UNIVERSITAT DE BARCELONA

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STRUCTURE AND FUNCTION IN FLUVIAL BIOFILMS

IMPLICATIONS IN RIVER DOC DYNAMICS

AND NUISANCE METABOLITE PRODUCTION

ESTRUCTURA I FUNCIÓ DELS BIOFILMS FLUVIALS

IMPLICACIONS EN LA DINÀMICA DEL DOC AL RIU

I LA PRODUCCIÓ DE METABÒLITS SECUNDARIS

ELISABET VILALTA BALIELLAS

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Dr. SERGI SABATER
Director

Dra. ISABEL MUÑOZ
Tutora

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Als meus pares

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ÍNDEX GENERAL

RESUM

I. INTRODUCCIÓ I OBJECTIUS

1. Ecosistemes Fluvials

Els rius i rieres han estat considerats com a centres integradors del paisatge, ja que funcionen com a font natural de riquesa per a la població i a més actuen de ronyons del sistema transportant i netejant l'aigua resultat de les activitat humanes. Els rius són sistemes oberts que requereixen una entrada constant d'energia en forma de matèria orgànica, la majoria produïda per fotosíntesi, que pot ser autòctona, produïda a dins del riu, o al.lòctona, provinent de fora el canal fluvial (Allan 1995, Wetzel 2001). La xarxa tròfica en ecosistemes fluvials pot ser complexa. La matèria orgànica és utilitzada per la biomassa microbiana, sobretot bacteris, pero també fongs i algues, que a la vegada són consumits pel zooplancton i aquest pels predadors. Exudats i productes de descomposició són utilitzats pels microorganismes completant així la xarxa tròfica.

2. Qualitat de l'aigua

Per una bona gestió de la qualitat de l'aigua de rius i rieres no solament hom ha de considerar el component hidrològic, sinó també la interacció amb el propi metabolisme biològic. L'us de rius com a subministrament d'aigua destinada al consum, i la necessitat de proveïr aigüa en bona qualitat, fa que l'aigua dels rius hagi de ser tractada i purificada cada vegada amb mecanismes més sofisticats. El fet

d'estudiar els processos naturals que es donen als sistemes fluvials i que affecten la qualitat de l'aigua, pot ajudar a elaborar tractaments de més baix cost. Aquest és el cas de la dinàmica del DOC (Carboni organic dissolt) a l'aigua i de la producció de metabòlits secundaris.

El paper del DOC en aigües destinades al consum té una important rellevància en el fet que pot causar el creixement de bacteris nocius en els sistemes de distribució (Kaplan & Newbold 1995). A més a més, pot reaccionar amb el procés de cloració de l'aigua i formar productes cancerígens com és el cas dels Trihalometans (THMs) (Bull et al. 1995). Els biofilms que creixen tan en sistemes naturals com en els filtres de carboni i sorra utilitzats en la purificació de l'aigua, poden ser responsables de la producció i consum del DOC a l'aigua. Per tant, un millor coneixement del funcionament dels biofilms i del seu paper com a autodepuració de l'aigua, pot ajudar en el tractament de l'aigua relacionat amb la dinàmica del DOC.

El cas de producció de metabòlits secundaris per part dels cianobacteris, tals com microcistines, han estat estudiats pel seu comportament tòxic (Dow & Swoboda 2000). Entre aquestes cianotoxines n'hi ha que no són considerades tòxiques pels mamífers, però que són d'important interès pel tractament d'aigües destinades al consum perquè produeixen un cert gust i olor de florit a l'aigua. Entre elles trobem el metabòlit de la geosmina, però també n'hi ha d'altres, com el 2-metilisoborneol (MIB) (Paerl & Millie 1996). En aquest cas, el coneixement de la dinàmica de creixement de les masses de cianobacteris i també de la producció de la geosmina, afavorirà un possible tractament previ de les aigües abans d'arribar a la planta de tractament.

Carboni orgànic dissolt (DOC) en els rius

La matèria orgànica dissolta (DOM) és la forma en que es troba majoritàriament la matèria orgànica en els rius, i es considera com la porció inferior de 0.5 μm de tamany de partícula. Dins aquesta, el DOC (carboni orgànic dissolt) és la que en forma la major part, i per tant, els dos termes es poden considerar intercanviables. Els compostos més abundants que s'han identificat i analitzat en les aigües fluvials són els hidrats de carboni, aminoàcids i àcids grassos, considerats biodegradables i retinguts fàcilment pels microorganismes. La resta (aproximadament del 50% al 75%) la componen àcids húmics, fúlvics i hidròfils, recalcitrants a la degradació biològica i d'alt pes molecular.

Metabòlits secundaris: el cas de la geosmina

La producció de metabòlits secundaris per part dels cianobacteris pot estar lligat a factors d'estrès ambiental, o també factors intrínsecs tals com la biosíntesis de pigments. La geosmina és un anell terpenoid bicíclic que és produït tant per cianobacteris com també actinomicets, i que produeix olor de florit a l'aigua (Aoyama 1990). La seva àmplia distribució i el seu baix llindar de detecció, fan que sigui considerat un principal problema per les aigües destinades al consum. Tot i així, la seva implicació en possibles casos de toxicitat per la salut és tema de discussió.

3. Biofilms fluvials

Biofilms fluvials i la seva significància ecològica

Els biofilms són comunitats estructurades compostes per bacteris, algues, fongs i protozous, englobades en una matriu polisacarídica i que viuen en una superfície sòlida i incloses en una fase aquosa (Lock et al. 1984). La seva importància s'ha centrat principalment en els biofilm hetrotròfics que creixen i malmeten superfícies artificials tals com tuberies, però també tenen una importància ecològica en els sistemes fluvials, ja que tenen la capacitat d'adsorció i retenció de nutrients, esdevenint un efecte natural d'autodepuració de l'aigua (Pusch et al. 1998). L'eficiència de retenció del DOC per part dels biofilms estarà afectada per processos abiòtics associats amb la matriu polisacarídica, tals com l'adsorció i emmagatzament del DOC, que més endavant serà degradat pels enzims extracel.lulars i assimilat pel compartiment heterotròfic del biofilm. Com a part del biofilm, la comunitat d'algues bentòniques han estat considerades bones indicadores de la salut dels ecosistemes aquatics (Stevenson et al. 1996), ja que són un grup important de productors primaris en els rius.

Condicions del creixement algal i cianobacteris en els rius

El creixement de les algues bentòniques en rius està afectat per la interacció de factors hidrològics i qualitat de l'aigua, com també factors biòtics. La disponibilitat de recursos, principalment la de nutrients i de llum, han estat considerats els principals responsables del seu creixement. A diferència de les algues planctòniques, el fet de créixer adherides a un substrat, poden acumular grans quantitat de biomassa, afectant la seva estructura i per tant el seu metabolisme.

4. Objectius

El propòsit d'aquesta tesi fou posar de manifest les característiques de l'estructura i funció del biofilm en relació amb el balanç del DOC a l'aigua i la dinàmica de la producció de geosmina en rius mediterranis. Els principals objectius de l'estudi van ser:

1) Determinar la capacitat dels biofilms en la retenció del DOC de l'aigua, describint la relació dels biofilms que creixen en condicions de llum i de foscor amb el nivell de DOC a l'aigua i la seva biodisponibilitat.

2) Relacionar el paper que pot exercir l'edat del biofilm (gruixaria, increment de biomasa) en la utilització del DOC de l'aigua.

3) Descriure els factors ecològics relacionats amb la dinàmica de la geosmina i determinar-ne els productors des d'una perspectiva d'escala fluvial, i conèixer si existeix un possible risc toxicològic associat a la producció de geosmina.

4) Determinar quines variables ambientals afecten el creixement i desaparició de les masses de cianobacteris al riu a nivell d'hàbitat, i relacionar-ho amb la dinàmica de la geosmina al riu.

5) Descriure l'estructura i funció dels diferents compartiments observats en el creixement de les masses de cianobacteris, i com les possibles diferències afecten a la producció de geosmina.

6) Estudiar la microestructura de les masses de cianobacteris, i com aquesta afecta al seu metabolisme, relacionat amb la aparició de la geosmina.

II. LLOC D'ESTUDI

Per tal d'assolir els objectius, l'estudi es portà a terme en dos rius. Primer de tot, la relació entre biofilms i la dinàmica del DOC es va estudiar en el tram situat als últims 50 km del riu Ebre. Es varen definir dos punts de mostreig en el riu, anomenats Riu 1 (R1) i Riu 2 (R2), distanciats 15 km riu avall. A més a més, a les proximitats del Riu 1, en divergeix un canal de reg, que a una distància de 15 km, l'aigua es canalitza en una tuberia fins arribar a la planta potabilitzadora del CAT (Consorci

d'aigües de Tarragona). Dos punts de mostreig, tant pel canal com per la tuberia es van situar al començament i al final de cada sistema, anomenats Canal 1 (C1), Canal 2 (C2), Tub 1 (P1) i Tub 2 (P2) respectivament.

La dinàmica de la geosmina es va estudiar en el riu Llobregat. Un riu amb un règim típicament mediterrani, amb un mínim de cabal a l'estiu, i amb crescudes durant la primavera i la tardor. Tres punts de mostreig es van localitzar al llarg del riu: S1 (Navàs), S2 (Pont de Vilomara) i S3 (Olesa de Montserrat). La planta potabilitzadora ATLL (Aigües Ter Llobregat) està localitzada just després del S3.

III. MATERIALS I MÈTODES

Riu Ebre

En el riu Ebre, a cada punt de mostreig, es van utilitzar substrats de vidre (1 cm^2) per tal de permetre la colonització dels biofilms. Es van dissenyar unes estructures metàl.liques especials per tal de submergir els vidrets a cada punt. Els vidrets colonitzats amb biofilms de 2 mesos es van recollir al setembre i novembre del 2000 i a l'abril, maig i juliol del 2001. A més a més, biofilms de 4 mesos van ser recollits al novembre del 2000 i juliol del 2001. I per últim, els biofilms de 12 mesos al juliol del 2001.

De cada mostreig es van recollir dades físico-químiques de l'aigua, incloent anàlisis de DOC i BDOC (DOC biodegradable), i dels biofilms se'n va estudiar l'estructura, incloent anàlisis de quantificació i determinació taxonòmica algal, quantificació bacteriana, clorofil.la-*a*, contingut de carboni i nitrogen i observacions al microscopi d'escàning (SEM) i al microscopi confocal (CLSM). Per la funció del biofilm es van mesurar diferents activitats enzimàtiques extracel.lulars.

Riu Llobregat

Al riu Llobregat es van utilitzar biofilms que creixien tant al litoral com a corrent i el mostreig es va portar a terme en diferents períodes. Primerament es va fer un anàlisi mensual en els tres punts d'estudi des del març del 2000 fins al maig del 2001, intensificant el mostreig setmanalment des del febrer fins al maig del 2001. Per altra banda, el punt S2 va ser mostrejat setmanalment durant l'hivern i primavera del 2002 (de febrer a maig del 2002). I ja per últim, coincidint amb el període de març i abril del 2003, es va realitzar un experiment utilitzant biofilms recollits a S2.

Es varen mesurar paràmetres físico-químics de l'aigua i es va analitzar la concentració de geosmina. Per recollir els biofilms es va utilitzar un raspat d'una superfície coneguda quan els biofilms cobrien pedres o còdols, o bé es va utilitzar un core de PVC (3.1 cm^2) que s'introduïa dins de la massa algal. Dels biofilms se'n va estudiar paràmetres estructurals i funcionals, tals com quantificació i composició algal, anàlisi de la geosmina dins el biofilm, clorofil.la-a, carbohidrats, contingut de carboni i nitrogen i també observacions amb el SEM, a més a més, anàlisi de l'activitat potencial enzymàtica extracel.lular i mesures de la capacitat fotosintètica màxima a la foscor utilitzant el PAM (Pulse Amplitude Modulation). Durant l'experiment es va estudiar la microestructura utilitzant microelèctrodes d'oxigen i redox.

CAPÍTOL 1. Estructura i funció del biofilm relacionat amb la dinàmica del DOC al riu Ebre

En aquest capítol es va estudiar la relació entre l'estructura i funció dels biofilms amb la dinàmica del DOC a l'aigua. Primer de tot es va estudiar la dinàmica del DOC i BDOC (DOC biodegradable) en el riu i també en el canal i en la tuberia al llarg d'un any, calculant-ne els balanços positius i/o negatius que van tenir lloc al llarg del canal i de la tuberia. El fet d'utilitzar sistemes artificials, com el canal i la tuberia, ens va permetre relacionar el paper dels biofilms responsables dels balanços de DOC i BDOC, ja que es va evitar la influència que hagués pogut tenir l'hiporreic i les aigües subterrànies.

La dinàmica de DOC es va relacionar amb l'estructura i el metabolisme del biofilms de 2 mesos que creixien al llarg de cada sistema. Les activitats enzimàtiques extracel.lulars utilitzades en aquest estudi ens van servir per relacionar l'activitat de descomposició i degradació de la matèria orgànica que té lloc en el biofilm, i que són una bona aproximació de l'activitat heterotròfica que és dóna en el biofilm. Com a activitats exoenzimàtiques, es van utilitzar les següents: β-glucosidasa i β-xilosidasa, que degraden els polisacàrids de la cel.lulosa i hemicel.lulosa respectivament; la fosfatasa, responsable de la degradació de monoésters ortofosfòrics per l'obtenció de fòsfor inorgànic; l'aminopeptidasa, que hidrolitza pèptids i proteïnes; i per últim, la lipasa, responsable d'hidrolitzar macromelècules de lípids complexes.

Els principals resultats obtinguts en aquest estudi van ser que els biofilms que van créixer sota condicions de llum (canal), tot i tenir unes variacions mensuals de producció/consum de DOC, la mitjana del balanç anual va ser de consum. A més a més, presentaven una major biomassa amb més complexitat estructural que els

biofilms de foscor (tuberia), cosa que permetria millorar el desenvolupament de la comunitat bacteriana, tant en biomassa com en activitat heterotròfica. La matriu polisacarídica que es desenvolupava en aquests biofilms, també podria haver ajudat en l'adsorció abiòtica del DOC, afavorida per una degradació del DOC fotoquímicament degut a la llum. Per altra banda, a la tuberia hi va haver un consum de DOC més constant al llarg de l'any. Els biofilms de tuberia eren majoritàriament dependents de la fracció biodegradable del DOC (BDOC). Tot i així, la relació positiva entre les activitats enzimàtiques extracel.lulars amb el DOC i BDOC trobats en els biofilms de tuberia, indiquen que el seu metabolisme contribueix a la dinàmica del DOC en aquest sistemes.

CAPÍTOL 2. Caracterització de l'edat del biofilm en relació amb la dinàmica temporal del DOC a l'aigua

La qualitat i quantitat de DOC influencia l'activitat heterotròfica del biofilm. Sota condicions de llum, la part autotròfica actua com a possible font de components biodegradables que seran utilitzats pels bacteris. Per tant, biofilms on coexisteixen el compartiment autotròfic i heterotròfic es veurà afectat per un alt reciclatge intern del carboni. L'edat del biofilm, i les corresponents característiques tals com la gruixària, el contingut de polisacàrids, la densitat algal i bacteriana, etc, determinarà el seu propi funcionament i per tant la capacitat de retenció i producció de DOC.

En aquest capítol es comparen dues situacions diferents en quant a la concentració de DOC (DOC alt i DOC baix), coincidint amb dos estacions diferents, tardor i primavera respectivament. No obstant, s'ha de tenir en compte que diferències en la composició del DOC i la seva biodegradabilitat, afectades pels canvis d'estacionalitat, també afectarien al metabolisme del biofilm. En tals situacions es van comparar biofilms de diferent edat i provinents dels tres sistemes descrits.

Es van observar diferències entre els biofilms que van créixer en el riu i en el canal, ja que degut a l'alta velocitat de l'aigua del canal (1 m s^{-1}), va impedir l'acumulació de gran biomassa, tot el contrari del litoral del riu. A més a més, tot i no haver moltes diferències en la composició algal, en el riu hi va haver més densitat de filaments d'algues verdes que en el canal, i el contingut de mucílag també va ser més gran. Aquestes condicions de creixement van afectar el metabolisme del biofilm. La principal conclusió extreta de l'estudi va ser que el metabolisme del biofilm no era proporcional a la quantitat de biomassa fixada, ja que els biofilms de 12 mesos provinents del riu, van presentar més baixes activitats exoenzimàtiques (referides

per mg C) que biofilms més joves sense tanta biomassa. Biofilms provinents de la tuberia tampoc van presentar un creixement del metabolisme en proporció a l'acumulació de biomassa en els biofilms de 4 i 12 mesos. Per tant, i com a conclusió, podem dir que biofilms gruixuts són menys eficients en la retenció del DOC de l'aigua, i que aquesta activitat es veurà altament afectada per la quantitat i qualitat del DOC i BDOC del sistema.

CAPÍTOL 3. Dinàmica temporal i espacial de la producció de la geosmina en el riu Llobregat

L'estudi de la dinàmica de la geosmina al riu Llobregat es va portar a terme en el tram mig i baix del riu, ja que s'havia descartat prèviament la seva presència en els trams de capçalera. La seva producció havia estat àmpliament descrita per part de cianobacteris planctònics, tot i que alguns estudis més recents la van associar a masses de cianobacteris bentònics (Izaguirre et al. 1992). A més a més, la majoria d'estudis van ser desenvolupats en cultius, fent que no es conegués en detall els principals factors ambientals relacionats amb la seva producció.

Aquest va ser el principal objectiu a l'hora de tractar el tema de la geosmina. Conèixer quins eren els factors ambientals que estaven lligats amb la dinàmica temporal i espacial de la seva producció, i també, determinar quins eren els productors en el riu Llobregat. A més a més, es va fer un anàlisi toxicològic de les masses productores de geosmina per tal de determinar si la seva producció anava lligada amb l'existència de toxicitat.

La dinàmica temporal del pics de producció de geosmina es repetia cada any des de finals de gener fins a principis de maig, coincidint amb unes temperatures de l'aigua que anaven dels 6.6ºC als 14.5ºC, i amb uns cabals mínims. La seva producció va ser relacionada amb el creixement d'unes masses bentòniques de cianobacteris, formats majoritàriament per *Oscillatoria limosa* i *Oscillatoria tenuis*, espècies que ja havien estat descrites prèviament com a productores de geosmina (Paerl & Millie 1996). Les masses creixien aderides al substrat, que havent assolit un cert gruix de biomassa, es desenganxaven i esdevenien flotants. Aquests flocs flotants eren arrossegats riu avall, provocant la dispersió de les espècies i de la geosmina. Per altre banda, els anàlisis de toxicitat que es van portar a terme en els laboratoris de RECETOX (Brno, República Txeca) van confirmar que no hi havia cap risc toxicològic associat a les masses productores de geosmina.

CAPÍTOL 4. Paràmetres ecològics i l'evolució de la comunitat algal relacionats amb la dinàmica de la geosmina a l'aigua

La producció de geosmina en el riu Llobregat va estar relacionada amb el creixement i ocupació de les masses de cianobacteris a les zones de litoral, i el posterior desenganxament i arrossegament riu avall. Per conèixer quines eren les causes que portaven al creixement massiu de les masses de cianobacteris i la conseqüent producció de geosmina, es va portar a terme un estudi detallat de l'hàbitat que afavoria aquest creixement. Es va dissenyar una cartografia setmanal que abarcava tota la llera del riu (30 x 45 m) en el punt S2, on es va recollir les dades de variables ambientals i el percentatge de cobertura de les diferents masses d'algues.

Els resultats de l'estudi van explicar quines variables ambientals afectaven aquest creixement. Les masses de cianobacteris creixien en unes condicions d'alta concentració de nutrients, sobretot de nitrogen i fòsfor, amb unes temperatures càlides i una baixa velocitat del corrent. A més a més, es va relacionar amb una proporció del N/P baix, suggerint que una possible limitació de nitrogen afavoria el seu creixement i possiblement la producció de geosmina. A finals de primavera, coincidint amb més disponibilitat de nitrogen i de llum, les masses d'*Oscillatoria* desapareixen i es veien afavorides masses de filamentoses verdes, com ara *Cladophora*, que són considerades fotosintèticament més competitives. Per altra banda, els flocs flotants contenien més concentració de geosmina que els adherits, i per tant, es van considerar els responsables de la distribució de la geosmina riu avall.

Per tal de gestionar la qualitat d'aigua destinada al consum, des del punt de vista de la producció de geosmina, és aconsellable tractar les aigües des del riu mateix, enlloc d'esperar el tractament de potabilització. El creixement de les masses de cianobacteris es veuria disminuït si hi hagués una disminució de la càrrega de nutrients de l'aigua, i el flux del riu fos normalitzat, evitant les preses i canalitzacions construïdes al llarg del riu i que interfereixen el seu flux.

CAPÍTOL 5. Estructura i funció dels biofilms i la seva implicació en la dinàmica de la geosmina a l'aigua

La producció de geosmina, i de metabòlits secundaris en general, ha estat relacionat en el context ecològic de competició i d'una possible limitació de nutrients a causa de l'acumulació de biomassa. Aquest pot ser el cas de les masses de cianobacteris, que el gruix i la densitat del biofilm pot dificultar la difusió d'oxigen i nutrients, i la

penetració de llum. Per tal de determinar quins factors estaven relacionats amb la dinàmica de producció de geosmina, la comunitat d'algues i cianobacteris van ser estudiats des del punt de vista estructural i funcional. Es va fer èmfasi en la possible limitació de nutrients dins del biofilm, sobretot del nitrogen, ja que el creixement de les masses d'*Oscillatoria* es donaven en condicions d'una baixa relació de N/P (veure Capítol 4).

Es varen utilitzar les activitats enzimàtiques per tal d'evidenciar una possible limitació de nutrients dins el biofilm, tals com l'aminopeptidasa (AMA), la fosfatasa (APA) i la β-glucosidasa. Els valors alts d'aminopeptidasa trobats en les masses de cianobacteris signifiquen que el nitrogen inorgànic és obtingut de fonts orgàniques, suggerint una limitació del nitrogen inorgànic. En canvi, la fosfatasa presentava valors més baixos, indicant que el fòsfor inorgànic no era limitant. A més a més, la relació entre APA:AMA va ser diferent per les diferents comunitats algals. Els flocs flotants van presentar una relació més baixa, indicant activitats d'aminopeptidasa més elevats, i per tant, més limitació de nitrogen dins el biofilm. Aquest fet podria explicar una més alta producció de geosmina, com a via de dissipació d'un excés de carboni durant canvis fisiològics (Naes et al. 1985). Tot i així, les masses de cianobacteris adherides també van presentar una relació APA:AMA més baixa que aquelles comunitats formades bàsicament per diatomees o algues verdes.

Els flocs flotants a part de presentar una activitat aminopeptidasa més alta, també presentaven alts valors de la β-glucosidasa. Això va suggerir que una possible degradació de les cèl.lules o lisis cel.lular estava afectant aquesta comunitat, ja que se n'alliberen productes proteïnics i polisacàrids. La molècula de geosmina es troba lligada a estructures cel.lulars, tals com les lamel.les dels cloroplasts i material cel.lular lipofílic (Wu & Jüttner 1987). Per tant, un alliberament de la molècula necessita una lisis cel.lular. La meiofauna també pot interferir en aquest procés, tant afavorint una degradació cel.lular com en l'alliberament i difusió de la geosmina a l'aigua, i el fet que major nombre de meiofauna habitava en el floc flotant, pot confirmar aquesta hipòtesi.

CAPÍTOL 6. Heterogeneïtat estructural associada a la dinàmica de les masses de cianobacteris en els rius

Les diferències estructurals i funcionals observades entre les comunitats de cianobacteris adherides i flotants (veure Capítol 5), va semblar estar relacionat amb la producció de geosmina a l'aigua. En aquest capítol es va tractar de fer una

aproximació de la microestructura de les diferents masses, per tal de relacionar-ho amb les condicions fisiològiques. Es van realitzar micro-perfils d'oxigen i del potencial de redox per tal de detectar l'evolució temporal i heterogeneïtats estructurals dins dels biofilms.

L'evolució dels perfils d'oxigen dins les masses van indicar que la dinàmica de flotabilitat era deguda a les dinàmiques de fotosíntesi i respiració. També presentaven diferències estructurals, en els quals els flocs flotants tenien més alta composició de cianobacteris que els adherits. Els flocs flotants presentaven una alta producció d'oxigen, el qual era retingut dins el floc, cosa que provocava una supersaturació d'oxigen amb la conseqüent formació de bombolles i la flotació. Durant la foscor, l'oxigen podia ser consumit i perdre la seva flotabilitat.

Es varen observar diferents micro-taques dins els floc flotant, en les quals filaments d'*Oscillatoria limosa* s'agrupaven i formaven masses denses de color negre, mentre que altres taques de color marró, majoritàriament formades per diatomees i detritus, cobrien les parts més superficials del floc, que estaven en contacte amb l'aire i que possiblement protegien al floc de possibles efectes negatius de fotooxidació. Les masses negres d'*Oscillatoria* consumien l'oxigen durant la nit fins a l'anòxia, i podien arribar a uns potencials de reducció molt baixos. Per altra banda, aquesta situació no es va trobar mai en les micro-taques marrones. *Oscillatoira limosa* ha estat descrita com una espècie sense heterocist fixadora de nitrogen atmosfèric (Villbrandt et al. 1990). Les condicions d'anoxia trobades a les taques negres afavoreixen a aquest activitat, ja que l'enzim responsable de la fixació, la nitrogenasa, és inhibit per l'oxigen. Aquest resultats van confirmar que hi havia problemes de difusió dins les masses d'*Oscillatoria limosa*, i que per tant, es podia donar una situació de limitació de nitrogen, afavorint la producció de geosmina.

IV. CONCLUSIONS

Les principals conclusions d'aquest estudi van ser:

I) La producció i retenció del DOC de l'aigua està relacionat amb el creixement dels biofilms. El metabolisme del biofilm (activitats enzimàtiques extracel.lulars) va estar relacionat amb el DOC i el BDOC de l'aigua. Els biofilms que van créixer en condicions de llum, tot i presentar variacions mensuals de retenció/producció de DOC, en general van presentar una major taxa de retenció que aquells que van créixer a la foscor. Això podia ser degut a la major biomassa algal acumulada en els biofilms

de llum, que afavoria el creixement de bacteris, i el desenvolupament de la matriu polisacarídica podia afavorir l'adsorció abiòtica del DOC. Per altra banda, els biofilms que creixien a la foscor contribuïen a la reducció de DOC, perquè presentaven una taxa més constant de retenció de DOC al llarg de l'any.

