concentració de nitrogen amoniacal, essent, per tant, un bon indicador del rendiment d'oxidació de l'amoni en el rang de paràmetres estudiats.

6.2. En el context abiòtic i biòtic del reactor PN, les espècies amb major potencial com a bioindicadores del procés han estat el ciliat *Cyclidium glaucoma*al licor mescla, i *Epistylis camprubii* i *Opercularia coarctata* al biofilm. Al licor mescla, la presència de *Cyclidium glaucoma* indica una baixa concentració d'àcid nitrós i de sDQO. Al biofilm, majors abundàncies d'*Epistylis camprubii* s'ha correlacionat amb majors concentracions d'amoníac lliure i baixes concentracions d'àcid nitrós i de sDQO, mentre que una major abundància de *Opercularia coarctata* s'ha correlacionat amb incrementsde la concentració d'àcid nitrós i de sDQO.

7. L'estudi integrat dels resultats obtinguts en el sistema SBNR i el reactor PN ha permès una anàlisi en més profunditat de l'efecte dels paràmetres fisicoquímics sobre els ciliats *Epistylis camprubii* i *Opercularia coarctata*. Arrel d'aquest nou enfoc de les dades s'ha pogut determinar que la relació de competència que s'estableix entre els dos sessílids als biofilms del sistema SBNR i reactor PN està determinada, en el rang de valors estudiats, per la interacció de l'efecte de l'àcid nitrós i del TAN sobre les seves poblacions.

8. S'ha posat de manifest la importància d'analitzar la concentració de les formes no ionitzades del nitrogen amoniacal i del nitrit (és a dir, l'amoníac lliure i l'àcid nitrós) en els sistemes de tractament d'aigües residuals per la influència que pot exercir sobre l'estructura de la comunitat microeucariota. Aquests paràmetres són especialment rellevants en sistemes que tracten aigua residual amb alta càrrega amoniacal i que no realitzen la desnitrificació via nitrat, ja que aquests sistemes es caracteritzen per mantenirelevades concentracions de nitrogen amoniacal i/o de nitrit al reactor.

9. S'han establert valors màxims i mínims de tolerància de les principals espècies del sistema SBNR i del reactor PN als paràmetres fisicoquímics nitrogen amoniacal, nitrit, amoníac lliure, àcid nitrós, nitrat, temperatura, pH i sDQO, que en alguns casos eixamplen àmpliament els rangs de tolerància coneguts fins al moment.

10. La comparació dels resultats obtinguts microscòpicament amb els obtinguts mitjançant Ilumina (tècnica molecular de *Next Generation Sequencing*) ha confirmat la major potència de Ilumina en la detecció d'organismes poc abundants a les mostres, tot i que també ha mostrat deficiències en la detecció d'alguns dels taxons més rellevants del reactor PN com *Opercularia coarctata*. Es confirma també que l'amplificació massiva de la regió V4 del gen 18S rDNA no és, de moment, una tècnica fiable per avaluar l'abundància dels organismes, com a mínim en els sistemes estudiats, a causa del diferent nombre de còpies del gen 18S rDNA que tenen els organismes dels diferents grups.

11. S'ha corroborat que la regió V4 del gen 18S rDNA no és un gen adequat per discriminar a nivell d'espècie en alguns clades de ciliats, tot i que és útil per a la determinació de la biodiversitat microeucariota a nivell de grans grups.

6. REFERÈNCIES VI

6. REFERÈNCIES

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7. ANNEX I. ALTRES PUBLICACIONS

VII

7.1. MICROBIAL AND PHYSICOCHEMICAL PARAMETERS ASSOCIATED WITH LEGIONELLA CONTAMINATION IN HOT WATER RECIRCULATION SYSTEMS

Alejandra Serrano-Suárez, Jordi Dellundé, Humbert Salvadó, Sílvia Cervero-Aragó, Javier Méndez, Oriol Canals, Silvia Blanco, Antoni Arcas, Rosa Araujo.

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RESEARCH ARTICLE

Microbial and physicochemical parameters associated with *Legionella* contamination in hot water recirculation systems

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Abstract Hot water recirculation systems (HWRS) in hotels and nursing homes, which are common in countries such as Spain, have been related to outbreaks of legionellosis. To establish the relationships of microbial and physicochemical parameters, especially protozoa, with the occurrence of *Legionella* in HWRS, 231 samples from hotels and nursing homes were analysed for *Legionella*, protozoa, heterotrophic plate counts (HPC) at 22 and 37 °C, *Pseudomonas*, metals, temperature and others. *Legionella pneumophila* was the dominant species isolated, and 22 % were sg. 1. The sampling method became particularly important in order to define which factors were involved on the occurrence of *Legionella*. Results showed that the bacteria and the accompanying microbiota were more abundant in the first flush water whose temperature was lower. The bacteria occurred in those samples with high HPC and were inversely correlated with *high temperatures*. Multivariate regression showed that a concentration above 1×10^5 CFU/100 mL of HPC at 37 °C, Fe above 0.095 ppm and the presence of protozoa increased significantly the risk of *Legionella* colonization, while univariant regression showed that the presence of Cu above 0.76 ppm and temperature above 55 °C diminished it. Therefore, to reduce the risk associated with *Legionella* occurrence in HWRS these parameters should be taken into consideration.

Keywords *Legionella* · Protozoa · Hot water · HPC · Temperature · Metals

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Introduction

Legionella pneumophila is an aquatic bacterium that was first linked with pneumonia in 1976 (McDade et al. 1977). Since then, many cases of legionellosis caused by different *Legionella* species and mostly related to contaminate man-made water systems have been reported worldwide.

These days, many countries have guidelines in place to prevent the growth of the bacteria and reduce the risk of outbreaks and transmission of *Legionella* spp. Despite this, new cases of legionellosis are reported every year. In 2009, 3,522 cases were reported in the USA with an incidence rate of 1.15 (CDC 2011). In Europe, most cases were reported in France, Spain and Italy, with an annual mean of 1,409,

1,214 and 906, and incidence rates of 2.26, 2.76 and 1.54, respectively (Joseph et al. 2010).

Legionella infections can occur sporadically and in outbreaks, but in both circumstances, determining the source of infection is difficult. A total of 501 outbreaks were reported in Spain between 1999 and 2009, 22 % of which were caused by sanitary hot water systems and 18 % by cooling towers or similar devices (Cano Portero et al. 2010). Even there is no information about the source of infection of most sporadic cases, it is likely that many of the cases could be related to sanitary hot water systems.

Hot water in hotels, nursing homes and hospitals may be a source of infection since *Legionella* spp. are often present in this type of environment (Borella et al. 2005a; Mouchtouri et al. 2007; Wadowsky et al. 1982) and can spread through aerosols generated by showers and taps. Furthermore, in countries like Spain that have limited energy and water resources, hot water systems in hotels and hospitals are often designed as a recirculating system with a storage tank, which increases the risk of legionellosis (Borella et al. 2005a). These systems have several characteristics that may favour *Legionella* growth. Such characteristics include long pipes, dead ends and the intermittent use of hot water, which results in differences in flow, speed and temperature in each part of the circuit.

In the environment, *Legionella* has various survival strategies, including multiplying in biofilms, where it can live alongside other bacteria such as *Pseudomonas* or multiply intracellularly in protozoan species such as *Acanthamoeba*, *Hartmannella* and *Tetrahymena* which feed and protect the bacteria (Abu Kwaik et al. 1998; Anand et al. 1983; Atlas 1999; Rowbotham 1980; Serrano-Suárez 2009; Taylor et al. 2009; Wadowsky et al. 1988). Moreover, *Legionella* density may vary in response to both the density and the composition of amoeba present which as well may play an important role on the reactivation of viable but nonculturable cells (Buse and Ashbolt 2011; Garcia et al. 2008) Thus, *Legionella* spp. have been isolated from many different water environments, especially from hot water systems and cooling towers, where the bacterium occurs within a wide range of physicochemical parameters. However, hot water recirculation systems have very different characteristics from cooling towers, so it is important to identify the ecological factors that favour the presence of *Legionella* in order to improve its control in this habitat.

The relationship between *Legionella* and the physicochemical factors and heterotrophic bacteria in hot water systems in Italian and Greek hotels has been studied previously (Borella et al. 2005b; Leoni et al. 2005; Mouchtouri et al. 2007). However, few works have considered the effect of amoeba on the occurrence of *Legionella* in hot water systems (Habicht and Muller 1988; Lasheras et al. 2006), despite the fact that it has been well demonstrated, especially under laboratory conditions, that protozoa enhance *Legionella* growth and provide protection in harsh environments (Abu Kwaik et al. 1998; Anand et al. 1983; Rowbotham 1980; Taylor et al. 2009; van der Kooij et al. 2005; Wadowsky et al. 1988).

The objective of the present study was to determine the environmental factors, including bacteria and protozoa, that may affect the distribution and abundance of *Legionella* spp. in hot water recirculation systems with storage tanks in hotels and nursing homes. The surveillance was carried out using two sampling methods to better characterize the *Legionella* environment. All of the samples were analysed for *Legionella* and related factors. Characterizing these niches that support *Legionella* will help develop more effective control measures.

Material and methods

Sampling analysis

A total of 231 samples from hot water continuous circulation systems with storage tanks were collected randomly from 30 hotels and nursing homes in Catalonia. All buildings in the study received water from the drinking water supplies with an average free chlorine content of 0.5 ppm and very low levels of bacterial contamination, which is in accordance with the Spanish regulations for this type of water (Boletín Oficial del Estado 2003a, b).

Two collecting methods were used A and B. (A) One hundred and seven samples were obtained in compliance with Spanish regulations (Boletín Oficial del Estado 2003a); in brief, a sample of 100 mL of the first water to flow from the tap was collected and approximately 3 min later, when the water had reached the maximum temperature, the rest of the sample—made up to 1 L—was added to the sample container. (B) One hundred and twenty-four samples were collected using a different sampling method; briefly, 1 L of the first flow was taken corresponding to proximal water, and an additional 1 L was collected after letting the water run for approximately 3 min, corresponding to the hottest water in the circuit, distal water. Water samples were collected using sterile plastic bottles containing 5 mL of 2 % sodium thiosulfate (Panreac, Spain). Samples were taken to the laboratory at 4 °C and processed a maximum of 24 h after collection (International Organization for Standardization 1998).

Reference bacterial strain

The reference strain used in this study, *L. pneumophila* subsp. *pneumophila* str. Philadelphia 1 (ATCC 33152) was cultured on BCYE agar (Oxoid LTD; Basingstoke, Hampshire, England) at 37 °C.

Microbial parameters

Heterotrophic bacterial counts (HPC) were determined in accordance with the ISO 6222:1999, standard on water quality; the enumeration of culturable micro-organisms: colony count by inoculation in a nutrient agar culture medium (International Organization for Standardization 2005; James et al. 1999). In summary, 100 μL of the direct sample and a tenfold diluted sample in Ringer 1/4 (Scharlau Chemie; Barcelona, Spain) were cultured in Plate Count Modified agar media (Scharlau Chemie; Barcelona, Spain) at 22 °C for 72 h and at 37 °C for 48 h, respectively. For the quantification of *Pseudomonas* sp., 500 μL of the filtration concentrate from the liter filtered for *Legionella* detection (described below) was cultured in Glutamate Starch *Pseudomonas* agar (Scharlau Chemie; Barcelona, Spain; Ribas et al. 2000).

For the analysis of protozoa, 5 ml of water was taken from the bottom of each sampling container, after 2 h of sedimentation. Samples were centrifuged at 800×*g* for 5 min and 50 μl of the pellet was observed with an optical microscope. Subsequently, 500 μl of wheat broth medium was added, and it was incubated at room temperature under dark conditions to avoid algae proliferation. After that, suspensions were observed at day 2, 5, 10 and 15. Wheat broth medium was prepared using the following method (López-Ochoterena and Serrano-Limón 1991), 50 g of *Triticum vulgare* was boiled for 20 min in 1 L of distilled water, then filtered through a 0.45-μm porous size membrane (EZ-Pak® Membrane Filters; Millipore, Molsheim, France) and adjusted to 1 L by adding more distilled water. Finally 1.3 g of $NaHPO_4$ 12 H_2O was added (Merck; Darmstadt, Germany) to the media before being sterilized by autoclave. Detection limit of the method is between 50 and 80 individuals per liter.

Detection of *Legionella* by culture

Detection and enumeration of *Legionella* in water samples were done by culture on BCYE agar supplemented with GVPC (Oxoid Ltd, Oxoid LTD; Basingstoke, Hants, England) following the ISO 11731:1998 standard on water quality; detection and enumeration of *Legionella* (International Organization for Standardization 1998). This method involves the filtration of a 1-L sample through a 0.45-μm porous size nylon membrane (Filter HNWP Millipore; Ireland); the retained material is then suspended in 10 mL of Ringer 1/40 by vortexing for 2 min. Concentrates were cultured either directly and after two treatments: a thermal treatment of 50 °C for 30 min and an acid treatment in which 100 μL of acid buffer is added to 900 μL of the sample concentrate, as described in the ISO 11731:1998 guidelines. The recovery of *L. pneumophila* using this method was determined experimentally and had an approximate detection limit equivalent to 44 CFU/L (data not shown; Serrano-Suárez 2009).

Legionella serology

An average of between three and five colonies of positive samples for *Legionella* were tested using the *Legionella* agglutination test (*Legionella* latex test; Oxoid, Basingstoke, Hampshire, England) following the manufacturer's instructions. This kit enables differentiation between *L. pneumophila* serogroup 1, *L. pneumophila* serogroups 2–14 and seven additional *Legionella* species.

DNA extraction

DNA extraction was performed by modifying the protocol described by Van Eys et al. (1989). Briefly, 1 mL of the concentrated water (as described above) was heated to 100 °C for 10 min and cooled for 10 min at−20 °C. A total of 0.015 g of the resin Molecular Biology Grade AG 501-X8 (Bio-Rad) was then added to the sample and mixed with the vortex for 30 s. Finally, the sample was centrifuged for 5 min at 10.000×*g*. The supernatant was then recovered and stored at −20 °C for further analysis.

Semi-nested PCR

A semi-nested PCR was also used for the detection of *Legionella* by amplifying the 16S rRNA gene, which is conserved in all species of the genera. The primers used for the semi-nested PCR were based on the method used in a study by Jonas et al. (1995). They are JFP (5′-AGGGTTGATAGGTTAAGAGC-3′) and JRP (5′-CCAACAGCTAGTTGACATCG-3′) for the first PCR round, and a reverse inner primer was designed for the second round ARS (5′-TTCCACTACCCTCTCCCATA-3′) which were used to generate a 234-bp amplicon. Ten microliters of the DNA extraction in a total reaction volume of 50 μL was employed for the first PCR round, while 1 μL of the first PCR product was used in the second round. All amplifications were performed in 1× PCR buffer, 1.5 mM $MgCl_2$, and 0.2 mM of a dNTPs mixture, 0.04 U of AmpliTaq® DNA polymerase (Applied Biosystems; Foster City, CA, USA) and 1 μM of each primer. The reaction parameters were as follows: denaturation at 95 °C for 5 min, followed by 40 cycles for the first round and 35 cycles for the second of 5 min at 95 °C, 90 s at 57 °C and 1 min at 72 °C. The amplification concluded with a final extension period of 10 min at 72 °C (GenAmp® PCR System 9700; Applied Biosystems). Ten microliters of the amplified PCR product was run (100 V) on 2 % agarose gel and stained with ethidium bromide. The detection limit of the first round of PCR for *L. pneumophila* was 6.80×10^2 Genome copies

(GC) for reaction, while the semi-nested PCR reached 6.80×10^{0} GC for reaction (data not shown; Serrano-Suárez 2009).

Physical and chemical analyses

Temperature was measured in situ during collection. Free chlorine concentration was determined using *N*, *N*-diethyl-*p*-phenylenediamine sulfate colorimetric method (Boletín Oficial del Estado 2003b). The value of pH was determined using the Micro pH2000 (*Crison Instruments* S.A, Spain) and turbidity using the Ratio/xr (*Hach Lange* France S.A.S). Concentrations of Zn, Fe and Cu were measured by inductively coupled argon plasma spectroscopy with a detection limit of 0.025, 0.01 and 0.01 ppm, respectively. Total organic carbon (TOC) was measured by high-temperature catalytic oxidation (HTCO) with a detection limit of 1 ppm. Heavy metal concentrations and HTCO data were measured by Serveis Cientificotècnics in the University of Barcelona.

Statistical analysis

Descriptive statistics, Wilcoxon matched pairs test and the Mann–Whitney *U* test were carried out using the statistical package software Statgraphics Centurion XVI (StatPoint Technologies). The statistical package Stata 11 (StataCorp, LP.) was used to estimate odds ratios and logistic regression; Pearson's and Spearman's rank correlations were also used to analyse the correlation between parameters. Odd ratios in 2×2 tables were calculated using the Woolf's approach, for purposes of comparison with logistic regression. All statistics were performed with the culture results of *Legionella*; otherwise, it is stated in the manuscript.

Results

Hot water characteristics

Over a period of 2 years, a total of 231 samples from hot water recirculation systems with storage tanks in 30 hotels and 3 nursing homes were analysed for the presence of *Legionella* and to determine the water quality parameters. *Legionella* was detected by PCR in 95 (41 %) samples but was isolated in only 64 (27 %) samples. Within this group, 77 % were identified as *L. pneumophila* serogroup 2–14, 22 % as *L. pneumophila* sg.1 and 1 % as *Legionella* spp. The accompanying microbiota was determined by HPC and cultivation of *Pseudomonas* and protozoa. The occurrence of HPC at 22 °C (84 %) was similar to HPC at 37 °C, as were the mean counts 5.68×10^{4} CFU/100 mL and 7.26×10^{4} CFU/100 mL, respectively. *Pseudomonas* was detected in 45 % of the total samples. Protozoa were observed directly and after culture in 29 % of the samples. Gymnamoebae were detected in 93 % of these samples, and only 26.8 % were flagellates and 3.6 % were ciliates.

The analysis of the physicochemical parameters revealed that levels of pH, turbidity and TOC were similar in all hot water samples. Water temperature was the most variable parameter, ranging from 16.3 to 71.7 °C. Despite the fact that mains water is always chlorinated in Spain, free chlorine was not detected in most of the samples. An analysis of trace metals showed its presence in most of the samples at low concentrations. Their concentration could have been affected by a variety of factors: corrosion of the alloy and welding of the pipes, tanks and taps; different pipeline compositions; the age of the systems; and the mains water characteristics.

Sampling effect

The surveillance was carried out using two sampling methods, and the data are shown in Table 1. During the initial sampling stage, 107 samples were collected in accordance with Spanish law (Boletín Oficial del Estado 2003a). These samples were representative of the entire circuit because each sample was a mixture of the first flush and the water that had reached the maximum temperature (recorded temperature). Results are shown in Table 1 as mixed samples. *Legionella* spp. was isolated in 28 samples (26 %) and usually accompanied by abundant microbiota. Temperature acted as the main biocide in these samples. However, in most of them, the temperature was not high enough to maintain a low level of microbiota throughout the circuit. This was firstly due to the dynamics of the water circuit and secondly to the fact that the temperature of the heater tank was not always high enough.

In the second stage, two samples were collected from the same tap: one proximal and one distal. Proximal samples corresponded to the first flush and distal samples to the hottest water. *Legionella* was detected in both sample types but was less prevalent in the distal (23 %) than in the proximal samples (35 %). Similarly, the counts were lower in the hottest water.