II) L'estructura del biofilm va interferir en el reciclatge del carboni, ja que el seu metabolisme es va veure afectat per les variacions d'estructura. Els biofilms autotròfics alliberen una alta quantitat de DOC de bona qualitat que és ràpidament consumit pels bacteris. Per tant, la biomassa algal i la gruixària del biofilm podrien afectar la retenció del DOC de l'aigua, indicant que els biofilms gruixuts en podrien ser menys eficients.

III) L'estudi de la dinàmica de la geosmina tant a l'escala de riu com fent una aproximació més detallada a l'hàbitat, ens va permetre relacionar quins factors ambientals afectaven el creixement de les masses de cianobacteris, i per tant, la producció de geosmina. Les masses formades bàsicament per *Oscillatoria limosa* creixien en zones de litoral, amb baix corrent, amb temperatures suaus i altes concentracions de nutrients, i en concret, amb una relació de N/P baix. El creixement de les masses presentava dues fases: l'adherida i la flotant, que mentre una és responsable localment de la producció de geosmina, l'altre s'encarrega de dispersar-la al llarg del riu. Per altra banda, els estudis de toxicitat fets amb els biofilms productors de geosmina van ser febles i no van estar relacionats amb la producció de geosmina, per la qual cosa, podem concluir que la producció de geosmina no implica un risc toxicològic per a la salut humana.

IV) Es van trobar diferències estructurals i funcionals entre els biofilms adherits i flotants. El floc flotant presentava més biomassa i les activitats exoenzimàtiques eren més altes. La relació entre les activitats fosfatasa/aminopeptidasa, indicadores d'una possible limiatació de nitrogen a dins el biofilm, també eren més altes en el floc flotant, i aquestes situacions semblaven afavorir la producció de geosmina. El creixement massiu de les masses podria afectar a la difusió de nutrients a dins el biofilm, creant situacions de limitació de nitrogen. Gràcies a les mesures realitzades amb els microelèctrodes d'oxigen i redox, es va observar com l'heterogeneïtat de l'estructura del biofilm podia afectar al seu metabolisme. En concret, es donaven unes situacions de micro-taques en que les masses d'*Oscillatoria* s'agrupaven i formaven taques denses, en que la difusió d'oxigen, i possiblement també la de nutrients, quedaven reduïts. Per altra banda, l'alliberament de la geosmina al medi aquatic necessita d'un procés de lisis cel.lular, provocada per una degradació del biofilm i afavorida també per la presència de meiofauna.

Implicacions en la gestió de l'aigua

La dinàmica del DOC

Els estudis realitzat en el riu Ebre, i en concret en els sistemes artificials del canal i tuberia, van indicar que els biofilms eren responsables de la producció i consum del DOC de l'aigua. Dels resultats obtinguts es podria suggerir la utilització de les tuberies tancades per tal de reduir la concentració de DOC abans d'arribar a la planta potabilitzadora. Ara bé, s'ha de tenir en compte que l'estudi es va portar a terme en unes situacions en què les concentracions de DOC no eren extremadament altes, i per tant, el metabolisme dels biofilms es veien altament relacionats amb l'aigua corrent.

Una qüestió que queda encara oberta és que passaria amb una entrada de DOC significativment més alta. Primer de tot cal precisar que no només la quantitat de DOC afectarà als biofilm, sinó també la qualitat de la seva composició i la fracció biodegradable (BDOC). Sembla ser que un augment de DOC afavoriria el metabolisme heterotròfic, i per tant una retenció. Ara bé, entrades continuades de DOC alts afectaria el creixement en biomassa i gruix del biofilm, i per tant, potenciaria el reciclatge de carboni intern, per la qual cosa no es veuria un efecte en la retenció de DOC. En aquestes situacions hauriem de considerar l'ús de sistemes naturals en que l'heterogeneïtat del substrat, on creixen els biofilm, afavoriria la interacció entre processos físics i químics amb els microorganismes, i per tant, el procés natural de reciclatge de la matèria orgànica. Per altra banda, l'ús de sistemes com el canal i la tuberia, en que la velocitat de l'aigua juga un paper important en el creixement dels biofilms, no permetent l'acumulació de grans quantitats de biomassa, el metabolisme heterotròfic del biofilm es podria veure efectat més pel DOC al.lòcton provinent de l'aigua que no pas el propi intern, i per tant, afavorint un consum net del DOC de l'aigua.

La producció de geosmina

La producció de geosmina al riu Llobregat va ser deguda al creixement de masses bentòniques de cinaobacteris, que coincidia amb una estacionalitat, és a dir, apareixien a finals de gener i desapareixien a principis de maig. Aquesta estacionalitat tant marcada pot ajudar a preveure i a prevenir els pics de geosmina, tot i que una gestió prèvia en el riu seria necessària i recomenada per evitar el creixement de les masses i així evitar la producció de geosmina. En el riu hi ha un munt de punts en que afavoreixen l'aparició de les masses, que coincideixen amb llocs de baix corrent,

just després de les petites preses que s'han construït al llarg del riu, a més a més, l'alta càrrega de nutrients que transporta el riu també afavoreix el seu desenvolupament. La millor gestió seria controlar la quantitat de nutrients del riu i evitar totes les construccions que efecten el flux del riu.

Els biofilms fluvials en el seu context ecològic

Els biofilms fluvials ja van ser descrits com a comunitats complexes caracteritzades per la interacció d'elements biotics, i per la seva compartimentalització estructural i funcional (Lock et al. 1984). La seva funció està afectada tant per elements abiòtics (paràmeteres físico-químics) com biòtics (contribució relativa dels elements autòtrofs i heteròtrofs, composició de la comunitat algal i bacteriana, matriu polisacarídica, gruix de la biomassa i meiofauna) i la interrelació entre ells. Per tal d'estudiar la funció dels biofilms s'ha de tenir en compte totes aquestes característiques. Una millor aproximació es podria aconseguir aplicant noves metodologies que permetessin l'avaluació del compartiment algal, bacterià, fongs, meiofauna i la matriu polisacarídica, i les interrelacions que tenen lloc dins el biofilms. Entre altres, seria important determinar la configuració espacial, la composició química, taxonòmica (tant algal com bacteriana), determinar l'activitat dels bacteris actius i no actius, quantificació de diferents pigments algals, i per últim, la determinació i quantificació de la meiofauna que afecta els biofilms.

I. INTRODUCTION AND OBJECTIVES

1. RIVER ECOLOGY

1.1 Streams and Rivers as integrators of landscape

Streams and rivers act as integrators and centres of organization within the landscape, providing natural resources, such as fish and clean water, as well as transportation, energy, diffusion of wastes and recreation. Today, freshwater utilization by human demands has been exponentially increased since human population has been growing in an exponential phase. Freshwater supply is constantly expanded in response to growing demands, and in addition, the consumption increases in response to that rising supply. The study of river ecology cannot ignore the role of human activities. It is important to understand the structure and function of running waters, as well as their metabolic response, to judge their resiliency and capacity for change in response to exponential utilization, and therefore, for formulating sound management and policy decisions (Naiman & Bilby 1998).

Freshwater ecology has been defined as the study of the structural and functional interrelationships of organisms of freshwaters as they are affected by their dynamic physical, chemical and biotic environments (Wetzel 2001).

The characteristics of streams and rivers serve as integrators of broader environmental conditions because they reflect the conditions of the surrounding landscape (Naiman 1992). Streams and rivers have been treated in various studies

(Hynes, 1970; Minshall, 1988; Calow & Petts, 1992; Allan, 1995; Giller & Malmqvist, 1998), which summarize and integrate the enormously diversity considering the ecological, hydrological and geomorphological understandings insight the functional and structural characteristics.

1.1 Structure and Productivity of Fluvial Ecosystems

Streams and rivers are open ecosystems which require a continual input of energy in the form of organic matter, most of it is produced by photosynthesis, either produced within the river or imported from terrestrial sources (Allan 1995, Wetzel 2001). Energy and nutrients must continually be replenished because they are being utilized and respired to inorganic compounds. Spatial and temporal heterogeneity is high, with longitudinal changes in flow and chemical conditions, where the biota have specialised adaptations to conditions within flowing water.

Trophic organization in river ecosystems can be both complex and indistinct (Allan 1995). Dissolved organic matter is utilized by microbial biomass, primarily bacteria, but also to some degree fungi and algae, inhabiting either the water column or the streambed on stones, wood, sand grains and other surfaces within the river. These microbes are consumed by flagellates and ciliates, which in turn are grazed by zooplankton such as rotifers, nematodes, micro-crustaceans and macroinvertebrates. Exudates, waste products and decomposing consumers are likely to be utilized by microbes, completing the ‘microbial loop’. Microbial loop, and particularly the benthic one, plays an important role in re-mineralizing organic matter in streams and rivers (Edwards et al. 1990).

2. WATER QUALITY

2.1 Water Quality Alterations

Contemporary management of freshwater resources has been often purely hydrological, without the interest in the biotic interactions of the water bodies themselves. Water quality is biologically mediated, since adjustments have occurred internally as organisms adapted to changes. Therefore, biological metabolism of aquatic ecosystems must be considered to assess water quality management (Welch et al. 1998).

Rivers have been regulated by dams, diversions, canalisations and other physical controls in all developed countries since the water supply have been increased, affecting the physical, chemical and biological characteristics of the water. In addition, human activities have transformed the landscape through which the river flows, increasing agricultural activities and removing riparian vegetation, which reduce the natural capacity to prevent sediments and nutrients from reaching stream channel. Moreover, human activities also increase the loading of organic matter, nutrients and chemical wastes that enters in the rivers, from industry, agricultural and urban sources. Changes in biodiversity in freshwater ecosystems will be arisen, affecting the ability of the ecosystem to recover after a disturbance.

2.2 Drinking water quality

Furthermore, rivers are one of the main sources of drinking water (Pimentel et al. 1997) and the necessity to provide drinking water of high quality is continuously challenged, since river water has to be treated by progressively more sophisticated and costly procedures to achieve full purification. The study of the natural processes that occur in flowing waters, affecting the water quality, can assess a cost-effective amelioration treatment. This is the case of water DOC dynamics and nuisance metabolite production in natural water systems.

The occurrence of DOC in drinking water is specially important, since it causes noxious bacterial re-growth in distribution systems (Kaplan & Newbold 1995). Furthermore, chlorination of water, used in some water-drinking treatment for control of water-borne infectious disease, can lead to the formation of organo-halides by the reaction of free chlorine with DOC in the water. The best known of these disinfection by-products (DBPs) are the trihalomethanes (THMs) and are considered carcinogens, so their presence in drinking water has given rise to health concerns (Bull et al. 1995, Nieuwenhuijsen et al. 2000). One of the methods of minimizing their formation would be the reduction or removal of DOC prior to chlorination, e.g. by biologically assisted granular activated carbon (BGAC) (Sketchell et al. 1995), ozone treatments (Siddiqui et al. 1997) and constructed wetlands (Rostak et al 2000) among others. Biofilms can be responsible for the production or absorption of DOC, both in natural systems and in the sand or active carbon filters which are used for purification in the drinking water plants. So, a better understanding of the biofilm functioning and its role in self-depurating process, can give a relevant information for the drinking water plant to better understand the dynamics of DOC.

On the other hand, in arid regions, such as Mediterranean countries, water sources become scarcer and the maintaining of the quality of raw water for drinking purposes is becoming increasingly critical. Although the toxicity of some cyanobacteria to mammals has been known for over a century, the risk to human health and possible environmental impacts have been considered recently, particularly those affecting drinking water and recreational use of waterbodies (Dow & Swoboda 2000). The most frequently mentioned groups of cyanotoxins include hepatotoxins (over 60 variants of microcystins), neurotoxins and cytotoxins compounds (Paerl & Millie 1996). Cyanotoxins can cause chronic health problems in humans and fatal poisonings in other animals, fish and birds (Carmichael 1992). The ecological and economic impact of cyanotoxins as blooms increase in many countries as a result of nutrient eutrophication (Dow & Swoboda 2000). On the other hand, unpleasant odours and tastes in drinking water are a common problem for water suppliers. Many cyanobacteria also produce geosmin and 2-methylisoborneol (MIB), secondary metabolites which produce earthy and musty tastes and odours (Paerl & Millie 1996). Taste and odours of organic origin in the raw water (e.g. geosmin metabolite) are some of the main problems arising from the use of surface waters. Conditions of high nutrient concentration allow huge development of nuisance algae (e.g. cyanobacteria) which produce these odours. To know the dynamics of production of geosmin, from cyanobacteria biofilms, in waters used for drinking water purposes, will permit anticipate episodes of high production.

2.3 Dissolved Organic Carbon (DOC) in rivers

Dissolved organic matter (DOM) is the major form of organic matter in almost all aquatic ecosystems (Wetzel 1992) and it is defined as the portion of organic matter smaller than 0.5 μm (Allan 1995). DOM typically is the largest pool of organic of organic carbon in running waters and originates as natural biological products from soil, plant or aquatic organic matter (Allan 1995). Some derives from instream processes such as leachate from leaves and other particulate organic matter (POM) as well as by extracellular release from plants. In addition, soil and groundwater are major sources of DOM in river water (Allan 1995).

Dissolved organic carbon (DOC) is typically the most abundant form of detrital organic matter and in rivers DOC typically accounts for ~60% of the total detrital carbon load (Benner 2003), and therefore, these terms can be used interchangeably. Approximately 10-25% of DOC consists of identifiable molecules of known structure: carbohydrates, proteins, and fatty, amino and hydroxy acids. These substances generally are labile and are relatively easily utilized and degraded by microorganisms.

The remainder (50-75%) can be placed in general categories such as humic and fulvic acids and hydrophilic acids, which are usually recalcitrant to biological degradation and high in molecular weight. The bulk composition of major bioelements (C, N, O and H) of DOM has been related to the bioavailability of DOM (Hunt et al. 2000). The term "labile" is used to describe DOM that is biodegradable by natural microbial assemblage (Benner 2003). However, there is also evidence that microorganisms must also be capable of immobilizing certain components of the higher-molecular weight fraction (Fiebig & Lock 1991).

2.4 Nuisance metabolites: The case of geosmin

Toxin production may be a mechanism for shunting excess metabolites during periods of environmental stress and may be metabolically linked to the production of other secondary metabolites, including those causing taste and odour problems. They may be closely linked with and/or regulated by complex environmental or endogenous factors affecting photopigment biosynthesis, physiological state and growth stage (Paerl & Millie 1996). A schematic diagram illustrating the range of environmental factors potentially affecting and influencing secondary metabolite and toxin production in aquatic cyanobacteria are shown in Fig.1.

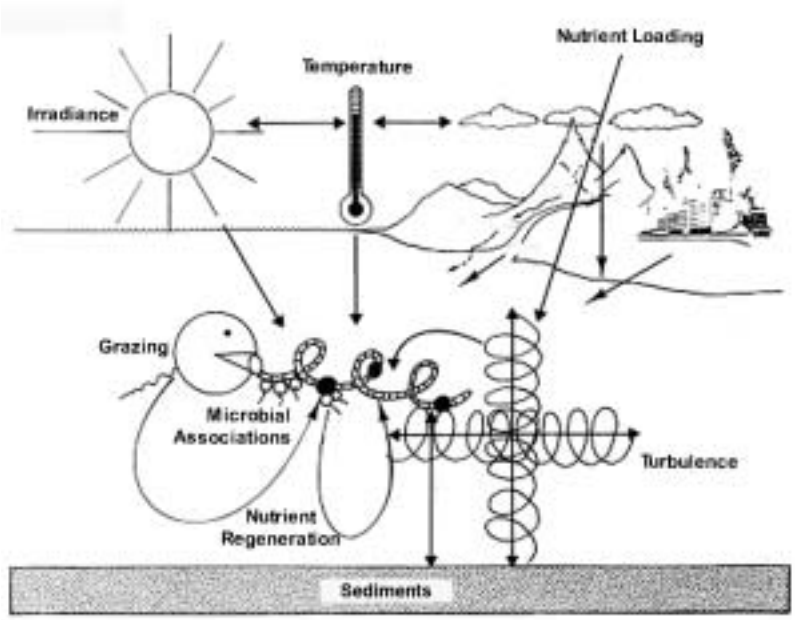

Fig.1. Schematic diagram illustrating possible interactions between environmental factors and their implications in secondary metabolite and toxin production by aquatic cyanobacteria (from Paerl & Millie 1996).

Geosmin is a bicyclic terpenoid by-product produced by cyanobacteria (Persson 1996) but also by actynomycetes (Aoyama 1990). Although the occurrence of the geosmin metabolite is relevant mainly with respect to water quality, since no toxicity has been reported for invertebrates (Nakajima et al. 1996) or mammals (Young et al. 1996, Paerl & Millie 1996), the low sensory threshold to this metabolite combined with its widespread occurrence have contributed to public concern that resources containing these compounds may constitute a toxicological risk. However, whether the occurrence of geosmin could be really an indicator of toxicity occurrence in river waters and related health risk problems is a matter of discussion.

3. FLUVIAL BIOFILMS

3.1 The ecological significance of the Fluvial Biofilms

Biofilms can be defined as a biological community of microorganisms and associated microfauna which are attached to a surface and embedded in an extracellular gellike matrix of polymeric substances (Lock et al. 1984). The succession of microorganisms upon a surface usually starts with the attachment of bacteria followed by algae, cyanobacteria, and protists. The extracellular mucilaginous material or exopolysaccharides (EPS) often occurs as coating around individual microbial cells and forms a matrix inhabited by a variety of microorganisms, particularly bacteria, algae, protists and fungi (Fletcher & Marshall, 1982). This matrix is excreted by the microorganisms themselves and may be enriched by molecules adsorbed from the surrounding water.

Studies have been focused to the heterotrophic biofilms that grow attached to surfaces of technical devices (e.g. pipes, ship hulls, and teeth) (MacLeod et al. 1990, Costerton et al. 1999), where growth occurs in darkness and heterotrophic biofilms, especially bacteria, affects this anthropogenic surfaces. On the other hand, in natural ecosystems, algal photosynthesis is coupled to heterotrophic attached microbial metabolism forming a much more complex communities than those that are only heterotrophic. The importance of the biofilms on ecological research in running waters has been focus in their capacity of adsorption and transformation of nutrients (Lock et al. 1984) and, therefore, in the self-purification capacity of streams and rivers (Cazelles et al. 1991, Grischek et al. 1998, Pusch et al, 1998). Biofilm function, and subsequent efficiency of biofilms to retain nutrient and pollutants, will be affected by physical (water current, temperature, light penetration), chemical (pH, nutrient availability) and biological parameters (relative contribution of autotrophs and

heterotrophs, community composition, biomass thickness and grazing) (Sabater et al. 2002).

The efficiency of water DOM immobilization by biofilms is increased markedly by abiotic processes associated with the polysaccharide matrix, such as the adsorption and storage of DOM and the later degradation of the high-molecular-weight DOM by extracellular enzymes, released by the heterotrophic microorganisms and accumulate within the polysaccharide matrix (Pusch et al. 1998). Metabolic pathways for organic matter immobilization by microbial biofilms is shown in Fig.2

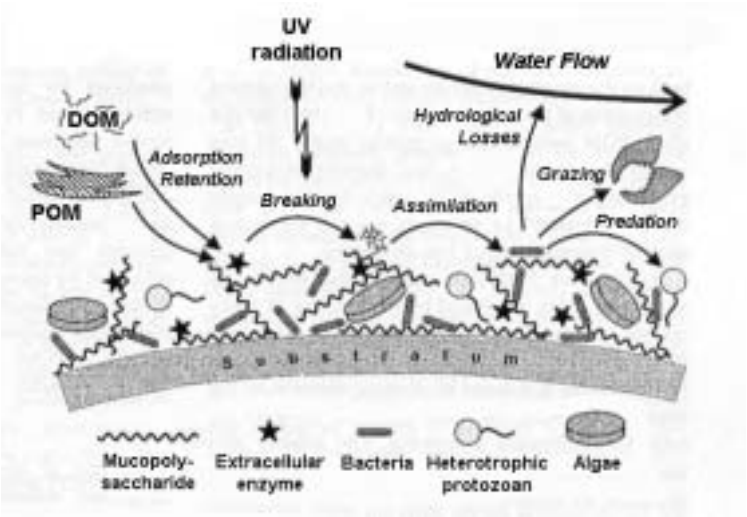

Fig.2. Metabolic pathways for organic matter in rivers mediated by microbial biofilms on and within the bed sediments (from Pusch et al. 1998).

Being part of the biofilm, benthic algae have been recognized as ideal indicators of the health of aquatic ecosystems (Stevenson et al.1996). Benthic algae are important primary producers in streams and rivers (Vannote et al. 1980). They are chemical modulators in aquatic ecosystems (Lock et al. 1984) since they transform many inorganic chemicals into organic forms (e.g. N_2 to NO_3 and amino acids by cyanobacteria; Peterson & Grimm 1992; DeYoe et al. 1992), and are primary harvesters of inorganic phosphorus and nitrogen in streams (Mullholland 1996). Moreover, they are considered to be important sinks for nutrients before release into the water column (Wetzel, 1996). On the other hand, benthic algae can stabilize substrata in many aquatic habitats, e.g. diatoms, filamentous blue-green algae, and Vaucheria can overgrow sands and sediments so that the substrata are less likely to move when current increases (Biggs, 1996), and they also can support many other organisms such as small invertebrates (Dodds & Gudder 1992).

3.2 Conditions for algal and cyanobacterial growth in rivers

The ability of benthic algae to grow in streams and rivers is the outcome of complex series of interactions between hydrological, water quality and biotic factors. The main factor that leads to accrual is the level of resources, particularly nutrient and light. Other environmental factors such as substrate stability, temperature and grazers may influence the distribution and abundance of benthic algae (Biggs 1996). Because photosynthesis responds quantitatively to changes in light, environmental variation in its quantity and quality potentially accounts for much of variation in the physiology, population growth, and community structure of benthic algae (Hill 1996). Eutrophication of streams and rivers have been also linked with an increase of algal biomass (Borchardt 1996). Cyanobacterial communities have been described to usually dominate zones where disturbance frequency is low, warm water temperatures and abundant nutrients (phosphate and often also nitrate) (Dow & Swoboda 2000). Moreover, differences in diversity of benthic cyanobacterial communities have been used as an alternative tool for monitoring water quality in rivers (Perona et al. 1998).

Unlike planktonic algae, which grow suspended in the water column, benthic algae grow attached on the substratum and often crate thick mats, giving a spatial structure to the algal community. The development of a thick mat, may alter the hydrodynamic environment and establish microenvironment conditions, and therefore, affect its metabolism, e.g. the capacity of nutrient uptake from water, and as a consequence, creating nutrient-limiting conditions (Borchardt 1996). Benthic communities may have problems of diffusion of nutrients, gases, and metabolic products across the biofilm and the overlying water. High production within these thick communities, may be maintained by an intensive internal recycling of nutrients (Wetzel 2001).

4. OBJECTIVES

The present study aims to elucidate the characteristics of the biofilm structure and metabolism in relation to water DOC budget and the geosmin dynamics in Mediterranean Rivers. The main objectives were:

1) To determine the capacity of DOC uptake by biofilms, in order to assess the implication of the natural biofilms as sinks or sources of water DOC. We aim to elucidate the differential role of light-growth biofilms and dark-growth biofilms, in relation to the water DOC level and bioavailability. We hypothesized that I) Light-

growth biofilms would have a low DOC consumption rate because exist a high internal DOC recycling from the high-quality algal-released DOC for heterotrophs inside the biofilm, II) Dark-growth biofilms would be net consumers of DOC, and III) DOC recycling may be affected not only by community composition but also by the structural components of the biofilm (C and N content).

2) Since water DOC composition and biodegradability could be related with seasonal differences, patterns are investigated in two different situations of DOC content. The role of biofilm age and its related characteristics (thickness, biomass increase) on biofilm function is also investigated, and therefore biofilms of different age are compared. The study aims to contrast the following hypotheses: I) which was the relationship between the water DOC level and bioavailability and biofilm structure and functioning along an annual period, and II) how the age of the biofilm may be determinant in defining the heterotrophic activities related to DOC utilization.

3) To address the ecological factors that co-occur with the production of geosmin by benthic cyanobacteria, the following questions were raised: I) Which are the main ecological factors related to geosmin production, II) Which are the geosmin producers amongst the biological community, and III) Is there co-occurrence of geosmin and toxicity in the benthic cyanobacterial mats from the Llobregat River.

4) Knowing the mechanisms involved in the formation and distribution of the benthic cyanobacterial masses could contribute to formulating the appropriate corrective measures for minimising the extraordinary abundance of geosmin in shallow river waters. Therefore, two questions were addressed: I) Which were the environmental conditions associated with the waxing and waning of the benthic cyanobacterial masses in the river, and II) Which were the factors that influenced geosmin occurrence and dispersion in the river.

5) The benthic cyanobacterial mat in the Llobregat River undergo a dynamic process of seasonal growth and subsequent detachment and drift, accompanied by a high production of geosmin. Therefore, to determine the relationship between the wax and wane of the benthic cyanobacteria in the river with the associated geosmin dynamics, some questions were addressed: I) Is there a relationship between the dynamics of the mats and their consumption of inorganic nutrients? II) Does such a relationship hold for the geosmin production dynamics? III) Is there any correlation between the apparently limiting nutrients and the physiological characteristics of these mats? and IV) What is the significance, if any, of the attached and unattached fractions of the cyanobacterial mat concerning geosmin occurrence? These questions

are placed in the context of a field-based study, where the community dynamics of algae and cyanobacteria are studied from both structural and functional perspectives.

6) In order to uncover the possible relationship between the structure of the mat and the physiological condition which leads to the production of geosmin, the functional structure of the mats was approached by means of microelèctrodes. The objectives of the present study were twofold; I) To determine whether the attachment/ detachment dynamics of the cyanobacterial mats was related with that of the mat metabolism, and II) To determine whether the microstructural heterogeneities within the mat were affecting the physiological response with regard of geosmin occurrence.

II. STUDY SITES

In order to asses the main objectives of the study, the sampling strategies were carried out in two different rivers. First of all, the relationship between the biofilms and the water DOC budget was analysed in the Ebre River, and on the other hand, the implication of the benthic cyanobacterial mats on the geosmin production was assessed in the Llobregat River.

Ebre River

The Ebre River, with a mean discharge of 500 $m^3 s^{-1}$, drains 85550 km^2 of the NE Iberian Peninsula (Fig.1). Because of the location of its headwaters and tributaries, this river has a nivo-pluvial regime with a low flow during the summer. The sampling area was located in the last 50 km of the river, where water is diverted through two main irrigation channels at a rate of 20 $m^3 s^{-1}$. Approximately 15 km downstream from the beginning of the open channels, a pipe, 15 km long, transports water from one of the channel to a treatment plant "Consorci d'Aigües de Tarragona" (CAT), which provides drinking water to approximately one million people.

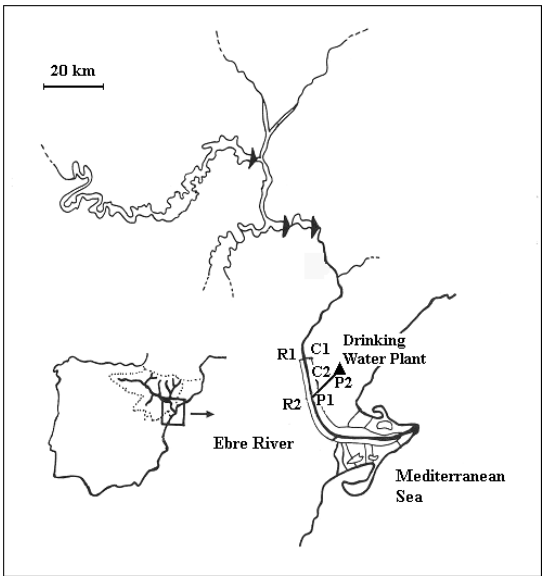

Fig. 1. Location of sampling stations and drinking water plant in the Ebre River. R1=River 1, R2=River 2, C1=Channel 1, C2=Channel 2, P1=Pipe 1, P2=Pipe 2.

Two sampling sites were placed in the river, R1 (River 1), located in Xerta, and R2 (River 2) in Campredó, distanced 15 km downstream (Fig.2). Sampling sites were also located at the beginning and at the end of the open channel, C1 (Channel 1) and C2 (Channel 2) respectively, and at the beginning and at the end of the pipe P1 (Pipe 1) and P2 (Pipe 2) (Fig.2). The water channel is 10 m wide and 2.65 m deep. The pipe measures 1.6 m in diameter. Water flow was on average 1 m s^{-1} in both systems. Mean residence time in the channel and the pipe was 4.2 h. Mean incident light at midday at 20-40 cm depth was 845.9 µmol photons m^{-2} s^{-1} (s.d.=493.3, n=16).

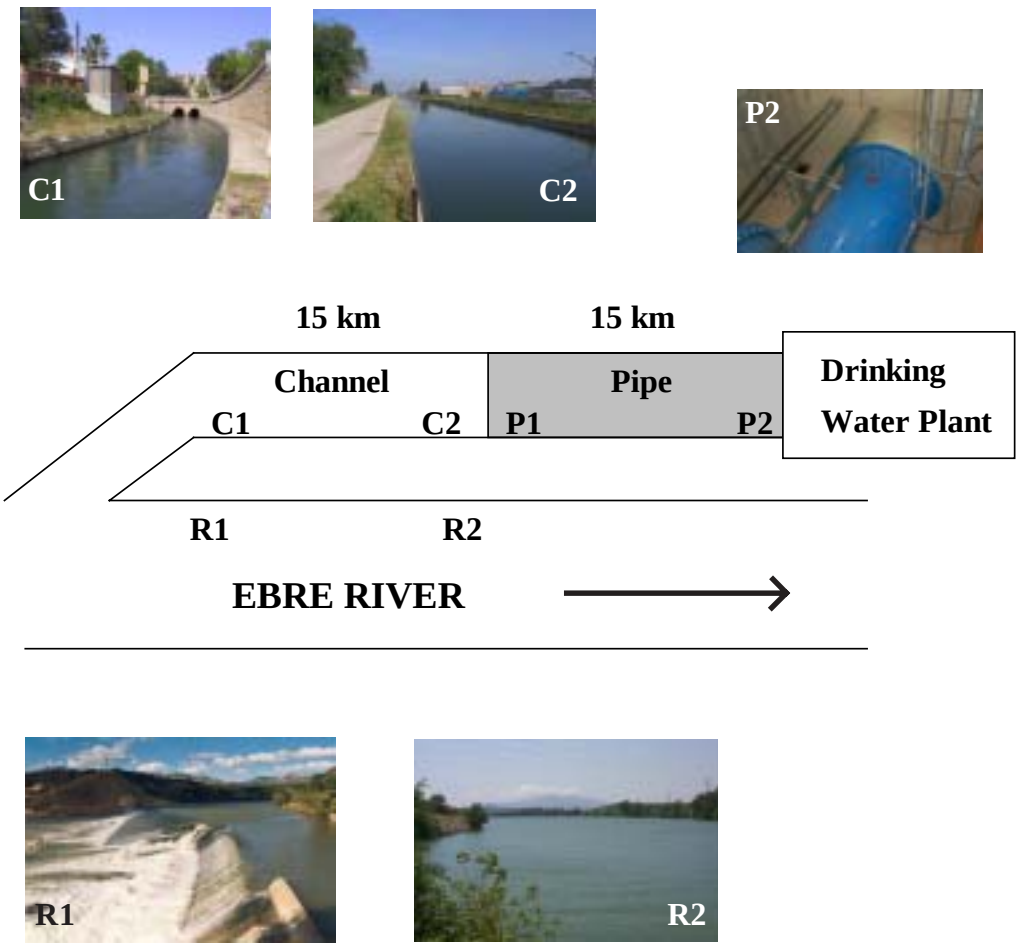

Fig. 2. Schematic diagram and corresponding pictures of the sampling sites locations. R1 = River 1, R2 = River 2, C1=Channel 1, C2=Channel 2, P1=Pipe 1, P2=Pipe 2. Arrow indicates the water flow direction.

Llobregat River

On the other hand, Llobregat River is located in Catalonia, NE Spain, in an area of intense water demand (Fig.3). The river has a calcareous geology and a typical Mediterranean regime, which causes frequent floods in spring and autumn and minimum flow in summer. A reservoir (La Baells) is located upstream and controls the water flow in the upper stretches. A major tributary enters in the mid stretch (Cardener River) and causes a dramatic increase in the water conductivity downstream. The main channel of the Llobregat is continuously interrupted by a series of small dams and derivation channels, which causes a further instability of the river water regime, especially during periods of low water flow. The river receives waste waters of industrial and urban origins in its mid and lower stretches, which greatly deteriorate the water quality. Moreover, the waters of the Llobregat are used for human consumption, and this creates a great deal of conflicts and makes the use of sophisticated water treatments necessary for acceptable standards to be reached.

Three sampling stations were placed to monitor the longitudinal variability in geosmin dynamics (Fig.3 and Fig.4). Site S1 (Navàs) was located 80 km upstream of the river mouth. Site S2 (Pont de Vilomara) was located 20 km downstream of the first site, just before receiving the Cardener River. Site S3 (Olesa de Montserrat) was located 40 km downstream of the first site and very close to the water drinking water plant "Aigües Ter Llobregat" (ATLL).

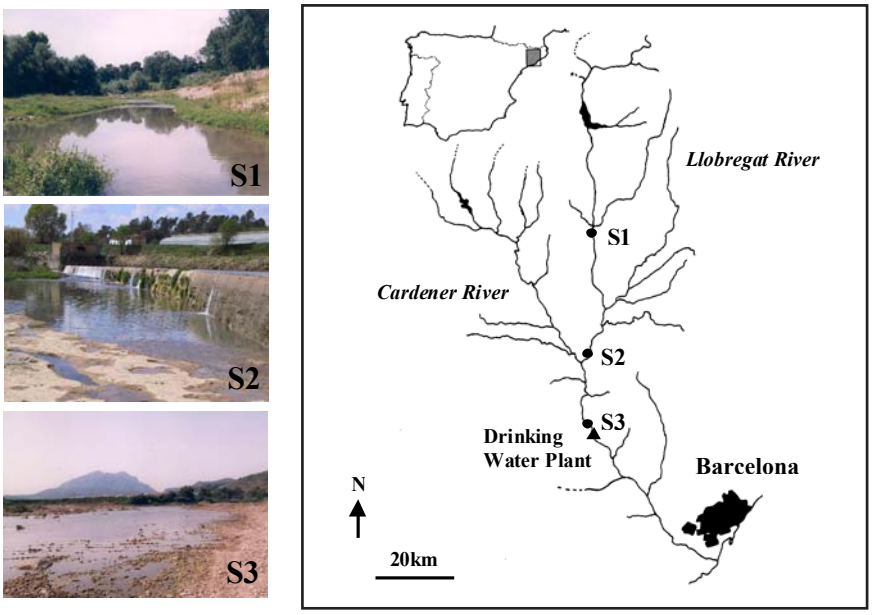

Fig. 3. Location and pictures of sampling sites and drinking water plant location in the Llobregat River. S1 (Navàs), S2 (Pont de Vilomara), S3 (Olesa de Montserrat).

III. MATERIALS AND METHODS

Sampling strategy

Ebre River. The sampling strategy in the Ebre River was performed for an annual assessment. Etched 1 cm^2 glass substrata were immersed at points R1, R2, C1, C2, P1 and P2, for two months before sampling to allow biofilm colonization. Special structures were designed to immerse the substrata within the river, the channel and the pipe. Substrata were inserted in Plexiglas racks (100 substrata per rack) which, in turn, were attached to a metal box (ca. 1 m^3) and immersed in the littoral of the river (R1 and R2) (Fig.1). In the channel (C1 and C2) (Fig.2), Plexiglas racks were attached to a metal plate fixed to the sides of the channel and immersed at a depth of 20-40 cm. And in the Pipe, at P1 the racks were immersed attached to a metal box (Fig.3), but at P2, a specific holder 1.6 m long (the whole pipe diameter) was used (Fig.4). All substrata were placed parallel to water flow. Substrata were collected in September and November 2000, and April, May and July 2001(with the exception of river July 2001).

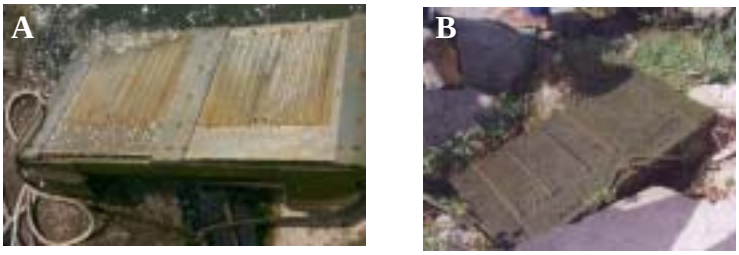

Fig. 1. Pictures of the racks (A) and glass substrata colonized (B) at site River 2.

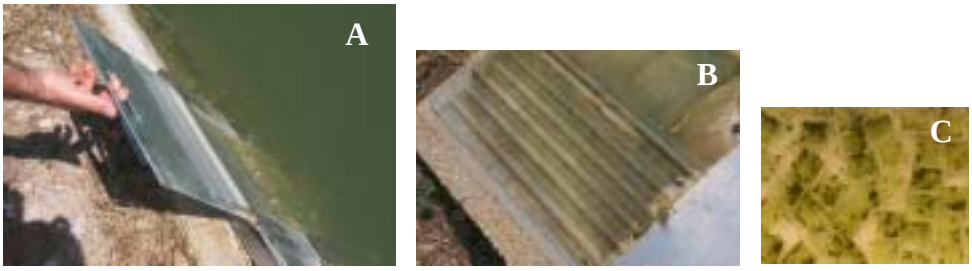

Fig. 2. Pictures of racks (A) and glass substrata colonized (B,C) at Channel 2.

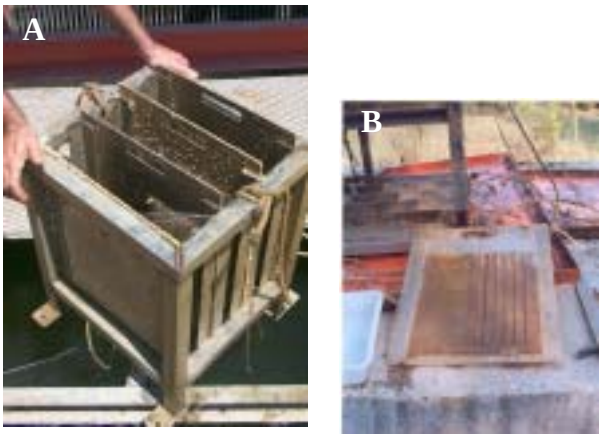

Fig. 3. Pictures of racks (A) and glass substrata colonized (B) at Pipe 1.

Fig. 4. Pictures of the Pipe 2 (A) and glass substrata colonized (B) at Pipe 2.

Samples were analysed for biofilm structure (bacterial density, algal density and composition, chlorophyll-*a*, C and N biofilm content and SEM observations) and function (extracellular enzymatic activities). Water samples were collected form each sampling point monthly from September 2000 to November 2001 to analyse physical and chemical parameters (conductivity, pH, carbonates, nitrate, nitrite, ammonium and dissolved inorganic phosphorus), as well as DOC and BDOC (biodegradable DOC). Notice that water samples from R1 and C1 were taken from an intermediate point, which was considered representative of both.

Furthermore, in order to study the biofilm age characteristics, 2 and 4 months colonized biofilms were collected from R2, C1 and P2 sampling points in November 2000 and July 2001, where, in addition, 12 months colonized biofilms were also taken from R2 and P2 in the latest. Biofilm structural and functional parameters (algal and bacterial composition, chl-a, C and N content and exoenzymatic activities), including CLSM image analyses, were performed in these biofilms.

Llobregat River. Water physical and chemical variables, including geosmin water analysis, as well as biofilm characterization, were sampled monthly in the three studied sites (S1, S2 and S3) from March 2000 to May 2001, increasing the intensity of sampling to a weekly basis during the geosmin peak (between February and May 2001). Algal samples were collected from littoral and riffles zones by scrapping a given surface area of rocks or cobbles, or introducing a small PVC corer (3.1 cm^2) into the algal mats. For toxicity assessment, biofilm samples were collected from various locations along the river on two different dates (January and March 2001). Furthermore, S2 was monitored weekly during winter and spring 2002 (January to May 2002), including physical and chemical parameters, as well as water geosmin analysis, biofilm structure (algal density and composition, geosmin content, chlorophyll-*a*, carbohydrates, C and N content as well as SEM observations) and function (extracellular enzymatic activities and PAM fluorescence).

An experiment set-up was performed using subsamples of cyanobacterial mats collected in S2 during the geosmin period in March and April 2003, where oxygen and redox microprofiles were used in combination with structural and functional parameters to ascertain the structural heterogeneity of the mats related with its physiology.

Sample collection

Physical parameters of the water were measured in the field and water samples for analysing chemical variables, such as DOC and BDOC concentration and geosmin content were kept in dark and at low temperature and analyse in few hours. Samples for determining inorganic nutrients were frozen in liquid nitrogen and stored at –20ºC in dark until analyse.

Samples for algal composition and bacterial density were filled with 10 ml of river water and fixed in 4% formaldehyde, while samples for chlorophyll-*a* concentration, geosmin content, total carbohydrate, phosphorous and C and N content, as well as samples for SEM observations were frozen in the field in liquid nitrogen.

Samples for CLSM were kept in dark and at low temperature and observed in few hours. Samples for measuring extracellular enzyme activities were preserved at low temperature until analysis. Additionally, samples for PAM fluorescence were measured in the field.

Physical and chemical parameters

All physical and chemical parameters were analysed following standard methods (APHA 1989). Water samples were analysed for temperature, pH, conductivity and dissolved oxygen (%) using a MultiLine F/SET-3 (WTW Multiline F/SET-3). Incident light was measured at the same frequency using a Li-Cor quantum sensor (Li-192SB). The light extinction coefficient (Beer-Lambert) was calculated by relating the light irradiance at the water surface and that reaching the streambed. Water velocity was measured with a current meter (MiniAir2 Schiltknecht 43221). Carbonate content was measured following the standard methods (APHA 1989). Filtered water samples (using Whatman GF/F filters; 3 replicates for each analysis) were taken in order to analyse inorganic nutrients. Nitrate, nitrite, ammonium, sulphate and chloride were analysed using an ion chromatograph (Kontron) equipped with an IC-Pack anion column (4.5 x 50 nm) and an ultraviolet detector. Soluble reactive phosphorous (SRP) was analysed spectrophotometrically (Perkin-Elmer Lamda 2 UV/VIS spectrophotometer) following the procedure described by Grasshoff et al. (1983).

DOC and BDOC analysis

DOC was measured using a total organic carbon analyser (TOC-5000, Shimadzu). BDOC was measured following the procedure described by Servais et al. 1989. Samples, 5 replicates for each water sample, were incubated immediately for 28 days at room temperature (20-24ºC) and in the dark. The glass flasks and ampoules used were previously heated for 4h at 550ºC to prevent the release of organic C. All DOC samples were fixed with sodium azide (2.7 mM) and preserved at 4ºC until analysis.

Bacterial density

Bacterial samples (3 replicates) were fixed with 4% formaldehyde and the bacterial density was estimated after 90 seconds of sonication. After appropriate dilution, fixed samples were stained for 5 min with DAPI (4,6-diamidino-2-phenyilindole; 2 $\mu g\ ml^{-1}$ final concentration) passed through 0.2 µm irgalan black-stained polycarbonate filters (Nuclepore). Bacteria were then counted under a

fluorescence microscope (Nikon) at 1250x magnification (Porter & Feig 1980). Fifteen fields were counted per filter for a total of 400-800 organisms.

Geosmin analysis in water and biofilm

Geosmin concentration in water was analysed by ATLL (Aigües Ter Llobregat) using 2 litres of water and determined after L/L Extraction Bases + Neutrals (Method 625 EPA) and chromatography by HRGC(MS) in SCAN mode; Extraction using Close Loop Striping Analysis (CLSA) and chromatography by HRGC(MS) in SIM mode; Extraction by Purge & Trap and chromatography by HRGC(MS) in SIM mode. Calibration curves for geosmin were produced with a commercial geosmin standard (Ultrafine Chemicals, England). In order to detect high concentrations of geosmin in the water and in the biofilm, screening using Purge & Trap extraction and HRGC (MS) chromatography in SIM mode was also used. Geosmin content in the biofilm was extracted after grinding and subsequent methanol extraction following the guidelines laid down by Durrel et al. (1999), and the concentrate was later analysed. Results were expressed in reference to litre (water measurements) and to chlorophyll content and dry weight (biofilms measurements).

Algal composition and abundance

Algal samples (3 replicates) were fixed with 4% formaldehyde and observed under a light microscope using a Reichert Polyvar at 500x to study the community composition and abundance. The abundance of algal cells was determined after scraping the glass substrata with a spatula, or segregating the algal mat corer, and dispersion of the sample using a sonication bath (3 min., Selecta 40W power, 40 kHz ultrasound frequency) and determining an aliquot of 0.2 ml, giving the results in number of cells per cm^2.

Chlorophyll-*a* concentration

Chlorophyll-*a* concentration (3 replicates) was measured after extraction in 90% acetone and sonication (4 min). A second and third extraction was necessary in some biofilms until all chlorophyll-*a* had been extracted. Chlorophyll-*a* concentration was measured spectrophotometrically (Perkin-Elmer, Lambda UV/VIS spectrophotometer) after filtration (Whatman GF/F) of the extract, following the method described by Jeffrey & Humprey 1975. The ratio of chlorophyll-*a* to carotenoids and/or chlorophyll-*a* degradation products (OD430/OD665) (Margalef 1983) was also calculated. Chlorophyll-*a* was expressed by surface area, dry weight (DW) and ash-free dry weight (AFDW).

Total carbohydrates, phosphorus, carbon and nitrogen content

Total carbohydrates content. Total carbohydrates in the biofilms were measured by the phenol-sulphuric assay (Dubois et al. 1956). About 10 mg DW of biofilm sample (3 replicates) was used for each analysis. After extraction, samples were filtered (precombusted, Whatman GF/F filters) and absorbance was measured at 485 nm against a reagent blank. Standards of glucose (0 to 200 mg ml^{-1}) were also prepared and the results were given as glucose equivalents.

Total phosphorus content. Total phosphorus content was measured by analysing the SRP (colorimetric ascorbic acid method) after the digestion of the sample with peroxidisulphate potassium in sodium hydroxide (Grasshoff et al. 1983). About 50 mg DW of ground sample (3 replicates) diluted with 30 ml of distilled water were used for each phosphorus analysis.

C and N content. 5 ml of distilled water was added to the glass substrata (3 replicates), which were then sonicated (4 min.) and scraped with a spatula. The extract was then filtered (precombusted filters Whatman GF/F). Successive extractions (2-3) were performed to ensure total extraction of the colonized biofilm. Filters were dried (2 days at 110ºC) and analysed for C and N content. On the other hand, about 5 to 10 mg DW of ground sample (3 replicates) from corers of algal mats were used for each analysis. Total C and N content was measured by a C/N analyser 1500 Carlo Erba using vanadium pentoxide as the oxidation catalyser. About 5 to 10 mg DW of ground sample (3 replicates) was used for each analysis.

Scanning Electron Microscope (SEM*)*

Samples of biofilms for SEM observations were freeze-dried and covered with carbon and gold, using a Hitachi S-2000 SEM was used, operated at 7 kV.

Confocal Laser Scanning Microscope (CLSM)

Samples of live biofilm were examined under the CLSM using a Leica True Confocal Microscope (TCS 4D). Algal biomass was quantified via chlorophyll autofluorescence (568 nm excitation, 590 nm emission). Extracellular polymeric substances (EPS) were stained using a fluorescein-conjugated lectin of Concanavalin A (Con-A, Molecular probes) at a final concentration of 80 mg ml^{-1}. Biofilms were incubated for 35 min at 20ºC and after staining, the samples were rinsed 3 times with phosphate buffered saline (PBS) solution (adjusted pH of 7.4). The intensity of

Con-A fluorescence (488 nm excitation, 520 nm emission) was used to quantify EPS. All observations were performed using a 20x objective (numerical aperture of 0.4) in order to include a higher area in the microscopic field. A series of optical sections were recorded at 1.5 μm intervals. Digital image analysis of CLSM sections was used to determine the relative area occupied by algae and EPS with respect to the total microscopic field area. The quantification was achieved by means of Metamorph software (v. 3.5, Universal Imaging Co.).

PAM fluorescence

The maximum photosynthetic capacity (photon yield) in the dark was estimated in the field using Pulse Amplitude Modulation (PAM) fluorescence (Hofstraat et al. 1994) (see Chapter 5).

Extracellular enzymatic activity

The extracellular enzymes lipase (ref. EC 3.1.1.3), leucine-aminopeptidase (ref. EC 3.4.11.1), β-glucosidase (ref. EC 3.2.1.21), β-xylosidase (ref. EC 3.2.1.37) and phosphatase (ref. EC 3.1.3.1-2) were measured using fluorescent-linked substrates (methylumbelliferyl [MUF], but aminomethyl-coumarin [AMC] for the peptidase). Immediately after sampling, samples were incubated with the substrates at river temperature for one hour and in dark conditions in a shaking bath. Incubations were performed at a range of substrate concentrations (0.1, 10, 300 and 600 μM) in order to calculate saturation curves, or at saturation conditions (300 μM) to calculate the potential extracellular enzymatic activity. Blanks and standard of MUF and AMC (0-100 μM) were also incubated. At the end of the incubation, glycine buffer at pH 10.4 was added (1/1, v/v) and fluorescence was measured at 365/455 nm excitation/emission for MUF and 364/445 nm excitation/emission for AMC (Kontron, SFM25). All substrata and standards were prepared with filter-sterilized river water (Whatman GF/F 0.2 μm pore-size cellulose nitrate membrane filters). At the end of the incubation, samples were dried (48 h at 110ºC), and burned (4h at 450ºC), to express the activity in μmol (of MUF or AMC released) per unit organic matter (OM) per hour. Activities were also expressed per cm^2 of biofilm surface or mgC and per hour.

Oxygen and Redox Microelectrodes

A minituarized Clark-type oxygen sensor with an internal reference and a guard cathode and a minituarized redox platinum electrode used with a reference elctrode (Unisense A/S, Denmark), were used to determine oxygen and redox microprofiles (see Chapter 6).

CHAPTER 1
BIOFILM STRUCTURE AND FUNCTION RELATED WITH DOC DYNAMICS IN THE EBRE RIVER

INTRODUCTION

Dissolved organic carbon (DOC), whatever its form or origin, represents the ultimate source of organic carbon for sustaining the heterotrophic metabolism (Fischer 2002). DOC is supplied from both external (allochtonous surface and subsurface inputs) and internal sources (autochthonous primary production) (Pusch et al. 1998, Findlay & Sinsabaugh 2003). DOC is removed from streamwater by both abiotic and biotic processes (Allan 1995) and the principal biotic processes are uptake by microorganisms, especially bacteria. In relation with this uptake, biofilms play an important role in the retention and storage of DOC (Marmonier et al. 1997; Battin et al. 1999), contributing to the self-purification capacity of streams and river (Pusch et al. 1998, Sabater et al. 2002).

The uptake rate of organic compounds is heavily related to their lability, and microorganisms show a faster and preferential use of the most labile and fresh molecules (Cherrier et al. 1999; Norrman et al. 1995). Although, bacterial utilization of DOC is determined by its size and diagenetic state (Amon & Benner 1996), the availability of DOC for the epilithic bacterial community may be more dependent on the composition of organic compounds rather than molecular weight (Ford & Lock 1985). The metabolic activity of biofilm bacteria can be influenced by the ambient concentration and composition of DOC (Fischer et al 2002). However, biofilms can buffer the supply of organic substrates so that short-term changes in the quality and quantity of DOC do not necessarily exert an immediate effect on their metabolism (Freeman & Lock 1995).