To determine whether the proximal and distal samples with different *Legionella* counts represented two different niches, the non-parametric Wilcoxon test was used. The *Legionella* counts were significantly higher in the proximal samples ($p<0.05$). As expected, the most distinctive physicochemical variable was temperature: in the proximal samples, the mean value was 27.6 °C (16.3 to 38 °C), and in the distal samples, it was 52.9 °C (38 to 66.6 °C). The concentrations of the other biological parameters, HPC at 22 and at 37 °C, and *Pseudomonas*, showed significantly higher numbers in the proximal samples ($p<0.05$). Also, there were significant differences in the presence and absence of protozoa: 43 % in the proximal and 24 % in the distal samples ($p<0.05$). However, there were no differences in terms of

Table 1 Microbiological and physicochemical parameters of hot water recirculation systems analysed

Parameter	Sample	Pos. (%)	Mean	Median	First quartile	Third quartile	Min.	Max.
Legionella CFU/100 mL	Mixed (107)	26	3.54[a]	<0.1	<0.1	2.00	$<5\times10^{0}$	2.95×10^{3}
	Prox. (62)	35	6.88 [a]	<0.1	<0.1	49.00	$<5\times10^{0}$	2.00×10^{3}
	Distal (62)	23	2.41 [a]	<0.1	<0.1	<0.1	$<5\times10^{0}$	5.30×10^{3}
HPC 22 °C CFU/100 mL	Mixed (107)	82	7.93×10^{4} [a]	4.40×10^{5}	8.50×10^{3}	6.17×10^{6}	$<5\times10^{2}$	8.80×10^{7}
	Prox. (62)	92	2.33×10^{5} [a]	5.50×10^{5}	6.73×10^{4}	4.80×10^{6}	$<5\times10^{2}$	1.31×10^{7}
	Distal (62)	77	7.78×10^{3} [a]	1.70×10^{4}	3.25×10^{2}	7.85×10^{5}	$<5\times10^{2}$	2.80×10^{7}
HPC 37 °C CFU/100 mL	Mixed (107)	85	1.15×10^{5} [a]	6.25×10^{5}	1.90×10^{4}	7.64×10^{6}	$<5\times10^{2}$	8.00×10^{7}
	Prox. (62)	92	2.43×10^{5} [a]	9.40×10^{5}	1.00×10^{5}	4.74×10^{6}	$<5\times10^{2}$	1.20×10^{7}
	Distal (62)	76	9.73×10^{3} [a]	3.50×10^{4}	1.25×10^{2}	9.78×10^{5}	$<5\times10^{2}$	3.00×10^{7}
Pseudomonas CFU/100 mL	Mixed (107)	44	47.20 [a]	<1	<1	4,250	$<5\times10^{1}$	2.00×10^{6}
	Prox. (62)	60	275.28 [a]	1,700	<1	2.64×10^{4}	$<5\times10^{1}$	1.20×10^{5}
	Distal (62)	31	18.62 [a]	<1	<1	975	$<5\times10^{1}$	1.20×10^{5}
Protozoa +/−	Mixed (107)	25	–	–	–	–	–	–
	Prox. (62)	43	–	–	–	–	–	–
	Distal (62)	24	–	–	–	–	–	–
Temperature °C	Mixed (107)	–	48.34	51.00	42.15	55.05	18.00	71.70
	Prox. (62)	–	27.60	28.50	23.05	32.00	16.30	38.00
	Distal (62)	–	52.9	52.00	50.00	56.68	38.00	66.60
pH	Mixed (107)	–	7.0	7.69	7.20	7.91	6.00	8.63
	Prox. (62)	–	7.4	7.41	7.10	7.82	6.36	8.76
	Distal (62)	–	7.8	7.47	7.09	7.92	6.58	8.45
Turbidity NTU	Mixed (107)	–	0.3	0.59	0.42	1.00	0.16	2.02
	Prox. (62)	–	0.1	0.60	0.40	0.90	0.12	5.00
	Distal (62)	–	0.7	0.58	0.36	0.87	0.12	2.00
Chlorine ppm	Mixed (107)	12	0.4	0.00	0.00	0.00	<0.01	2.00
	Prox. (62)	10	0.3	0.00	0.00	0.00	<0.01	0.52
	Distal (62)	2	0.0	0.00	0.00	0.00	<0.01	0.02
TOC ppm	Mixed (107)	100	2.3	1.64	1.29	2.20	0.42	29.60
	Prox. (62)	94	1.2	1.35	0.81	2.14	<1	9.55
	Distal (62)	90	1.4	1.43	0.91	2.00	<1	20.25
Fe ppm	Mixed (107)	78	0.3	<0.01	0.00	0.03	<0.01	0.42
	Prox. (62)	71	0.03	<0.01	0.00	0.02	<0.01	0.20
	Distal (62)	77	0.04	<0.01	0.00	0.03	<0.01	0.45
Zn ppm	Mixed (107)	94	0.18	0.13	0.05	0.24	<0.01	0.90
	Prox. (62)	100	0.25	0.18	0.08	0.33	0.03	1.30
	Distal (62)	100	0.26	0.07	0.05	0.13	0.01	4.01
Cu ppm	Mixed (107)	93	0.35	0.13	0.04	0.44	<0.01	2.10
	Prox. (62)	98	0.17	0.05	0.02	0.12	<0.01	1.54
	Distal (62)	97	0.15	0.03	<0.01	0.13	<0.01	1.25

The results are distributed according to sampling methods: mixed (n=107), proximal (n=62) and distal (n=62) samples

[a] Geometric mean

pH, turbidity, free chlorine, TOC and iron concentration levels. Copper and zinc concentrations were slightly higher and differed significantly in the proximal samples ($p<0.05$), probably because these metals are common compounds used in tap alloys and appear in higher concentrations due to leaching. In summary, the characteristics of the distal and proximal samples corresponded to two different niches with different microbial loads.

Characteristics of hot water contaminated with *Legionella*

The analytical results from those samples that contained *Legionella* are shown in Table 2. These include 28 mixed, 22 proximal and 14 distal samples. In these samples, HPC cultivated at 37 or 22 °C always occurred above 10^{3} CFU/100 mL, regardless of the sampling protocol, with mean values between 1.93×10^{6} CFU/100 mL and $1.62\times$

Table 2 Microbiological parameters (CFU/100 mL) and temperatures of hot water samples contaminated with *Legionella*

Parameter	Type	Detection	Geom. mean	Max.	Min.	*r*
Legionella spp.	Mixed	28/28	1.18×10^{2}	2.95×10^{3}	2.00×10^{0}	1.00
HPC 22 °C	Mixed	28/28	1.62×10^{6}	8.64×10^{7}	4.00×10^{3}	0.31
HPC 37 °C	Mixed	28/28	1.93×10^{6}	2.80×10^{7}	2.00×10^{3}	−0.04
Pseudomonas spp.	Mixed	13/28	2.14×10^{1}	1.84×10^{4}	0	−0.04
Protozoa (+/−)	Mixed	13/28	–	–	–	
Temperature (°C)	Mixed	28/28	42.42	65.00	25.00	−0.34*
Legionella spp.	Proximal	22/22	2.26×10^{2}	2.00×10^{3}	1.80×10^{1}	1.00
HPC 22 °C	Proximal	22/22	1.01×10^{6}	1.10×10^{7}	2.40×10^{4}	0.31
HPC 37 °C	Proximal	22/22	1.45×10^{6}	9.60×10^{6}	1.50×10^{4}	0.16
Pseudomonas spp.	Proximal	14/22	1.04×10^{2}	1.20×10^{4}	0	0.40*
Protozoa (+/−)	Proximal	12/22	–	–	–	–
Temperature (°C)	Proximal	22/22	27.31	36.00	16.30	0.31
Legionella spp.	Distal	14/14	4.48×10^{1}	5.30×10^{3}	3.00×10^{0}	1.00
HPC 22 °C	Distal	14/14	2.18×10^{5}	2.80×10^{7}	1.10×10^{3}	0.46*
HPC 37 °C	Distal	14/14	3.97×10^{5}	3.00×10^{7}	5.00×10^{3}	0.39
Pseudomonas spp.	Distal	5/14	1.17×10^{1}	1.00×10^{4}	0	0.75**
Protozoa (+/−)	Distal	4/14	–	–	–	–
Temperature (°C)	Distal	14/14	50.85	60.00	45.00	−0.65**

Correlation coefficients according to Spearman's test
*$p<0.1$; **$p<0.05$

10^{6} CFU/100 mL. *Pseudomonas* was detected in 46 % of the mixed samples, 64 % of the proximal samples and 36 % of the distal samples. Protozoa with a detection limit of 50 cells/100 mL were detected in 46 % of the mixed, 54 % of the proximal and 29 % of the distal samples, with a major abundance in samples where *Legionella* was present. The greater abundance of microbiota in water where *Legionella* was detected indicated that the presence of bacteria was probably associated with the presence of biofilms in the hot water circuit pipes.

Physicochemical data from the mixed, proximal and distal samples with *Legionella* (data not shown) were very similar to the totals shown in Table 1, with the exception of temperature because *Legionella* was not isolated in samples taken above 60 °C. The presence of the bacteria was distributed unequally at different temperatures: 24 % of the samples analysed were positive when the temperature was below 25 °C, 44 % were positive between 25 and 35 °C, 23 % were positive between 35 and 45 °C, 28 % were positive between 45 and 55 °C, and 12 % were positive at temperatures above 55 °C.

To establish whether there was any correlation between *Legionella* and any of the other parameters, Spearman's correlation was applied (Table 2). Results showed a negative correlation between *Legionella* and temperature in the mixed and distal samples, and a positive correlation with *Pseudomonas* in the proximal and distal samples, plus a positive correlation with HPC at 22 °C in the distal samples.

Although the proximal and distal samples were obtained from the same tap, and therefore corresponded to one single circuit, *Legionella* was sometimes detected in one, two or any of the 62 taps sampled. Figure 1 shows the results of the *Legionella* counts, HPC at 37 °C and temperature in distal and proximal samples collected from contaminated circuits. In the proximal water samples with moderate temperatures, microorganisms were significantly more abundant than in the distal samples, although there were some exceptions. In 5 out of 22 distal samples, there were higher counts of heterotrophic bacteria, and in two distal samples, there were higher numbers of *Legionella*. Remarkably, when *Legionella* was present in a distal sample, the corresponding proximal sample was always positive and showed higher numbers. With few exceptions, *Legionella* was more abundant in the distal samples when the maximum temperature was around 45 °C.

Relation of the microbial and physicochemical parameters with *Legionella* colonization in hot water

To establish the relations of water parameters with the presence of *Legionella*, results from samples with the bacterium were compared to those without the bacterium using the Mann–Whitney *U* test. To prevent a statistical bias due to the distal and proximal samples not being independent, given that they came from the same tap, data from the distal samples were not analysed. Only the mixed and proximal samples were considered. Non-significant differences were found when we compared the parameters in the mixed and proximal samples using the Mann–Whitney *U* test, and therefore both sample types (169 samples) could be considered for further analysis.

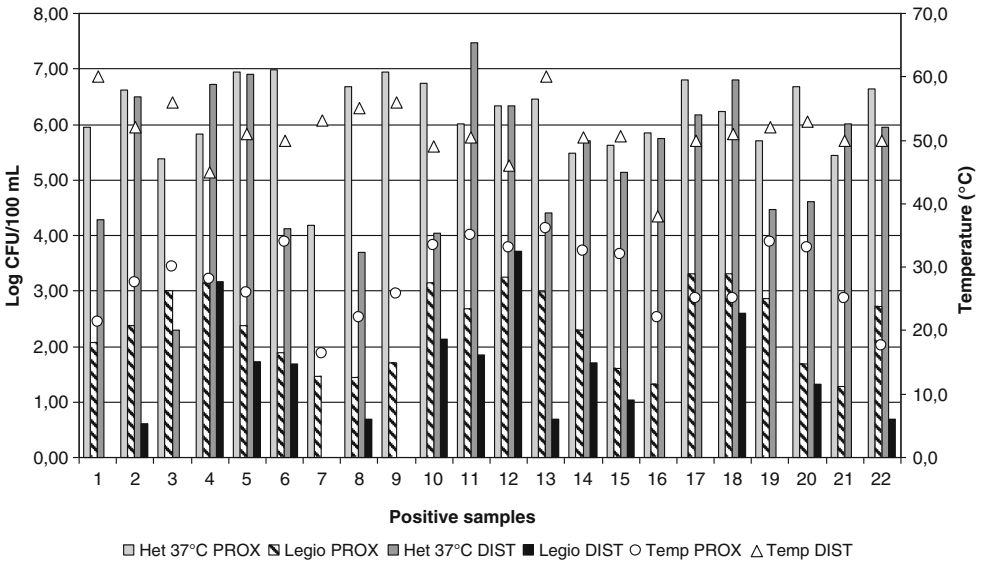

Fig. 1 *Legionella* counts, HPC at 37 °C and temperatures of proximal and distal samples collected from contaminated circuits (n=22)

In Table 3, the mean values for microbiological and physicochemical parameters from the 169 samples were compared based on the presence or absence of *Legionella*. The statistical test revealed that when *Legionella* was present, the mean values of HPC at 22 and 37 °C were significantly higher, and temperature was significantly lower (p<0.05). As well, the presence of protozoa was higher when the bacteria were present, 53 versus 23 %.

To evaluate the risk of *Legionella* colonization in the hot water circuits with recirculation, the odds ratio for each parameter was calculated. The cutoff levels for the parameters were chosen based on the most specific and sensitive. The odds ratios (OR) are shown in Table 4 and indicate that the risk of *Legionella* colonization in the circuits significantly increased in hot water and exceeded the cutoff values for concentration of HPC at 22 °C (OR=14), HPC at 37 °C (OR=18) and protozoa (OR=3), and significantly decreased with respect to Cu concentration. Temperature varied significantly, but no association was found with the presence of *Legionella*. This was probably due to an insufficient number of samples.

Finally, the variables that were considered more closely related to *Legionella* colonization, according to the odds ratios and previous studies, were tested using multiple logistic regression analysis (see Table 4). The regression analysis showed that the values of HPC at 37 °C, protozoa and Fe increased the risk of *Legionella* colonization.

Discussion

Hotels and nursing homes in Spain have hot water recirculation systems. These systems are regularly controlled to prevent

Table 3 Mean values and ranges of the analysed parameters in hot water samples, mixed and proximal, related with *Legionella* spp. occurrence, and significant differences between means according to Student's t test

Parameters	Total (n=169)		*Legionella* occurrence		
	Mean range		Negative (n=119) Mean	Positive (n=50) Mean	p value
HPC 22 °C (CFU/100 mL)	5.72×10^{6} [a]	(< 5×10^{2}–8.80×10^{7})	4.27×10^{4} [a]	1.32×10^{6} [a]	<0.05
HPC 37 °C (CFU/100 mL)	5.21×10^{6} [a]	(<5×10^{2}–8.00×10^{7})	5.5×10^{4} [a]	1.7×10^{6} [a]	<0.05
Pseudomonas (CFU/100 mL)	2.99×10^{4} [a]	(<5×10^{1}–2.00×10^{6})	7.24×10^{1} [a]	1.51×10^{2} [a]	ns
Temperature	40.73	(16.30–71.70)	42.20	37.23	<0.05
pH	7.5	(6.0–8.8)	7.54	7.56	ns
Turbidity (NTU)	0.8	(0.1–5.0)	0.79	0.69	ns
TOC (ppm)	2,284.2	(1–29,600)	2,523	1,716	ns
Fe (ppm)	28.32	(<0.01–418)	26	33	ns
Zn (ppm)	206.65	(<0.01–1,300)	205	209	ns
Cu (ppm)	283.91	(<0.01–2,101)	330	173	ns

ns non-significant

[a] Geometric mean

Table 4 Association between *Legionella* and hot water parameters according with the univariant and multivariate odds ratio regression

		Univariate		Multivariate	
Parameter	Cutoff	χ^2 p value	Odds ratio (95 % CI)	[z]p value	Odds ratio (OR)
HPC 22 °C	5×10^3	0.001	14.09 (1.86–106.85)		
HPC 37 °C	1.4×10^4	0.0002	18.42 (2.44–138.92)	0.018	14.17 (1.57–128.27)
Pseudomonas	1×10^5	$p>0.05$	3.82 (0.62–23.58)		
Temp.	55 °C	0.045	0.33 (0.11–1.02)	0.520	0.66 (0.18–2.37)
pH	6.92	$p>0.05$	3.00 (0.36–25.053)		
TURB.	2.02	$p>0.05$	2.49 (0.34–18.19)		
Cl	Presence	$p>0.05$	0.62 (0.19–1.96)		
TOC	0.25	$p>0.05$	2.55 (0.30–21.74)		
Fe	0.095	$p>0.05$	2.23 (0.71–7.02)	0.025	5.10 (1.23–21.13)
Zn	0.391	$p>0.05$	1.36 (0.51–3.64)		
Cu	0.764	0.044	0.11 (0.053–0.98)	0.349	0.46 (0.089–2.35)
Protozoa	Presence	0.0002	3.67 (1.82–7.42)	0.000	4.05 (1.82–8.88)

legionellosis outbreaks (Boletín Oficial del Estado 2003a). Despite this, *Legionella* may be isolated from hot water and has sometimes been related to human infections. The risk associated with contaminated hot water could decrease if a better understanding of *Legionella* ecology in these systems was reached. This work was focused on the biological and physicochemical parameters that provide the most favourable conditions for *Legionella* colonization and proliferation in hot water recirculation systems.

Legionella was detected by culture and PCR in 28 and 41 % of the samples, respectively. The DNA of *Legionella* was detected in many samples from which the bacteria could not be isolated in culture media, as observed by other authors (Catalan et al. 1997; Devos et al. 2005; Edagawa et al. 2008). Differences between detection and isolation were especially high in the distal samples, where temperature was high. This is probably because the bacteria are stressed in extreme conditions thus transforming into a non-culturable state or becoming inactivated. The *Legionella* isolated from the hot water systems analysed were mainly identified as *L. pneumophila*, in line with other authors such Bargellini et al. (2011). Although more than 50 species of the genus *Legionella* have been found in natural environments, most of the species isolated from sanitary hot waters systems are *pneumophila*, and much lower recoveries of other species such as *anysa*, *longbeachae* and *micdadei* have been described (Alary and Joly 1991; Edagawa et al. 2008; Fields et al. 2002; Leoni et al. 2005; Mathys et al. 2008; Mouchtouri et al. 2007).

The presence of *Legionella* was related to the presence of microbiota. *Legionella* and accompanying microbiota, both bacteria and protozoa, were more abundant in the first flush (proximal water). The results indicate that *Legionella* was largely isolated when the HPC mean was between 5 and 6 logs CFU/100 mL, indicating that *Legionella* may only appear when there is a biofilm on which it can grow. HPC obtained at 22 and 37 °C were very similar, in contrast with data published by Bargellini et al. (2011). However, there is no correlation between the heterotrophic bacteria counts and the *Legionella* counts (Bargellini et al. 2011; Edagawa et al. 2008) in our work or in other published reports. Nevertheless, logistic analysis showed that the risk of the presence of *Legionella* increased 18-fold when the HPC at 37 °C exceeded 3.4 logs per 100 mL. This is probably because the presence of bacteria is essential for the survival of *Legionella* in water systems, but their presence alone does not determine the occurrence of the pathogen (Temmerman et al. 2006). *Pseudomonas*, a group within heterotrophic bacteria, has previously been linked with the presence of biofilms and regrowth of *Legionella* in water pipes (Paszko-Kolva et al. 1991; Ribas et al. 2000). Therefore, we were interested in looking for a possible relationship between the presence of the two bacteria types. Data published on this subject are inconclusive; some authors consider that *Pseudomonas* favours the presence of *Legionella*, and others state that it inhibits it (Borella et al. 2005a). Our results did not clarify this point, although there was a correlation between them at high temperatures. Nevertheless, further research is needed.

One of the objectives of this study was to determine the relationship between the presence of *Legionella* and protozoa. Since the studies by Rowbotham in 1980, multiple studies showing the growth and survival of the pathogen within protozoa have been described, especially in in vitro conditions (Rowbotham 1980; Thomas et al. 2006). Some authors, such as Rohr et al. (1998) and Declerck et al. (2007), have detected the presence of amoebas in water containing *Legionella*. Their association in man-made water systems has been studied in different environments, including spring waters, tap water and treated water, for example from cooling towers (Marciano-Cabral et al. 2010; Ohno et al. 2003; Valster et al. 2011; Yamamoto et al. 1992). However, there are few studies on the presence of protozoa and *Legionella* in characterized hot water systems, as described in this work. We

observed protozoa in 45 % of the hot water samples contaminated with *Legionella*, and amoebas were present in nearly all of them. These data are similar to those reported by Henke and Seidel (1986), who detected amoeba and *Legionella* in 38 % of warm drinking water samples, and are higher to 13 % detected by Habicht et al. (1988). According to these results and those of other authors (Ohno et al. 2003; Paszko-Kolva et al. 1991), *Legionella* can survive as a free organism for long periods of time by maintaining cultivability under non-strict environments. However, our data show that the presence of protozoa increases the risk of *Legionella* occurrence (odds ratio, 3). This is probably because protozoa facilitate bacterial growth and survival in hot water systems, similar to the way Yamamoto et al. (1992) demonstrated in cooling towers.

The data range of physicochemical characteristics of hot water circuits, pH, turbidity and TOC was enough to facilitate *Legionella* growth, and no significant differences were found between samples with *Legionella* and those without the bacteria. May be the analysis of other parameters such as natural organic matter or Mn which have been also related with the presence of Legionella in water by other authors (Bargellini et al. 2011; Wullings et al. 2011) would expand the information of the water system.

In agreement with the literature (Groothuis et al. 1985; Lee et al. 1988; Mathys et al. 2008), the presence of *Legionella* was affected by temperature. *Legionella* was isolated within a wide range of temperatures. Most of the isolates were between 25 and 45 °C, its growth temperature, but there were also some under 25 and above 55 °C, as reported by other authors (Borella et al. 2005a; Dennis et al. 1984; Hruba 2009; Mathys et al. 2008). This could be because those cells were protected by other microorganisms, which were probably organized in biofilms in pipelines. Thus, in hot water recirculation systems with varying temperatures, warm temperatures stimulate bacterial multiplication and high temperatures act as a biocide (Darelid et al. 2002). However, it is difficult to determine an exact temperature because it depends on how protected the bacteria are on a biofilm or as a protozoan endosymbiont. Therefore, further research is needed on the biocidal effect of temperature on *Legionella* in relation to biofilms, with or without the presence of protozoa.