Uptake of DOC can be directly diffused via membrane permeases or via diffusion through pores if the molecules are small enough (Nikaido and Vaara 1985). However, bacteria can afford extracellular enzymes to degrade and effectively use large molecules (Fischer 2003). High molecular weight compounds (>10.000 Da), comprising macromolecules such as proteins, polysaccharides and lipids complexes, forms the major part of the organic matter in aquatic environments (Cole et al. 1984, Lock 1990). Otherwise, DOC of low molecular weight, such as monosaccharides and certain 'high quality' sources, are taken up most rapidly by heterotrophic bacteria (Kapplan & Bott 1982, Meyer et al. 1987, Kaplan and Bott 1989).

Extracellular enzymes are required to initiate the remineralization of high-molecular-weight organic matter, since heterotrophic bacteria can not take them directly from the surrounding medium into the cell, and therefore, they need to be hydrolysed outside the cell to sizes small enough to permit transport across the outer membrane (Arnosti 2003). Therefore, extracellular enzymatic approach provides a powerful information for understanding basic processes of decomposition and microbial activity in freshwater ecosystems (Chróst 1990, Lock 1990). Extracellular enzymes are either attached to the outer membrane or released ("free") into the surrounding solution (Chróst 1991). Their production and activity are in general tightly controlled by a microbial cell, because represents and investment of energy, and therefore, constant synthesis in the absence of substrates is unnecessary (Chróst 1991). Some enzymes are expressed constitutively, but many more are induced under specific circumstances (Chróst 1991, Arnosti 2003). Extracellular enzyme production can be further regulated by catabolite repression, in which the presence of a substrate (usually an easily metabolised carbon source) may prevent production of enzymes required for metabolism of a more complex substrate (Chróst 1991, Priest 1992). In general, hydrolytic enzymes can be classified based on the type of reaction catalysed. In this study 5 extracellular enzymes were used: β-glucosidasa and β-xylosidase, involved in the degradation of polysaccharides, cellulose and hemicellulose respectively (Desphande & Eriksson 1988, Lachke 1988). Alkaline phosphatase is the responsible for the degradation of orthophosphoric monoesters obtaining inorganic phosphorous (Klotz 1992). Leucine-aminopeptidase hydrolyses peptides and protein, which comprise the largest part of the organic nitrogen pool (Halemejko & Chróst 1986). Finally, lipase activity hydrolyses macromolecules of lipids complexes.

Although few studies have examined biofilms DOC uptake rates in rivers either in situ or by laboratory experiments (Fishcer et al. 2002, Kaplan & Bott 1983, Kuserk et al. 1984), little information is available on the effect of biofilm structure and metabolism on uptake. Here, we elucidate whether biofilms act as sinks or sources

of fluvial DOC, depending on their structure and biomass accumulation. To contrast these hypotheses, a field study was performed along a natural river, an open channel and a dark pipe. The channel biofilms will not be influenced by hyporheic or groundwater inputs, and therefore represent a relevant contrast with the natural river biofilms. Overall, the three compartments represent a variety of situations useful to describe the relationships between the biofilms and the DOC available in the system.

MATERIALS AND METHODS

Historical records

The historical physical and chemical variables were recorded by CAT (Consorci d'Aigües de Tarragona) at site R2. The first set included historical data of discharge from October of 1960 until December 2000. The second data set included information of physical and chemical parameters (chlorophyll-*a*, suspended solids, temperature, conductivity, nitrate, ammonium, dissolved phosphorus and DOC) from January of 1998 until May of 2001.

Sampling strategy

Physical and chemical parameters, as well as DOC and BDOC, were analysed monthly from September 2000 to November 2001, from each sampling site (see Materials & Methods). Algal samples, 2 months colonized, were also collected in the 5 studied periods (see Materials & Methods) to study biofilm structure and function.

River DOC budget

The DOC and BDOC uptake or release rates of the channel and pipe were calculated by means of the DOC and BDOC balance between sites C1 and C2, and P1 and P2, respectively, at the sampling times when differences between sites were significant. The DOC and BDOC balance was transformed to mg cm^{-2} h^{-1} by knowing the total water volume and surface area potentially covered by biofilm in each system (357750 m^3 and 204900 m^2 for the channel, 30159 m^3 and 75398 m^2 for the pipe), and the travel time of certain water parcel from C1 to C2 and P1 to P2, respectively. Mean annual uptake or release rates were calculated from monthly results (November 2000-October 2001) and a value of zero was assigned when non-significant differences were obtained between sampling sites.

Extracellular enzymatic activities

The extracellular enzymes β-glucosidase, β-xylosidase, phosphatase, leucine-aminopeptidase and lipase were measured at different range of substrate concentrations (0.1, 10, 300 and 600 μM). The enzymatic activities were expressed by nmol (of MUF or AMC released) per mgC or cm^2 and per hour.

Statistical analyses

For the comparison of DOC and BDOC between C1 and C2, and P1 and P2, from the same sampling time, a 2-tailed *t*-test was used. A correlation analysis (Pearson coefficient) was performed to determine possible relationships between the DOC and BDOC parameters and the extracellular enzymatic activities.

RESULTS

Historical records

Monthly values of discharge had a highly fluctuation, coinciding with a high period in late autumn and winter and a low period during spring and summer (Fig.1). However, some punctual values of high discharge could be also found in spring. Annual means of discharge, although having some annual fluctuations, showed a tendency to be reduced since 1961 to 2001 (Fig.1).

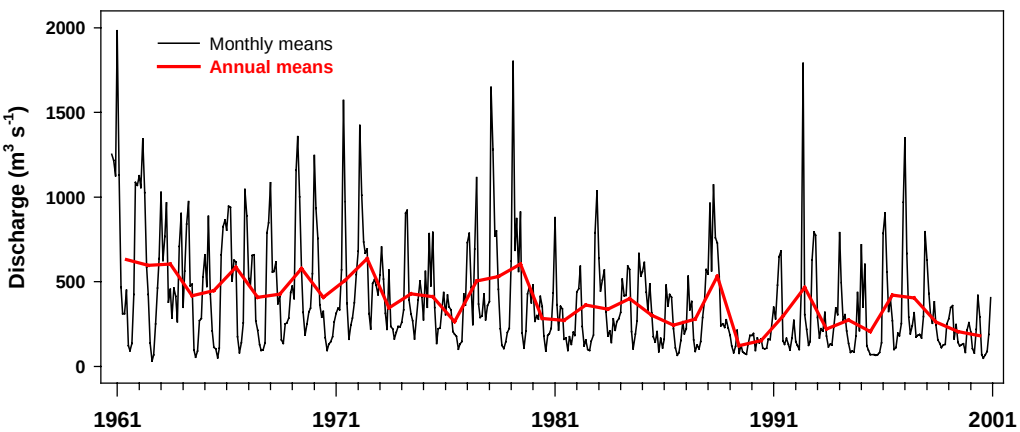

Fig.1. Discharge values of Ebre River from 1961 to 2001.

Physical and chemical parameters studied from a more recent period (from 1998 to 2001) showed the monthly fluctuations of discharge, reaching a peak of discharge from late November to March (Fig.2). A second peak of discharge, was also found during May (Fig.2).

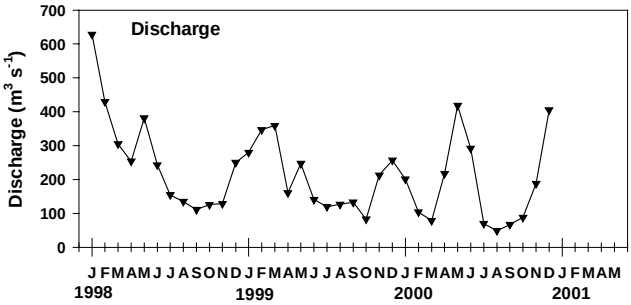

Fig.2. Discharge values of Ebre River from January 1998 to January 2001.

Higher chlorophyll-*a* concentration in the water were found when discharge values were lower, coinciding with March and April months, as well as summer season (Fig.3). Water temperature had the maximum values in the summer season, from June to August, reaching to 25ºC, while the minimum during winter was about 9ºC (Fig.3). Maximum conductivity was found during late summer and autumn, just before to find the maximum discharges (Fig.3). Suspensid solids had an intraannual fluctuations as well as between studied years (Fig.3).

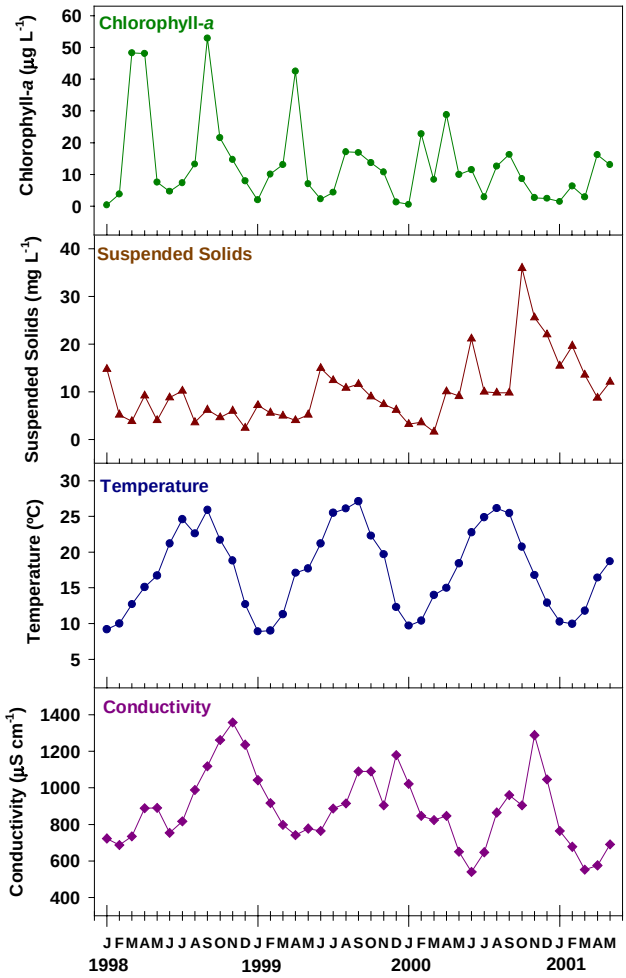

Fig.3. Chlorophyll-a, suspended solids, temperature and conductivity dynamics from the water of the Ebre River measured from Januray 1998 until May 2001.

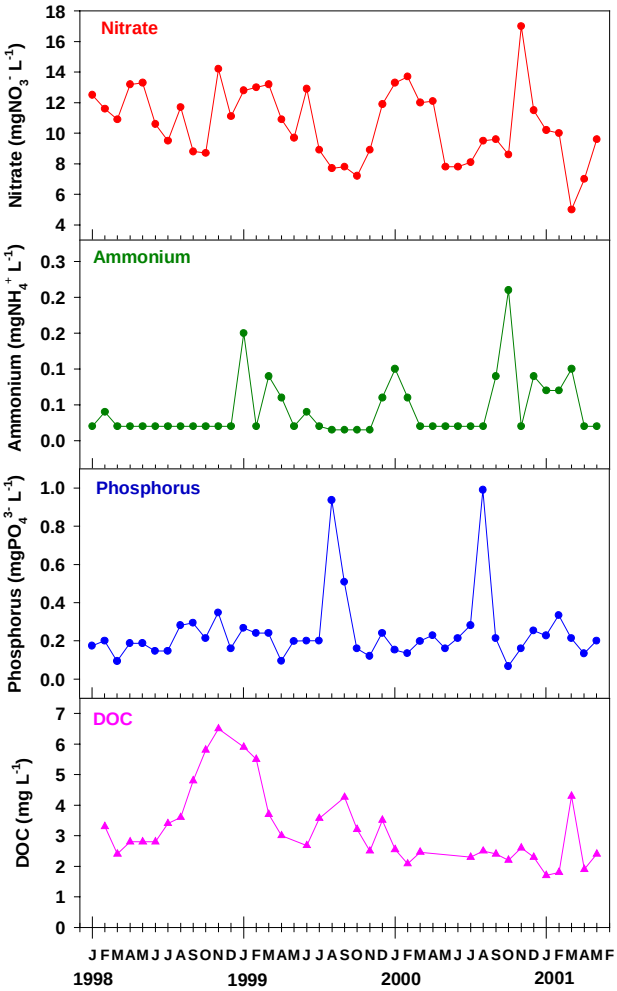

Fig.4. Nitratre, ammonium, phosphorus and DOC dynamics from the water of the Ebre River measured from Januray 1998 until May 2001.

Nitrate concentration was usually high, and some peaks coincided with late autumn and winter seasons (Fig.4), when in addition some peaks of ammonium were found (Fig.4). Dissolved phosphorous fluctuates between 0.1 and 0.3 mg L^{-1}, although 2 peaks were found during July reaching to 1.0 mg L^{-1} (Fig.4). Finally, DOC concentration had some fluctuations (Fig.4), usually followed a dynamics where autumn had the maximum levels, reaching to 6.5 mg L^{-1} during autumn and winter of 1999 (Fig.4).

The relationship between DOC concentration and the environmental variables measured during the studied period (from January of 1998 to May of 2001) was described by the following expression obtained by means of a multiple regression analysis:

DOC = 0.60 Conductivity – 0.60 Suspended Solids (R^2 = 0.55, p-value<0.05)
Significant correlation (p<0.05): DOC-Cond (R^2 = 0.55); SS-NH_4 (R^2 = 0.52)

Physical and chemical parameters of the flowing water

Values of physical and chemical parameters of the Ebre River, during the studied period (from September 2000 to November 2001) are summarized in Table 1. The Ebre River water was characterised by high nutrient content, especially for dissolved inorganic nitrogen level (Table 1).

Mean water pH was 8.2 and temperatures range between 8.9ºC in winter and 25ºC in summer (annual mean = 18.1, s.d = 5.7). The chemical characteristics of the river water showed no significant differences between the sampling points, except for DOC concentration, which were higher in river than the channel and pipe systems (t-test, $p<0.05$). Annual mean values of DOC concentration in river systems accounted for 2.60 mg L^{-1} (s.d = 0.98), while in channel and pipe systems were 2.33 mg L^{-1} (s.d = 0.43) and 2.23 mg L^{-1} (s.d = 0.52) respectively. Annual mean values of BDOC concentration were found higher at the beginning than the end of the channel and pipe system, although significant differences were found only in the latter (t-test, $p=0.05$) (Table 1).

Table 1. Physical and chemical characteristics at the six sampling sites of the river, channel and pipe systems in the Ebre River during the studied period (September 2000-November 2001). Values are means of monthly values and standard deviations in parentheses (n=14).

		River 1 (n=14)	River 2 (n=14)	Channel 1 (n=14)	Channel 2 (n=14)	Pipe 1 (n=14)	Pipe 2 (n=14)
Conduct	[µS cm^{-1}]	**832.7 (199.5)**	**829.4 (194.1)**	**832.7 (199.5)**	**829.6 (199.8)**	**833.3 (199.1)**	**833.5 (195.3)**
pH		**8.18 (0.14)**	**8.14 (0.12)**	**8.18 (0.14)**	**8.17 (0.15)**	**8.17 (0.13)**	**8.15 (0.08)**
$CaCO_3$	[mg L^{-1}]	**161.3 (11.4)**	**162.2 (11.3)**	**161.3 (11.4)**	**161.2 (10.8)**	**161.0 (10.4)**	**159.9 (11.1)**
NO_3^-	[mg L^{-1}]	**9.18 (2.26)**	**9.44 (2.37)**	**9.18 (2.26)**	**9.38 (2.82)**	**9.46 (2.76)**	**9.48 (2.28)**
NO_2^-	[mg L^{-1}]	**0.06 (0.03)**	**0.05 (0.03)**	**0.06 (0.03)**	**0.06 (0.03)**	**0.06 (0.04)**	**0.05 (0.04)**
NH_4^+	[mg L^{-1}]	**0.06 (0.06)**	**0.07 (0.06)**	**0.06 (0.06)**	**0.05 (0.04)**	**0.04 (0.02)**	**0.03 (0.03)**
PO_4^{3-}	[mg L^{-1}]	**0.29 (0.24)**	**0.23 (0.09)**	**0.29 (0.24)**	**0.28 (0.22)**	**0.24 (0.21)**	**0.26 (0.26)**
N:P		**81.03 (60.89)**	**83.32 (51.76)**	**81.03 (60.89)**	**87.57 (75.62)**	**86.79 (54.27)**	**81.80 (50.42)**
DOC	[mg L^{-1}]	**2.31 (0.47)**	**2.59 (0.74)**	**2.31 (0.47)**	**2.29 (0.44)**	**2.24 (0.68)**	**2.19 (0.42)**
BDOC	[mg L^{-1}]	**0.41 (0.29)**	**0.43 (0.30)**	**0.41 (0.29)**	**0.34 (0.24)**	**0.50 (0.24)**	**0.34 (0.23)**

DOC and BDOC dynamics in channel and pipe systems

DOC and BDOC concentrations showed no differences between the three studied systems when mean values of each month were compared (Fig.5). However, monthly results for DOC and BDOC showed some significant differences between the beginning and the end of the channel (channel 1 vs. channel 2) and the pipe (pipe 1 vs. pipe 2) systems (Fig.6 and Fig.7 respectively).

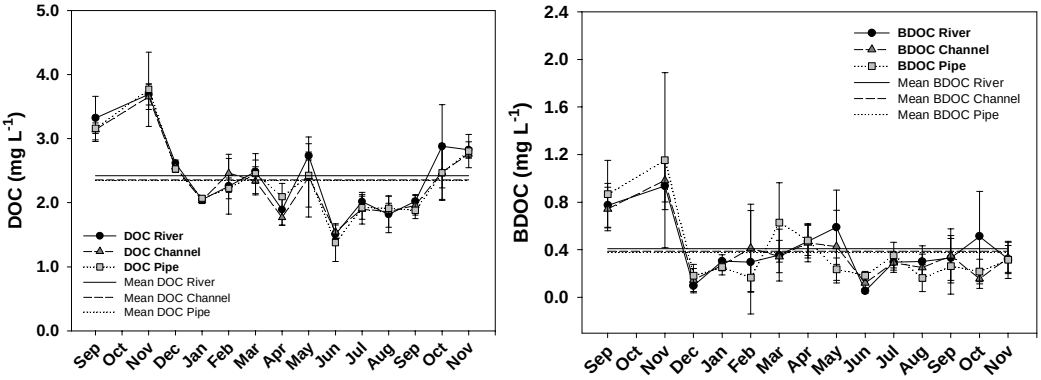

Fig.5. DOC and BDOC dynamics from river, channel and pipe systems during the studied period (September 2000-November 2001). Monthly values are means of each systems and standard deviations (n=6).

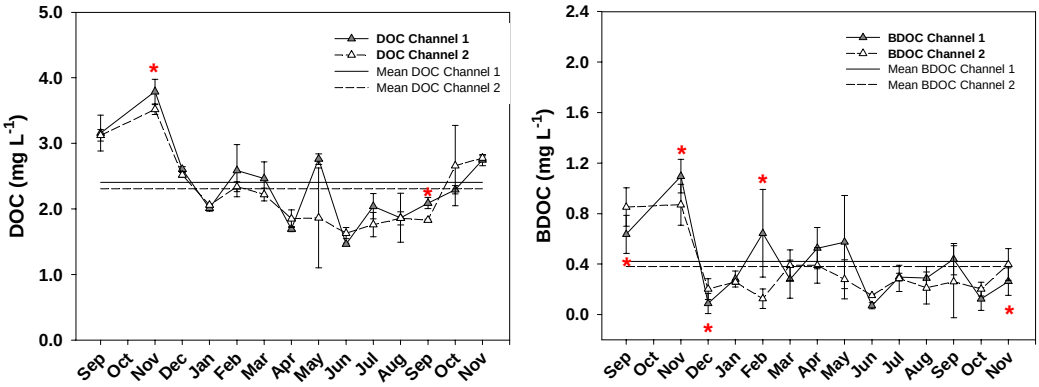

Fig.6. DOC and BDOC dynamics along the channel system (channel 1 vs. channel 2) during the studied period (September 2000-November 2001). Monthly values are means and standard deviations (n=3). Significant differences between channel 1 and channel 2 are indicated by asterisks (t-test, $p<0.05$). Horizontal lines indicate annual means.

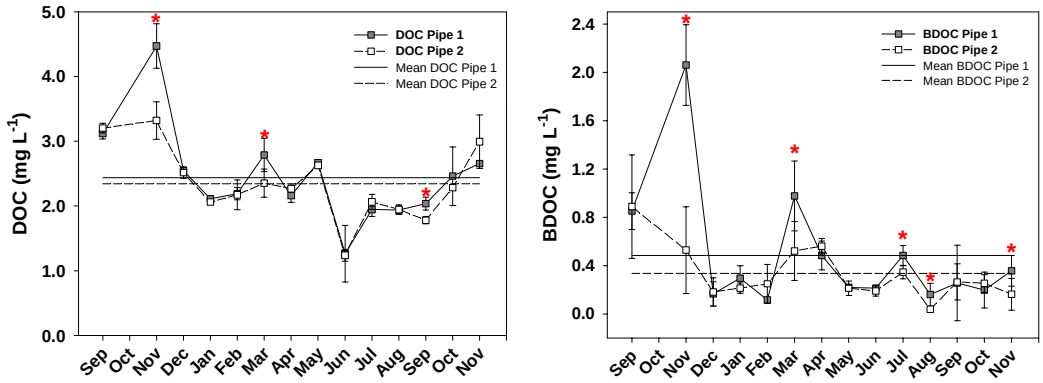

Fig.7. DOC and BDOC dynamics along the pipe system (pipe1 vs. pipe2) during the studied period (September 2000-November 2001). Monthly values are means and standard deviations (n=3). Significant differences between pipe 1 and pipe 2 are indicated by asterisks (t-test, $p<0.05$). Horizontal lines indicate annual means.

The production of BDOC along the channel was observed in September and December 2000 and in November 2001, while a consumption was observed in November 2000 and February 2001 (Fig.6). On the other hand, significant consumption of BDOC along the pipe was observed at five sampling times (Fig.7).

DOC and BDOC budget in channel and pipe systems

An increase in BDOC between 5.7 and 9.0 µg per cm^2 of channel surface per hour was estimated in the channel on four occasions. However, a decrease occurred in two other periods, thereby producing a consumption of 9.4 - 21.7 µg BDOC cm^{-2} h^{-1}. Therefore, the mean annual DOC uptake rate was 1.86 µg DOC cm^{-2} h^{-1} and 2.06 µg BDOC cm^{-2} h^{-1} (Table 2).

Table 2. DOC/BDOC biofilm uptake rates as annual C balance express the annual average uptake rate in the whole channel and pipe systems (mean values and standard deviations, n=12)

Biofilm C uptake rate [µg cm^{-2} h^{-1}]	**Annual C-Balance**	
	DOC	**BDOC**
Channel	**-1.86 (4.34)**	**-2.06 (7.07)**
Pipe	**-1.24 (2.64)**	**-1.52 (3.39)**

The pipe showed a net decrease of BDOC in five sampling times. The uptake rate ranged between 1.4-11.7 µg cm^{-2} h^{-1}, while the mean annual DOC uptake rate for this system was 1.24 µg DOC cm^{-2} h^{-1} and 1.52 µg BDOC cm^{-2} h^{-1} (Table 2).

Biofilm structure

Light-growth biofilms from river and channel were characterized by having higher chlorophyll-*a* density with lower OD430/OD665 ratio than dark-growth biofilms from pipe system, which P2 presented the highest values of OD430/OD665 ratio (Table 3). However, chlorophyll-*a* did not significantly differ between river and channel biofilms and between C1 and C2. Moreover, light-growth biofilms presented high carbon and nitrogen content (C/N ratio of 13.4 in river biofilms and 11.2 in channel biofilms, Table 3).

Pipe biofilms had a significantly lower C content than river and channel biofilms, however, biofilms from P2 showed a higher C and N content than P1 (Table 3). Light-growth biofilms were composed mainly by benthic cyanobacteria, but river biofilms showed higher percentage of filamentous green algae than channel biofilms. Pipe biofilms were composed mainly by diatoms and P1 showed higher percentage of green algae than P2, most of them from planktonic origin. Bacterial density was significantly higher in C1 than C2 (t-test, $p<0.05$), furthermore, channel biofilms presented significantly higher density than pipe biofilms, (t-test, $p<0.02$).

Table 3. Composition and structure of the biofilms grown on the glass substrata in the River (means of R1 and R2, n=6), Channel 1 (n=4), Channel 2 (n=4), Pipe 1 (n=4) and Pipe 2 (n=5). Values are means and standard deviations in parentheses.

	River (n=6)	Channel 1 (n=4)	Channel 2 (n=4)	Pipe 1 (n=4)	Pipe 2 (n=5)
Chlorophyll-*a* [μg cm^{-2}]	6.6 (4.8)	4.5 (2.7)	6.1 (4.4)	0.4 (0.3)	0.2 (0.1)
OD 430/665	2.5 (1.5)	2.9 (1.2)	2.6 (0.9)	5.1 (3.8)	11.9 (17.3)
C [μg cm^{-2}]	816.8 (863.3)	524.2 (313.6)	600.8 (557.0)	86.8 (49.8)	179.3 (296.2)
N [μg cm^{-2}]	57.7 (57.0)	47.5 (32.5)	47.1 (28.8)	10.3 (9.8)	58.2 (110.8)
C/N	13.4 (3.0)	11.3 (3.1)	11.1 (4.3)	10.3 (3.3)	9.7 (5.0)
Bacteria [cell cm^{-2}] x10^{7}	n.m	12.4 (5.3)	6.4 (2.9)	2.7 (1.3)	2.7 (1.2)
Algae [cell cm^{-2}] x10^{6}	9.1 (20.9)	4.5 (7.1)	31.9 (33.9)	0.11 (0.14)	0.07 (0.11)
% cyanobacteria	53.3	72.4	76.2	23.8	22.1
% filamentous green algae	10.7	0.2	4.0	12.5	-
% green microalgae	4.8	6.3	1.7	16.4	9.4
% diatoms	31.2	21.1	18.0	47.3	68.5

Photographs of SEM (Scanning Electron Microscope) are shown in Figure 8. Light-growth biofilms from Channel 2 showed the presence of some diatoms (Fig.8 A), as well as, filamentous of Cladophora with the presence of some epiphytes (Fig.8 B). On the other hand, biofilms growing in dark conditions, Pipe 2, showed some bacteria with mucopholysaccharide (Fig.8 C) and sporadically some presence of diatoms (Fig.8 D).