Metals such as Fe or Zn in the environment are important parameters for bacterial growth and virulence (Reeves et al. 1981; States et al. 1985; Yaradou et al. 2007). Bacteria cannot grow in culture media without Fe. The logistic analysis showed that the presence of Fe above 0.095 ppm is associated with *Legionella*. Moreover, the bacteria were not isolated from samples with copper levels above 0.76 ppm, which is in agreement with other authors, such as Rogers et al. (1994) and Borella et al. (2004), who proposed that Cu inhibits its growth.

Although the mains water that supplies the buildings had free residual chlorine (data not shown) in the majority of the hot water samples, it was not detected. This was probably because it had evaporated, was under the detection limit or had already transformed into by-products. Similarly, other authors who have studied chlorinated hot water systems did not detect residual free chlorine at tap level (Plouffe et al. 1983).

In summary, the presence and growth of *L. pneumophila* in the environment is multifactorial and dependent on the microbial and physicochemical characteristics of each system. Therefore, to understand its behaviour it is very important to define the characteristics of the habitat, as we have done in the present work. The physicochemical parameters of the hot water recirculation sanitary systems, usually without free chlorine, were compatible with bacterial presence. Temperature, defined as a key factor for many authors (Hruba 2009), had two different effects as the results have shown; it promoted the multiplication of the bacteria, something that often occurred in the stagnant water nearest to the tap, or it acted as a biocide when the system achieved a temperature above 55 °C. The presence of metals derived from pipelines and fittings had an important effect on the bacteria, Fe favouring bacterial growth and copper inhibiting it. Likewise, the most determinant factors in these hot waters systems have been the accompanying microbiota. High counts of heterotrophic bacteria and the presence of protozoa favoured the growth of *Legionella* and protected it from harsh conditions. Probably these microorganisms were structured in biofilms from where the bacteria were detached contaminating running water.

Finally, it should be highlighted that the first flush of the hot water tap may be the most susceptible source of legionellosis infection because microorganisms, both bacteria and protozoa, have accumulated at this point in the circuit where biocides, specially temperature, are not particularly active. Therefore, this first water should be specifically considered when considering the health risk associated to hot water recirculation systems and when monitoring them to analyse the occurrence of *Legionella*.

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7.2. EFFECT OF THERMAL TREATMENT ON FREE-LIVING AMOEBA INACTIVATION

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ORIGINAL ARTICLE

Effect of thermal treatment on free-living amoeba inactivation

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Acanthamoeba, amoeba, dialysis, disinfection, *Hartmannella*, thermal.

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Abstract

Aims: To evaluate the effect of temperature on two amoeba strains of the genera *Acanthamoeba* and two amoeba strains of the genera *Hartmannella* separately treated depending on their life stage, trophozoite or cyst, when cells are directly exposed under controlled conditions.
Methods and Results: For thermal treatments, three temperatures were selected 50, 60 and 70°C, and a microcosm was designed using dialysis bags. The inactivation of each strain was determined using a method based on the most probable number quantification on agar plates. The results showed that for all amoeba strains, thermal treatment was more effective against trophozoites compared with cyst stages. The inactivation patterns showed statistical differences between the two genera analysed at temperatures above 50°C. The effectiveness of the thermal treatments at 60 and 70°C was higher for both life stages of *Hartmannella vermiformis* strains compared with *Acanthamoeba* strains, being the most resistant *Acanthamoeba* cysts.
Conclusions: Free-living amoebae have been isolated in a wide range of environments worldwide due to their capacity to survive under harsh conditions. This capacity is mainly based on the formation of resistant forms, such as double-walled cysts, which confers a high level of resistance as shown here for thermal treatments.
Significance and Impact of Study: Free-living amoebae survival can promote a rapid recolonization of drinking water systems and is a likely source of emerging opportunistic pathogens such as *Legionella*. Because of that a better understanding of the factors that affect micro-organism inactivation in water systems would allow more efficient application of disinfection treatments.

Introduction

Free-living amoebae (FLA) are widespread protozoa and normal inhabitants of soil, and in particular, fresh-water sources (Rodriguez-Zaragoza 1994). These aquatic sources include environmental water systems such as lakes, ponds (Barbeau and Buhler 2001) and man-made water systems, including water supplies, hospital water networks, cooling towers, swimming pools and hydrotherapy baths (Schmitz-Esser *et al.* 2008; Thomas *et al.* 2010).

Amoebae are a group of unicellular micro-organisms that have a two-stage life cycle: a replicating and feeding trophozoite stage and a latent cyst stage. In the trophozoite stage, FLA are important predators of prokaryotic and eukaryotic micro-organisms with much influence on the composition of the microbial community feeding on biofilms and detritus (Rodriguez-Zaragoza 1994; Ramaley *et al.* 2001; Schmitz-Esser *et al.* 2008). Specifically, they establish symbiotic relationships with other microbes including bacteria of the genera *Legionella, Mycobacterium*, *Campylobacter* and *Listeria*, which, apart from being a nutritional source, are able to survive amoeba digestion and multiply within them (Greub and Raoult 2004; Schmitz-Esser *et al.* 2008). Moreover, some FLA species can infect humans and other mammals, causing serious illnesses like ocular and central nervous system

infections (Kollars and Wilhelm 1996; Schuster and Visvesvara 2004).

Among FLA species, *Acanthamoeba* and *Hartmannella* are two of the most widely distributed genera. *Acanthamoeba* spp. are the most prevalent protozoa in aquatic environments, including fresh and marine waters (Marciano-Cabral and Cabral 2003; Behets *et al.* 2007). The cyst of *Acanthamoeba* species is characterized by a double-walled envelope, an outer exocyst and an inner endocyst (Hirukawa *et al.* 1998). The major components of cyst walls are proteins and cellulose, which are differentially located in the trophozoite stage and constitute an important characteristic of the genus (Rubin *et al.* 1976; Stewart and Weisman 1974). On the other hand, *Hartmannella vermiformis* has become an important species in the recent years due to its role as a bacterial pathogen host (Dykova *et al.* 2005). As a gymnamoebae, it has two life stages: trophozoite with an elongated cylindrical monopodial locomotive shape, and a double-walled cyst, the structure of which varies from strain to strain, mainly in terms of the distance between the ectocyst and the endocyst (Smirnov and Michel 1999). Unlike *Acanthamoeba* strains, the presence of cellulose in *H. vermiformis* cystic walls has not been described.

The distribution of amoeba species relies on their capacity to survive in front of a wide range of environmental factors, with the cysts being able to tolerate changes in salinity, dessication, temperature and osmotic pressure, as well as exposure to chemicals and prolonged starvation (Rodriguez-Zaragoza 1994; Greub and Raoult 2004). These structures also confer greater resistance to many commonly used biocides than typically found in bacteria (Taylor *et al.* 2009). FLA are also able to persist within biofilms and may act as environmentally shielded reservoirs and vectors for pathogens, especially when they are encysted (Winiecka-Krusnell and Linder 1999; Kuiper *et al.* 2004; Thomas *et al.* 2006).

Free-living amoebae usually grow at temperatures between 10 and 30°C. Below 4°C, only cold-resistant strains survive, such as *Acanthamoeba polyphaga* (Rodriguez-Zaragoza 1994). However, several amoeba species are also capable of growing at or above 40°C, such as *Naegleria fowleri* (De Jonckheere 2006). Rohr *et al.* (1998) isolated some *Hartmannella* and *Saccamoeba* species from hospital hot water systems that were able to grow at 53°C, and other authors such as Serrano-Suarez *et al.* (2013) also reported their presence in hot water systems of several hotels and nursing homes (Serrano-Suarez *et al.* 2013). Pathogenic amoebae have also been observed by Ramaley *et al.* (2001) in Yellowstone National Park (Ramaley *et al.* 2001; Baumgartner *et al.* 2003). These amoeba species have been classified by some authors as thermotolerant (Storey *et al.* 2004).

Although relationships between FLA and bacteria are frequent in natural environments, these are a cause of concern when they occur in drinking or sanitary water. These types of water are subject to national guidelines to maintain microbial populations under control using biocide treatments. In Spain, one of the most commonly used disinfection systems in hot water sanitary systems is thermal treatment (BOE 2003); however, regulations are based on its effect on bacteria with no reference to other micro-organisms that may be present in the same water system. In consequence, although thermal treatments are generally considered trophocidal and cysticidal, it is important to determine the effect of the treatments applied on FLA (Griffin 1972; Storey *et al.* 2004; Coulon *et al.* 2010; Pumidonming *et al.* 2010).

The purpose of the study was to evaluate the effect of thermal treatments at three different temperatures 50, 60 and 70°C on the inactivation of four FLA strains specifically considering the differences in the inactivation pattern between life stages (trophozoites and cysts). Of the four strains used in the study, two *Acanthamoeba* strains and two *Hartmannella* strains, one strain of each genus was isolated from hot tap water in Catalunya. The test was performed in laboratory microcosms using dialysis bags, which ensured that the temperature effect was applied homogenously to cell suspensions. Amoeba inactivation after thermal exposure was measured as the loss of culturability according to the most probable number (MPN) method.

Material and methods

Amoeba strains

Inactivation studies were conducted on four amoebal strains: two strains obtained from the Culture Collection of Algae and Protozoa: *Acanthamoeba castellanii* CCAP 1534/2 and *Hartmannella vermiformis* CCAP 1534/7A; and two environmental strains *Hartmannella vermiformis* 195 and *Acanthamoeba* sp. 155 previously isolated by our group from hot tap water in Catalunya (described below). The four strains were stored at the cyst stage at −80°C in Ringer 1/40 [Scharlau] with 20% glycerol [Panreac].

Isolation of environmental amoeba strains

Environmental strains were isolated and identified using an adaptation of the method of Thomas *et al.* (2006). One litre of hot water sample was filtered through a 0·45-μm nylon membrane. The membrane was then resuspended in 10 ml of Ringer 1/40 from there, 200-μl aliquots were spread on non-nutritive agar plates (NNA)

(1·5% Agar [Pronadisa], 0·5% NaCl [Panreac]) (Sanden *et al.* 1992) that had previously been seeded with a layer of living *Escherichia coli* WG5, ATCC 25922. NNA plates were incubated at 30°C and examined daily for the presence of amoebal cells and subcultured again on new NNA plates with *E. coli*. After three subcultures, under the same conditions, amoebae were harvested by scraping and resuspended in 1 ml of Ringer 1/40. For amoebae identification, DNA extraction was performed by following the protocol described by Serrano-Suarez *et al.* (2013). A PCR amplifying the 18S rRNA was performed following the protocol described by Thomas *et al.* (2006) and Serrano-Suárez (2009). PCR products were then sequenced using the same primers as in the PCR. Reactions were performed with the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction kit version 3.1 with Ampli Taq® DNA polymerase FS (Applied Biosystems, Austin, TX, USA) according to the manufacturer's instructions. Nucleotide sequences were analysed using the basic BLAST tool (Altschul *et al.* 1990). Two amoeba isolates were identified as *H. vermiformis* and a species of the genera *Acanthamoeba*. Both were included in the inactivation studies.

Differentiated FLA suspensions

The strains of amoebae were cultured on NNA plates (Sanden *et al.* 1992) seeded with fresh *E. coli* WG5 at 30°C for 8–10 days. For each strain, two thermal exposure tests were performed according to the life stage. For the *Acanthamoeba* trophozoite experiments, axenic cultures were obtained according to the method described by Walochnik *et al.* (2000) using the liquid medium ATCC 1034 PYG (proteose peptone-yeast extract-glucose). Briefly, double-walled cysts from NNA plate cultures were harvested and incubated in 3% HCl overnight to eliminate possible accompanying bacteria. Two washing steps with Ringer 1/40 were performed by centrifuging the cysts at 800 ***g*** for 15 min and then transferring them to 10 ml of the liquid culture medium in 25 cm^3 tissue culture flasks [Nunc]. Trophozoite cultures were maintained axenically by subculturing them in PYG in 25 cm^3 culture flasks. After the trophozoites were grown to confluence for 2–3 days at 30°C, they were recovered from the tissue culture flasks by shaking softly. *Acanthamoeba* cysts were obtained by culturing amoebal strains on NNA agar plates seeded with fresh *E. coli* at 30°C for 10 ± 2 days. At that time, cultures composed of 90% of double-walled cysts were harvested with Ringer 1/40. *H. vermiformis* strains were not grown in PYG due to their special nutritional requirements, so both cysts and trophozoites were cultured on NNA plates seeded with fresh *E. coli*. Trophozoites were harvested from the plates on day 2 before the population encysted, while cysts were obtained after 10 days.

Finally, trophozoite and cyst suspensions obtained from the four FLA strains were centrifuged at 800 ***g*** for 15 min and resuspended in Ringer 1/40, adjusting to a final concentration of 1×10^5 amoeba cells ml^{-1} using a Neubauer chamber.

Exposure of amoeba strains to varying temperatures

To study amoebal inactivation by thermal treatment, a microcosm system was designed using dialysis bags (Medicell International Ltd., London, UK). The dialysis bags were pretreated to ensure a uniform pore size (molecular weight cut-off of 14 kDa) and sterilized before use.

Dialysis bags containing 2 ml of each amoebal suspension were sealed with a knot and placed in a bath (Wasserbad Water Bath 1002–1013, GFL). Three experimental temperatures were tested: 50, 60 and 70°C at several exposure times. Trophozoites and cysts were exposed at 50°C for, 5, 10, 20 and 30 min, at 60°C for 2, 5, 10, 15, 20 and 30 min and at 70°C for 0·5, 1 and 2 min. The data reported in this study were obtained independently in triplicate. Once the thermal treatment was performed, 10-fold dilution series in Ringer 1/40 were transferred to NNA plates previously seeded with five spots of 20 μl of fresh *E. coli* WG5. For every *E. coli* spot, 10 μl of the diluted treated sample was added. The plates were incubated at 30°C for 8 days and checked every 2 days using inverted microscopy (Axiovert 25, Zeiss, Oberkochen, Germany). The presence of trophozoites in a dilution spot was considered positive. The MPN values were obtained from MPN tables (ISO 8199:2005) (Anonymous 2005).

Statistical data analysis

The results are reported as the log reduction mean ± standard deviation (SD). The experimental conditions were statistically analysed using full factorial ANOVA tests (Statgraphics® Plus 5.1, Rockville, MD, USA); P values <0·05 were considered to indicate statistical significance. After the ANOVA test, the pairwise Fisher's Least Significant Difference (LSD) test was used to discern between the means in cases of significant difference (Statgraphics® Plus 5.1). Before any statistical analysis, the data were checked for compliance with ANOVA assumptions. The graphs were plotted using GraphPad Prism 4 (GraphPad Software, San Diego, CA, USA).

Free-living amoebae inactivation was calculated as the decrease in $\log_{10}$ units (log reduction Nt/N_0), where N_0 is the value at the beginning of the experiment and Nt is the value at the times indicated (t). Inactivation kinetics were modelled using a monophasic exponential decay

and a polynomial first-order regression (straight line) following Chick Watson's law (Chick 1910):

$$\log_{10}(Nt) = \log_{10}(N_0^{-\lambda t})$$

where λ is the inactivation kinetic constant. The model parameters were estimated by the least squares estimation, and the best fitting model was chosen using the Akaike Information Criterion (Hurvich and Tsai 1989). A correlation coefficient of R^2 was added. The times required to achieve a 3-log reduction were estimated by solving (t) with the equations mentioned above using the most likely parameter values. All data were fitted to the experimental data using the program GraphPad Prism 4.

Results

Effect of thermal treatment on FLA strains

A thermal treatment at three different temperatures (50, 60 and 70°C) was applied to four free-living amoeba (FLA) strains: *A. castellanii* CCAP 1534/2 strain, *Acanthamoeba* sp 155, *H. vermiformis* CCAP 1534/7A strain and *H. vermiformis* 195. Each experiment was performed based on the amoebal life stage: trophozoite or cyst. Comparison of the inactivation pattern between life stages showed that for all amoeba strains, the thermal treatments were significantly more effective against trophozoites compared with cysts (Fig. 1).

Effect of thermal treatment on FLA trophozoites

The analysis of trophozoite inactivation of the four FLA strains showed no significant differences between strains when cells were exposed to 50°C. Thermal treatments at 60°C and 70°C for <2 min reduced the viability of both *Hartmannella* strains by more than 4 logs. In the case of *Acanthamoeba* strains, the viability of *Acanthamoeba* sp. 155 was reduced by more than 4 logs after being exposed to 60°C for more than 5 min and to 70°C for more than 0·5 min, whereas it was necessary to expose trophozoites of *A. castellanii* CCAP 1534/2 to 60°C for more than 10 min to obtain the same result. Moreover, *A. castellanii* CCAP 1534/2 was the only strain in which some trophozoites remained viable after being treated at 70°C for 0·5 min (Fig. 1).

The inactivation kinetics of trophozoites was calculated at 50, 60 and 70°C. The results showed that trophozoites of all FLA strains fitted a monophasic exponential decay model when amoeba cells were exposed to 50°C. High R^2 values supported the robustness of the model (Table 1). The estimated inactivation times required to obtain a 3-log reduction at 50°C revealed that *H. vermiformis* 195 was the most resistant strain, requiring 32·5 min exposure, whereas the other strains required between 21·7 and 27·6 min exposure. The inactivation kinetics of *H. vermiformis* at 60 and 70°C could not be estimated due to strains reached the maximum log reduction at the lowest times analysed. On the other hand, *Acanthamoeba* strains achieved a 3-log reduction at 60°C in 1·01 min for *Acanthamoeba* sp. 155 and 4·06 min for *A. castellanii* CCAP 1534/2 (Table 1).

Effect of thermal treatment on the FLA cysts

The viability of cysts of all strains was reduced by <1 log after 30 min at 50°C, with no significant differences between strains at this temperature. Amoebal cyst inactivation clearly increased when cells were treated at 60 and 70°C. These temperatures significantly affected the two *Hartmannella* strains, the viability of which was reduced by more than 4 logs after being treated for 2 min at 60°C or 0·5 min at 70°C. In the case of *Acanthamoeba* cysts, although significant differences were found between strains, both *A. castellanii* CCAP 1534/2 and *Acanthamoeba* sp. 155 were more resistant to thermal treatment than the *Hartmannella* strains. While the viability of *Acanthamoeba* sp. 155 was reduced by 4·5 logs after exposure to 60°C for 20 min, *A. castellanii* CCAP 1534/2 showed just a 2-log reduction under the same conditions. *A. castellanii* CCAP 1534/2 was the only strain in which cysts remained viable after thermal treatment at 70°C for 1 min.

Cyst inactivation at 50°C fitted a first-order polynomial model (Table 1). The time required to reduce the viability of the initial amoeba suspensions by 3 logs varied from one strain to another. Thermal treatment was most effective against *H. vermiformis* 195, the viability of which decreased by 3 logs in 89 min; the same loss of viability required exposure for 122 min for *Acanthamoeba* sp. 155, 206 min for *H. vermiformis* CCAP 1534/7A and 227 min for *A. castellanii* CCAP 1534/2. At 60°C, the time required to undergo a 3-log reduction was 14·2 min for *Acanthamoeba* sp. 155 and 32·1 min for *A. castellanii* CCAP 1534/2.

Discussion

This work focused on the effect of thermal treatments at 50, 60 and 70°C on amoeba cells. To observe how the effect of thermal treatment varies between life stages, trophozoites and cysts of CCAP and environmental FLA strains were treated separately. The lack of official standards available to assess the effectiveness of disinfectant treatments against amoebae (Coulon *et al.* 2010) led us to design a microcosm system using dialysis bags to ensure that the thermal effect was homogenously applied to all

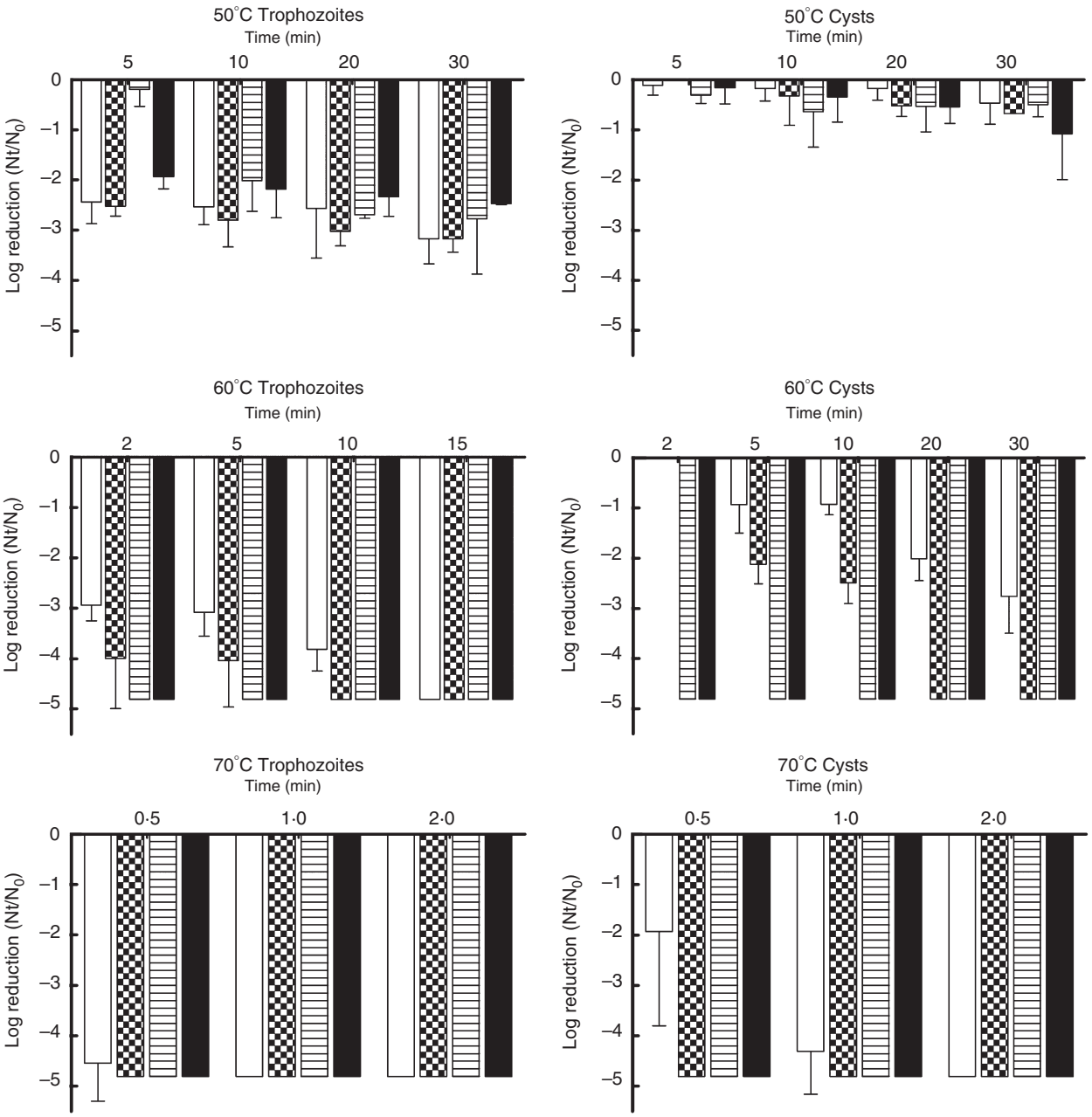

Figure 1 Effect of thermal treatments at 50, 60 and 70°C on the viability of trophozoites and cysts of four free-living amoeba strains, (□) *Acanthamoeba castellanii* CCAP 1534/2, (▩) *Acanthamoeba* sp. 155, (▤) *Hartmannella vermiformis* CCAP 1534/7A and (■) *H. vermiformis* 195. Inactivation was determined using an adaptation of the MPN method. Data are presented as logarithmic reduction mean ± standard deviation (SD), where N_0 is the initial cell number and Nt is the final number of cells. Time is presented in minutes.

amoeba cells. Moreover, to quantify amoeba survival, an adaptation of the MPN method was implemented. This method proved to be easy to perform and showed a high reproducibility. Although previous studies on the effect of temperature on FLA have been reported (Griffin 1972; Storey *et al.* 2004; Coulon *et al.* 2010; Pumidonming *et al.* 2010), the current study is the first in which the effect of thermal treatments at 50, 60 and 70°C was investigated in four FLA strains at different life stages.

The results showed that the thermal treatments applied were significantly more effective at inactivating trophozoites compared with cysts. Thermal treatment at 50°C showed a 2 to 3-log reduction in the viability of trophozoites after

Table 1 Parameters of FLA strain inactivation kinetics and time needed (min) to achieve a 3-log reduction in viability after being exposed to thermal treatments at 50, 60 and 70°C.

	Strain	Best fitting model (50°C)	R^2	3-log inactivation time (min)		
				50°C	60°C	70°C
Trophozoites	*A. castellanii* CCAP 1534/2	Monophasic	0·96	24·23	4·66	<0·5
	Acanthamoeba sp. 155	Monophasic	0·99	21·77	1·01	<0·5
	H. vermiformis CCAP 1534/7A	Monophasic	0·90	27·62	<2	<0·5
	H. vermiformis 195	Monophasic	0·99	32·59	<2	<0·5
Cysts	*A. castellanii* CCAP 1534/2	Polynomial first order	0·86	227·49	32·14	0·71
	Acanthamoeba sp. 155	Polynomial first order	0·91	122·06	14·02	<0·5
	H. vermiformis CCAP 1534/7A	Polynomial first order	0·41	206·38	<2	<0·5
	H. vermiformis 195	Polynomial first order	0·97	89·01	<2	<0·5

30 min of exposure, whereas cyst inactivation remained below 1 log. No significant differences were found between strains for either cysts or trophozoites at this temperature.

Trophozoite inactivation fitted a monophasic exponential model. The duration of thermal exposure at 50°C required to reduce the viability of FLA trophozoites by 3 logs was similar for all strains: 21 min for *Acanthamoeba* sp. 155, 24 min for *A. castellanii* CCAP 1534/2, 27 min for *H. vermiformis* CCAP 1534/7A and 32 min for *H. vermiformis* 195. On the other hand, cyst inactivation fitted a first-order polynomial model. The time required to achieve a 3-log reduction in viability was 89 min for *H. vermiformis* 195, 122 min for *Acanthamoeba* sp. 155, 206 min for *H. vermiformis* CCAP 1534/7A and 227 min for *A. castellanii* CCAP 1534/2. Cysts suspensions used had at least a 90% of mature cysts; thus, some trophozoites might still be present on the suspensions. The fact that all amoeba cysts fitted a first-order polynomial model with high R^2 values except for *H. vermiformis* CCAP 1534/7A suggest that remaining trophozoites did not alter cysts inactivation.

The World Health Organization (WHO) recommendations on drinking water quality state that water temperature should be kept above 50–55°C in systems in large buildings (WHO 2011). Based on the current study, this temperature might not be high enough to reduce FLA populations in drinking water systems. However, other water treatments such as pasteurization, which involves keeping the water temperature at 63°C for 30 min, could provide a greater level of safety.

Thermal treatment at 60 and 70°C was radically more effective against the four FLA used, and significant differences were found between species, especially *H. vermiformis* compared with *Acanthamoeba* strains. Cysts and trophozoites of both CCAP and environmental *H. vermiformis* strains showed a similar pattern of inactivation at these higher temperatures, with a reduction in viability of more than 4 logs in both stages when temperatures of 60 and 70°C were applied over 2 min and 0·5 min, respectively. This finding differed from the results of Kuchta *et al.* (1993), who reported that a temperature of 60°C was required for 30 min to achieve a cysticidal and trophocidal effect in an *H. vermiformis* environmental strain (Kuchta *et al.* 1993). It should be noted that in this case, the inactivation study was performed using a different strain in a real water system, where other environmental factors may have influenced the temperature effect (Kuchta *et al.* 1993).

In the case of *Acanthamoeba* strains, thermal treatment was more effective against *Acanthamoeba* sp. 155 than the CCAP strain. A temperature of 60°C applied for 2 min reduced the viability of A*canthamoeba* sp. 155 trophozoites by 4 logs, whereas exposure to the same temperature was required for more than 10 min to obtain the same result in the cyst stage. No viable cysts or trophozoites were recovered after thermal treatment at 70°C. Thermal treatment was less effective against *A. castellanii* CCAP 1534/2, the cysts of which showed less than a 4-log reduction in viability after exposure to 60°C for 30 min, and still remained viable after thermal treatment at 70°C for 1 min. So, among the four FLA strains used in the current study, *A. castellanii* CCAP 1534/2 was significantly more resistant to thermal treatment than the others. The differences between life stages shown by the *Acanthamoeba* strains used were consistent with the results of Turner *et al.* (2000), who observed that trophozoites of *A. castellanii* were inactivated following treatment at 46°C for 30 min, whereas it was necessary to increase the temperature to 56°C to obtain the same level of inactivation for cysts (Turner *et al.* 2000). The morphology and structure of *Acanthamoeba* cysts and trophozoites have been studied by several authors (Khunkitti *et al.* 1998; Smirnov and Michel 1999; Coulon *et al.* 2010), who reported a higher cellulose concentration in the inner wall of the cystic stages (Turner *et al.* 2000). Even the information obtained is limited due to the fact that just two species and four strains of FLA were used,

variations in the inactivation pattern between life stages as well as between strains in response to thermal treatment could be attributed to differences in membrane composition, especially as components such as cellulose have been used as fire insulation material (Keerthan and Mahendran 2013).

Comparison of our results with those found in the literature showed heterogenic results. While Ludwig *et al.* (1986) reported a cysticidal effect of thermal treatment on cysts of *A. castellanii* and *A. polyphaga* strains after exposure to 80°C for 10 min (Ludwig *et al.* 1986), Aksozek *et al.* (2002) reported the same effect in an *A. castellanii* strain after exposure to 65°C for more than 5 min (Aksozek *et al.* 2002). The *Acanthamoeba* cysts used in the current study were more resistant than the strain used by Aksozek *et al.* (2002) but more sensitive than that used by (Ludwig *et al.* 1986; Aksozek *et al.* 2002). In the case of *Hartmannella* strains, our results conflicted with the hypothesis of authors such as Rohr *et al.* (1998), who suggested that this genus is more heat tolerant than *Acanthamoeba* strains due to its higher prevalence in hot waters, as thermal treatments were more effective against *Hartmannella* strains than *Acanthamoeba* strains (Rohr *et al.* 1998).

The diversity of results could be attributed to differences in the intrinsic characteristics of each strain and the lack of official standards for inactivation assays, which makes it difficult to compare different studies about the disinfectant effect of temperature, especially when authors use different culture media, initial cell concentrations and methods for quantifying cell viability (Mercer 2008; Thomas *et al.* 2010; Coulon *et al.* 2010). As an example, the method used in the current study using dialysis bags ensured that all cells were quick and evenly subjected to the chosen temperature; therefore, thermal treatment was more effective at inactivating amoeba cells than the other studies mentioned above.

The variability of the results was even higher when comparing with those obtained in real water systems, where the inactivation of micro-organisms could not be analysed as an isolated event but as a complex event influenced by many factors such as the presence of biofilms, pipeline design, water stagnation, etc. (Kuchta *et al.* 1993; Mathers *et al.* 1996; Ahearn and Gabriel 1997; Taylor *et al.* 2009). Even so, it is generally agreed that FLA play an important role in water systems and should be taken into consideration when applying disinfection treatments because as Mathers *et al.* (1996) reported that sublethal disinfectant doses can stimulate encystations of *Acanthamoeba*, which may be a source of thermotolerant strains (Mathers *et al.* 1996; Ahearn and Gabriel 1997; Winiecka-Krusnell and Linder 1999). Furthermore, FLA survival can promote rapid recolonization of these systems and is a likely source of emerging opportunistic pathogens such as *Legionella* (Swanson and Hammer 2000; Thomas *et al.* 2004, 2010; Loret and Greub 2010).

A better understanding of the factors that affect micro-organism inactivation in water systems would allow more efficient application of treatments. For this reason, 'in vitro' experiments such as those reported herein are necessary, and the first step, to find the optimal temperature at which FLA are completely inactivated and at the same time avoid high temperatures that could damage water system supplies, thus helping to optimize energy expenditure (Taylor *et al.* 2009).

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Conflict of interest

The authors declare that they have no conflict of interest concerning this publication.

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7.3. EFFECT OF CHLORINE AND TEMPERATURE ON FREE-LIVING PROTOZOA IN OPERATIONAL MAN-MADE WATER SYSTEMS (COOLING TOWERS AND HOT SANITARY WATER SYSTEMS) IN CATALONIA

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RESEARCH ARTICLE

Effect of chlorine and temperature on free-living protozoa in operational man-made water systems (cooling towers and hot sanitary water systems) in Catalonia

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Abstract In recent decades, free-living protozoa (FLP) have gained prominence as the focus of research studies due to their pathogenicity to humans and their close relationship with the survival and growth of pathogenic amoeba-resisting bacteria. In the present work, we studied the presence of FLP in operational man-made water systems, i.e. cooling towers (CT) and hot sanitary water systems (HSWS), related to a high risk of *Legionella* spp. outbreaks, as well as the effect of the biocides used, i.e. chlorine in CT and high temperature in HSWS, on FLP. In CT samples, high-chlorine concentrations (7.5±1.5 mg chlorine L^{-1}) reduced the presence of FLP by 63.8 % compared to samples with low-chlorine concentrations (0.04±0.08 mg chlorine L^{-1}). Flagellates and amoebae were observed in samples collected with a level of 8 mg chlorine L^{-1}, which would indicate that some FLP, including the free-living amoeba (FLA) *Acanthamoeba* spp., are resistant to the discontinuous chlorine disinfection method used in the CT studied. Regarding HSWS samples, the amount of FLP detected in high-temperatures samples (53.1±5.7 °C) was 38 % lower than in low-temperature samples (27.8±5.8 °C). The effect of high temperature on FLP was chiefly observed in the results obtained by the culture method, in which there was a clear reduction in the presence of FLP at temperatures higher than 50 °C, but not in those obtained by PCR. The findings presented here show that the presence of FLP in operational man-made water systems should be taken into account in future regulations.

Keywords Free-living amoebae · Ciliates · Chlorine · Temperature · Disinfection · Free-living protozoa

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Introduction

In recent decades, free-living protozoa (FLP) have gained greater prominence as the focus of research studies in a wide variety of man-made water systems (Lasheras et al. 2006; Ménard-Szczebara et al. 2008; Patterson et al. 1997; Rohr et al. 1998; Thomas et al. 2008; Yamamoto et al. 1992) due to their pathogenicity to humans (Trabelsi et al. 2012; Visvesvara et al. 2007) and their close relationship with the survival and growth of pathogenic amoeba-resisting bacteria such as *Legionella* spp., *Listeria monocytogenes* and nontuberculous mycobacteria (Cateau et al. 2014; Fields et al. 1984; Greub and Raoult 2004; Rowbotham 1980; Thomas et al. 2006).

In order to control and remove pathogenic microorganisms from engineered water systems (e.g. tap water installations, distribution systems and cooling towers), a wide variety of sanitation practices such as chemical disinfection, UV light and temperature has been employed worldwide (Kim et al. 2002; Sutherland and Berk 1996). However, certain FLP, specifically the free-living amoeba (FLA) *Acanthamoeba* spp., are resistant to most treatments, especially during the cyst stage (Cervero-Aragó et al. 2013; Storey et al. 2004; Thomas et al. 2004). The cysts play a protective role against disinfectants by harbouring amoeba-resisting bacteria, thus

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leading to the multiplication of bacteria and serving as a transmission vector. Regarding the pathogenicity of FLA, amoeba infections such as *Acanthamoeba* keratitis, granulomatous amoebic encephalitis and primary amoebic meningoencephalitis are considered to be rare diseases in Europe. Nevertheless, studies focusing on FLP are required in order to prevent the development of these pathogenic FLA, especially *Acanthamoeba* spp., due to the increasing number of contact-lens wearers.

Here, we studied the presence of FLP in several cooling towers with chlorine as disinfection method in the city of Barcelona, Spain, and in hot-water recirculation systems with storage tanks in 30 hotels and nursing homes around Catalonia (Serrano-Suárez et al. 2013). Further information on the presence and diversity of FLP in man-made water systems with a high risk of *Legionella* spp. infection, as well as their resistance to disinfection treatment methods, is needed in order to develop better management systems and improve disinfection methods. Accurate knowledge of the tolerance and resistance of FLP to disinfection treatment methods in current water facilities would lead to a reduction in the number of infections and outbreaks.

The main objectives of the present study were to detect and determine the prevalence and diversity of FLP present in the water sources most commonly at risk of *Legionella* contamination in Spain (specifically in Catalonia), i.e. cooling towers (CT) and hot sanitary water systems (HSWS) in hotels and nursing homes, and determine the in situ effect of their disinfection methods, i.e. chlorine and high temperature, on FLP.

Materials and methods

Sampling and sample treatment

A total of 47 samples from cooling towers and 124 samples from hot sanitary water systems were collected to conduct this study. The CT samples were collected from the city of Barcelona. The studied systems presented a discontinuous addition of chlorine for disinfection through a Redox control system, ranging from 0 to 8 mg chlorine L^{-1} several times a day. The HSWS samples were collected from public buildings in Catalonia, including hotels, hospitals and nursing homes (Serrano-Suárez et al. 2013). Both CT and HSWS samples were collected in 2-L sterile plastic bottles containing 5 mL of 2 % sodium thiosulphate (PanReac, Barcelona, Spain).

In order to determine the optimal conditions for the development of protozoa in HSWS, two samples were collected from each sampling point, as follows: 1 L from the initial flow and an additional 1 L after leaving the water to run for approximately 3 min, since this was the hottest water in the circuit. In the laboratory, all samples were left to stand for 2 to 8 h to facilitate sedimentation. Next, 5 mL was collected in a 10-mL sterile plastic tube from the sediment for culture and microscopic observation.

Heterotrophic plate counts

Heterotrophic bacterial counts were determined in accordance with ISO Standard 6222:1999: Water quality—Enumeration of culturable microorganisms—Colony count by inoculation in a nutrient agar culture medium (International Organization for Standardization 2005). In summary, 100 μL of the direct sample and a tenfold diluted sample in Ringer 1/4 (Scharlau Chemie, Barcelona, Spain) were cultured in plate count modified agar (Scharlau Chemie) and incubated at 22 °C for 72 h and at 37 °C for 48 h, respectively.

Culture of protozoa

Samples were inoculated with 500 μL of a bacteria growing medium of *Triticum vulgare* using an adapted protocol for liquid cultivation described by Lee and Soldo (1992). Briefly, 50 g of seeds of *T. vulgare* were boiled in 1 L of distilled water for 30 to 45 min. The liquid was then filtered through a 0.45-μm pore diameter filter and 1.3 g of Na_2HPO_4 $12H_2O$ was added. Finally, distilled water was added to compensate for the water lost through evaporation. The samples were grown at room temperature (20–25 °C) in the dark for 15 days.

It is important to mention that the objective of the study was to detect FLP, and no cysts were counted or detected during the sampling process. All samples were cultured to allow the cyst-forming FLP species to eclose. The absence of FLP or the presence of unenclosed cysts in the cultured samples was considered negative results.

Microscopic detection and identification of protozoa

Two 25-μL replicates of each sample were observed in vivo at 0, 3, 5, 10, and 15 days. Before the microscopic observation, samples were centrifuged at 360×*g* for 5 min, and 25 μL was then extracted from the pellet. Samples were analysed using a Leitz Dialux 20 microscope (bright field and phase contrast).

Different guides were used to identify the protist species. Amoebae were determined using the method proposed by Page (1988), ciliates were identified in accordance with the method proposed by Foissner (1993), and flagellates were identified using *The Illustrated Guide to the Protozoa* (Lee et al. 2000). The silver impregnation method (Fernández-Galiano 1994) was carried out for an accurate determination of the ciliate species.

DNA extraction

DNA extraction was performed as described by Serrano-Suárez et al. (2013). Briefly, 1 mL of the concentrated water

was heated to 100 °C for 10 min and then cooled for 10 min at −20 °C. To eliminate PCR inhibitors, 0.015 g of the resin Molecular Biology Grade AG 501-X8 (Bio-Rad, Hercules, CA, USA) was then added to the sample and mixed with a vortex mixer for 30 s. Finally, the sample was centrifuged for 5 min at 10,000×*g*. The supernatant was then recovered and stored at −20 °C for further analysis.

Detection of protozoa by PCR

PCR to amplify the 18S rRNA was performed in accordance with the protocol described by Serrano-Suárez (2009). The PCR was performed with the reverse primer Ami6R (5′-CCAGCTCCAATAGCGTATATT-3′) described by Thomas et al. (2006) and a forward degenerate primer designed by Serrano-Suárez (2009), and the two forward primers were combined as previously described by these authors: Ami6DEG (5′-CCAGCTCCAAKAGYGTATATT-3′). Next, 10-μL of the DNA extraction was tested in a total reaction volume of 50 μL that included 1× PCR buffer, 1.5 mM MgCl2, 0.2 mM dNTPs, 0.05 U of Taq DNA polymerase (Applied Biosystems, Foster City, CA, USA) and 0.5 μM of each primer. Following denaturation at 95 °C for 5 min, 40 cycles (5 min at 94 °C, 30 s at 55 °C, 2 min at 72 °C) were performed. The amplification was completed with an extension period of 10 min at 72 °C. The PCR products were then sequenced using the primers mentioned above. Reactions were performed with the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction kit v.3.1 with AmpliTaq DNA polymerase FS (Applied Biosystems, Austin, TX, USA) in accordance with the manufacturer's instructions. Nucleotide sequences were analysed using the basic BLAST tool (Altschul et al. 1990).