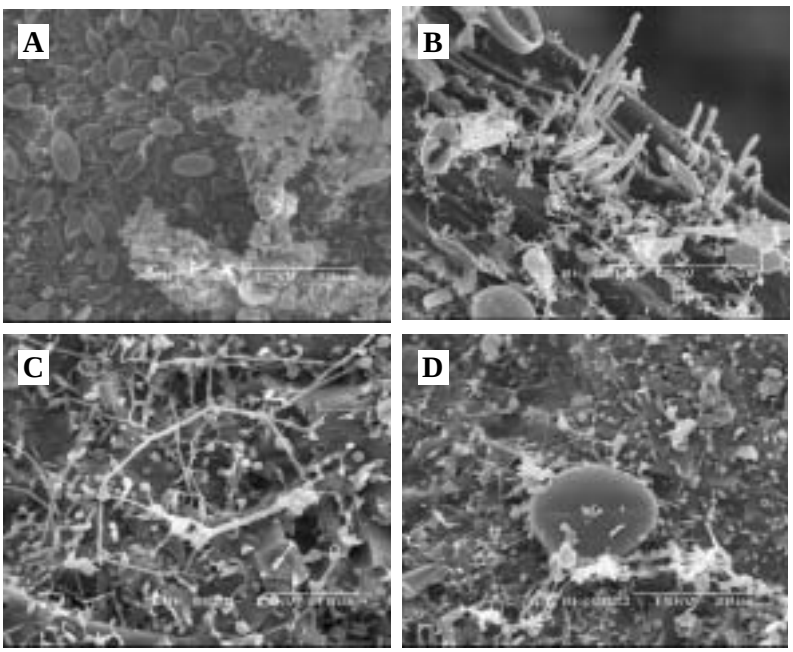

Fig. 8. SEM photographs of 2 months biofilms from Channel 2 (A,B) and Pipe 2 (C,D) (April 2001).

Biofilm function

The extracellular enzymatic activities per cm^2 of biofilm were lower in the pipe than in the light-growth biofilms (river and channel) (Table 4). However, when enzymatic activities were calculated per mg of C differences were less obvious. Higher values of β-glucosidase (expressed by mgC) were found in river and C1 biofilms, which were decreased along C2, P1 and P2 (Table 4). In contrast, β-xylosidase values were high in river biofilms but low in C1 biofilms. However, β-xylosidase were increased again along the C2 and P1 biofilms. Phosphatase and peptidase activities were lower in river than channel biofilms and furthermore, they were found high in pipe biofilms, especially for peptidase activity. Finally, lipase activity was also found high in river biofilms and decrease in C1 biofilms. However, values of lipase were found high again in C2 and P1 and decrease in P2 biofilms (Table 4).

Table 4. Extracellular enzymatic activities measured in biofilms from River (R1 and R2, n=5), Channel 1 (n=4), Channel 2 (n=4), Pipe 1 (n=4) and Pipe 2 (n=5). Values are means and standard deviations in parentheses.

	River (n=5)	**Channel 1** (n=4)	**Channel 2** (n=4)	**Pipe 1** (n=4)	**Pipe 2** (n=5)
[nmol cm^{-2} h^{-1}]					
β-Glucosidase	**32.7 (32.6)**	**56.4 (69.1)**	**16.4 (10.6)**	**9.4 (10.2)**	**2.5 (1.3)**
β-Xylosidase	**17.4 (19.2)**	**6.8 (3.9)**	**11.4 (5.7)**	**4.0 (4.6)**	**1.2 (1.2)**
Phosphatase	**193.7 (203.7)**	**451.3 (320.4)**	**250.8 (109.8)**	**77.7 (64.3)**	**18.6 (13.3)**
Peptidase	**301.9 (214.7)**	**564.0 (291.3)**	**601.3 (167.5)**	**136.4 (68.7)**	**48.9 (27.2)**
Lipase	**1.7 (1.4)**	**1.5 (1.2)**	**1.1 (1.3)**	**0.8 (0.7)**	**0.8 (1.1)**
[nmol mgC^{-1} h^{-1}]					
β-Glucosidase	**126.0 (187.7)**	**144.2 (191.7)**	**89.0 (139.4)**	**95.4 (47.9)**	**54.1 (47.2)**
β-Xylosidase	**79.3 (138.5)**	**20.8 (22.7)**	**51.5 (55.2)**	**41.5 (25.5)**	**11.8 (12.7)**
Phosphatase	**524.0 (493.5)**	**1156.4 (998.2)**	**1121.6 (1577.0)**	**1003.1 (877.7)**	**261.0 (154.0)**
Peptidase	**818.3 (664.5)**	**1229.7 (645.7)**	**2136.5 (2319.5)**	**2102.2 (1295.5)**	**1258.6 (1288.7)**
Lipase	**12.1 (18.1)**	**3.4 (2.8)**	**8.3 (14.0)**	**9.3 (7.7)**	**2.2 (2.2)**

Relationship between DOC and exoenzymatic activities

Exoenzymatic activities (considered by cm^2 and mg C) from light-growth biofilms (river and channel) were significantly correlated with DOC and BDOC water concentrations (Table 5). Significant correlation was observed between β-glucosidase and β-xylosidase activities to BDOC concentration in light-growth biofilms (Table 5). In contrast, exoenzymatic activities of dark-growth biofilms were correlated with BDOC concentration, but not with DOC concentrations (Table 5). Phosphatase and lipase were the only activities that were related with BDOC concentration in dark-growth biofilms (Table 5). Furthermore, pipe biofilms showed a positive correlation between most of the enzymatic activities and accumulated chlorophyll-*a* (Table 5).

β-glucosidase and β-xylosidase activities (nmol mgC^{-1} h^{-1}) from light-growth biofilms (river and channel) fitted with BDOC concentration in a positive non-linear regression expression (adjusted R^2 = 0.79 and 0.35 respectively) (Fig.9A,B). Phosphatase and lipase (nmol mgC^{-1} h^{-1}) fitted with BDOC concentration only in dark-growth biofilms (adjusted R^2 = 0.71 and 0.80 respectively) (Fig.9C,D).

Table 5. Significant Pearson correlations between the extracellular enzymatic activites and DOC and BDOC water content and chlorophyll-a biofilm content for light-growth biofilms (River and Channel) and dark-growth biofilms (Pipe). Significance is indicated by asterisks: ***p<0.001, **p<0.01, *p<0.05.

	LIGHT BIOFILMS			**DARK BIOFILMS**		
	DOC	**BDOC**	**Chl-*a***	**DOC**	**BDOC**	**Chl-*a***
[nmol cm^{-2} h^{-1}]	(n=13)	(n=13)	(n=13)	(n=9)	(n=9)	(n=9)
β-Glucosidase		**0.61***				**0.81****
β-Xylosidase		**0.56***				**0.76***
Phosphatase					**0.70***	**0.92*****
Peptidase	**-0.67***	**-0.61***				
Lipase	**0.65***	**0.64***			**0.80***	
[nmol mgC^{-1} h^{-1}]	(n=13)	(n=13)	(n=13)	(n=8)	(n=8)	(n=8)
β-Glucosidase	**0.65***	**0.74****				**0.87****
β-Xylosidase		**0.56***				**0.75***
Phosphatase					**0.75***	**0.73***
Peptidase						
Lipase					**0.85****	**0.75***

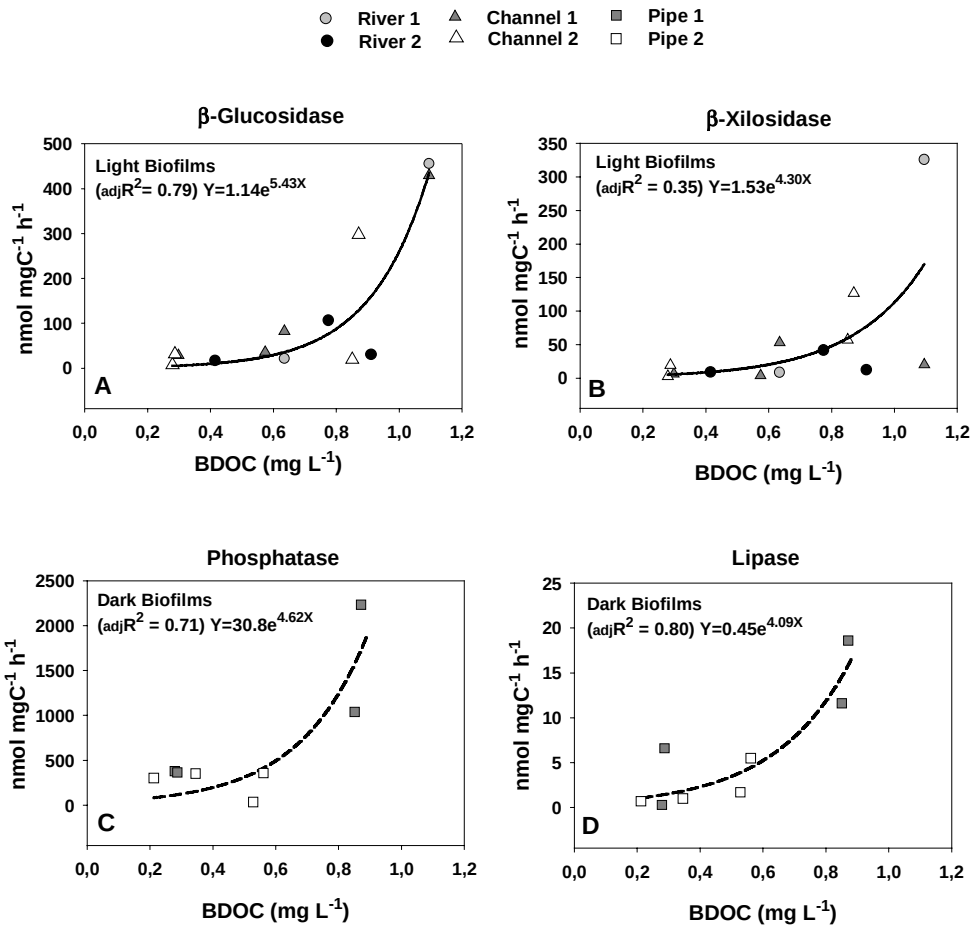

Fig. 9. Relationship between BDOC content in the water and β-glucosidase and β-xilosidase activities in light-growth biofilms (River and Channel) (A,B) and phosphatase and lipase activities in dark-growth biofilms (Pipe)(C,D).

DISCUSSION

The use of the artificial channel and pipe systems allowed us to circumscribe the effect of DOC transport and storage in the water mass and the biofilms, since we avoided the influence of the hyporheic zone and groundwater. On the other hand, the study of the natural river system, let us to compare the results with a natural, open light system.

Annual DOC budgets

The higher DOC and BDOC uptake rate in the channel than the pipe system may be due to several processes that act simultaneously: 1) biotic factors, that is, major heterotrophic activity, and 2) abiotic factors, that is, biofilm DOM adsorption and photochemical transformation of DOM. The development of an active autotrophic community in a light biofilm might provide appropriate colonization sites for heterotrophs and may also increase the amount of organic C available (Jones & Lock, 1993; Romaní & Sabater, 1999). Moreover, a further biotic mechanism could be enhancement of DOM uptake or photoheterotrophy by exposure to light (Paerl et al. 1999). The photochemical degradation of DOM (Moran & Zepp 1997) may also contribute to the higher DOC uptake rate of the autotrophic biofilm in channel system, although Wiegner & Seitzinger (2001) conclude that microbial degradation is more important than photochemical processes in degrading river dissolved organic matter. Finally, biofilm adsorption may produce an abiotic retention of organic matter (Fischer 2002).

The annual average rates of DOC uptake in the channel and pipe system (2.1 and 1.5 $\mu g\ cm^{-2}\ h^{-1}$ respectively) observed in our study are consistent with the range of values reported for other river systems (Kaplan & Bott, 1983; Kuserk et al. 1984; Fischer et al. 2002). Some considerations should be taken from the riverine DOC dynamics analysed by the field results (DOC budget): the net uptake rate calculated from the DOC budget not only includes the effect of the biofilm but also the variations derived from the flowing water, since, in the light, BDOC might increase in several periods of the year because of phytoplankton activity, especially in spring. In the case of large rivers, such as the Ebre, the phytoplankton community is well developed (Sabater & Muñoz 1990). However, the high current velocity, such as that in the Ebre River channel, produces the least favourable conditions for the phytoplankton development (Friedrich & Viehweg 1984). Part of the high density of phytoplankton in the channel could be due to the drift of benthic algae, which accounted for a large proportion (Romaní et al. 2004). However, the uptake rate calculated from the DOC

balance could underestimate the true DOC uptake rate of the channel biofilm, especially in periods of increased phytoplankton production. Furthermore, this real uptake rate value is complicated by the DOC released by the biofilms (Kuserk et al. 1984). The joint variation of the two sources of DOC, i.e. fluvial DOC and that released from the biofilm, could explain the large seasonal variability observed in the DOC budget in the channel. In the dark pipe, the more constant DOC balance throughout the study period could be related to the less significant effect of the variations in the flowing water and the lower effect of the molecules released by algae from the biofilm.

Relation between DOC and exoenzymatic activities

Exoenzymatic activities and BDOC water concentrations were significantly correlated both in light-growth and dark-growth biofilms, suggesting that the labile fraction of DOC was quickly uptake by the biofilms, compared to more refractory compounds which have a slower uptake (Norrman et al. 1995). Moreover, within the light-growth biofilms, correlations were more significant in channel than river biofilms, indicating that differences were due to BDOC uptake efficiency.

β-glucosidase and β-xylosidase activities correlated better with water BDOC concentration in light-grown biofilms, whereas the correlation between BDOC and phosphatase and lipase activities fitted better in dark-grown biofilms. This suggests that light-grown biofilms were involved in the degradation of polysaccharides, cellulose degradation by β-glucosidase (Desphande & Eriksson 1988) and hemicellulose degradation by β-xylosidase (Lachke 1988), a major terrestrial plant constituent. Furthermore, the accumulation of algal biomass in the biofilm, and its polysaccharide released during photosynthesis, might enhance the β-glucosidase and β-xylosidase activities (Romaní & Sabater 1999; Espeland et al. 2001). This supports the idea that thicker biofilms with higher biomass, 4- and 12-months biofilms, will be less efficient in the retention of water BDOC since they will be regulated by their own internal carbon recycling.

Phosphatase and peptidase activities were not correlated with BDOC water content in light-grown biofilms, suggesting that the regulation of these exoenzymes was complex, since both algae and bacteria were involved. Cellular excretion by algae may be an important source of phosphorous for biofilm bacteria, furthermore, algal photosynthesis could indirectly affect the phosphate uptake by the biofilm uptake (Espeland & Wetzel 2001). Leucine-aminopeptidase activity may be affected by the supply of dissolved inorganic nitrogen, labile organic carbon as well as the active

periphytic photosynthesis (Francoeur & Wetzel 2003). Dark-grown biofilms had a quite constant DOC uptake rate during the year, suggesting a limited availability of labile DOC molecules, and moreover, a rapid response to the input of allochthonous DOM sources. In addition, this is confirmed by the high correlation between lipase and phosphatase activities and BDOC water content in pipe biofilms, suggesting that the quality of water inputs of DOM affected the biofilm metabolism. The metabolic response of microbial communities to quantity and quality of DOM has been described elsewhere (Hunt et al. 2000; Findlay et al. 2003). Phosphatase activity in pipe biofilms may be related with the inputs of some algae and diatoms, mainly planktonic, which could contribute with the labile fraction of DOC (Cole et al. 1982; Norrman et al. 1995). Furthermore, the high percentage of diatoms found in the pipe could contribute with the lipid content of DOM inputs, since it has been reported that they accumulate triglycerides as a cellular storage product, meanwhile Chlorophyceae accumulate mainly carbohydrates (Tuchman 1996). On the other hand, the accumulation of algal biomass in the light-grown biofilms, and thereby its cellular excretion as well as cellular lysis, might be an important source of phosphorus for biofilm bacteria (Espeland & Wetzel 2001).

The function of the dark-growth biofilm (efficient uptake of labile molecules and constant DOC consumption along the year) permits the maintenance of low DOC levels. This is important for water management since the presence of DOC in drinking water allows noxious bacterial re-growth in water pipes (Kaplan & Newbold 1995). From these results we may suggest that use of covered conduits may be advisable to substantially diminish the DOC concentration in the water. At the river ecosystem level, the low availability of organic carbon for the microbial community might reinforce the relationships between the biofilm and the flowing water, since there is low DOC content in Ebre River water in comparison to other river systems (Thurman 1985).

We can conclude that the DOC uptake/release in channel and pipe systems is due to the biofilms growth, which will be analysed in the Chapter 2. Moreover, the results of the field annual DOC budget are that light-growth biofilms growing in channel system are, on annual average, a net DOC consumer. Although they present monthly variability in DOC uptake/release rates, the annual uptake rate was greater than that of the dark-growth biofilm.

CHAPTER 2
BIOFILM AGE CHARACTERIZATION IN RELATION TO TEMPORAL WATER DOC DYNAMICS

INTRODUCTION

Quality and quantity of DOC influences bacterial activity in the biofilm. Under light conditions, autotrophic algae in the biofilm are a possible source of labile compounds that may be used by biofilm bacteria living in close proximity to the algae. Therefore, bacterial activity often correlated with algal biomass and production in the biofilm (Chappell & Goulder 1994). The high productivity of attached microcommunities of algae and bacteria can be due to efficient internal recycling of carbon and nutrients, whereas heterotrophic bacterial biofilms would be largely dependent upon external importation of DOC, the mixed community of auto- and heterotrophs decreased this dependency upon importation from the ambient environment (Wetzel 1993).

The biofilms have the potential to play an important role in the retention and transformation of organic matter from the water column on an ecosystem scale (Ribas et al. 1995, Volk et al. 1997, Fischer et al. 2002). Because of the high area of solid surfaces covered with biofilms, these biofilms dominate the heterotrophic metabolism in many aquatic ecosystems and are major sites for the uptake and storage of fluvial DOC (Battin et al. 1999; Kaplan & Bott 1983) and contribute significantly to C cycling in rivers and streams.

Biofilm age and the corresponding changes in structure (thickness, exopolysaccharide content (EPS), algal and bacterial density, etc) might determine

the biofilm functioning and at the end the biofilm capacity of DOC uptake and/or release, since biofilm structure affects the biofilm activity (Sabater et al 2002). Uptake of DOC can be directly diffused via membrane permeases or via diffusion through pores if the molecules are small enough (Nikaido and Vaara 1985). It is assumed that bacterial activity is stimulated by the available fractions of DOC (Fischer et al. 2002), even though bacteria can afford extracellular enzymes to degrade and effectively use larger molecules (Chróst 1991), which therefore play a key role in the organic matter process of river ecosystems.

The present study compares two different situations in terms of DOC content in the river, a high-DOC period and a low-DOC period, coinciding with autumn and spring-summer respectively. Since DOC composition and biodegradability could be related with seasonal differences, especial attention has been paid to the biodegradable fraction (BDOC). We aim to elucidate the differential role of light-growth biofilms, obtained from a natural river and an open channel, and dark-growth biofilms, from a dark pipe, in relation to the water DOC availability. Furthermore, water velocity also influenced the growth regime of the biofilms, since river water velocity was lower and more fluctuant than channel and pipe water systems. Since it is known that biofilm age and its related characteristics (thickness, biomass increase) may influence the biofilm function (Sabater et al. 2002), biofilms of different age are compared.

MATERIAL AND METHODS

Sampling strategy

Biofilm age characterization was carried out with 2, 4 and 12 months colonized biofilms collected in the river, channel and pipe, in the 5 studied periods (see Materials & Methods).

Confocal Laser Scanning Microscope (CLSM)

Live biofilm samples (2 months and 4 months old) which were collected from the R2, C1 and P2 systems in November 2000 were examined under the CLSM. A series of optical sections were recorded at 1.5 μm intervals and the maximum observable depths were 89 μm for river biofilms, 41 μm for channel biofilms and 38 μm for pipe biofilms.

RESULTS

Temporal DOC dynamics

Two distinct periods in Ebre River were detected in relation to DOC and BDOC concentration (Table 1). The higher DOC concentration period (average water river value of 3.58 mg L^{-1}) occurred during September and November 2000, while the lower DOC concentration (average water river value of 2.26 mg L^{-1}) period coincided with Spring and Summer (April, May and July 2001). Similar values were found in channel and pipe water systems (Table 1).

Table 1. Physical and chemical characteristics from River 2, Channel 1 and Pipe 2 during high-DOC period (September and November 2000) and low-DOC period (April, May and July 2001). Values are means and standard deviations in parentheses.

		River 2		Channel 1		Pipe 2	
		high DOC (n=2)	low DOC (n=3)	high DOC (n=2)	low DOC (n=3)	high DOC (n=2)	low DOC (n=3)
Conduct	[µS cm^{-1}]	1111.0	691.0 (115.5)	1124.0	679.3 (107.5)	1121.0	702.0 (125.0)
pH		8.03	8.12 (0.12)	8.02	8.15 (0.12)	8.11	8.14 (0.06)
$CaCO_3$	[mg L^{-1}]	171.0	152.7 (2.9)	165.0	152.3 (4.16)	165.0	154.0 (2.65)
NO_3^-	[mg L^{-1}]	12.30	8.17 (1.42)	12.20	8.27 (1.23)	11.85	8.43 (1.45)
NO_2^-	[mg L^{-1}]	0.08	0.05 (0.04)	0.08	0.06 (0.05)	0.04	0.07 (0.07)
NH_4^+	[mg L^{-1}]	0.43	0.02 (0.00)	0.16	0.02 (0.00)	0.08	0.02 (0.00)
PO_4^{3-}	[mg L^{-1}]	0.17	0.24 (0.13)	0.23	0.18 (0.15)	0.26	0.14 (0.05)
N:P		91.0	70.9 (51.9)	83.99	110.2 (80.4)	90.1	96.6 (22.5)
DOC	[mg L^{-1}]	3.58	2.26 (0.38)	3.47	2.16 (0.54)	3.26	2.32 (0.29)
BDOC	[mg L^{-1}]	0.84	0.44 (0.16)	0.87	0.47 (0.15)	0.71	0.37 (0.18)

Maximum DOC and BDOC concentrations in river water occurred in November 2000 reaching 3.79 mg L^{-1} and 1.10 mg L^{-1} (29.0%) respectively. Minimum concentrations were recorded in July 2001 reaching 1.99 mg L^{-1} of DOC and 0.29 mg L^{-1} of BDOC (14.6%). River water from high-DOC period was also characterised by higher conductivity (1111.0 vs. 691.0 µS cm^{-1}) and nitrate concentration (12.30 vs. 8.17 mg L^{-1}) than low-DOC period.

Differences on DOC and BDOC between compartments (River, Channel and Pipe) in some of the studied periods (see Chapter 1) were smaller than the corresponding differences between periods (Fig 1). Moreover, differences between systems were not observed for the rest of physical and chemical parameters (Table 1).

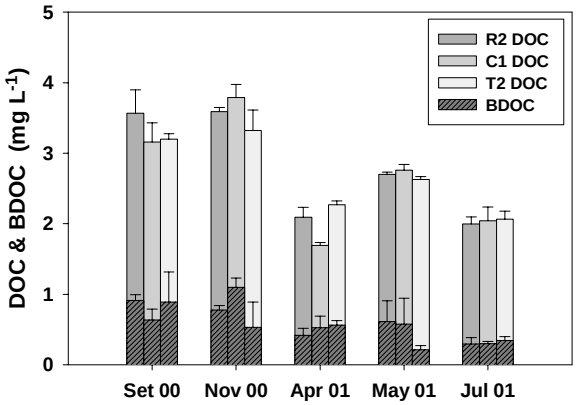

Fig.1. DOC and BDOC water concentrations form River 2, Channel 1 and Pipe 2 systems during the study period (September and November 2000, and April, May and July 2001). Monthly values are means with standard deviations (n=3).

Biofilm characteristics related to age and growth regime

Two-month biofilms from the R2 and C1 systems had higher chlorophyll-*a* concentration and lower OD430/OD665 than 2-month dark-growth biofilms (P2) (Table 2).

Table 2. Means values of chlorophyll-*a*, OD 430/665, carbon, nitrogen and C/N (molar ratio) from the 2, 4 and 12 months biofilms from River 2, Channel 1 and Pipe 2 during high-DOC and low-DOC periods. Values not measured are indicated with -.