Other physicochemical and biological parameters

Temperature was measured in situ during sampling. Free chlorine concentration was also determined using the *N*,*N*-diethyl-*p*-phenylenediamine sulphate colorimetric method (Boletín Oficial del Estado 2003). The pH value was determined using the Micro pH2000 (Crison Instruments S.A, Barcelona, Spain) and turbidity using the Ratio/XR Turbidimeter (Hach Lange France S.A.S, Lognes, France). Zn, Fe and Cu concentrations were measured by inductively coupled argon plasma spectroscopy with a detection limit of 0.025, 0.01 and 0.01 ppm, respectively. Total organic carbon (TOC) was measured by high-temperature catalytic oxidation (HTCO) with a detection limit of 0.1 ppm. Heavy metal concentrations and HTCO data were measured by the University of Barcelona Scientific and Technological Centres.

Mathematical and statistical analysis

K-means cluster analysis (using the Bray-Curtis distance) was conducted using the Ginkgo software, a subset of the VegAna package. The Mann–Whitney *U* test was carried out using the Statgraphics Centurion XVI software package. Odds ratio and ROC curve analyses were carried out using the Stata 12 software package. In order to evaluate how high-chlorine concentration and high temperature affect the presence of FLP, the following formula was used: [100 – ("% FLP high chlorine or hot temperature" / "% FLP low chlorine or cold temperature")*100], in which a result of 100 % would mean a total reduction in FLP and 0 % would indicate that the biocide used was harmless.

Results

Over a period of 2 years, a total of 47 samples from CT and 124 samples from HSWS were analysed by culture and molecular methods to assess the diversity of FLP in man-made water systems in Catalonia.

Cooling tower samples

Cooling tower samples were collected from buildings in the city of Barcelona. In order to determine the effect of chlorine on protozoan communities, the CT samples were divided into two groups: high-chlorine CT samples (7.5±1.5 mg L^{-1}) and low-chlorine CT samples (0.04±0.008 mg L^{-1}). The chlorine, temperature, pH, TOC, turbidity, Fe, Zn and Cu values of the CT samples are shown in Table 1. Other than chlorine concentration, the physicochemical parameters did not show any statistically significant differences (Mann–Whitney *U* test, 95 % confidence) between the low-chlorine and high-chlorine CT samples.

FLP were detected in 10 and 13 out of 47 CT samples (21.3 and 27.7 %) by culture and PCR methods, respectively. By combining both methods, FLP were detected in 17 out of 47 CT samples (36.1 %). However, regardless of the detection method used, higher amounts of FLP were reported in the low-chlorine CT samples than in the high-chlorine ones (Table 2). Specifically, the higher chlorine concentrations resulted in reductions in the amount of FLP detected of 87, 67.8 and 63.8 % in the high-chlorine samples compared to the low-chlorine ones using culture, PCR and a combination of both methods, respectively.

Microscopic analyses were carried out to determine FLP diversity. Free-living amoebae were the most common group and were observed in all samples where FLP were detected. *Acanthamoeba*, *Vermamoeba* and other unidentified species belonging to the family Paramoebidae were classified in

Table 1 Physicochemical parameters of cooling towers samples

	Low-chlorine CT (n=16)			High-chlorine CT (n=31)		
	Mean±Std	Max	Min	Mean±Std	Max	Min
Het 23 °C (cfu 100 mL^{-1})	2.2E+06±4.8E+06	1.9E+07	0	4.4E+03±1.8E+04	1.0E+05	0
Het 37 °C (cfu 100 mL^{-1})	1.3E+06±2.9E+06	1.1E+07	0	8.0E+04±4.4E+05	2.4E+06	0
Temperature (°C)	22.0±4.8	30.3	10.0	24.3±2.9	30.5	14.2
Chlorine (mg L^{-1})	0.04±0.08	0.20	<0.01	7.5±1.5	8.0	2.5
pH	7.5±0.7	8.6	6.0	8.0±0.8	8.8	6.0
Total organic carbon (mg L^{-1})	1.6±1.0	3.7	0.5	0.9±0.5	2.7	0.3
Turbidity (NTU)	12.5±24.6	100.0	2.5	8.5±10.3	43.4	0.5
Fe (mg L^{-1})	0.02±0.04	0.15	<0.01	0.04±0.11	0.57	<0.01
Zn (mg L^{-1})	0.11±0.17	0.62	<0.025	0.19±0.31	1.43	<0.025
Cu (mg L^{-1})	0.29±0.84	3.40	<0.01	0.05±0.08	0.43	<0.01

Mean; *Std* standard deviation, *Max* maximum value, *Min* minimum value

samples from cooling tower systems. Flagellates were observed in 4 out of 16 (25 %) and 1 out of 31 (3.2 %) low- and high-chlorine CT samples, respectively (Table 2). The flagellates identified belonged to the *Bodo* genus. Ciliates were only detected in low-chlorine samples (4 out of 16 samples), and the species identified corresponded to the *Colpoda* genus (*Colpoda steinii*). A greater FLP diversity, understood as the number of different taxa, was detected in low-chlorine samples than in high-chlorine ones (Table 2).

Table 2 Number of samples in which FLP were detected using both PCR and culture methods and list of the protozoan taxa in the cooling towers

	Cooling towers (n=47)	
	Low-chlorine (n=16) *	High-chlorine (n=31) **
FLP detection (PCR+culture)	10 (62.5 %)	7 (22.6 %)
FLP detection by PCR	8 (50 %)	2 (6.5 %)
FLP detection by culture	8 (50 %)	2 (6.5 %)
Free-living amoebae	8 (50 %)	2 (6.5 %)
Acanthamoeba spp.	1	2
Vermamoeba spp.	2	–
Non identified	5	–
Flagellates	4 (25 %)	1 (3.2 %)
Bodo spp.	3	–
Non identified	2	1
Ciliates	4 (25 %)	0 (0 %)
Colpoda steinii	3	–
Non identified	1	–

n Number of samples; *mean chlorine concentration of 0.04 mg L^{-1}; **mean chlorine concentration of 7.5 mg L^{-1}

Hot sanitary water system samples

Two samples were collected from each sampling point, one corresponding to the first flow and the second corresponding to the hottest water in the circuit. A K-means cluster analysis was conducted using the Bray-Curtis distance in order to statistically cluster the samples into two groups considering all the physicochemical variables. The analysis grouped the samples according to temperature, but it revealed that one of the samples from the hottest water flow did not reach enough temperature to be considered as hot sample. Therefore, two groups were obtained: one of 63 samples (mean of 27.8±5.8 °C) named "cold HSWS" and the other of 61 samples (mean of 53.1±5.7 °C) named "hot HSWS". The chlorine, temperature, pH, TOC, turbidity, Fe, Zn and Cu values of the HSWS samples are shown in Table 3. Other than temperature, the physicochemical parameters did not show any statistically significant differences (Mann–Whitney U test, 95 % confidence) between the cold and hot HSWS samples.

FLP were detected in 29 and 27 out of 62 HSWS sampling points (46.8 and 43.5 %) by culture and PCR methods, respectively. Out of all of the positive results, regardless of the detection method, FLP were detected in 34 out of 62 sampling points (54.8 %). The results of FLP detection according to temperature can be seen in Table 4. Briefly, FLP were detected in 28 and 19 out of 63 cold HSWS samples (44.4 and 30.2 %) and 10 and 13 out of 61 hot HSWS samples (16.4 and 21.3 %) by culture and PCR methods, respectively (Table 4). Higher temperatures resulted in reductions in the amount of FLP detected of 63.1, 29.5 and 38 % in hot samples compared to cold ones using culture, PCR and a combination of both methods, respectively.

As occurred with the CT samples, microscopic analyses revealed that FLA appeared in all positive results. The FLA

Table 3 Physicochemical parameters of hot sanitary water system samples

	Cold HWSW (n=63)			Hot HSWS (n=61)		
	Mean±Std	Max	Min	Mean±Std	Max	Min
Het 23 °C (cfu 100 mL^{-1})	2.7E+06±3.5E+06	1.3E+07	0	1.8E+06±5.2E+06	2.8E+07	0
Het 37 °C (cfu 100 mL^{-1})	2.6E+06±3.3E+06	1.2E+07	0	1.7E+06±4.8E+06	3.0E+07	0
Temperature (°C)	27.8±5.8	38.0	16.3	53.1±5.7	66.6	42.0
Chlorine (mg L^{-1})	0.03±0.10	0.52	<0.01	<0.01	0.02	<0.01
pH	7.4±0.5	8.8	6.4	7.5±0.5	8.5	6.6
Total organic carbon (mg L^{-1})	1.5±1.4	9.6	<0.1	1.8±2.6	20.3	<0.1
Turbidity (NTU)	0.8±0.7	5.0	0.1	0.7±0.4	2.0	0.1
Fe (mg L^{-1})	0.03±0.05	0.20	<0.01	0.04±0.08	0.45	<0.01
Zn (mg L^{-1})	0.25±0.25	1.30	0.03	0.26±0.74	4.01	<0.025
Cu (mg L^{-1})	0.17±0.32	1.54	<0.01	0.15±0.26	1.25	<0.01

Mean; *Std* standard deviation, *Max* maximum value, *Min* minimum value

identified belonged to the genera *Acanthamoeba*, *Vahlkampfia*, *Vannella* and *Vermamoeba*. Other unidentified amoebae belonging to the family Paramoebidae were also detected. The presence of both *Acanthamoeba* and *Vermamoeba* was also confirmed by sequencing. Flagellates were observed in 5 out of 63 cold HSWS samples (7.9 %) and 2 out of 61 hot HSWS samples (3.3 %). The observed taxa corresponded to the *Bodo* genus, the Chrysomonadida class and a flagellate morphologically similar to the flagellate phase of *Paratetramitus*. No ciliate species developed in the HSWS samples, regardless of their temperature. A higher number of FLP taxa was observed in cold samples than in hot ones (Table 4).

Figure 1 represents the percentage of FLP detected by culture, PCR and a combination of both methods, in different temperature ranges. The results obtained using the culture method showed that temperatures above 50 °C negatively affected the prevalence of FLP in HSWS. FLP were not detected by the culture method in samples above 52 °C. However, the amount of FLP detected using the PCR method remained constant, regardless of temperature. A negative, statistically significant relationship was observed between temperature and FLP presence detected by both the culture and PCR+culture methods (OR=0.1317, confidence interval from 0.04 to 0.37 and OR=0.3162, confidence interval from 0.14 to 0.70, respectively; cut-off 50 °C, obtained by ROC curve analysis). However, no statistically significant relationship was observed between temperature and FLP presence detected by PCR.

Discussion

A diverse variety of FLP, specifically FLA, has been reported to inhabit a wide range of man-made water systems such as eyewash stations, cooling towers, transplant unit water supplies, hot sanitary water systems and drinking water supplies (Behets et al. 2007; Loret and Greub 2010; Ménard-Szczebara et al. 2008; Paszko-Kolva et al. 1991; Patterson et al. 1997). It is important to note that most of the studies concerning FLP in man-made water systems have focused on the detection of FLA, and few authors have also studied or reported the presence of flagellate and ciliate species (Barbeau and

Table 4 Number of samples in which FLP were detected using both PCR and culture methods and list of the protozoan taxa in hot sanitary water systems

	Hot sanitary water systems (n=124)	
	Cold HSWS (n=63) *	Hot HSWS (n=61) **
FLP detection (PCR+culture)	30 (47.6 %)	18 (29.5)
FLP detection by PCR	19 (30.2)	13 (21.3 %)
FLP detection by culture	28 (44.4 %)	10 (16.4 %)
Free-living amoebae	28 (44.4 %)	10 (16.4 %)
Acanthamoeba spp.	4	1
Vermamoeba spp.	5	1
Vahlkampfia spp.	5	1
Vannella spp.	1	–
Non identified	15	8
Flagellates	5 (7.9 %)	2 (3.3 %)
Bodo spp.	1	–
Chrysomonadida	1	1
Paratretamitus spp.-like	1	–
Non identified	3	1
Ciliates	0 (0 %)	0 (0 %)

n Number of samples, *mean temperature of 27.8 °C, **mean temperature of 53.1 °C

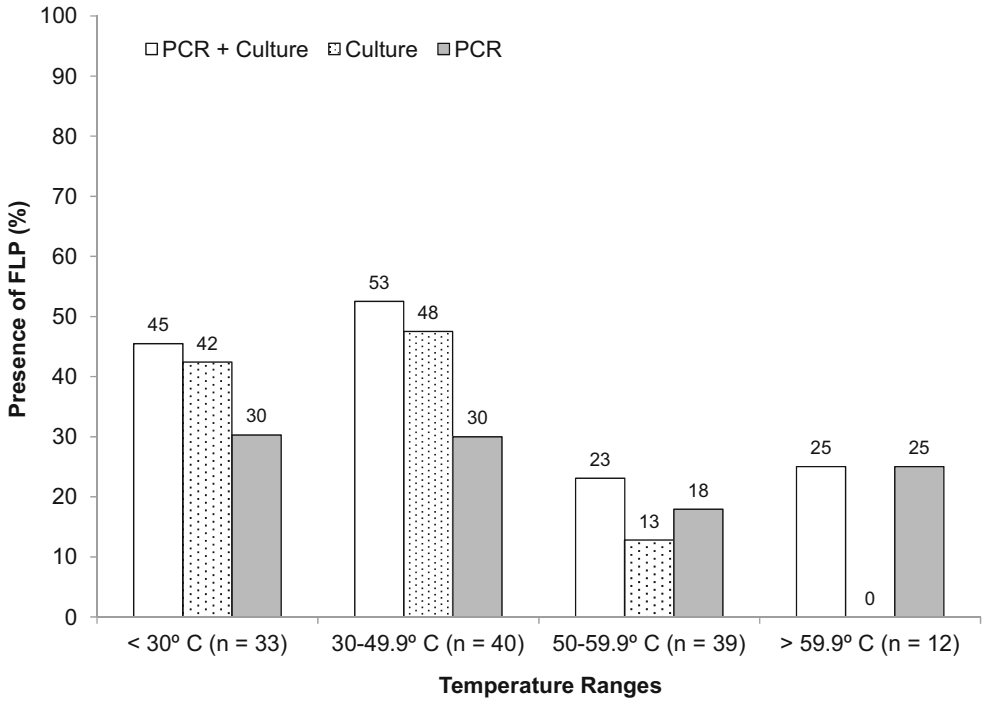

Fig. 1 Percentage of samples that presented FLP throughout temperature ranges. *First bar* at each temperature range represents the results obtained by combination of PCR and culture methods, *second bar* shows the results of culture and *third bar* the results of PCR method (*n* number of samples within each temperature range)

Buhler 2001; Patterson et al. 1997; Sutherland and Berk 1996; Valster et al. 2009; Yamamoto et al. 1992), so information regarding the flagellate and ciliate species that inhabit man-made water systems and how disinfection treatments affect them is more scarce than information regarding amoebae. It is expected that the use of metagenomic analysis will help increase this knowledge.

During the present study, the observed FLA genera in HSWS and CT were *Acanthamoeba*, *Vahlkampfia*, *Vannella* and *Vermamoeba*, genera that have been repeatedly reported in several studies using culture and molecular methods to inhabit a wide variety of man-made water systems (Barbeau and Buhler 2001; Delafont et al. 2013; Rohr et al. 1998; Shoff et al. 2008; Thomas et al. 2008; Valster et al. 2009). The flagellates identified corresponded to *Bodo* spp., the Chrysomonadida class and a flagellate similar to the flagellated phase of *Paratetramitus* spp. Most of the flagellates observed in our study have been reported previously by other authors: *Bodonidae* flagellates have been reported in cooling tower systems (Yamamoto et al. 1992), transplant unit water supplies (Patterson et al. 1997), dental unit waterlines (Barbeau and Buhler 2001) and drinking water supplies (Valster et al. 2009), and *Monas* sp., a Chrysomonadid flagellate, was observed by Paszko-Kolva et al. (1991) in eyewash stations. In the present work, ciliates were only observed in CT samples, specifically in those with low chlorine concentrations, and in three cases, the observed ciliate corresponded to *C. steinii*. In line with our findings, Yamamoto et al. (1992) also observed Colpodid ciliates in Japanese CT, and Sutherland and Berk (1996) isolated individuals belonging to the *Colpoda* genus from CT in Tennessee, USA.

Effect of chlorine on protozoa presence and diversity in CT

Chlorine is a commonly used biocide in cooling tower systems. Some studies regarding the effectiveness of chlorination on FLP, specifically FLA, are available, but most of them were conducted under laboratory conditions (Kuchta et al. 1993; Mogoa et al. 2010; Storey et al. 2004) or in pilot-scale domestic water systems (Thomas et al. 2004). As far as we know, the present results are the first concerning the in situ effect of chlorine concentration on the presence of FLP in operational CT.

Our results show a clear negative effect of chlorine concentration on FLP presence in CT systems (63.8 % lower in high-chlorine samples than in low-chlorine samples). *Acanthamoeba*, an amoeba genus known to be highly resistant to chlorine concentration, and an unidentified flagellate were detected by the culture method from samples with a chlorine concentration of 8 mg chlorine L^{-1}. *Acanthamoeba* spp. has been reported to resist up to 100 mg chlorine L^{-1} during the cyst stage (Storey et al. 2004), and other studies conducted on *Acanthamoeba castellanii* trophozoites have stated that a 3-log reduction is reached at 5 mg chlorine L^{-1} (Mogoa et al. 2010). Based on these references, it is logical to assume that *Acanthamoeba* spp. from 8 mg chlorine L^{-1} samples were probably collected at the cyst stage and developed when cultured.

Fields et al. (1984) demonstrated that, in addition to FLA, ciliates such as *Tetrahymena pyriformis* and *Tetrahymena thermophila* may act as hosts for *Legionella* spp., thus ensuring the survival and reproduction of the bacterium within these ciliates. In the present study, ciliates were observed in 25 % of the CT samples. The presence of *C. steinii*, a very common cyst-forming soil ciliate, in the CT samples could be explained by the dispersive role of air, since the CT systems analysed were not watertight and most protozoan cysts are easily transported by air due to their small size and weight. In our results, *C. steinii* only appeared in low-chlorine samples, which would suggest that, in contrast to some FLA, high chlorine concentrations, above 2.5 mg chlorine L^{-1} in the CT under study, are effective at preventing the development of ciliate species.

Effect of temperature on protozoa presence and diversity in HSWS

Temperature is a widely used method for disinfecting man-made water systems, especially HSWS, worldwide, thus several studies regarding the tolerance of protozoa, especially amoebae, to temperature have been carried out (Cervero-Aragó et al. 2013; Kuchta et al. 1993; Ménard-Szczebara et al. 2008; Rohr et al. 1998; Storey et al. 2004; Thomas et al. 2006). In the present study, the results obtained on FLP presence in HSWS (detected in 46.8 % of sampling points) are similar to those reported in other man-made systems with similar temperatures, with percentage values ranging from 47 to 68 % (Patterson et al. 1997; Rohr et al. 1998). The presence of FLP showed a negative, statistically significant relationship with temperature; this is consistent with previous data demonstrating that higher temperatures negatively affect the presence of FLP (Cervero-Aragó et al. 2013; Ménard-Szczebara et al. 2008). The amount of FLP detected was lower when the temperature of the samples exceeded 50 °C. However, the reduction in the amount of FLP presence obtained by culture was higher and, in contrast to that obtained by the PCR method, statistically significant, since PCR detects the presence of eukaryotic DNA in samples but does not report information about the viability of the FLP. Using the culture method, the maximum temperature from which FLP developed was 51.2 °C. The species observed corresponded to *Vermamoeba*, a common thermophilic amoeba (Rohr et al. 1998; Thomas et al. 2006). The results obtained support those of previous studies, which reported that the prevalence of FLA decreases considerably above 50 °C, and that they are practically undetected by the culture method at temperatures exceeding 60 °C (Kuchta et al. 1993; Lasheras et al. 2006). Nevertheless, it has been demonstrated that, under laboratory conditions, the cysts of some strains of *Acanthamoeba* spp. can resist an exposure to a temperature of 60 °C for 20 min (Cervero-Aragó et al. 2013) and 65 °C for 10 min (Coloun et al. 2010). Even other authors observed cyst resistance to higher temperatures (Storey et al. 2004).

To correctly interpret the results of the present study, it should be noted that 62 out of the 63 cold HSWS samples corresponded to the first litre that flowed from the sampling point. Thus, our results are consistent with those of Ménard-Szczebara et al. (2008), who observed a higher prevalence of FLA in the taps than the water at the inlet or within the domestic water network. This can be explained by the fact that, in HSWS systems with recirculation, water can be retained in the distal part of the domestic water network if the tap is not used for a long period of time; this allows the retained water to cool and biofilms to form. Biofilm formation can cause an increase in the amount of FLA detected, as Barbeau and Buhler (2001) reported in dental unit waterlines. The importance of biofilms for FLP survival should be studied in more depth.