2 months biofilms	**River 2**		**Channel 1**		**Pipe 2**	
	high DOC (n=2)	low DOC (n=2)	high DOC (n=2)	low DOC (n=2)	high DOC (n=2)	low DOC (n=3)
Chl-*a* [µg cm^{-2}]	**8.1**	**6.3**	**2.2**	**6.6**	**0.2**	**0.1**
OD 430/665	**3.0**	**2.2**	**3.7**	**2.2**	**24.2**	**3.7**
C [µg cm^{-2}]	**1214.3**	**925.3**	**295.6**	**752.7**	**623.6**	**31.2**
N [µg cm^{-2}]	**76.9**	**71.6**	**29.3**	**65.7**	**224.4**	**2.8**
C/N	**16.1**	**16.9**	**11.4**	**15.1**	**3.2**	**14.0**
4 months biofilms	**River 2**		**Channel 1**		**Pipe 2**	
	high DOC (n=1)	low DOC (n=1)	high DOC (n=1)	low DOC (n=1)	high DOC (n=1)	low DOC (n=1)
Chl-*a* [µg cm^{-2}]	**6.5**	**37.2**	**3.6**	**2.3**	**0.3**	**0.1**
OD 430/665	**2.0**	**1.9**	**2.4**	**2.1**	**3.5**	**4.0**
C [µg cm^{-2}]	**737.3**	**1945.0**	**419.6**	**419.2**	**107.4**	**69.6**
N [µg cm^{-2}]	**31.1**	**146.0**	**48.4**	**48.7**	**15.3**	**5.1**
C/N	**27.5**	**15.6**	**10.3**	**10.8**	**8.1**	**15.3**
12 months biofilms	**River 2**		**Channel 1**		**Pipe 2**	
	high DOC (n=1)	low DOC (n=1)	high DOC (n=1)	low DOC (n=1)	high DOC (n=1)	low DOC (n=1)
Chl-*a* [µg cm^{-2}]	**-**	**50.1**	**-**	**-**	**-**	**0.1**
OD 430/665	**-**	**1.9**	**-**	**-**	**-**	**4.1**
C [µg cm^{-2}]	**-**	**4594.4**	**-**	**-**	**-**	**80.8**
N [µg cm^{-2}]	**-**	**272.4**	**-**	**-**	**-**	**7.3**
C/N	**-**	**19.6**	**-**	**-**	**-**	**13.0**

Table 3. Total algal cell density, algal community composition (in percentage of the total density), protozoa density (number of individuals cm^{-2}) and total bacterial cell density of the 2 and 4 months biofilms from River 2, Channel 1and Pipe 2 systems during hihg-DOC and low-DOC periods and 12 months biofilms from River 2 and Pipe 2 during low-DOC period.

	2 months biofilms						4 months biofilms						12 months	
	River 2		Channel 1		Pipe 2		River 2		Channel 1		Pipe 2		River 2	Pipe 2
	high DOC (n=2)	low DOC (n=2)	high DOC (n=2)	low DOC (n=2)	high DOC (n=2)	low DOC (n=3)	high DOC (n=1)	low DOC (n=1)	high DOC (n=1)	low DOC (n=1)	high DOC (n=1)	low DOC (n=1)	low DOC (n=1)	low DOC (n=1)
Algal density [cells cm^{-2}] x10^6	**26.2**	**0.5**	**1.1**	**8.0**	**0.1**	**0.01**	**1.6**	**16.4**	**0.5**	**2.8**	**0.001**	**0.01**	**33.4**	**0.03**
CYANOBACTERIA														
Heteroleibleinia sp	**78.2**		**10.9**	**10.9**			**36.6**	**66.1**	**10.7**	**57.0**			**81.2**	
Lyngbya martensiana (Menegh. ex Gom.)	**11.9**		**8.0**	**3.3**	**42.4**		**57.3**		**16.8**					
Merismopedia sp	**0.2**				**13.0**									
Oscillatoria sp								**0.8**					**0.1**	
Oscillatoria tenuis (Agardh ex Gom.)														
Phormidium ambiguum (Gom.)	**5.4**							**23.9**					**16.8**	
Phormidium subfuscum (Kütz.)				**47.3**						**34.0**				
Pleurocapsa sp			**64.3**				**2.0**	**3.3**	**49.0**	**5.5**				
Pseudoanabaena sp								**0.7**						
ALGAE (non diatoms)														
Cladophora glomerata ((Linn.) Kütz.)	**0.2**	**2.9**		**0.3**			**0.2**	**0.1**		**0.6**			**0.1**	
Micractinium sp			**2.7**		**4.5**									
Oedogonium sp	**1.4**	**10.8**					**0.4**	**0.6**					**1.1**	
Pediastrum sp	**0.2**		**7.8**		**15.9**			**0.4**						
Scenedesmus sp	**0.1**		**1.8**	**0.4**	**3.0**			**0.3**		**0.3**				
Stigeoclonium tenue (Kütz.)		**12.9**												
Ulothrix zonata ((Weber et Mohr.) Kütz.)										**0.6**				
DIATOMS	**2.3**	**73.4**	**4.5**	**37.8**	**21.2**	**100.0**	**3.6**	**3.9**	**23.5**	**2.1**	**100.0**	**100**	**0.7**	**100**
Protozoa density [ind. cm^{-2}]														
Amoeba group					**885**						**6759**			
Peritrichia (*Vorticella* like)					**6422**	**966**								
Bacterial density [cell cm^{-2}] x10^7	**n.m**	**n.m**	**18.4**	**9.4**	**1.7**	**3.1**	**n.m**	**n.m**	**11.9**	**n.m**	**1.9**	**6.2**	**n.m**	**4.8**

Maximum chlorophyll-*a* was reached in September in the R2 (14.9 μg cm^{-2}) and in May in C1 biofilms (7.6 μg cm^{-2}). Chlorophyll-*a* in P2 biofilms were maximum in July (up to 0.3 μg cm^{-2}). Accordingly, algal densities were higher in light than in dark-growth biofilms (see Table 3, 2-months biofilms). However, annual mean values of chlorophyll-*a* did not significantly differ between 2-months biofilms of river and channel (7.7 ± 6.8 μg cm^{-2} and 4.5 ± 2.7 μg cm^{-2} respectively). Otherwise, chlorophyll-*a* and carbon content from 4 months biofilms were higher in the R2 than in the C1 biofilms (Table 2). Four- and 12-months biofilms from the R2 were characterised by maximum values of chlorophyll-*a* in July (37.2 μg cm^{-2} and 50.1 μg cm^{-2}) as well as carbon content (1945.9 μg cm^{-2} and 4594.4 μg cm^{-2}) (Table 2). Otherwise, 4-months biofilms from C1 presented annual means of chlorophyll-*a* lower than 2-months biofilms (2.9 μg cm^{-2} vs. 4.5 μg cm^{-2}), as well as carbon content (419.4 μg cm^{-2} vs. 524.2 μg cm^{-2}).

The taxonomic composition of 4 month-old biofilms was not remarkably different than that of the 2 month-old biofilms (Table 3), the cyanobacteria and filamentous green algae (mainly *Cladophora glomerata* and *Oedogonium* sp) being analogously present in the two phases of growth. Four-month biofilms in the P2 system generally showed a higher bacterial density than two-month biofilms (Table 3). However, the bacterial density from the 12-month old biofilms in the pipe did not present significant differences from that of the 4 months. Pictures of DAPI staining of bacterial and protozoa community (*Vorticella*) collected from Pipe 2 (November 2000) are shown in Figure 2.

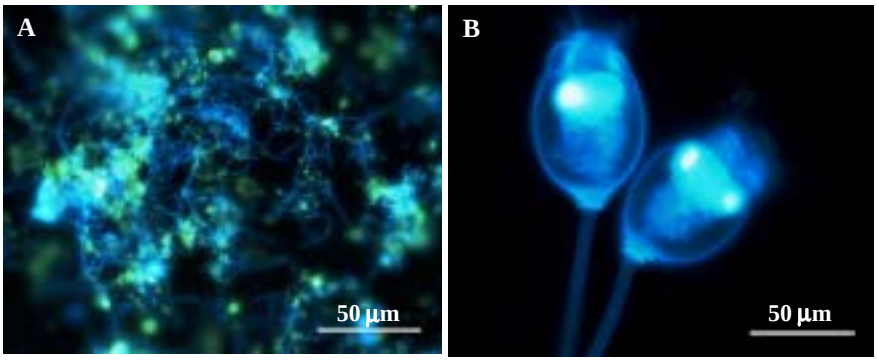

Fig. 2. DAPI staining of bacteria (A) and *Vorticella* (B) from 2 month biofilms collected from Pipe 2 (November 2000)

The structure of two- and four-month old biofilms from the R2, C1 and P2 systems (November 2000) were analysed by CLSM (Fig. 3). Lower density of filaments (measured as chlorophyll autofluorescence) was characteristic of the 2 month-old river biofilms (Fig.3A).

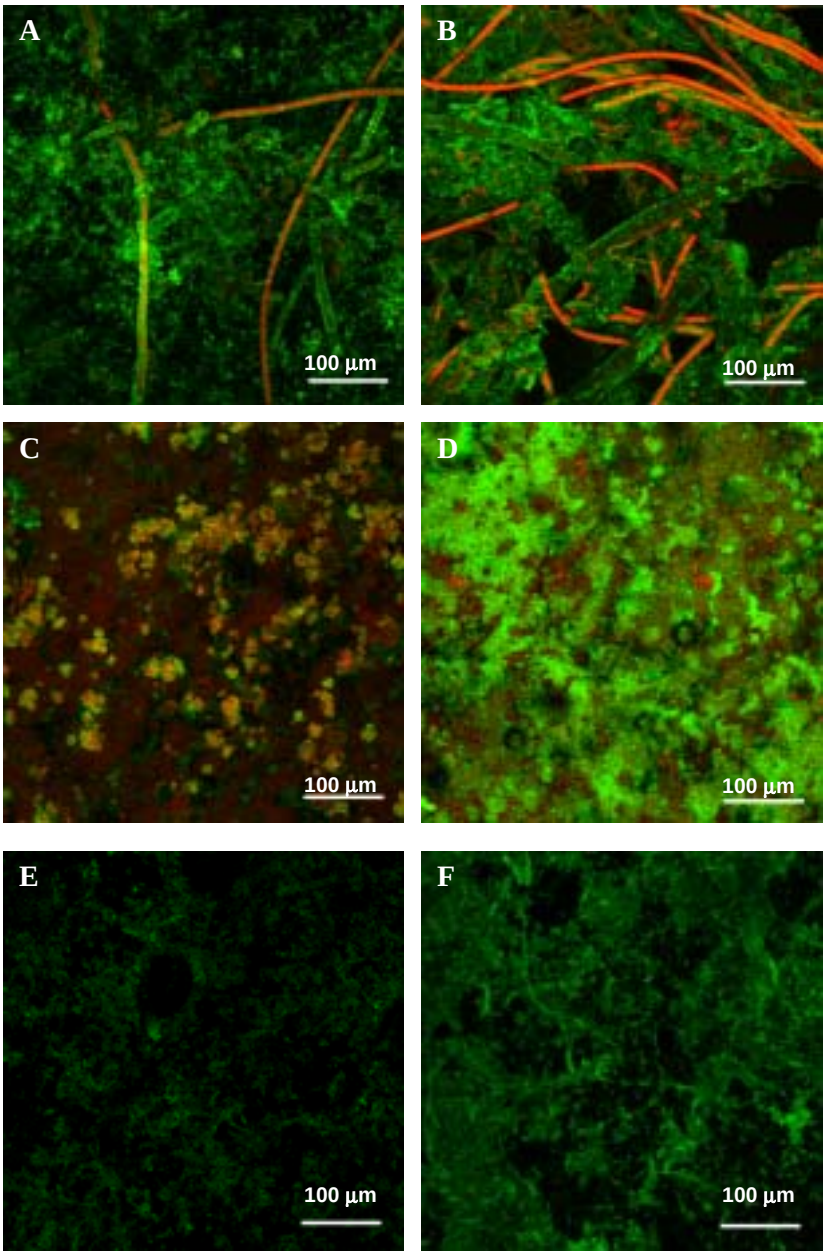

Fig. 3. CLSM (Confocal Laser Scanning Microscope) images corresponding to 2 and 4 months respectively, from biofilms collected at River 2 (A,B), Channel 1 (C,D) and Pipe 2 (E,F) during November 2000. Red-autofluorescence (algae); green-Concanavalin-A (mucopolysaccharide).

The area occupied by autofluorescence accounted for 3.6% in 2-months biofilms (Fig.3A) and increased up to 15.9% in 4-months biofilms (Fig.3B). The EPS products (Con-A staining) also increased their proportion with colonisation time (41.7% to. 61.2%, from 2-months to 4-months old). Algal cells and mucilage formed a more compact biofilm in channel than in river biofilms. In the cannel system, 4-months biofilms (Fig.3D) also presented higher algal biomass and mucilage abundance than 2-months biofilms (Fig.3C). In this case, both autofluorescence (from 18.6% to 49.3%), and EPS increased (from 20.8% to 50.7%) with biofilm age. Therefore, in river biofilms, mucilage occupied a higher area than algal cells, with a proportion of 12-fold in 2-months biofilms and more moderate but still high in 4-months biofilms (61.2 vs. 15.9%). On the other hand, the channel biofilms presented a similar proportion of mucilage vs. algal cells, both in 2-months (20.2 vs.18.6%) and 4- months biofilms (50.7 vs. 49.3%). In the pipe biofilms (dark-grown conditions) only Con-A fluorescence was detected, being the mucilage proportion higher in 4-months (Fig.3F) than in 2-month biofilms (Fig.3E) (39.7% and 10.3% respectively).

Relationships between temporal DOC dynamics and biofilm structure

Algal and cyanobacterial composition differed from high-DOC and low-DOC periods (Table 3). In the high-DOC period benthic cyanobacteria (mainly *Heteroleibleinia* sp ((Geitler) Hoffman) and *Pleurocapsa* sp) were dominant in light-grown systems. During the low-DOC period, filamentous green algae and diatoms increased their contribution in detriment of cyanobacteria. Some cyanobacteria, diatoms and green algae occurred in the pipe biofilms during the high-DOC period. Moreover, a high number of protozoa (*Amaeba* sp and *Vorticella* sp) per surface area were found also during that period colonising the pipe.

Bacterial density was higher during the high-DOC period (Table 3). Bacterial density was significantly higher in channel than in pipe biofilms. Bacterial density in the former biofilm reached a maximum of 18.4 $x10^7$ bacterial cell cm^{-2} in November 2000.

During the high-DOC period, C/N ratio was higher in river biofilms than channel biofilms, both in 2-months biofilms (C/N ratio of 16.1 vs. 11.4) and 4-months biofilms (27.5 vs. 10.3) (Table 2). Moreover, C/N ratio from channel biofilms were significantly higher than pipe biofilms, also both in 2-months (11.4 vs 3.2) and 4-months biofilms (10.3 vs. 8.1). Otherwise, during the low-DOC period, 2-months biofilms from channel were characterized by a higher C and N content and high C/N ratio than those from high-DOC period, but there were no differences when 4-months biofilms were compared.

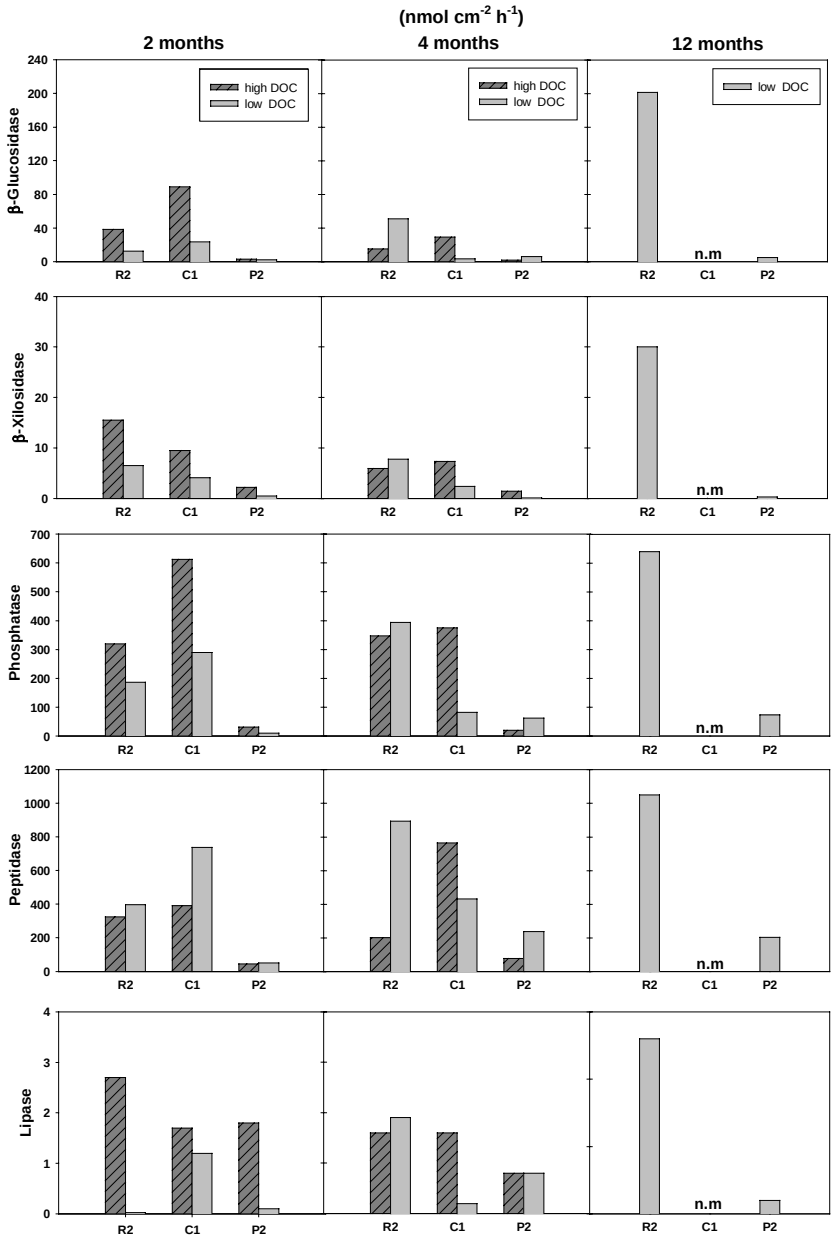

Fig. 4. Mean values of extracellular enzymatic activities expressed as nmol cm^{-2} h^{-1} from 2 month biofilms (n=2), 4 month biofilms (n=1) and 12 month biofilms (n=1), collected in River 2 (R2), Channel 1 (C1) and Pipe 2 (P2) systems during high-DOC period (September and November 2000) and low-DOC period (April, May and July 2001). Not measured values are indicated by n.m.

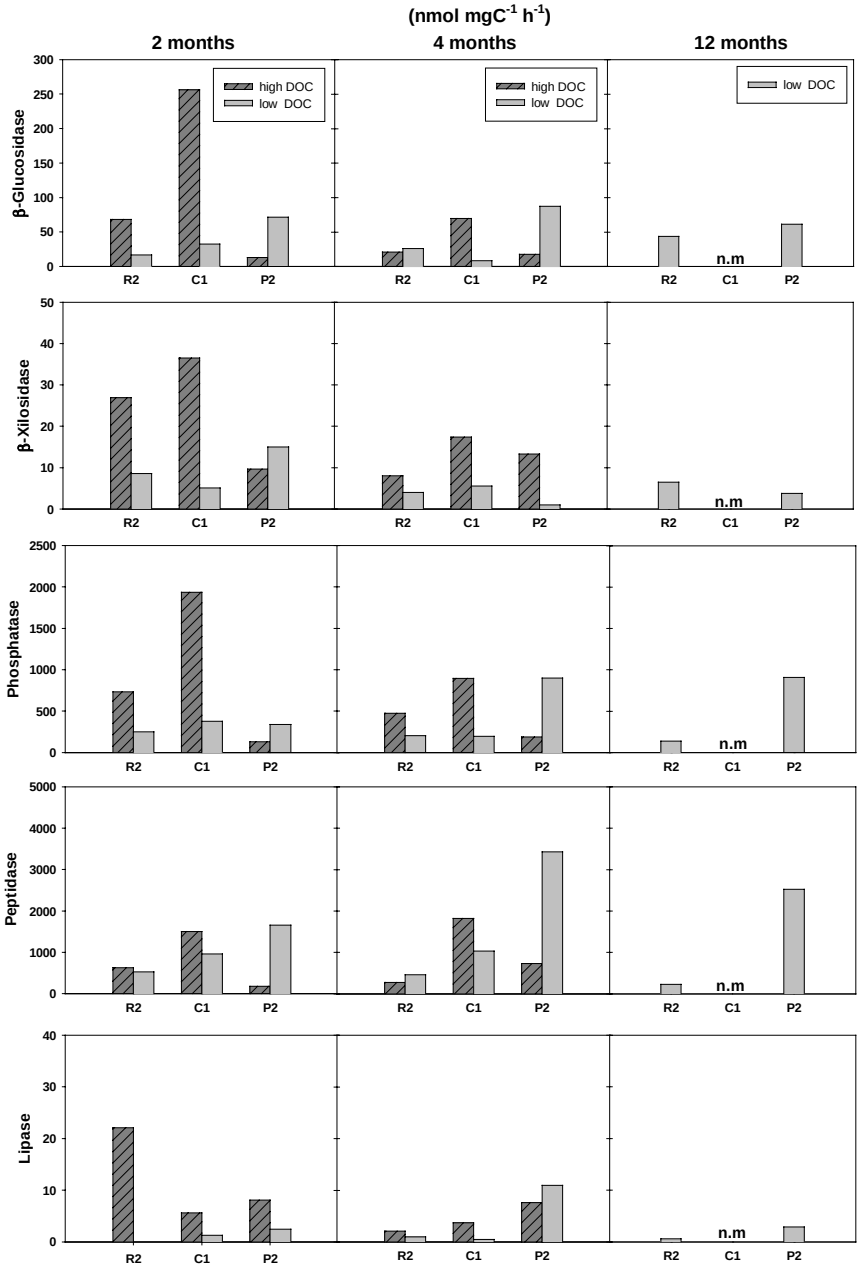

Fig. 5. Mean values of extracellular enzymatic activities expressed as nmol mgC^{-1} h^{-1} from 2 month biofilms (n=2), 4 month biofilms (n=1) and 12 month biofilms (n=1), collected in River 2 (R2), Channel 1 (C1) and Pipe 2 (P2) systems during high-DOC period (September and November 2000) and low-DOC period (April, May and July 2001). Not measured values are indicated by n.m.

Variation of exoenzymatic activities with biofilm age

With the exception of peptidase, the remaining measured activities were higher in biofilms from high-DOC period than in low-DOC period (Figs. 4 and 5). These differences were more obvious when activities were expressed by carbon weight (Fig.5) rather than by surface area (Fig.4). River biofilms presented higher activities in older (4 and 12 months) than younger biofilms (Fig. 4) when these activities were expressed by surface area. However, these differences were significantly reduced when activities were expressed by carbon weight (Fig. 5). Channel and pipe showed similar activities between 2 and 4 month-old biofilms.

Between compartments, activities per surface area were higher in biofilms in the river and channel than in the pipe system, with exception of lipase activity (Fig. 4). These differences were smaller when activities were expressed by carbon weight (Fig. 5). Values of β-glucosidase and β-xylosidase activities (expressed by cm^2) where similar in river than channel systems, but once expressed by mgC, channel biofilms presented higher values. On the other hand, phosphatase and peptidase activities were usually higher in channel biofilms than in river biofilms. Notice that peptidase activity during low-DOC period (expressed by mgC) was even higher in pipe system than channel and river.

DISCUSSION

Relation between DOC and biofilm structure

Differences in structure and algal composition were observed in biofilms collected from each compartment. Light-grown biofilms (river and channel) had higher chlorophyll-*a* concentration and higher algal densities than dark-grown biofilms (pipe). Furthermore, river biofilms can support higher algal biomass (chlorophyll-*a*) with higher C and N content than channel biofilms, since 4- and 12-months biofilms from the river presented the highest values. In contrast, channel biofilms presented annual means of chlorophyll-*a* and carbon content significantly lower in 4-months than 2-months biofilms. Although there were not significant differences in taxonomic composition between river and channel systems, filamentous green algae were found in higher percentage in river than in channel biofilms, where cyanobacteria were the dominant. The algae detected in the pipe biofilm (mainly diatoms, some cyanobacteria and green algae) had their origin in the seston compartment. Accordingly, chlorophyll-*a* concentration was very low with high OD430/OD665 ratio.

These differences have been also observed by CLSM. CLSM has been utilized in a number of studies describing biofilms structure (Lawrence et al. 1988; Sabater 2000; Barranguet et al. 2004). Digital image analyses from CLSM showed that in river biofilms, mucilage occupied a higher area than algal cells, while in channel biofilms presented a similar proportion of mucilage than algal cells. River biofilms were growing in shallow littoral areas while channel biofilms were growing against the vertical walls of the artificial channel being directly exposed to the high flow of water. The effect of high flows abrading the biofilm matrix, and preventing the EPS accumulation, has been described when frequent storm flows occurs and "channel processes" takes place (Blenkinsopp & Lock 1994). Pipe biofilms were also affected by high water velocities, but it is true that 4-months biofilms accumulated more mucopolysaccharide than 2-month biofilms. This difference was a likely result of increasing complexity in the biofilm, in spite of the high water velocity.

Differences in structure and composition in the biofilm between the two described periods (high-DOC and low-DOC) may be affected by the differences in seasonality (autumn vs. spring-summer). The increase of green algae filaments (e.g. *Cladophora glomerata*), found in light-growth biofilms during the spring and summer, could be a response of an increase of temperature and light irradiance regime (Lester et al. 1988). As a result of this algal biomass increase, a higher carbon and nitrogen content (and higher C/N ratio) was found in biofilms from the low-DOC period, which could affect the internal carbon recycling within the biofilm (Romaní & Sabater 2000). Because of this, results of exoenzymatic activities were expressed by mgC to account for likely implications of the carbon content between the compartments.

Relation between biofilm age and exoenzymatic activities

The differences found in biofilm age between systems had implications in the biofilm activities, expressed as exoenzymatic activities, and these had subsequent implications in the DOC uptake efficiency. One of the results observed in our study has been that biofilm activity was not proportional to the quantity of fixed biomass, since the exoenzymatic activities expressed by mg of C decreased considerably with the accumulation of biomass, especially in the 12-months biofilms from river system. Furthermore, channel biofilms presented high exoenzymatic activities despite accumulating less biomass than river biofilms. On the other hand, 4- and 12-months biofilms from pipe system, although accumulating higher bacterial density as well as carbon and nitrogen content, did not increase significantly the exoenzymatic activities (expressed by mgC) than those of 2-months old. This was supported by the idea that the activity increases with the thickness of biofilm up to a determined level,

(termed the "active thickness", Kornegay & Andrews 1968; LaMotta 1976). Above this level of biomass, the diffusion of nutrients becomes a limiting factor, thus differentiating an "active" biofilm from an "inactive" biofilm. A stable, thin and active biofilm thus offers numerous advantages in the water and wastewater treatment (Lazarova & Manem 1995). Otherwise, the effect of algal biomass enhancing the heterotrophic metabolism has been reported in several studies (Romaní & Sabater 1999; Romaní & Sabater 2000; Sekar et al 2002). This could explain why the high algal biomass accumulated per surface area in river biofilms supported elevated exoenzymatic activities compared to channel biofilms.