Ciliates were not observed in either hot or cold HSWS samples. The absence of ciliates in HSWS could be explained by the watertight characteristics of HSWS that would impede the colonization of ciliates by air or other vectors (as occurred in CT), or by the potentially lower resistance of ciliates' feeding and cysts stages to biocides compared to amoebae, as observed and reported by Sutherland and Berk (1996). Our results suggest that ciliates might not survive the water treatment plant process, unlike some amoebae (Loret and Greub 2010 and Thomas et al. 2008). If they do survive, the high temperature of the water within the domestic water network would prevent their colonization.

Final remarks

Regardless of the biocide used and its effectiveness, our results showed that FLP diversity, understood as the number of taxa, is negatively affected when a biocide is applied to the system.

Concerning CT systems, our results suggest that the discontinuous chlorine disinfection method used in some operational CT in the city of Barcelona to prevent the colonization of *Legionella* spp. is not guaranteed to prevent the presence of FLP. The survival capacity of FLA in high-chlorine concentrations, presumably during the cyst stage, may be considered as a way for amoeba-resisting bacteria to prevail in CT systems.

With respect to HSWS, the results obtained provide helpful data to optimize the performance and efficiency of high-temperature disinfection methods used in water network systems in Catalonia. Our results suggest that a water temperature within the domestic water network above 55 °C could improve the prevention of FLP development, including FLA, and thus reduce the risk of possible outbreaks of *Legionella* spp. and other pathogenic bacteria, and prevent infections caused by opportunistic pathogenic FLA. Although there are

no regulations regarding the presence of FLP in man-made water systems in Spain, the regulation for controlling *Legionella* in HSWS with recirculation (Boletín Oficial del Estado 2003) recommends a temperature of 60 °C for the water in the storage tank and a minimum of 50 °C for the return water. Even if the recommendations are followed, our results suggest that the current regulation does not completely guarantee the prevention of FLP development in HSWS in hotels and nursing homes in Catalonia.

In order to improve the systems to prevent pathogenic bacteria and amoebic diseases in operational man-made water systems, future regulations concerning water quality health standards should make provisions for FLP.

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7.4. CHRONIC EFFECTS OF TEMPERATURE AND NITRATE POLLUTION ON DAPHNIA MAGNA: IS THIS CLADOCERAN SUITABLE FOR WIDESPREAD USE AS A TERTIARY TREATMENT?

Alberto Maceda-Veiga, Gordon Webster, Oriol Canals, Humbert Salvadó, Andrew J. Weightman, Jo Cable.

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Chronic effects of temperature and nitrate pollution on *Daphnia magna*: Is this cladoceran suitable for widespread use as a tertiary treatment?

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ABSTRACT

Effluent clarification and disinfection are major challenges in wastewater management. The cladoceran *Daphnia magna* has been proposed as a cost-effective and ecosystem-friendly option to clarify and disinfect secondary effluents, but its efficacy has not been fully tested under different sewage conditions. The present study explores the effects of temperature and nitrate on the efficacy of *D. magna* as a tertiary treatment at two different scales (individual assays and microcosms). Individual assays were employed to determine direct effects of temperature and/or nitrate on *D. magna* cultured in a suspension of organic matter. Using microcosms under the same environmental conditions, we explored the clearing efficacy of *D. magna* interacting with a natural microbial community. Individual assays revealed that *D. magna* mortality increased by 17% at 26 °C, 21% at >250 mg NO_3^-/l and by 60% at 26 °C and at >250 mg NO_3^-/l, and individuals displayed reduced body size, filtering rates and fecundity when compared to those at 21 °C and <40 mg NO_3^-/l. Improved performance under these conditions was also mirrored in the microcosms, with a higher density of *D. magna* (>100 ind/l) at 21 °C and <40 mg NO_3^-/l compared to the number (0–21 ind/l) at 26 °C and/or >250 mg NO_3^-/l. In the microcosms at 21 °C and <40 mg NO_3^-/l, turbidity and the density of bacteria, protists and micro-metazoa decreased in relation to those at 26 °C and/or >250 mg NO_3^-/l. Each treatment developed a unique and characteristic microbial assemblage, and *D. magna* was identified as the major driver of the community structure of protists and micro-metazoa. This enabled us to determine taxa vulnerability to *D. magna* grazing, and to re-define their tolerance thresholds for nitrate. In conclusion, this study increases our knowledge of how microbes respond to temperature and nitrate pollution, and highlights that *D. magna* efficacy as a tertiary treatment can be seriously compromised by variable environmental conditions.

1. Introduction

Improving the quality of sewage effluents is a priority in wastewater management. In water scarce regions, this interest increases given the current and predicted future dependency on reclaimed water (Vörösmarty et al., 2010; Halaburka et al., 2013). Besides achieving safe health thresholds, recycled water must meet public standards of water quality, including transparency (Levine and Asano, 2004; Crook, 2005). However, turbidity reduction is a major challenge in sewage treatment (Pau et al., 2013; Serra et al., 2014). Conventional settling tanks and filters cannot trap small particles, such as microbes and fine suspended solids that give water a cloudy appearance (Madoni, 2003; Pau et al., 2013). Water clarification and disinfection can be achieved with subsequent tertiary treatments, such as ultrafiltration, ozonation and chlorination (Gómez et al., 2007, 2010). These procedures, however, are not always cost-effective and/or ecosystem-friendly (Joss et al., 2008; Rosal et al., 2010; Grant et al., 2012). Thus, research into more ecologically sound, cheaper and effective tertiary treatments is needed, particularly given the growing popularity of wastewater treatment processes

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integrated into natural systems (Hering et al., 2013; Garcia-Rodríguez et al., 2014).

Cladocerans are keystone grazers (Jürgens, 1994; Berger et al., 2007; Schalau et al., 2008), and growing evidence indicates that species of the genus *Daphnia* could be effective at reducing fine suspended solids and microbes in sewage effluents (e.g. Roche, 1998; Filella et al., 2008; Compte et al., 2009; Pau et al., 2013). Besides cost reduction, daphnids cultured in sewage effluents can serve as indicators of effluent quality (see Pellegri et al., 2014), and provide raw material for biofuel (Kring et al., 2013) or prey for fish and birds (Kampf et al., 1999; Patil et al., 2010). Nonetheless, daphnids including the widespread *Daphnia magna* have still not been adopted as a tertiary treatment, possibly because their efficacy has not been fully tested under different sewage conditions. Many compounds present in secondary effluents such as drugs, pesticides, metals and nutrients can be toxic to *D. magna* (see Heugens et al., 2003; Compte et al., 2009). Further, such toxicity may be modulated by climatic variables such as temperature (e.g. Heugens et al., 2003), which in turn exerts a direct effect on *D. magna* metabolism and hence filtering rate (Burns, 1969). *D. magna* populations can, however, survive, and even acclimate, to changes in water quality (e.g. Zeis et al., 2004; Seidl et al., 2005; Coors et al., 2009). Nonetheless, the effects of typical secondary effluent pollutants, such as nitrates, are still largely unexplored (Camargo et al., 2005). Thus, studies on the response of *D. magna* to nitrates are essential to optimise the efficacy of *D. magna* at reducing turbidity and microbial load.

Water clarification and disinfection can be achieved by *D. magna* directly grazing on a wide range of particles, including microbes (Jürgens, 1994), but also indirectly via the complexity of microbial food-webs (Zöllner et al., 2003). By affecting the relative abundances of microbial taxa, *D. magna* can influence processes such as flocculation and sedimentation that strongly depend on microbial activity (Lapinski and Tunnacliffe, 2003; Pajdak-Stós and Fiałkowska, 2012). Temperature and the complex mixture of sewage contaminants can also alter flocculation and sedimentation via direct effects on microbes (e.g. Puigagut et al., 2005, 2007; Liao et al., 2011) and on particle aggregation (Bratby et al, 2006). In this context, since sludge production is a major concern in sewage treatment (Wei et al., 2003; Khursheed and Kazmi, 2011), the ideal tertiary treatment is one that improves water clarity whilst reducing sediment production. To better assess the potential of *D. magna* as a tertiary treatment, it is necessary to understand of how environmental stressors and microbial food-webs influence its efficacy (Compte et al., 2009).

The present study explores the efficiency of *D. magna* as a sewage treatment under variable biotic and abiotic conditions at two different scales. *D. magna* were cultured individually in a suspension of organic matter to determine direct effects of temperature and nitrate on key *D. magna* life-history traits influencing its filtering efficacy. Then within a natural microbial community, we explored the long-term grazing effects of *D. magna* under the same environmental conditions using microcosms. If *D. magna* is effective in water treatment, without grazing, we expected a reduction in water quality, evidenced by an increase in bacteria abundance, suspended solids and sedimentation. Moreover, if *D. magna* is suitable for widespread use as tertiary treatment, we expected *D. magna* treatments to perform well and show similar results under all experimental conditions. We also predicted that the microbial community structure would reflect taxa vulnerability to *D. magna* grazing along with microbes' tolerance to the experimental environmental conditions.

2. Materials and methods

2.1. General experimental design

The impact of temperature and nitrate pollution on the performance of *D. magna* as a tertiary treatment was investigated in both individual trials and microcosms. Organisms were exposed for 30 days to 21 or 26 °C, and/or two nitrate concentrations (<5 or 250 mg NO_3^-/l) using potassium nitrate (KNO_3) as a nitrate source, following a two fully factorial design for the *D. magna* individual assays (n = 30 replicates) and a three fully factorial design (including the grazing by *D. magna* effect) for the microcosms (n = 6 replicates). Experimental temperatures were chosen based on the optimal growth and filtering temperature for *D. magna* laboratory cultures (Burns, 1969; Gielbelhausen and Lampert, 2001), and an upper value expected to increase its filtering rates (Burns, 1969). Temperatures are also within the values observed in secondary effluents (e.g. mean temperature of 20.8 °C and maximum value of 28 °C in a sewage treatment plant in Girona, H. Salvadó *unpublished data*) for temperate regions or throughout the year in tropical countries (20–35 °C, van Haandel and Lettinga, 1994). The lowest nitrate concentration represents UK dechlorinated tap water, whereas the highest nitrate concentration is within the range (100–1000 mg NO_3^-–N/l) reported in agricultural or aquaculture effluents (see Camargo et al., 2005; Hamlin, 2006).

The *D. magna* population was obtained from a wild-type culture, originally from a UK pond, and maintained at Cardiff University for ca. 5 years in four 40 l tanks under 21 ± 1 °C and 12 h light:12 h dark cycle and a gentle air supply. During the experiments, the input of organic matter was constant for all treatments and consisted of a mixture of freeze-dried *Arthrospira platensis* (also known as *Spirulina*; Organic Spirulina Powder®, Indigo Herbs of Glastonbury) and yeast (*Saccharomyces cerevisae*, Easy Bake Yeast®, Allinson) at 4.4×10^4 cells ml/l and 0.4×10^6 cells ml/l, respectively, determined using a haemocytometer. This mixture simulates an input of organic matter with particles ranging from 5 to 300 μm, which are within the values observed in secondary effluents or in stabilisation ponds (Ekama, 1997; Asano, 1998).

2.2. Individual Daphnia assays

Thirty gravid females were isolated in 50 ml tubes to give birth. Four neonates (<24 h) of each female were randomly distributed in 50 ml tubes following a two fully factorial experimental design and acclimatised for 24 h. Fresh culture media was renewed every third day and mortality recorded and offspring counted when they occurred. Using a spectrophotometer at 665 nm, the filter efficiency of each adult *D. magna* was estimated on termination of the exposure period by comparing the water turbidity in a 10 ml tube with the above mentioned mixture of *Spirulina* and yeast before and after grazing by a single individual of *D. magna* for 9 h. Each individual was removed with a Pasteur pipette and the tube was manually inverted 6 times to homogenise the sample before the water sample was processed for the turbidity analysis. Finally, each *D. magna* was fixed in formaldehyde 4% (w/v) for 48 h, transferred to ethanol 70% (v/v) and the body size (total length, mm) was measured under a stereo-microscope with an ocular micro-meter at ×20 magnification.

2.3. Microcosms

For the inoculation of microcosms, water was collected from a pool in a tributary of the River Taff, Cardiff, UK (51° 29′ 20.4″ N, 3° 11′ 20.4″ W), receiving the input of an urban collector. One litre pots were filled with 700 ml dechlorinated tap water and 200 ml of

inoculum, and left for 3 days for the establishment of the community at 21 ± 1 °C. Crustacean zooplankton were not observed in the inoculum, but the water sample was sieved through 50 μm to ensure their absence. Each pot had an air supply (~6 bubbles per second using a 5 mm ø plastic tube) to provide a well oxygenated but stable environment for the biota. To prevent algal growth during the experiment, microcosms were kept under diffuse light (10 lux), which also simulated the darkness of high turbidity water. The microbial assemblage of time 0 was determined by collecting 1 l for the bacterial, protist and micro-metazoa community (see details below). On the 4th day, the nitrate level was increased in each microcosm, drop by drop (~24 h) via a 5 mm ø tube connected to a reservoir of concentrated potassium nitrate solution. Likewise, microcosms reached the experimental temperatures after 24 h by a hot water bath using an aquarium heater. When temperature and nitrate conditions were reached, ten *D. magna* neonates were introduced after 24 h into the *D. magna* treatments. We replaced 10% of the water in each microcosm with fresh solutions every three days. This is equivalent to a hydraulic retention time of 30 days, which is within the range (5–40 days) observed in wastewater stabilisation ponds (Tchobanoglous and Burton, 1991; Nelson et al., 2004). At the start and end of the experiment, pH, carbonate (°dKH) and the concentrations of ammonia (mg NH_3^+/l), nitrite (mg NO_2^-/l) and nitrate (mg NO_3^-/l) were measured using colorimetric test kits Sera®. After 30 days, a water sample was taken from the water column to determine water transparency using a spectrophotometer as detailed above. Then, a lid was placed on each microcosm and the content was homogenised by 10 manual inversions before water samples were collected and immediately processed for the characterization of the microbial community as detailed below. Sedimentation was estimated by the difference in absorbance between water samples collected before and after inverting each microcosm. The final number of *D. magna* per treatment was recorded by counting individuals after sieving the water through a 100 μm mesh.

2.3.1. Protist and micro-metazoa community characterization

The density of protists and micro-metazoa was determined by examining 50 μl of a homogenised water sample from each of the three 50 ml tubes collected per microcosm under the microscope at ×100 and ×400 as previously described by Canals et al. (2013). Identification of the organisms was confirmed at the University of Barcelona by sending photographs and videos of specimens recorded *in-vivo* at Cardiff University as well as sending duplicates of the water samples fixed in formaldehyde 4% (final concentration) and acetic Lugol 2% (final concentration). All organisms were identified based on morphological traits following Foissner (1991), Foissner et al. (1994) and Curds et al. (2008) for ciliates and Streble and Krauter (1987) for micro-metazoa. Abundance data was available to the genus level for all organisms with the exception of amoeba (grouped as testate amoebae or gymnamoebae), flagellates and gastrotrichs. The results were expressed as individuals/ml averaged per microcosm.

2.3.2. Bacterial community profiling and quantitative estimation of bacterial numbers

The density and diversity of bacteria were determined by collecting three 100 ml water samples from three individual microcosms per treatment after 30 days, and a 500 ml water sample from Time 0. Microbial biomass was collected from each water sample by repeated centrifugation at 14,000 g for 15 min and the cell pellet was stored at −20 °C until required for DNA extraction. Genomic DNA was extracted from microbial cell pellets using the FastDNA® SPIN Kit for Soil as described by Webster et al. (2003). As a proxy for total bacterial cell numbers, quantitative real-time PCR (qPCR) was used to quantify bacterial 16S rRNA gene copy numbers in all water samples. All qPCRs were run on an Agilent Mx3000P QPCR System (Agilent Technologies UK Ltd.) with a standard curve derived from serial dilutions of a full length *Anaerolinea thermophila* DSM1453 16S rRNA gene PCR product. All samples were analysed in triplicate and qPCR results were rejected if the r^2 value of the standard curve was below 0.95 or the efficiency of the reaction was not >80%. Using 16S rRNA gene primers 534F-907R (Wilms et al., 2007), each qPCR mixture comprised a total volume of 20 μl with 400 nM of each primer (Eurofins MWG Operon) and 1 μl of DNA in 1× qPCRBIO SyGreen Lo-ROX Mix (PCR Bio systems Ltd.) made up with molecular grade water (Severn Biotech Ltd.). The protocol was 95 °C for 7 min, 40 cycles of 95 °C for 30 s, 52 °C for 30 s, 72 °C for 60 s, followed by a melting curve from 55 °C to 95 °C. Each cycle was followed by data acquisition at the elongation step.

To obtain information on the bacterial community structure, 16S rRNA genes were amplified directly with the PCR primers 357FGC/518R using PCRBIO *Taq* DNA Polymerase (PCR Bio systems), and analysed by denaturing gradient gel electrophoresis (DGGE; Gorra et al., 2012) with a 30–60% denaturing gradient. DGGE gels were stained with SYBRGold nucleic acid gel stain (Invitrogen) for 30 min and gel images were captured under UV with a Molecular Imager VersaDoc™ System (Bio-Rad Laboratories, Inc.).

2.4. Statistical analysis

All analyses were performed in R version 2.14.2 (R Development Core Team, 2012) using the libraries 'stats', 'MASS', 'survival', 'car', 'lawstat' and 'vegan'. Data normality and homogeneity of variances were checked using Shapiro–Wilk and Levene tests, respectively, and then parametric or non-parametric statistics were applied accordingly. Counts and continuous variables were log-transformed to satisfy statistical requirements. *D. magna* survival (%) was examined using the 'surfit' function. Differences in water chemical and physical properties and the density of bacteria between treatments were compared using a Kruskal–Wallis test followed by Mann–Whitney U tests for pair-wise comparisons. Mean values of life-history traits of *D. magna* for individual assays, and density of *D. magna* for microcosms were compared between treatments using an analysis of variance (ANOVA) followed by a Tukey's honest significant difference (HSD) test for pair-wise comparisons. Differences in protists and micro-metazoa community composition across treatments were visualised through a detrended correspondence analysis (DCA). This form of unconstrained ordination was preferred to constrained methods (e.g. RDA and CCA) because it produced easily interpretable plots, and accounted for the influence of explanatory factors not considered in the study (see details in ter Braak and Šmilauer, 2014). Also, unlike other unconstrained methods (e.g. NMDS), DCA axes can be interpreted as changes in units of beta diversity, indicating a full taxa replacement (no taxa in common) when the distance separating samples is 4 SD.

Treatment effects on protists and micro-metazoa assembly were appraised using permutational multivariate analysis of variance (PERMANOVA, nperm = 999) and the influence of dispersion effects was tested using the permutation test of multivariate homogeneity of group dispersions (betadisper test). Variation partitioning (VP) was then used to assess to which extent each treatment combination was related to the variation in protists and micro-metazoa composition. VP decomposes the variation of dependent variables in unique and shared (joined) effects of a set of factors (Peres-Neto et al., 2006). Similarity percentages (SIMPER) tests were used to identify the protists and micro-metazoa taxa responsible for the dissimilarities in the community between temperature, nitrate and *D. magna* treatments. Overall differences in mean density of protists

and micrometazoa taxa between treatments were examined using an ANOVA followed by Games Howell (GH) posthoc test for pairwise comparisons. Unlike TukeyHSD, GH deals with heterogeneity of variances. Distance matrices for PERMANOVA and SIMPER were constructed using the Bray–Curtis dissimilarity index. DGGE gel images were analysed using GelCompar II software version 6.5 (Applied Maths NV) and cluster analysis was performed using Pearson correlation coefficient and UPGMA. Treatment effects on cluster groupings were assessed using a PERMANOVA as indicated above. The relationship between water chemical and physical properties, and the densities of microbes and *D. magna* was explored using a Spearman's rank correlation analysis (Spearman's *rho*). Significant thresholds were established at *P*-values $\leq$0.05. In multiple comparisons (repeated Mann–Whitney U tests), *P*-values were corrected using the Benjamini–Hochberg procedure to control false discovery rate (Benjamini and Hochberg, 1995).

3. Results

3.1. Daphnia magna individual assays

D. magna mortality significantly increased at 26 °C (21%) or high nitrate level (17%) with evidence of interactive effects (60%, surfit function, All $P < 0.01$). No deaths occurred at the lowest nitrate and temperature conditions throughout the entire study period. The filter efficiency and growth of *D. magna* significantly decreased at both 26 °C and high nitrate level (Table 1, Fig. 1) and was particularly marked for the former under the combined action of elevated temperature and nitrate (Fig. 1). There was also a significant reduction in the mean number of offspring produced by *D. magna* exposed to high temperature or nitrate conditions but these factors did not show interactive effects (Table 1, Fig. 1).