Biofilms growing under a persistent current (channel system) have the direct effect of turbulent mixing on nutrient transport, which stimulate the nutrient and carbon uptake (Stevenson 1996). On the other hand, low EPS accumulation may allow biofilm bacteria to immediately respond to unpredictable carbon pulses in the water (Claret & Fontvieille 1997; Battin et al, 1999). In contrast, biofilms growing in absence of strong physical disturbance (the river biofilms), not only let the accumulation of high cell density, but also allows the biofilm matrix to grow and retain the autochthonous DOC, which will be catabolic processed by extracellular enzymes prior to bacterial uptake (Chróst 1990). Since the development of a polysaccharide matrix might act as a retention of carbon, as well as of extracellular enzymes, giving the biofilms the capacity to buffer the supply of dissolved organic substrates and enhancing the internal DOM recycling (Freeman & Lock 1995; Thompson & Sinsabaugh 2000).

In conclusion, this study underlies the importance of biofilms in the efficiency of DOC and BDOC uptake from the water, both in natural and artificial systems. The evidence that water DOC and BDOC levels enhance the heterotrophic metabolism of the biofilms supports this conclusion. Furthermore, the importance of the biofilm age on the carbon recycling is also shown in the Ebre biofilms, indicating that thick biofilms could have less efficiency in the water DOC uptake.

CHAPTER 3
TEMPORAL AND SPATIAL DYNAMICS OF GEOSMIN PRODUCTION IN THE LLOBREGAT RIVER

INTRODUCTION

Unpleasant odours and tastes in drinking water are a common problem for water suppliers. Sources of contaminants that produce off-flavours can be natural as well as anthropogenic (Zoeteman & Piet 1973). Among the substances produced by biological agents, geosmin is commonly responsible for earthy and musty odour in drinking water (Young et al. 1996).

Several studies have described geosmin production by planktonic cyanobacteria (such as *Anabaena sp*) (Jüttner 1987, Hayes & Burch 1989, Wu et al. 1991, Ploeg & Denis 1992), but records of its production by benthic cyanobacteria are more recent (Izaguirre 1992, Evans 1994, Izaguirre & Taylor 1995, Sugiura et al. 1998). Although it has been described that production of this metabolite is dependent on environmental conditions (Persson 1996), the main factors associated with its production are still unclear. Adverse growth conditions are determinants for geosmin production (Wu & Chou 1991, Jüttner1995). Wu & Jütnner (1988) observed that production was minimal at optimum temperatures, while Naes et al. (1987) determined that transition from light to nutrient-limited growth caused a decrease in production. Nevertheless, most of these studies were carried out in culture conditions, and there is no report which deal with the factors that co-occur with geosmin production in the field. For a clear link to be established between the organisms causing the problem and the dynamics of the metabolite (Persson 1983) it is essential to reveal the conditions allowing mass production of the cyanobacteria and the related spread of the metabolite.

Although geosmin is considered not toxic (in the context of impairing mammalian health), its presence has been described as a growth enhancer for green algae, possibly because its presence could decrease the number of competing bacteria or fungi (Sklenar & Horne 1999). Questions still remain of whether the presence of this metabolite is indicative of more serious problems, e.g. the co-occurrence of toxicants, which could affect consumers' health.

The aim of this study was to determine the ecological factors related with the geosmin production in the Llobregat River, describing the spatial and temporal dynamics of geosmin metabolite all along the river. Different sampling points located along the river were studied and compared, giving an overview of the river section scale. Furthermore, taxonomic determination of the algal species of the benthic cyanobacteria was carried out to determine the producers of geosmin, and in addition, a posterior toxicity assessment was carried out to determine if the co-occurrence of geosmin and toxicity existed in cyanobacterial mats growing in the Llobregat River.

MATERIALS AND METHODS

Historical records

Historical data sources were completed by ATLL (Aigües Ter Llobregat) from January 1998 to May 2001, which covers physical and chemical variables of the raw water used by the water treatment plant (pH, water temperature, conductivity, dissolved oxygen [%], discharge, ammonium, nitrate), collected in the vicinity of site S3. However, monthly values of geosmin were recorded until May 2002.

Sampling strategy

The three sampling stations (S1, S2 and S3), placed along the Llobregat River (see Study Site), were monitored to assess the longitudinal variability of the geosmin dynamics. Physical, chemical and biological data were collected monthly since March 2000 to May 2001. Futhermore, during the geosmin peak (between February and May 2001) sampling intensity was increased to a weekly basis at site S2.

Physical and chemical parameters

Water samples were collected from each sampling site, to analyse physical and chemical parameters (geosmin, pH, water temperature, conductivity, dissolved oxygen concentration and percentage [%], light extinction, water velocity, chloride, sulphate, nitrate and phosphate).

Algal composition and abundance, chlorophyll-a and geosmin content

Samples for algal identification and counting, chlorophyll-a extraction and geosmin analysis in the biofilm were collected by scraping a given surface area of rocks or cobbles. The algal masses developing in littoral, pool and riffle zones at each site were sampled separately.

Statistical analyses

Stepwise multiple regression with forward selection ($p<0.05$) was used to relate geosmin content in the water at ATLL entrance with the physical and chemical variables from the historical record (1998-2001). Direct gradient analysis was used to relate the algal and cyanobacterial taxa with geosmin and the environmental variables of site S2. Analyses were carried out using the package of programs CANOCO 4.0 (Ter Braak 1987). The environmental data collected at site S2 were included in a Redundancy Analysis (RDA) on a correlation matrix of species. Data were logarithmically transformed to enforce linearity of the relationship between species and environmental variables. This type of constrained ordination assumes a linear response of the species along the environmental gradients. RDA was selected because the length of the gradient of the species matrix was less than 5 times the standard deviation (Ter Braak & Prentice 1988). Forward selection of environmental variables was applied to select the minimum set of variables that explained significantly ($p<0.05$) the distribution of algal and cyanobacterial species. Finally, non-linear regression analysis was carried out to elucidate the relationship between the scores of the first RDA axis and the geosmin concentration in the algal mats.

Toxicity assessment

Biofilms were collected by scraping of the benthic biomass from various locations scattered along the main stretch of the Llobregat River, including S1, S2 and S3, but also an extra point called La Rabeia, located between S1 and S2. Collection was made on two different dates (31 January 2001 and 28 March 2001), coinciding with periods known to be of geosmin-free and geosmin-rich waters. Samples for cyanobacterial community analysis were collected and stored in 4% formaldehyde and observed under light microscope, counted and related to percent composition. Other samples were also collected, stored in liquid nitrogen and analysed for geosmin concentration in the biofilm. Finally, separate samples were also stored in liquid nitrogen and lyophilized and sent to RECETOX, Masaryk University, in Brno, Czech Republic, where samples were analysed for health risk assessment.

RESULTS

Historical records of geosmin concentration

Geosmin in water showed alternate periods of high and low concentration since 1998 (Fig.1). The peaks consistently occurred from early winter (late January or February) to late spring (May or June). Absolute geosmin maxima differed between years, the highest concentration occurring at March 2002 (190 ng/L). During the low periods of production (summer and fall) geosmin ranged from nearly zero values in 1998 to below 20 ng/L in 1999 and 2000.

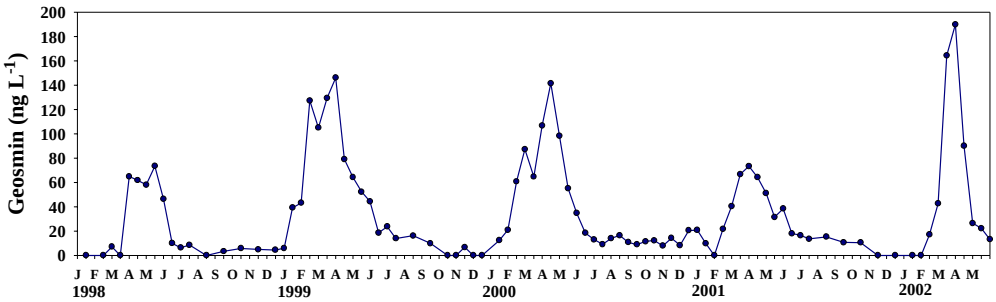

Fig. 1. Annual pattern of geosmin concentration in the water of the Llobregat River the vicinity of site S3, from January 1998 to May 2002.

Table 1. Mean and standard deviation (in parentheses) of physical and chemical characteristics of the Llobregat River at the vicinity of site S3 from January 1998 to May 2002.

		Winter (n=25)	Spring (n=60) *(n=55)	Summer (n=14)	Autumn (n=18)
Geosmin	[ng L^{-1}]	39.4 (37.3)	54.5 (34.5)	10.7 (4.7)	8.1 (6.4)
pH		7.9 (0.2)	7.9 (0.1)	7.9 (0.2)	7.9 (0.2)
Temperature	[ºC]	11.2 (2.9)	18.3 (2.7)	23.9 (1.1)	15.1 (4.6)
Conductivity	[µS cm^{-1}]	1415.4 (238.3)	1492.5 (381.9)	1418.6 (148.2)	1358.0 (207.4)
O_2 (percent.)	[%]	68.1 (14.1)	52.7 (14.1)	50.4 (14.3)	69.1 (9.5)
Discharge	[m^3 s^{-1}]	8.1 (3.6)	8.0* (2.6)	6.7 (1.2)	8.2 (1.6)
NH_4^+	[mg L^{-1}]	0.61 (0.42)	0.20 (0.11)	0.13 (0.07)	0.38 (0.37)
NO_3^-	[mg L^{-1}]	12.43 (2.06)	10.13 (1.51)	6.08 (1.65)	10.05 (1.30)

The relationship between geosmin and the environmental variables (Table 1) measured during the period between 1998-2001 was described by the following expression obtained by means of a multiple regression analysis:

Geosmin = 263.11 – 1.19 Dissolved oxygen (%) – 6.63 Temperature – 75.65 Ammonium –2.37 Discharge (adjusted R^2 = 0.47, p-value = 0.00001).

Ammonium and oxygen were highly correlated to temperature (R = -0.72, and -0.39, respectively, p <0.001).

Temporal and spatial changes of geosmin concentration

Physical and chemical characteristics of the water. There were some characteristics which changed downstream (Table 2). Conductivity reached 1897 µS cm^{-1} in S3 while in S1 was about 600 µS cm^{-1}. Temperature was 5-7ºC higher in S3 than S1. Nutrient concentration in S3 was the highest of the three sites. Seasonal patterns coincided with a geosmin production (GP) period (winter and spring) and a no GP period (summer and autumn). Differences between seasons were noticed by a decrease of Nitrates and N/P ratio after winter, which occur at the three sites.

Geosmin concentration dynamics. The geosmin production period (GP) had distinct temporal patterns in the three study sites (Fig.2). Production was noticeable first at the downstream site (S3), where it had a longer duration. The highest concentration (75ng L^{-1}) at this site occurred on 21st March 2001. In contrast, the most upstream site (S1) had the shortest and most intense period of production, the highest peak occurring on 18th April 2001 and reaching 155 ng L^{-1} (Fig.2). Evolution at site P2 was intermediate between the two other sites, a maximum of 110 ng L^{-1} occurring on 28th March 2001. During the GP period the water temperature ranged between 11.6 and 15.2ºC and the N/P ratios were low (Table 3).

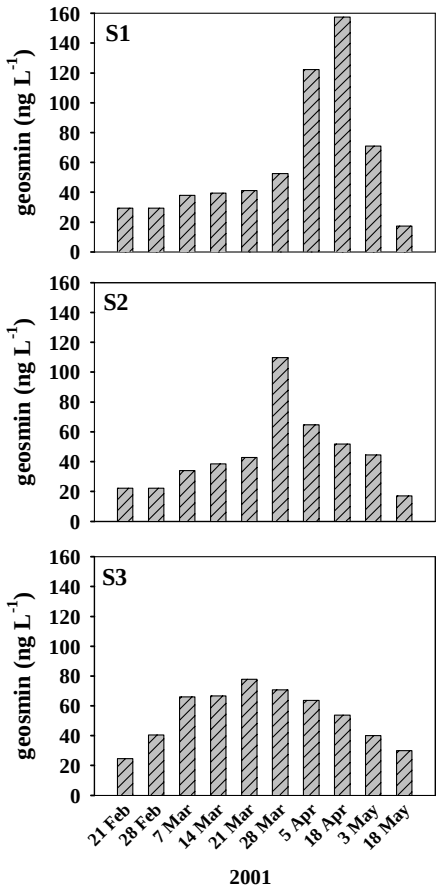

Fig. 2. Geosmin concentration at the study sites along the Llobregat River (sites S1, S2 and S3) during the period of geosmin production from February to May 2001.

Table 2. Physical and chemical characteristics of the water along the Llobregat River (sites S1, S2 and S3) from March 2000 to May 2001. Values are means and standard deviations (in parentheses). N:P is expressed as the molar ratio.

		Winter	Spring	Summer	Autumn
S1		(n=8) *(n=5)	(n=7) *(n=4)	(n=3)	(n=3)
Geosmin	[ng L^{-1}]	30.3 (9.9)	71.5 (52.7)	16.2 (6.0)	15.4 (1.4)
pH		8.5 (0.2)	8.4 (0.1)	8.2 (0.1)	8.4 (0.1)
Temperature	[ºC]	8.3 (2.2)	12.3 (2.0)	16.5 (0.9)	9.7 (5.1)
Conductivity	[µS cm^{-1}]	614.9 (55.8)	624.1 (31.6)	566.0 (11.1)	626.7 (59.0)
O_2 (conc.)	[mg L^{-1}]	12.0 (1.5)	10.3 (1.0)	7.5 (1.2)	10.6 (1.5)
O_2 (percentage)	[%]	105.9 (13.2)	98.7 (7.1)	78.5 (12.3)	97.0 (5.8)
Light extinction	[m^{-1}]	0.49 (0.10)	0.51 (0.31)	0.45 (0.15)	0.32 (0.03)
Velocity	[cm s^{-1}]	42.3 (7.1)	33.8 (12.3)	33.0 (2.8)	50.3 (45.2)
Cl^-	[mg L^{-1}]	34.1 (13.4)	42.9 (10.1)	44.4 (23.9)	43.2 (14.5)
SO_4^{2-}	[mg L^{-1}]	91.3 (13.9)	84.4 (33.8)	133.9 (13.4)	102.4 (3.4)
NO_3^-	[mg L^{-1}]	3.64 (0.98)	2.17 (0.85)	2.30 (0.80)	1.01 (0.29)
PO_4^{3-}	[mg L^{-1}]	0.06 *(0.03)	0.13 *(0.03)	n.m	0.17 (0.06)
N:P		118.3 *(81.9)	32.4 *(25.6)	n.m	9.3 (0.6)
S2		(n=8) *(n=7)	(n=7) *(n=4)	(n=3)	(n=3)
Geosmin	[ng L^{-1}]	40.8 (35.4)	105.2 (129.8)	13.1 (1.2)	13.3 (5.8)
pH		8.8 (0.1)	8.5 (0.2)	8.3 (0.1)	8.7 (0.1)
Temperature	[ºC]	9.8 (3.2)	16.2 (2.4)	21.6 (1.5)	10.8 (5.4)
Conductivity	[µS cm^{-1}]	1241.0 (97.6)	1278.6 (189.1)	1281.0 (270.1)	1275.3 (218.6)
O_2 (conc.)	[mg L^{-1}]	13.7 (1.2)	11.1 (1.5)	8.8 (1.2)	12.8 (1.8)
O_2 (percentage)	[%]	122.5 (9.3)	115.0 (13.4)	103.2 (16.1)	116.6 (8.2)
Light extinction	[m^{-1}]	0.47 (0.06)	0.44 (0.189	0.39 (0.1)	0.39 (0.16)
Velocity	[cm s^{-1}]	65.6 (11.1)	59.9 (14.4)	54.0 (17.0)	66.3 (12.8)
Cl^-	[mg L^{-1}]	176.5 (20.7)	224.7 (35.6)	276.0 (89.7)	220.9 (56.6)
SO_4^{2-}	[mg L^{-1}]	137.5 (6.3)	134.1 (34.0)	164.0 (25.0)	123.8 (10.5)
NO_3^-	[mg L^{-1}]	3.86 (1.73)	3.13 (0.63)	2.52 (0.59)	1.22 (0.39)
PO_4^{3-}	[mg L^{-1}]	0.09 (0.04)	0.34 *(0.04)	n.m	0.20 (0.05)
N:P		82.7 (62.3)	13.8 *(2.8)	n.m	9.3 (0.8)
S3		(n=6) *(n=5)	(n=5) *(n=3)	(n=3)	(n=3)
Geosmin	[ng L^{-1}]	58.5 (47.3)	58.3 (45.3)	12.4 (1.4)	16.8 (4.9)
pH		8.4 (0.2)	8.4 (0.2)	8.4 (0.2)	8.3 (0.1)
Temperature	[ºC]	11.0 (2.5)	19.5 (3.2)	24.0 (2.2)	12.4 (5.6)
Conductivity	[µS cm^{-1}]	1421.2 (50.0)	1766.2 (501.2)	1897.3 (911.7)	1320.3 (100.8)
O_2 (conc.)	[mg L^{-1}]	11.5 (1.6)	10.7 (2.6)	10.2 (3.0)	10.9 (1.7)
O_2 (percentage)	[%]	105.8 (16.7)	114.5 (22.0)	122.9 (41.4)	101.6 (7.0)
Light extinction	[m^{-1}]	0.50 (0.10)	0.50 (0.13)	0.50 (0.18)	0.36 (0.30)
Velocity	[cm s^{-1}]	68.4 (12.7)	51.3 (7.4)	47.7 (30.9)	78.0 (14.6)
Cl^-	[mg L^{-1}]	224.8 (25.3)	349.6 (223.1)	487.3 (328.5)	226.6 (20.4)
SO_4^{2-}	[mg L^{-1}]	142.1 (6.9)	130.2 (52.5)	188.9 (36.2)	130.7 (5.2)
NO_3^-	[mg L^{-1}]	7.60 (1.66)	6.98 (1.24)	4.86 (1.28)	3.62 (1.3)
PO_4^{3-}	[mg L^{-1}]	0.30 *(0.10)	0.47 *(0.06)	n.m	0.50 (0.07)
N:P		44.7 *(21.7)	20.2 *(2.1)	n.m	11.0 (2.8)

Table 3. Means and standard deviations (in parentheses) of physical and chemical characteristics of the water along the Llobregat River (sites S1, S2 and S3) during the peak of geosmin (geosmin concentration > 60 ng L^{-1}) and before the peak phases, during the geosmin production period of 2001 (from January to May 2001). N:P is expressed as the molar ratio.

		S1		S2		S3	
		before peak 10 Jan - 28 Mar (n=8)	**during peak** 5 - 18 Apr (n=2)	**before peak** 10 Jan - 21 Mar (n=7)	**during peak** 28 Mar - 5 Apr (n=2)	**before peak** 10 Jan - 21 Feb (n=3)	**during peak** 7-Mar - 5 Apr (n=3)
Geosmin	[ng L^{-1}]	32.7 (12.7)	139.8	28.7 (9.6)	87.2	22.1 (2.2)	69.3 (7.6)
pH		8.5 (0.2)	8.4	8.8 (0.1)	8.6	8.5 (0.2)	8.5 (0.2)
Temperature	[ºC]	8.4 (0.2)	12.2	9.6 (3.4)	15.7	9.1 (1.3)	14.8 (2.1)
Conductivity	[µS cm^{-1}]	624.9 (56.5)	650.0	1245.4 (104.6)	1508.5	1399.0 (37.6)	1504.3 (163.4)
O_2 (conc.)	[mg L^{-1}]	11.9 (1.7)	11.2	13.8 (1.3)	12.6	12.0 (0.3)	12.9 (1.3)
O_2 (percentage)	[%]	104.0 (14.1)	103.8	122.9 (9.9)	129.2	103.7 (4.0)	130.8 (14.7)
Light extinction	[m^{-1}]	0.46 (0.11)	0.28	0.48 (0.05)	0.35	0.45 (0.13)	0.44 (0.14)
Velocity	[cm s^{-1}]	41.0 (9.4)	28.3	68.5 (7.9)	56.3	70.2 (16.3)	62.3 (18.3)
Cl^-	[mg L^{-1}]	37.60 (13.4)	53.8	172.30 (18.4)	268.9	211.60 (11.7)	195.8 (46.2)
SO_4^{2-}	[mg L^{-1}]	86.10 (10.1)	98.0	136.50 (6.1)	130.9	141.10 (10.5)	117.9 (41.4)
NO_3^-	[mg L^{-1}]	3.88 (0.73)	1.80	3.99 (1.82)	3.6	8.61 (1.17)	6.35 (1.52)
PO_4^{3-}	[mg L^{-1}]	0.07 (0.03)	0.13	0.09 (0.04)	0.36	0.24 (0.04)	0.44 (0.12)
N:P		110.20 (75.9)	22.4	82.70 (62.3)	15.1	57.80 (17.5)	22.7 (4.3)

Development and taxonomic composition of algal mats

Development of the algal mats. The riverbed became progressively covered by thick algal mats during the geosmin episode. These algal mats mainly developed on sediments and cobbles, especially in pools and littoral zones (Fig.3A). The mats were primarily benthic (Fig.3B), but they reached a point when they collapsed, detached and drifted downstream, becoming free-floating mats (Fig.3C).

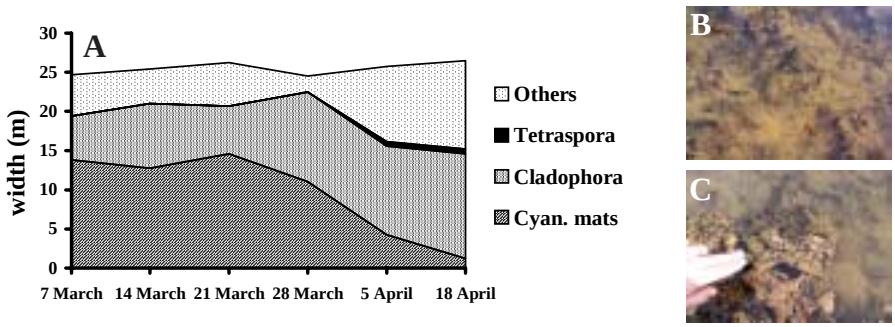

Fig. 3. Algal cover at the site S2 during the period of geosmin production of 2001 (A), indicating distinct patches: cyanobacterial mats (cyan. mats), *Cladophora glomerata*, *Tetraspora sp.* and others. Pictures of the attached cyanobacterial compartment (B) and free-floating cyanobacterial mats (C).

Table 4. Total density, chlorophyll-*a* content (chl-*a*) and species composition (in %) of the benthic community at site S2, from March 2000 to May 2001.

	2000										2001											
	6 Mar	4 Apr	2 May	1 Jun	6 Jul	10 Aug	13 Sep	4 Oct	13 Nov	18 Dic	10 Jan	7 Feb	21 Feb	28 Feb	7 Mar	14 Mar	21 Mar	28 Mar	5 Apr	18 Apr	3 May	18 May
Total density [cells cm^{-2}] x10^{6}	1.5	1.0	0.5	0.4	0.9	0.2	0.9	0.5	1.4	0.9	0.9	0.4	0.6	10.4	11.5	5.6	1.7	39.4	7.4	9.7	3.5	0.3
Chl-*a* [µg cm^{-2}]	17.1	6.5	11.2	11.3	3.9	6.7	3.5	21.0	28.9	43.0	109.4	38.6	22.8	19.6	73.3	13.8	32.7	80.9	25.8	28.3	13.8	100.0
CYANOBACTERIA																						
Dermocarpa sp			8.4					0.3														
Heteroleibleinia sp					40.0																	
Lyngbya martensiana (Menegh. ex Gom.)				5.1																		
Oscillatoria limosa (Agardh ex Gomont)	10.4	24.0	9.6							0.3		9.0	6.1	15.5	69.2	42.2	58.3	95.0	86.0	81.3	84.8	9.0
O. aff *tenuis* (morph 1) (Ag. ex Gom.)	2.7		9.6		6.5	41.4	77.0	77.8	57.2	18.4	1.1	3.8	1.5		19.0	1.7			3.5	7.7	6.5	
O. aff *tenuis* (morph 2) (Ag. ex Gom.)	2.8	15.3	11.6		32.9				6.3	4.6	0.7	7.5			8.9	39.5	5.2	2.8	3.4	3.9	2.7	
Phormidium sp				1.9		27.3	7.2															10.1
Pleurocapsa fluviatilis (Lagerheim)				17.7																		
ALGAE (non diatoms)																						
Cladophora glomerata ((Linn.) Kütz.)		2.3	1.4	9.4				0.3	0.1				1.0	0.1		0.1		0.04		0.4	0.2	17.1
Klebsormidium sp														75.6		7.4	10.8					
Oedogonium sp	0.9	3.0	1.8										2.9				5.2	0.4				4.2
Pleurodiscus sp														0.3								
Spirogyra sp									0.04												0.3	
Ulothrix zonata ((Weber et Mohr.) Kütz.)									0.1	0.05				0.5			1.3					
DIATOMS	83.1	55.4	57.6	65.9	20.5	31.3	15.8	21.6	36.3	76.6	98.2	79.7	88.6	8.0	2.9	9.1	19.2	1.7	7.1	6.7	5.5	59.6

Taxonomic composition. The dominant taxa in the mats were *Oscillatoria limosa* (Agardh ex Gomont) (cell dimensions of 15 µm width x 2.5-4 µm height) (Fig.4B) and two morphotypes of *Oscillatoria* aff. *tenuis* (Agardh ex Gomont) (cell dimensions of morph.1: 7.5-9.6 µm width x 4-5 µm height , and of morph.2: 5-8 µm width x 1.5-2.4 µm height). While *Oscillatoria limosa* did not occur in the river during the no GP period, *O.* aff. *tenuis* occurred in summer and autumn (Table 4). Algal cell densities were maximal during the mat development, reaching a maximum of 40 10^{6} cells cm^{-2}. During the no GP period, diatoms, green algae and other cyanobacteria were dominant on the river bed substrata, but their cell density was much lower (Table 4).