3.2. Microcosms

As expected from individual assays, the density of *D. magna* differed between microcosms after 30 days (Table 1), with the highest density at 21 °C and low nitrate level (Fig. 1). In contrast, all *D. magna* individuals died in microcosms at 26 °C and high nitrate,

Table 1
ANOVA output for the mean body length and offspring counts of *Daphnia magna* exposed to two nitrate concentrations (<5 mg NO_3^-/l or 250 mg NO_3^-/l) and two temperatures (21 °C or 26 °C) and the mean change in water turbidity of experimental tubes exposed to the filter action by *Daphnia* from these treatments for 9 h. The effect of temperature and nitrate on mean *D. magna* density in microcosms after 30 days is also shown (n = 6 replicates/treatment). For individual assays, note that differences in the number of degrees of freedom (df) are due to the fact that offspring counts are referred to all individuals used (n = 30/treatment), whereas body size and clearing rate only applies to those individuals alive after 30 days.

Factors	SS	df	*F*	*P* value
Body size				
Temperature	0.04	1, 70	12.92	<0.01
Nitrate	0.05	1, 70	15.17	<0.01
Nitrate × Temperature	0.001	1, 70	0.52	0.47
Offspring counts				
Temperature	35.16	1, 116	48.92	<0.01
Nitrate	39.20	1, 116	54.55	<0.01
Nitrate × Temperature	1.50	1, 116	2.09	0.15
Clearing rate (water turbidity)				
Temperature	0.02	1, 68	81.72	<0.01
Nitrate	0.01	1, 68	67.93	<0.01
Nitrate × Temperature	0.00	1, 68	10.46	<0.01
D. magna density (microcosms)				
Temperature	42.56	1, 20	697.66	<0.01
Nitrate	26.56	1, 20	435.44	<0.01
Nitrate × Temperature	0.91	1, 20	14.89	<0.01

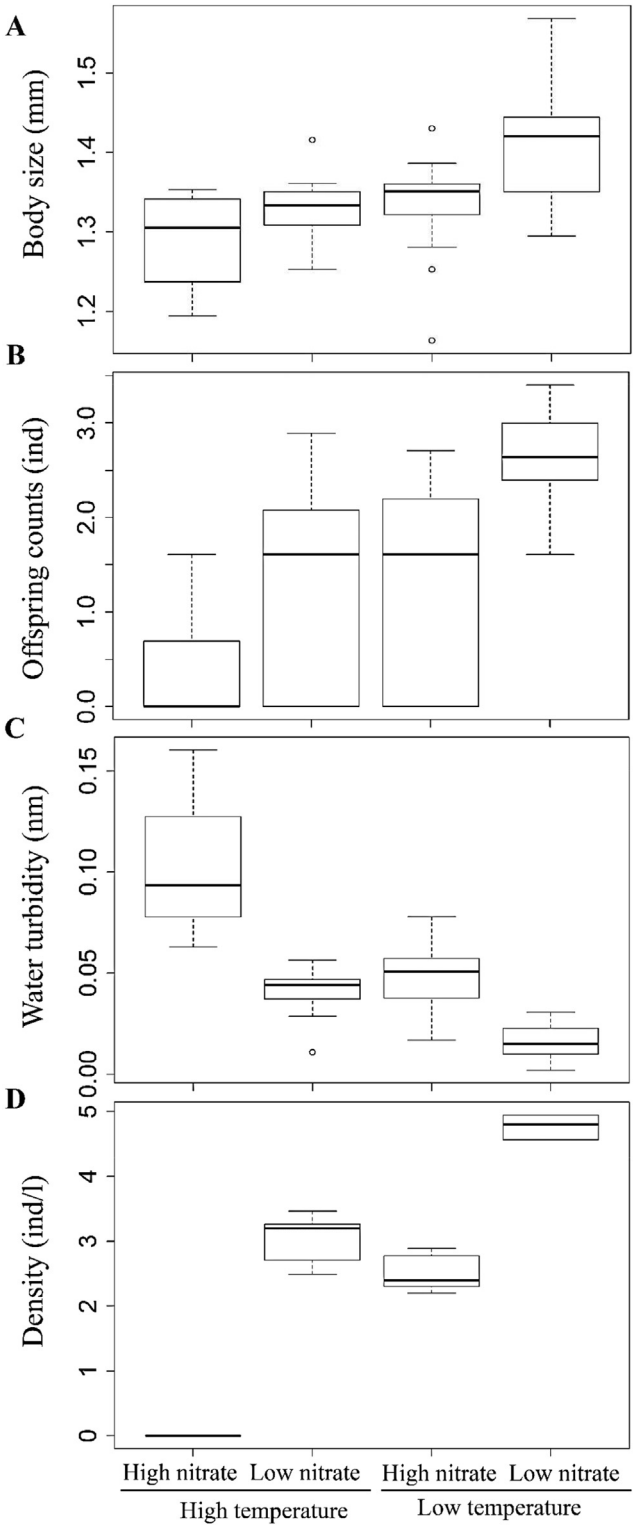

Fig. 1. Box-plots of changes in body size (A) and offspring counts (B) of *Daphnia magna* in individual assays after 30 days exposure to nitrate and temperature treatments, and in the water turbidity (C) of the experimental tubes after the filtering action of *D. magna* from the four experimental conditions (see methods for details). Differences in density of *D. magna* between treatments in microcosms are also shown (D). Note that data are log-transformed to highlight the differences between treatments. Each box corresponds to 25th and 75th percentiles; the dark line inside each box represents the median; error bars show the minima and maxima except for outliers shown as open circles.

possibly due to a build-up of metabolic waste products (Fig. 1, Table 2).

3.2.1. Water physical and chemical properties and bacterial load

As expected, water turbidity and sedimentation was higher in all microcosms after 30 days compared to the baseline at time zero (Table 2). The lowest turbidity values occurred in *D. magna* treatments at 21 °C and low nitrate level, whereas the highest turbidity was observed in microcosms without *D. magna* at 26 °C and/or high nitrate (Table 2). No differences were observed in sediment load among all 6 treatments (Table 2), and for chemical variables, only nitrate significantly increased in all treatments (Table 2). Bacterial cell numbers, as estimated by qPCR, varied little during the 30 days incubation (Table 2) with this proxy of cell numbers in most treatments ranging from 2.6 to 3.9 × 10^7 16S rRNA genes/ml, similar to those observed at time zero (3.90 × 10^7 16S rRNA genes/ml). However, *D. magna* at 21 °C and low nitrate level reduced bacterial cell numbers by 100 fold (Table 2).

In contrast to bacterial cell numbers, bacterial diversity as assessed by DGGE profiling of bacterial 16S rRNA genes varied with nitrate, temperature and *D. magna* treatments (Table 3, Fig. 2). Each treatment had a different and unique DGGE profile, indicating selection for a particular bacterial community. This was further supported by cluster analysis (Fig. 2) which consistently grouped DGGE profiles from the same microcosm treatment together, with the exception of replicates from the high nitrate at 21 °C without *D. magna*. Interestingly, the presence of *D. magna* at 21 °C and low nitrate level reduced bacterial diversity (14 bands) compared to time zero (21 bands) and all other treatments (mean number of bands = 22), confirming that grazing of bacteria by *D. magna* in this treatment was particularly important. Nonetheless, some bacterial taxa appeared not to be affected by these experimental conditions, since some DGGE bands were clearly present in all treatments while other bands were only found in *Daphnia* treatments (Fig. 2).

3.2.2. Protist and micro-metazoa community structure

Similar to the response by the bacterial community, changes in temperature, nitrate and/or *D. magna* grazing resulted in a different community structure of protists and micro-metazoa for each treatment (Table 3, Fig. 3). In the absence of *D. magna*, particular taxa (flagellates, gymnamoebae, testate amoebae, the rotifers *Lecane* spp., the pelagic ciliates *Uronema* spp. and *Cyclidium* spp. and the benthonic ciliates *Vorticella* spp. and *Euplotes* spp.) contributed disproportionally to the dissimilarities in the community structure between temperature and nitrate conditions (Table 4). Warming affected positively the density of the rotifers *Lecane* spp., but with a negative effect on the density of flagellates and the ciliates *Cyclidium* spp. (Games Howell, both $P < 0.01$; Table 4). By contrast, flagellates and the ciliates *Uronema* spp. increased in density at high nitrate (Games Howell, $P = 0.01$), while no effect was observed in rotifer populations (Games Howell, $P > 0.05$). Under the nitrate-warming interaction, flagellates density remained similar to that at high nitrate (Games Howell, $P = 0.80$) but the planktonic filter feeders *Vorticella* spp. increased in density (Games Howell, $P = 0.01$; Table 4). Besides these major changes in planktonic grazers, there was a decrease in the density of benthonic organisms, such as amoebae, at 26 °C and/or at high nitrate level alone with an increase in the density of the crawling ciliates *Euplotes* spp. at 26 °C and high nitrate (Games Howell, $P = 0.01$; Table 4).

The effects of grazing by *D. magna* on protist and micro-metazoa communities varied with both temperature and nitrate levels (Tables 3 and 4), and dispersion was not a confounding factor (betadisper test, $P = 0.10$). Changes in community assemblies were visualised in a detrended correspondence analysis (DCA), which indicated that 19% of the variance in composition was explained by axis 1, and 13% by axis 2 (Fig. 3). DCA also showed that a full taxa replacement did not occur across treatments (<4 SD), and that *Daphnia* treatments were mostly segregated along axis 1 (Fig. 3). Variation partitioning supported the fact that *D. magna* exerted a disproportionate effect on the community structure of protist and micro-metazoa compared to nitrate and/or temperature (Fig. 4). At 21 °C and low nitrate, as for bacteria and turbidity, *D. magna* grazing significantly reduced the density of all protists and rotifers (Games Howell, All $P < 0.01$ in Fig. 5 and Table 4). This negative trend, however, only occurred in the density of flagellates and crawling ciliates under *D. magna* grazing at high nitrate levels (Games Howell, All $P < 0.01$; Fig. 5). In contrast, these conditions favoured

Table 3
PERMANOVA output for the protist, micro-metazoa and bacterial community exposed to two initial nitrate concentrations (<5 mg NO_3^-/l or 250 mg NO_3^-/l), two temperatures (21 °C or 26 °C) and grazing by *Daphnia magna*.

Factors	Protists and micrometazoa			Bacteria		
	SS	df	*F*	SS	df	*F*
Nitrate	0.87	1, 40	24.34	0.26	1, 16	6.14
Temperature	0.69	1, 40	19.3	0.86	1, 16	20.06
Daphnia	1.46	1, 40	40.83	0.38	1, 16	0.09
Temperature × Nitrates	0.52	1, 40	14.43	0.24	1, 16	6.03
Temperature × *Daphnia*	0.55	1, 40	15.24	0.56	1, 16	12.99
Nitrates × *Daphnia*	0.79	1, 40	21.96	0.53	1, 16	12.28
Temperature × Nitrates × *Daphnia*	0.71	1, 40	19.93	0.38	1, 16	8.75

P value for all factors <0.001.

Table 2
Physical and chemical properties of water, and bacterial density (mean ± SE) in microcosms in the beginning and after 30 days exposure to two initial nitrate concentrations (<5 mg NO_3^-/l or 250 mg NO_3^-/l) and two temperatures (21 °C or 26 °C). Letters grouped treatments considered homogeneous at $P < 0.001$ following parametric or non-parametric analyses of variance (see methods).

Treatments	Turbidity	Bacteria (cells/ml)	Sedimentation	Temp. (°C)	pH	KH (°dKH)	NH_3^+(mg/l)[a]	NO_2^- (mg/l)[a]	NO_3^- (mg/l)
Time 0	0.02 ± 0.001[a]	3.90·10^7 ± 2.50·10^{5a}	0.00 ± 0.00[a]	21 ± 0.8[a]	7.2 ± 0[a]	5 ± 0[a]	0.25 ± 0[a]	0.10 ± 0[a]	5 ± 0[a]
Without *D. magna*									
Control	0.11 ± 0.001[b]	2.95·10^7 ± 4.81·10^{5b}	0.05 ± 0.02[b]	21 ± 0.8[a]	7.2 ± 0[a]	5 ± 0[a]	0.25 ± 0[a]	0.10 ± 0[a]	35 ± 4.08[b]
Temperature	0.10 ± 0.001[c]	4.53·10^7 ± 7.83·10^{5b}	0.03 ± 0.02[b]	26 ± 0.8[b]	7.2 ± 0[a]	5 ± 0[a]	0.25 ± 0[a]	0.10 ± 0[a]	30 ± 4.08[b]
Nitrate	0.11 ± 0.002[b]	9.56·10^7 ± 6.93·10^{6b}	0.07 ± 0.02[b]	21 ± 0.8[a]	7.2 ± 0[a]	5 ± 0[a]	0.25 ± 0[a]	0.10 ± 0[a]	280 ± 4.08[c]
Temperature × Nitrate	0.10 ± 0.001[c]	4.43·10^7 ± 1.69·10^{6b}	0.04 ± 0.02[b]	26 ± 0.8[b]	7.2 ± 0[a]	5 ± 0[a]	0.25 ± 0[a]	0.10 ± 0[a]	270 ± 4.08[c]
With *D. magna*									
Control	0.06 ± 0.003[d]	2.07·10^5 ± 2.12·10^{3c}	0.04 ± 0.01[b]	21 ± 0.8[a]	7.2 ± 0[a]	5 ± 0[a]	0.25 ± 0[a]	0.10 ± 0[a]	40 ± 4.08[b]
Temperature	0.10 ± 0.002[c]	3.05·10^7 ± 4.36·10^{6b}	0.06 ± 0.01[b]	26 ± 0.8[b]	7.2 ± 0[a]	5 ± 0[a]	0.25 ± 0[a]	0.10 ± 0[a]	35 ± 4.08[b]
Nitrate	0.10 ± 0.001[c]	2.61·10^7 ± 3.11·10^{5b}	0.06 ± 0.03[b]	21 ± 0.8[a]	7.2 ± 0[a]	5 ± 0[a]	0.25 ± 0[a]	0.10 ± 0[a]	275 ± 4.08[c]
Temperature × Nitrate	0.10 ± 0.001[c]	5.47·10^7 ± 1.19·10^{6b}	0.04 ± 0.02[b]	26 ± 0.8[b]	7.2 ± 0[a]	5 ± 0[a]	0.25 ± 0[a]	0.10 ± 0[a]	280 ± 4.08[c]

[a] Were below the detection limits.

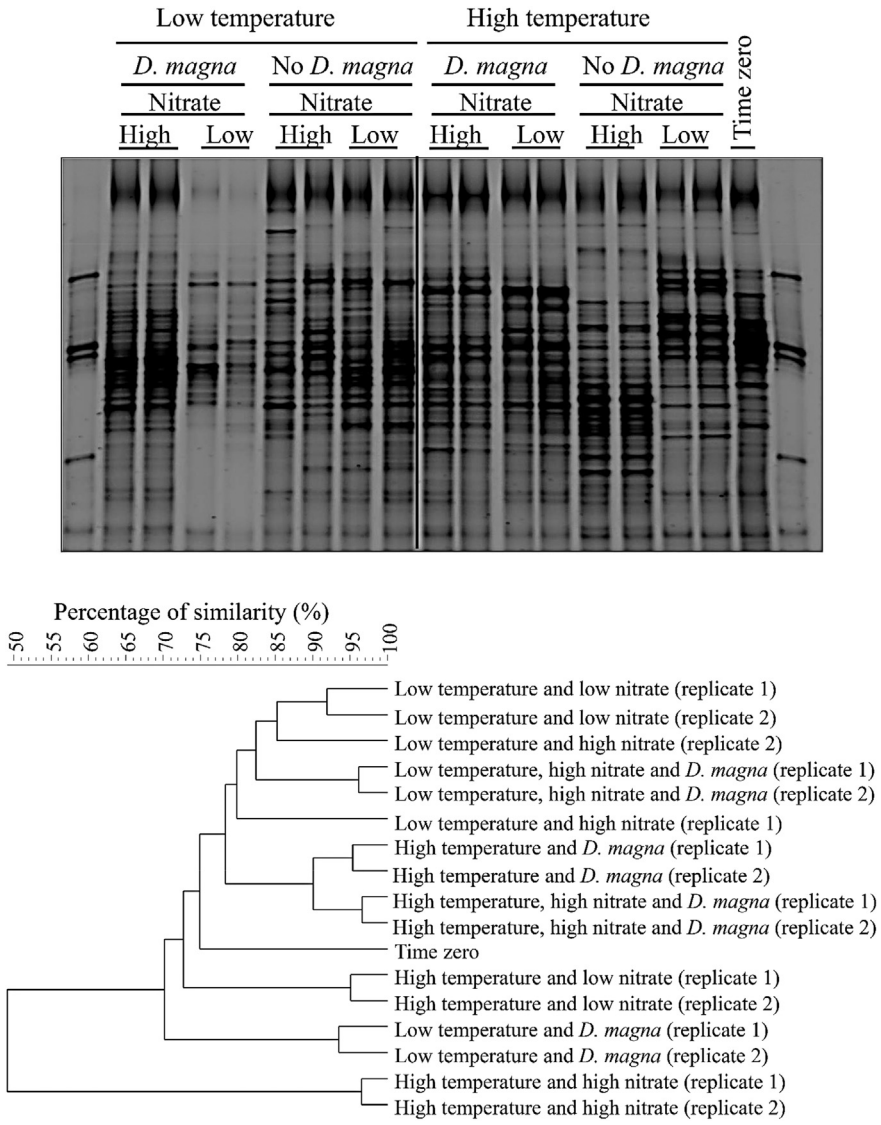

Fig. 2. PCR-DGGE analysis of bacterial 16S rRNA gene diversity in microcosms before and after 30 days exposure to elevated temperature, differing nitrate concentrations, and with and without grazing by *Daphnia magna*. Cluster analysis of DGGE profiles using Pearson correlation coefficient and UPGMA.

sessile suspensivorous ciliates, which also increased in density in the *D. magna* treatment at 26 °C (Games Howell, All $P = 0.01$; Fig. 5). Interestingly, microcosms grazed by *D. magna* at 26 °C had significantly low densities of rotifers *Lecane* spp. and micro-metazoa other than rotifers compared with the un-grazed microcosms (Games Howell, $P < 0.01$; Fig. 5). In microcosms at different temperature and nitrate conditions, *D. magna* did not affect the density of other micro-metazoa than rotifers (Games Howell, All $P > 0.05$). Although *D. magna* was extirpated at high nitrate and 26 °C, there was a strong reduction in the density of pelagic ciliates (Games Howell, $P < 0.01$; Fig. 5). These conditions also favoured other suspensivorous filter-feeders, such as rotifers and sessile filter-feeder ciliates (Games Howell, both $P < 0.01$; Fig. 5).

3.3. Relationship between microbes, water transparency, sedimentation and D. magna

As predicted, *D. magna* density was negatively correlated with water turbidity and the densities of bacteria and protists (Table 5). Interestingly, water turbidity was positively related to the densities of flagellates and the ciliates *Cyclidium* spp. (Table 5), which may have been a direct effect or related to the fine particle suspension upon which they were feeding. A significant positive relationship was also observed between the density of these taxa and that of the planktonic filter-feeders *Uronema* spp. (Table 5). The density of the ciliates *Uronema* spp. was also positively associated with the densities of benthonic suspensivorous taxa such as the rotifers *Rotaria* spp. and the ciliates *Epistylis* spp. (Table 5). All these bacterivorous taxa, however, showed no significant relationship with bacterial density, which was only positively associated with gymnamoebae density (Table 5). The density of several planktonic grazers was also positively related to that of amoebae and crawling ciliates (Table 5), but none of them was significantly associated with sediment content. A negative but highly significant relationship was found between sediment load and micro-annelid density (Table 5).

4. Discussion

The present study shows the high efficacy of *D. magna* in water clarification and disinfection, but highlights its variability under

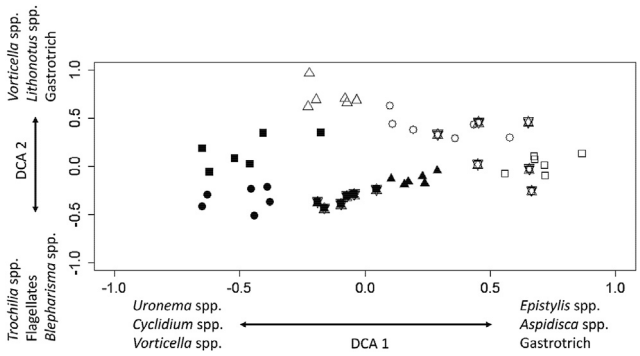

Fig. 3. Detrended correspondence analysis (DCA) plot for protists and micro-metazoa assemblages in the microcosms after 30 days exposure to two temperatures and nitrate concentrations, and grazing by *Daphnia magna*. Black symbols correspond to treatments without *Daphnia*, squares shows the highest nitrate and temperature conditions, triangles portray the highest temperature and the lowest nitrate levels and circles correspond to the highest nitrate and the lowest temperature levels. Stars illustrate the grazing by *D. magna* under low nitrate and temperature conditions. Note that the taxa with the highest contribution to DCA are shown in each axis (see Appendix S1 for further details). Given the community structure at Time 0 differed strongly from that of treatments, Time 0 is omitted to facilitate visualisation of the differences between experimental conditions.

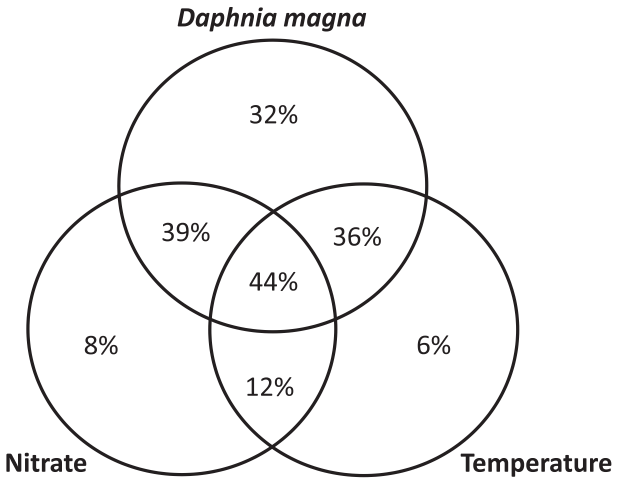

Fig. 4. Venn diagram showing the results of variation partitioning analyses performed on the protists and micro-metazoa data after 30 days exposure to two temperatures and nitrate concentrations, and grazing by *Daphnia magna*. Each circle indicates the independent and joint contributions of each treatment (%) to the explained variation in protists and micrometazoa community composition.

different environmental conditions. Our results therefore are discussed in relation to the possible climatic constraints on the use of *D. magna* as a tertiary treatment and the need of a nitrate pretreatment to increase its efficacy. Nonetheless, regardless of the nitrate and temperature conditions, *D. magna* was identified as the major driver of protist and micro-metazoa community structure in the microcosms. Our findings also identify the taxa most vulnerable to *D. magna* grazing, and increase our knowledge of how microbial communities, possibly including *D. magna* epibionts, respond to warming and/or nitrate pollution.

As expected, optimal performance of *D. magna* occurred in individual assays at 21 °C and low nitrate (Burns, 1969; Giebelhausen and Lampert, 2001). Elevated temperature was a major constraint for *D. magna* survival, but we observed mortality at a lower temperature (26 °C) than that (29 °C) reported by Heugens et al. (2003). Differences in the lethal temperature value for *D. magna* may be explained by the longer duration of our experimental assay (30 d vs. 48 h for Heugens et al., 2003). Such differences may also be attributed to inter-population variability of *D. magna* in response to stressful environmental conditions (Boersma et al., 1999; Mitchell

Table 4
SIMPER analysis of dissimilarity in protists and micro-metazoa communities between temperature, nitrate and grazing by *Daphnia* treatments compared to the controls. The taxa contributing most (>3%) to the dissimilarity between treatments are shown together with their percentage of contribution in dissimilarity and their trend in density (D, decrease; I, increase). Bold values indicated the taxa with the highest contribution to the dissimilarities between communities under warming and/or nitrate conditions without (A) and with *D. magna* grazing (B).

(A) Without *D. magna*

Temperature			Nitrate			Temperature × Nitrate		
Taxa	%	Trend	Taxa	%	Trend	Taxa	%	Trend
Testate amoebae	5.12	D	Gymnamoebae	4.11	D	Gymnamoebae	3.62	D
Flagellates	**5.61**	**D**	**Testate amoebae**	**6.01**	**D**	**Testate amoebae**	**5.17**	**D**
Pelagic ciliates			Flagellates	3	I	Flagellates	4.1	D
Cyclidium spp.	3	D	Pelagic ciliates			Pelagic ciliates		
Rotifers			*Uronema* spp.	4.65	I	*Uronema* spp.	4.86	I
Lecane spp.	3.4	I				Crawling ciliates		
						Euplotes spp.	3.1	I
						SPF[a] ciliates		
						Vorticella spp.	3.9	I

(B) With *D. magna*

Temperature			Nitrate			Temperature × Nitrate			Grazing by *Daphnia* alone		
Taxa	%	Trend	Taxa	%	Trend	Taxa	%	Trend	Taxa	%	Trend
Gymnamoebae	7.2	D	Gymnamoebae	5.22	D	**Gymnamoebae**	**5.04**	**I**	**Gymnamoebae**	**27.34**	**D**
Flagellates	**8.4**	**D**	**Flagellates**	**20**	**D**	Pelagic ciliates			Testate amoebae	12.44	D
Crawling ciliates			Pelagic ciliates			*Uronema* spp.	4.74	D	Flagellates	23.88	D
Blepharisma spp.	3.22	D	*Uronema* spp.	6.01	D	*Cyclidium* spp.	3.25	D	Pelagic ciliates		
Euplotes spp.	3	D	Crawling ciliates			SFF[a] ciliates			*Cyclidium* spp.	7.45	D
SFF[a] ciliates			*Euplotes* spp.	3.04	D	*Epistylis* spp.	4.56	I	Crawling ciliates		
Vorticella spp.	6.80	I	SFF[a] ciliates			*Vorticella* spp.	3.8	D	*Cinetochillum* spp.	3.01	D
Rotifers			*Vorticella* spp.	4.83	I	Rotifers					
Lecane spp.	4.01	D				*Rotaria* spp.	4.24	I			

[a] SFF = sessile planktonic filter feeders ciliates.

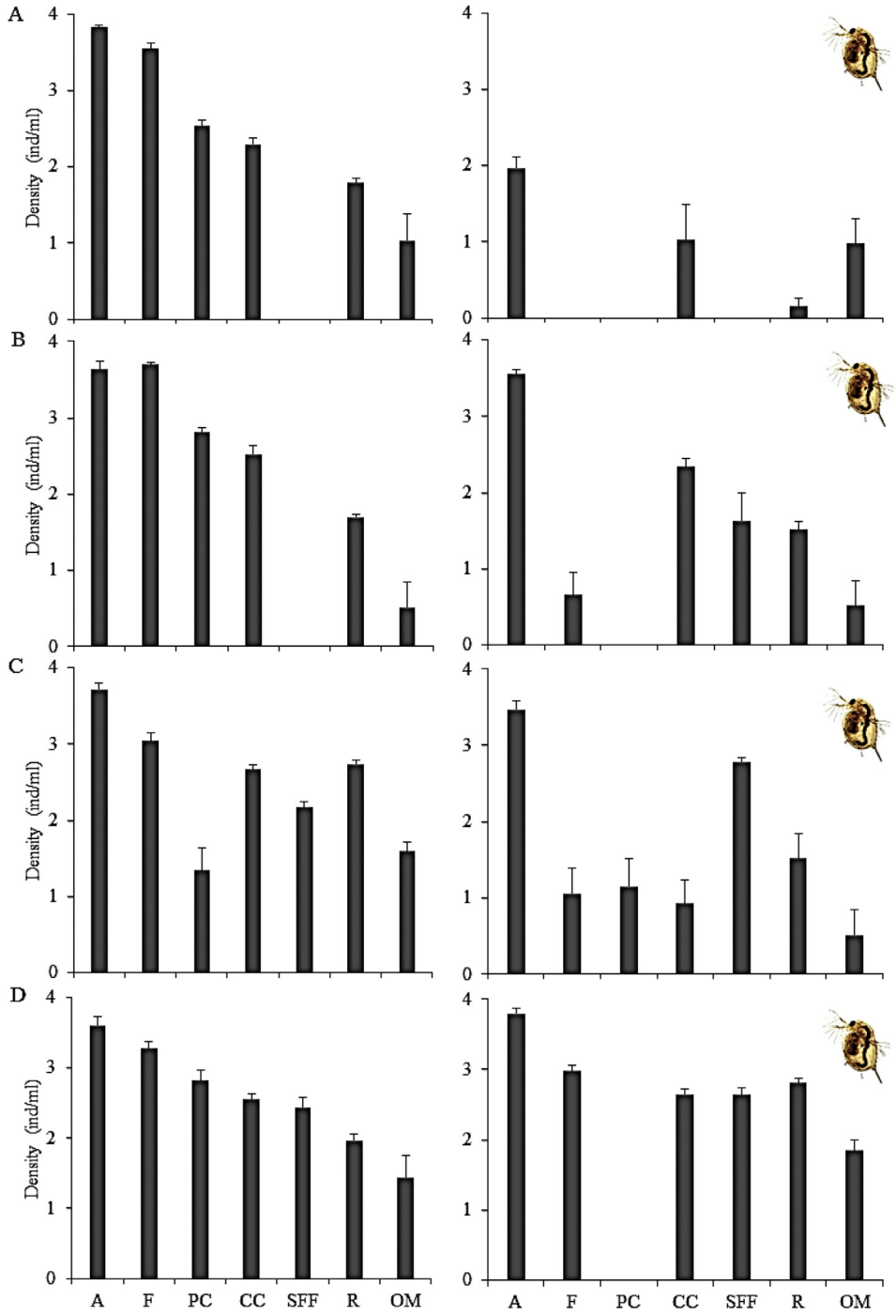

Fig. 5. Mean and standard error (±SE) of densities of the taxa (ind/ml) present in microcosms with (right column) and without (left column) *Daphnia magna* grazing under the same environmental conditions: A, 21 °C and low nitrate level; B, 21 °C and high nitrate level; C, 26 °C and low nitrate level; and D, 26 °C and high nitrate level. Data is presented log-transformed to facilitate comparisons between taxa with large differences in their abundances (e.g. flagellates versus rotifers). Note that taxa are grouped into broad categories (amoebae, A; flagellates, F; pelagic ciliates, PC; crawling ciliates, CC; suspensivorous filter feeders ciliates, SFF; rotifers, R; and micro-metazoa other than rotifers, OM) and include all representatives from each group regardless of their significant trends in the SIMPER analyses (Table 4) to better assess the disinfection ability of *D. magna* treatments.

and Lampert, 2000). These two factors could also explain why the lethal nitrate value for *D. magna* observed in the current study was much lower (250 mg NO_3^-/l) than that (3155 mg NO_3^-/l) reported in acute toxicity tests by Scott and Crunkilton (2000). As documented for nitrates in other aquatic species (Camargo and Alonso, 2006), foraging rate, offspring counts and body-size of *D. magna* were reduced in the current study by nitrate. The reduction in *D. magna* fecundity, however, has been reported previously in other populations associated with warming based on short-term individual assays (Garbutt et al., 2014). The observed increased mortality of *D. magna* under nitrate-warming conditions could be attributed to the fact that temperature raises oxygen demand but oxygen transport was disrupted because nitrate oxidises haemoglobin (Sandstedt, 1990). Thus, our findings suggest that *D. magna* may be unsuitable for treating secondary effluents in warm climates, and that its filter efficacy can increase if nitrates are removed

Table 5
Spearman rank correlation coefficients (*r*) between microbe densities, turbidity and sedimentation ranked by *Spearman's rho*. Note that only high correlated (*rho* > 0.5) and significant relationships at $P < 0.05$ are shown.

		r			R
Gymnamoeba	*Euplotes* spp.	0.53	*D. magna*	Gymnamoeba	−0.55
				Flagellates	−0.83
Flagellates	*Uronema* spp.	0.61		*Rotaria* spp.	−0.56
	Cyclidium spp.	0.78		*Cyclidium* spp.	−0.57
				Blepharisma spp.	−0.61
Lecane spp.	*Euplotes* spp.	0.52		*Euplotes* spp.	−0.69
	Stentor spp.	0.58		Bacteria	−0.50
Rotaria spp.	Gastrotrich	0.74	*Uronema* spp.	Flagellates	0.61
	Aspidisca spp.	0.65		*Cyclidium* spp.	0.61
	Blepharisma spp.	0.53		*Euplotes* spp.	0.58
	Epistylis spp.	0.66			
Gastrotrichs	*Aspidisca* spp.	0.86	Turbidity	Flagellates	0.60
	Epistylis spp.	0.86		*Cyclidium* spp.	0.60
				D. magna	−0.64
Aspidisca spp.	*Epistylis* spp.	0.99			
Blepharisma spp.	*Vorticella* spp.	−0.60	Sediment	Microannelidae	−0.78

from effluents using specific pre-treatments (Masters, 2012; Siegrist et al., 2008; Garcia-Rodríguez et al., 2014). Nonetheless, *D. magna* populations can display strong adaptive capacities to changes in temperature (van Doorslaer et al., 2009) or in nitrate and phosphate levels (Decaestecker et al., 2015).

The direct effects of temperature and/or nitrate observed in *D. magna* individual assays were mirrored in microcosms with major changes in *D. magna* density and hence water quality. At 21 °C and low nitrate, the highest density of *D. magna* occurred (>100 ind/l) and consequently, the strongest increase in water transparency and reduction in bacteria, protist and rotifer density. Our results, however, suggest that grazing pressure differed amongst taxa, with the pelagic ciliates *Cyclidium* spp. (15–32 μm) and flagellates (<5 μm) being more affected than bacteria. This can be attributed to the fact that many bacteria are too small (0.1–1 μm, Hobbie, 1979) for the filter-mesh size of *D. magna* (1–35 μm) (Geller and Müller, 1981). Indirectly, *D. magna* grazing on bacterivorous groups, such as flagellates, ciliates and rotifers, may have also favoured bacteria density (Zöllner et al., 2003). Whilst the rotifers *Lecane* spp. are too large (>100 μm) for *D. magna*, daphniids can reduce rotifer populations by the combined effects of interference and exploitative competition (Gilbert, 1988). Besides trophic competition, the observed decrease in amoebae density could be attributed to direct predation according to a prey selectivity assay (Compte et al., 2009). Thus, our findings are consistent with the generalist foraging behaviour of *D. magna* (see Lampert, 1987; Jürgens, 1994).

At 26 °C or high nitrate, a low density of *D. magna* occurred in microcosms (11–21 ind/l) compared to controls and the cleaning effect of *D. magna* was only observed on protist density. These results support that a high density of *D. magna* is required to reduce total microbial numbers (Degans et al., 2002). Interestingly, protists within the upper limit for the filter-mesh size of *D. magna*, such as the ciliates *Cyclidium* spp., showed a higher density in microcosms with *D. magna* at 26 °C compared to those at 21 °C. Whilst *D. magna* experienced reduced body-size in individual assays at 26 °C or high nitrate, this is unlikely to have altered its prey selectivity. Small-sized protists, such as flagellates, were less affected by *D. magna* grazing at 26 °C than controls, and *D. magna* filter-mesh size reportedly does not vary with body size (Geller and Müller, 1981). Thus, differences in *D. magna* density between treatments are likely to have made a major contribution to the observed differences in protist density. In this regard, our results suggest that more than 20 individuals of *D. magna* per litre are needed to effectively control flagellates and ciliates (see Degans et al., 2002). However, our experimental design does not enable us to determine direct effects of *D. magna* grazing on particular taxa, and we cannot rule out any potential physiological alteration in protists exposed to warming and/or nitrate that may have affected their fitness and vulnerability to grazing. Also, these experimental conditions may have indirectly reduced *D. magna* fitness via the observed increased abundance of the ciliates *Epistylis* spp. or *Vorticella* spp. which can be epibionts (Chatterjee et al., 2013). Either free living or as epibionts, these ciliates and the large rotifers *Rotaria* spp. may have represented strong trophic competition for *D. magna* (see Decaestecker et al., 2005; Aalto et al., 2013; Pauwels et al., 2014). Also, warming and/or nitrate enrichment might have altered the prey-consumer food stoichiometry leading to possible nutrient deficiencies (e.g. phosphorous, fatty acids) that can seriously affect the growth and development of *D. magna* (see Persson et al., 2011; Sperfeld and Wacker, 2012).

Under relaxed or no *D. magna* grazing, turbidity and bacterial density did not differ between microcosms despite bacterivorous protists (e.g. flagellates and ciliates) and other generalist filter-feeders (rotifers) being present (Jürgens, 1994; Arndt, 1993; Simek et al., 1994). The observed plateau in bacterial density could be attributed to anti-grazing mechanisms for protists related to bacteria motility, physicochemical properties and anti-grazing morphologies (reviewed by Jürgens and Matz, 2002). Bacteria-detritus complexes (flocs) are one of these anti-grazing morphs, which influence sedimentation (Bratby et al., 2006). In our study, sediment did not vary between treatments but a negative relationship was found with micro-worm density, supporting the use of micro-worms to reduce sludge accumulation in STP (Tamis et al., 2011; Zhao and Wen, 2012). Also, the rotifers *Lecane* spp., common in some of our microcosms (44–477 individuals/ml), are known to consume flocs (Puigagut et al., 2007). In activated sludge systems, however, 400 lecanid rotifers per ml reduced floc area but did not affect floc settling (Puigagut et al., 2007). By grazing on flocs, micrometazoa and hypotrich ciliates (e.g. *Euplotes*, *Aspidisca*) also ingest and release bacteria into the water column (Madoni, 2003). This might favour a more effective bacteria control but in our microcosms bacteria grazing seemed to be mostly coupled to bacteria productivity.

Whilst the direct contribution of all these prey-consumer interactions to our results is unknown, the shifts in bacterial assemblages observed have been associated with changes in the grazing community (e.g. van Hannen et al., 1999; Degans et al., 2002; Zöllner et al., 2003). Nonetheless, part of the bacterial community appeared to be unaffected by the experimental conditions,

as suggested by the fact that some bands on the DGGE were present in all treatments. It is possible that these bands may represent bacteria taxa that typically escape from bacterivory (Jürgens and Matz, 2002). Additionally, in the *D. magna* treatments, the observed bacterial DGGE pattern may also be attributed to the release of specific *Daphnia* gut bacteria into the water column, as it has been reported that the gut microbiome of *D. magna* is dominated by only a few bacterial taxa that are unaffected by shifts in free-living bacterial communities (Freese and Schink, 2011). Finally, grazers themselves and/or their activity (e.g. release of excretes) could explain the microcosms' turbidity as food items (*Spirulina* and yeast) were not observed under microscope, and turbidity was not correlated with bacteria density.

Despite the fact that our study cannot determine direct effects of temperature and nitrates on protists and micro-metazoa, it provides an indication of taxa tolerance to these conditions (e.g. Salvadó et al., 1995, 2004; Canals et al., 2013). Our results suggest that *Rotaria* spp. are less sensitive to nitrate pollution than species of the genus *Lecane* and that both taxa perform better at 26 °C. This pattern is consistent with previous data showing that warming favours rotifer populations (Andrew and Andrew, 2005; Burdis and Hoxmeier, 2011), and our own observations that *Lecane* spp. are rare in polluted effluents (unpublished data). Our results also indicate that *Uronema* spp. tolerate a higher nitrate level (280 mg NO_3^-/l) than that (47.5 mg NO_3^-/l) reported by Bick (1972). For *Vorticella* spp., our results agree with Canals et al. (2013) who showed that a *Vorticella* species tolerates at least up to 199 mg NO_3^-/l. Likewise, the presence of *Epistylis* spp. at high nitrate levels supports previous data on their nitrate tolerance (Canals et al., 2013). The presence of amoebae and flagellates in both clean and nitrate-polluted microcosms supports their use as indicators of both good (Pérez-Uz et al., 2010) and bad effluent quality (Arévalo et al., 2009). In this regard, it is remarkable that our microcosms nitrified well in all the experimental conditions, suggesting the presence an efficient nitrifying bacteria colony either in water, flocs or the biofilm.

5. Conclusions

Although the cladoceran *D. magna* has great potential for use in natural wastewater treatment processes at 21 °C and low nitrate, its efficacy can differ under variable sewage conditions. Individual assays revealed that an increase in temperature and/or nitrate concentration negatively affected the life-history traits of *D. magna*. Such pernicious effects were mirrored in microcosms with major differences in *D. magna* density and hence cleaning efficacy. A high *D. magna* density (>100 ind/l) appears necessary to effectively control total microbial density and noticeably increase water transparency. Lower densities (up to 20 ind/l) only partially reduce ciliates and flagellate populations in a natural microbial food-web. However, regardless of the nitrate and temperature conditions, *D. magna* was identified as the major driver of the community structure of protists and micro-metazoa in our study. It was also remarkable that bacterial density stabilised under relaxed or no *D. magna* grazing pressure despite the presence of major bacterivores such as flagellates, ciliates, amoebae and rotifers. Thus, reducing bacterial load is likely to be challenging using bio-treatments given the complexity of microbial food-webs. Although this study suggests caution for the widespread use of *D. magna* as a tertiary treatment, further investigations may reveal the suitability of other strains since the response of this species to environmental stressors presents a high phenotypic and genotypic variability.

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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.watres.2015.06.036.

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