The RDA based on algal and cyanobacterial taxa performed with data from site S2 (including the GP and no GP periods of 2000-2001) accounted for 28.4% of the species variance. This RDA showed a gradient of velocity and dissolved geosmin in the water which was associated with the occurrence of distinct taxa of cyanobacteria. *Oscillatoria limosa* and *O.*aff *tenuis* (morph. 2) were related to high geosmin concentration and low water velocity (Fig.4A). In contrast, other cyanobacteria (*Pleurocapsa fluviatilis*, *Phormidium* sp. and *Lyngbya martensiana*) were correlated with higher water velocity and absence of geosmin. Axis 1 of the RDA was significantly regressed with geosmin concentration inside the mat. The regression expression fitted an exponential decay relationship (adjusted R^2 = 0.387), which emphasised the swift decrease in dissolved geosmin when water velocity increased and *O.limosa* disappeared (Fig.5).

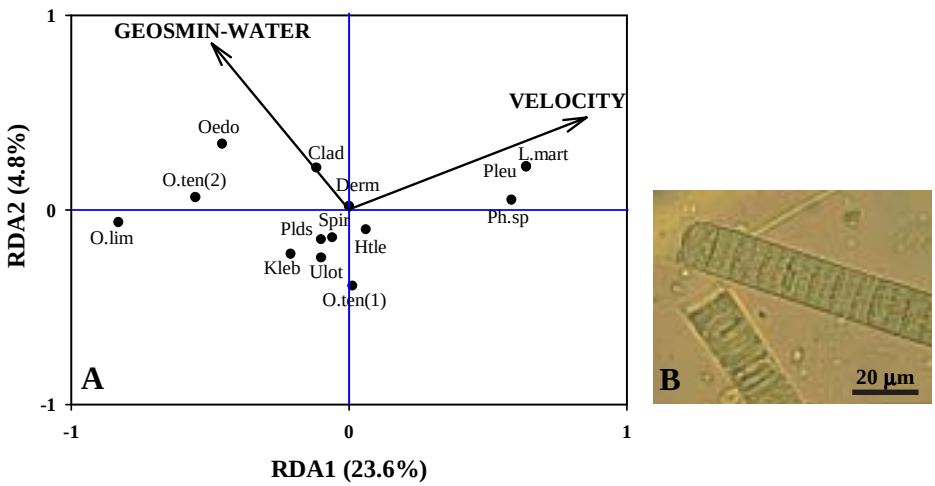

Fig. 4. (A) Redundancy analysis based on algal and cyanobacterial taxa in site S2. (Derm = *Dermocarpa sp*; Htle = *Heteroleibleinia sp*; L.mart = *Lyngbya martensiana*; O.lim = *Oscillatoria limosa*; O.ten(1) = *Oscillatoria* aff *tenuis* (morph.1); O.ten (2) = *Oscillatoria* aff *tenuis* (morph.2); Ph.sp = *Phormidium sp*; Pleu = *Pleurocapsa fluviatilis*; Clad = *Cladophora glomerata*; Kleb = *Klebsormidium sp*; Oedo = *Oedogonium sp*; Plds = *Pleurodiscus sp*; Spir = *Spirogyra sp*; Ulot = *Ulothrix zonata*).
(B) Picture from light microscope of *Oscillatoria limosa*.

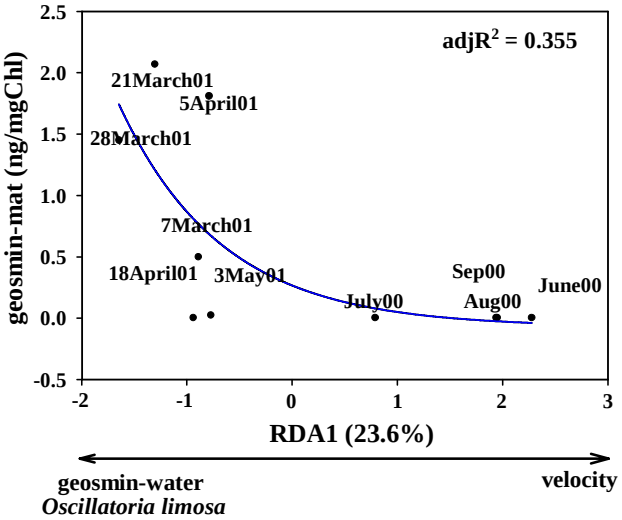

Fig. 5. Regression of geosmin concentration in the mat with respect to the first axis of the RDA (see Fig.4)

Toxicity assessment of cyanobacterial mats

The community composition of non-geosmin producer samples was dominated by diatoms, but complete dominance by cyanobacteria characterised the geosmin-producing biofilms (Table 5). Differences in organic matter content between the biofilms considered were not substantial (Table 5). In periods of occurrence of the non-geosmin-producing biofilms (0 ng geosmin $mgChl^{-1}$), geosmin in the water ranged from 16.1 to 20.3 ng L^{-1}. However, when geosmin was detected in the biofilms (0.34-1.45 ng geosmin $mgChl^{-1}$), geosmin in the water reached between 61 to 110 ng L^{-1}.

The results of HPLC analyses for microcystins and the toxic effects of biofilm extracts are summarized in Table 5. HPLC analyses showed that microcystins were absent both in samples with and without geosmin. Screening of the toxicities showed that biofilms elicit relatively weak effects in most of the in vitro assays (Table 5). Three out of five tested biofilms had no significant effect in any of assays. Only partial inhibitions or weak toxicities (+/-) were observed at two other biofilm samples (Table 5).

Table 5. Proportion of organic matter (in %), geosmin concentration inside the mat, algal community composition and abundance (in %) and toxicity assessment of biofilm samples. Non-geosmin samples from S1, S2 and S3 (31/01/2001) and geosmin producer samles from 'La Rabeia' and S2 (28/03/2001). (+) traces of microcystin-like peptides detected (microcystin absorbtion spectra), (+/-) weak toxic effect in the highest doses tested, (-) no toxic effect. (Results of toxicity assessment from RECETOX laboratories)[1].

	geosmin-free 31/01/2001			**geosmin-rich** 28/03/2001	
	S1	**S2**	**S3**	**"La Rabeia"**	**S2**
Organic Matter (%)	9.6	16.2	36.4	19.6	12.7
Geosmin (ng µgChl^{-1})	0.0	0.0	0.0	0.34	1.45
% CYANOBACTERIA					
Oscillatoria limosa + O. tenuis	-	-	-	97.0	98.0
Phormidium sp	1.0	3.0	1.0	-	-
% ALGAE					
Cladophora glomerata	1.0	2.0	-	-	-
Vaucheria sp	-	-	-	-	1.0
% DIATOMS	98.0	95.0	99.0	3.0	1.0
(toxicity assessment)					
HPLC - MCYSTIN	-	-	-	-	-
Cytotoxicity	-	-	-	+/-	-
Hepatotoxicity	-	-	+/-	+/-	-
GSH	-	-	+	+	-
Neurotox (cells)	-	-	-	-	-
Neurotox (AcE)	-	-	-	+/-	-
Immunotox (MTT)	-	-	+/-	-	-
Immunotox (Thy)	-	-	+/-	+/-	-
Mutagenicity	-	-	-	-	-

DISCUSSION

Environmental factors related with geosmin production

Several environmental factors have been identified to co-occur with the increase of geosmin in the Llobregat River. Temperature and water flow (and its related parameter water velocity) are the most critical physical factors that determine the

[1] RECETOX laboratories, Masaryk University, Brno, Czech Republic

wax and wane of geosmin occurrence in the river. The exponential phase of the GP period (between January and February of each year) occurred at a river water temperature between 6.6 and 14.5 ºC and water discharges approaching basal flow. The combination of these two factors, together with the mass development of *Oscillatoria limosa,* mats may define the timing of the geosmin maximum which was characteristic of the sites.

Geosmin producers

The analyses carried out in mats of *O. limosa* and *O.* aff. *tenuis* showed higher geosmin concentration inside the mat than in others mats where these taxa were not dominant. These two taxa have previously been described as geosmin producers (Paerl & Millie 1996). The significant correlation between the geosmin content in the biofilm and that occurring in the water and with the community inhabiting the river at that time (summarised by the first axis of the RDA, Fig.4) implies that *Oscillatoria limosa* were responsible for the geosmin production which deteriorates the quality of the Llobregat waters. On the other hand, a background geosmin concentration was detected throughout the year (Table 1 and 2), when *O. limosa* was absent but *O.* aff. *tenuis* was present. It can be assumed that the continuous presence of this cyanobacteria may have produced this background concentration.

Geosmin is retained in cells and released upon their degradation (Wu & Jüttner 1988). Although the factors that cause the degradation of the cells are still unknown, we believe that they may be biological. The high proportion of Nematoda in the geosmin-producing mat (Gaudes et al., unpublished) indicate that these organisms may contribute to dislodging the mat, thereby significantly affecting cell integrity. The masses therefore slough off and drift downstream in a process which is characteristic of *Oscillatoria* mats (Komárek 1992). This detachment and further transport may have major consequences for the dynamics of geosmin in the river. Water velocity, which was determined by the RDA as one of the environmental factors that could affect geosmin concentration in water, and the occurrence of geosmin-producing cyanobacterial mats could be coupled to the biological processes behind the wax and wane of these masses in the river. These mats developed in pools located in the littoral zone where the water velocity was very low (2.3 ± 5.0 cm s^{-1}). The significance of this for the entire river system is obvious given that analogous zones are scattered along the river (Muñoz & Prat 1996), and therefore constitute potential areas for geosmin production. The temporal pattern of geosmin occurrence in the water at the most downstream site (S3) indicate the relevant function of water transport

while at upstream production predominated transport. The later occurrence of GP period at upper sites also underlies the preference for lower temperatures at a particular date and provides further insight into the role of this parameter in determining geosmin occurrence.

In conclusion, this study shows the importance of several physical factors in favouring the mass development of geosmin producing cyanobacteria in littoral areas of the river, which may decisively affect its water quality. While the ecological causes surrounding the thriving of geosmin producing cyanobacteria are clear, the reasons for their increase and decline in the areas and periods will require a scale of observation that includes physiological and microstructural processes (Sabater et al. 2001). Laboratory studies have indicated that nutrient stress (Naes et al. 1988, Wu & Chou 1991) and interactions with grazers could be influential for geosmin to be produced (Paerl & Millie 1996). Therefore subsequent approaches should focus on the interactions occurring at the habitat scale, in order to produce reliable predictions of geosmin production in the river.

Toxicity assessment

On the other hand, the toxicological assessment carried out with cyanobacterial mat samples from Llobregat River, concluded that no significant health toxicity was found in these mats. The toxicity effects were weak, regardless of whether they were producers of geosmin or not, therefore, they can not be related to the occurrence of the geosmin metabolite production. No microcystin-like compounds were detected by HPLC in any of the biofilm samples. The mutagenicity and neurototoxicity was negligible, and weak adverse effects of biofilm extracts detected in the assays. Only two out of the five samples presented traces of GSH levels (a marker of the oxidative stress) and elicited weak or partial toxicity patterns in several assays, however, they were not environmentally relevant. The potential adverse effects of external pollutants which are known to accumulate in the periphyton biomass should also be considered when estimating the overall toxicity of complex biofilms (Whitton & Kelly 1995). In conclusion, the production of the geosmin metabolite can not be taken as a given indication of the existence of toxicity of any kind.

CHAPTER 4
ECOLOGICAL PARAMETERS AND ALGAL COMMUNITY EVOLUTION RELATED TO WATER GEOSMIN DYNAMICS

INTRODUCTION

While factors associated with cyanobacteria occurrence and its wax and wane in the water column have been exhaustively studied in lakes (Reynolds 1999), much less information has been gathered on benthic mass growths. While planktonic cyanobacteria have often been found to be related to toxin occurrence (Codd 1995), this link is much less common in benthic habitats (but see Mez et al. 1998, Baker et al. 2001, Hamill 2001). Cyanobacterial mass growths in rivers may be associated with water quality problems (Sabater et al. 2000). In many instances, mass development of cyanobacteria have been associated with odours and flavours in the water (Izaguirre & Taylor 1995, Young et al. 1996). Benthic mats may undergo a process involving growth, the occupation of the river surface area and subsequent detachment and drift.

Geosmin dynamics in the Llobregat River was related to the waxing and waning of benthic cyanobacteria mats developing along the river, and Oscillatoria limosa being as the main suspected responsible for the geosmin production (see Chapter 3). The geosmin episodes coincided with the progressive development of thick cyanobacterial mat on sediments and cobbles, especially in pools and littoral zones, and a posterior phase of detachment and transport downstream, affecting the dispersion of the metabolite.

For a clear link to be established between the organisms causing the problem and the dynamics of the metabolite (Persson 1983), it is essential to reveal the conditions allowing mass production of the cyanobacteria and the related spread of the metabolite. However, it is not yet clear in which way the dynamics of the cyanobacterial mats are related to the spread of the metabolite throughout the river.

This study is addressed to know the mechanisms involved in the formation and distribution of the cyanobacterial masses, contributing to the formulation of the appropriate corrective measures for minimising the extraordinary abundance of geosmin in shallow river waters. The field study was carried out in one sampling point located in the middle stretch of the Llobregat River. Within this scale, a section of the river was analysed in detail, covering all the habitats, in order to assess the ecological parameters related with the wax and wane of the benthic cyanobacterial masses.

MATERIALS AND METHODS

Sampling strategy

At site S2 in the Llobregat River, geosmin, cyanobacterial communities and environmental factors were monitored weekly during winter and spring of 2002 (January to May 2002). Monitoring looked into the possible differences between habitats in the river (riffles vs. littoral areas).

Physical and chemical parameters

Physical and chemical measurements were performed separately in the littoral and current (riffle) areas. At each sampling date, geosmin, pH, water temperature, dissolved oxygen, light extinction, discharge, water velocity, DOC, chloride, sulphate, nitrate, nitrite, ammonium and phosphorus were thus measured in both habitats.

Algal composition and abundance, chlorophyll-a and geosmin content

Algal mat samples were collected with a small corer (3.1 cm^2) which was protruded in the attached and the free-floating biofilms. Samples (three replicates) were fixed in 4% formaldehyde to study the community's composition and abundance. Other three replicates were collected for chlorophyll-a density and geosmin content inside the biofilm.

River cartography

A rectangular region, comprising 30 m downstream and 45 m river width, was monitored weekly at site S2 from 8 January to 28 May 2002, excepting one week in April and two weeks in May due the high discharge of water, and as a consequence was not possible to cover the measures of the river. Measures of depth, water velocity and water temperature were recorded every 10 m downstream and every meter covering the whole river width, including all the littorals, pools and riffles zones of the river. In addition, algal distribution, expressed as percentage of river cover, was recorded at every point of the grid. Algal monitoring included attached and free-floating cyanobacteria, diatoms, mixed (green algae + diatoms) and green algae.

Environmental parameters and algal distribution were subsequently mapped using SURFER 6.0 computer software (Golden Software, Inc., USA), which plotted the points and generated a satisfactory contour map.

Statistical analyses

A direct ordination analysis (Redundancy Analysis, RDA) was carried out on the percentage surface area covering the various algal patches and the environmental variables. Redundancy Analysis (RDA) was selected because the species matrix length was lower than 5 times the standard deviation. 47 samples, 8 biological variables and 10 environmental variables were involved in the analysis. The data for the species matrix were log-transformed. The algal communities were considered separately between the littoral and riffle zones.

RESULTS

Physical and chemical characteristics of the water

Geosmin production period at site S2. Even though basal geosmin concentration (ca. 15 to 20 ng L^{-1}) occurred throughout the year (see Chapter 3), the geosmin episode was defined by values from 60 to 200 ng L^{-1}. The geosmin production (GP) period in 2002 occurred from 19 February to 2 April and reached up to 204 ng L^{-1} (Fig.1). The temperature during the studied period (from 8 January until 20 May 2002) ranged between 5.6 and 15.3ºC (Table 1). Water flow during the GP period was low (0.25-0.27 $m^3 s^{-1}$) and waters were characterised by high phosphorous and low dissolved inorganic nitrogen levels (low N/P ratios) (Table 1).

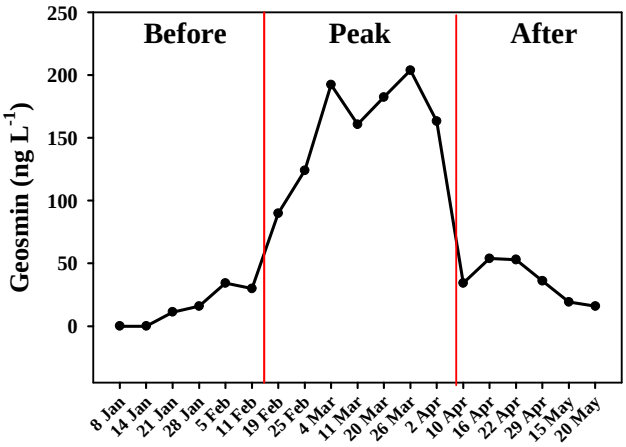

Fig. 1. Geosmin concentration at site S2 during the geosmin production period of 2002 (from January to May). Geosmin peak phase defined as geosmin concentration > 60 ng L^{-1}.

Table 1. Mean and standard deviations (in parentheses) of physical and chemical characteristics at site S2 during the peak (geosmin concentration > 60 ng L^{-1}), before and after the peak phases of 2002 (from January to May). N:P is expressed as the molar ratio. Significant differences between periods are indicated by asterisks: ***p<0.001, **p<0.01, *p<0.05.

		Before 8 Jan - 11 Feb (n=6)		**Peak** 19 Feb - 2 Apr (n=7)		**After** 10 Apr - 20 May (n=6)	
Geosmin	[ng L^{-1}]	15.3	(14.6)	159.5***	(40.2)	35.4	(16.1)
pH		8.3	(0.1)	8.3	(0.2)	8.2	(0.3)
Temperature	[ºC]	5.6***	(1.1)	10.8***	(2.8)	15.3***	(2.4)
O_2 (conc.)	[mg L^{-1}]	12.8***	(0.8)	10.6	(0.8)	9.8	(0.8)
O_2 (percentage)	[%]	101.6	(4.7)	97.1	(8.5)	99.2	(7.6)
Light extinction	[m^{-1}]	0.56	(0.03)	0.65	(0.24)	0.94	(0.29)
Discharge	[$m^3 s^{-1}$]	0.25	(0.04)	0.27	(0.04)	0.43*	(0.18)
Velocity	[cm s^{-1}]	38.8	(7.1)	33.0	(9.4)	38.1	(6.5)
DOC	[mg L^{-1}]	1.75	(0.2)	3.64	(2.7)	3.41	(0.60)
Cl^-	[mg L^{-1}]	302.5**	(19.9)	244.1**	(30.9)	185.5**	(48.0)
SO_4^{2-}	[mg L^{-1}]	98.0	(34.5)	101.0	(12.4)	76.6	(13.2)
NO_3^-	[mg L^{-1}]	6.08	(0.64)	3.95***	(0.84)	5.49	(0.91)
NO_2^-	[mg L^{-1}]	0.33***	(0.13)	0.12	(0.02)	0.16	(0.03)
NH_4^+	[mg L^{-1}]	0.18	(0.12)	0.11	(0.03)	0.41	(0.64)
PO_4^{3-}	[mg L^{-1}]	0.41	(0.07)	0.41	(0.08)	0.39	(0.06)
N:P		27.3	(5.4)	17.4**	(3.7)	27.3	(5.2)

There were some differences between the phases before and after the peak (Table 1). Nitrites were higher before the peak, and the temperature increased during the studied period and reached 10.8ºC at the peak. Nitrates and N/P ratio decreased at the peak. A high geosmin concentration in the water was negatively related to all forms of inorganic nitrogen (nitrate, nitrite and ammonia). Geosmin values above 60ng L^{-1} were related to N/P values below 25 (Fig.2). Oxygen concentration was higher before the peak and an increase of discharge (average 0.43 $m^3 s^{-1}$) co-occurred during the phase of decrease.

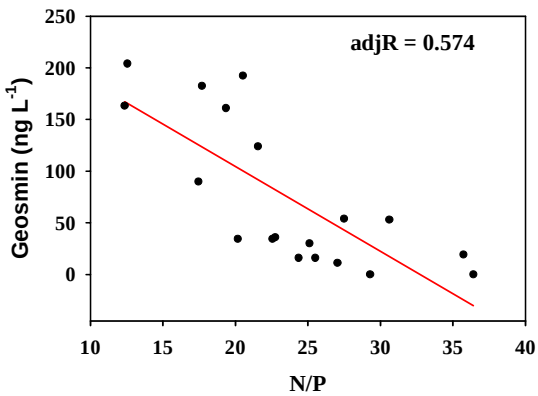

Fig. 2. Relationship between geosmin concentration and N/P ratio in water from Llobregat River at site S2.

Littoral and Riffles zones. Differences in physical characteristics between habitats (riffle and littoral areas) were reflected in significantly lower water velocity in the littoral zones (one-way ANOVA, p=0.00001) and lower dissolved oxygen in riffles than in littoral zones (one-way ANOVA, p=0.034) (Table 2). However, no significant differences were observed with the rest of chemical parameters (Table 2). Light availability was slightly higher in the littoral than in the riffle areas (Table 2).

Table 2. Physical and chemical characteristics of riffles and littoral zones from Llobregat River at site S2 during the geosmin peak period of 2002 (from 19 February to 2 April). Values are means and standard deviations (in parentheses). N:P is expressed as the molar ratio. Significant differences between riffle and littoral zones are indicated for each variable by asterisks: ***p<0.001, **p<0.01, *p<0.05.

		Riffle (n=7)		**Littoral** (n=7)	
Geosmin	[ng L^{-1}]	159.5	(40.2)	159.5	(40.2)
pH		8.3	(0.2)	8.3	(0.4)
Temperature	[ºC]	10.8	(2.8)	13.2	(4.0)
O_2 (conc.)	[mg L^{-1}]	10.6*	(0.8)	14.3*	(3.3)
O_2 (percentage)	[%]	97.1*	(8.5)	141.1*	(45.6)
Light extinction	[m^{-1}]	0.65	(0.24)	1.11	(0.74)
Velocity	[cm s^{-1}]	33.0***	(9.4)	1.0***	(0.7)
DOC	[mg L^{-1}]	3.64	(2.70)	2.79	(0.96)
Cl^-	[mg L^{-1}]	244.1	(30.9)	247.5	(26.0)
SO_4^{2-}	[mg L^{-1}]	101.0	(12.4)	99.3	(6.5)
NO_3^-	[mg L^{-1}]	3.95	(0.84)	3.78	(0.97)
NO_2^-	[mg L^{-1}]	0.12	(0.02)	0.11	(0.02)
NH_4^+	[mg L^{-1}]	0.11	(0.03)	0.09	(0.02)
PO_4^{3-}	[mg L^{-1}]	0.41	(0.08)	0.38	(0.05)
N:P		17.4	(3.7)	17.3	(4.5)

Evolution of the algal mats communities during the geosmin period

The evolution of the environmental parameters (water depth, velocity and temperature) as well as algal distribution, week by week / fortnightly, from 8 January to 28 May 2002 are represented in the contour maps showed in Fig.3. The geosmin production peak period (geosmin concentration > 60 ng L^{-1}) corresponds the maps from 19 February to 2 April 2002. The depth contour maps (Fig.3A) represented the depth evolution of the river. Water velocity in riffles reached up to 110 cm s^{-1}, while in littoral zones it did not overcome 1 cm s^{-1}. Water temperature (Fig.3C) was characterised by values under 10ºC from 8 January to 11 February 2002, coinciding with the phase before the GP period. These values started to rise after 19 February, reaching up to 20ºC in the littoral zones by the end of March. Water temperature in

riffles were around 14ºC. At the beginning of April, temperatures were more uniform between riffles and littoral, coinciding with a period of rainfall and a slight increasing of discharge. By the end of April and May, water temperatures increased significantly, up to 24ºC in the littoral zones and around 18ºC in riffles.

Algal distribution was represented by the percentage of cover of the different algal masses (Fig.3D-H). The onset of benthic cyanobacteria started in mid-January and lasted until the end of May. Attached cyanobacteria (mainly formed by *Oscillatoria limosa* and *O. tenuis*) grew in thickness and extension, already covering some areas of the littoral in early January. However, it was not until late February when the cyanobacteria covered up to 100% surface of some riverbed areas (Fig.3D). Their growth began in the shallow areas (current velocity not much higher than 1 cm s^{-1}) and extended progressively towards the riffle zones (avoiding, however, the areas with fast water current). Attached cyanobacteria extensively covered the littoral zones until early April, later on starting to diminish.

The growth dynamics of the benthic cyanobacterial mats meant that significant fractions of the attached mats became unattached and free-floating. These fractions later drifted downstream and were observed throughout the downstream area of the studied site (Fig.3E). This detachment process increased as cyanobacterial growth increased. Therefore the free-floating mats had a similar behaviour than the attached ones, which covered the maximum water surface during the period between 19 February to 2 April (Fig.3E). Free-floating mats were retained in some areas where macrophytes were growing, this enhancing their occupation of the littoral zones. Finally, the cyanobacterial mass growth collapsed when waters became warmer and flow increased towards the end of May.

Prior to the cyanobacterial mass development, diatom communities predominated, producing ‘brown mats’ which covered the riverbed (Fig.3F). Diatoms were dominant at the beginning of winter, where the temperatures were colder and they were covering especially the riffle zones (Fig.3F). These ‘brown mats’, composed by diatoms (mainly *Navicula sp* and *Nitzschia sp*), started to be replaced by "mixed patches" (Fig.3G), which were composed by green filamentous algae, such as *Vaucheria sp* or *Chladophora glomerata*, plus diatoms. These masses covered the riffles zones during the winter season (Fig.3G), but they started to be replaced by "green filamentous patches" after 20 March (Fig.3H). Green algae, dominated by *Cladophora* in riffle zones and Zygnametales in littoral ones, were covering all the riverbed surface, and therefore, replaced the other communities, including benthic cyanobacteria, coinciding with an increase of water temperature (Fig.3H).

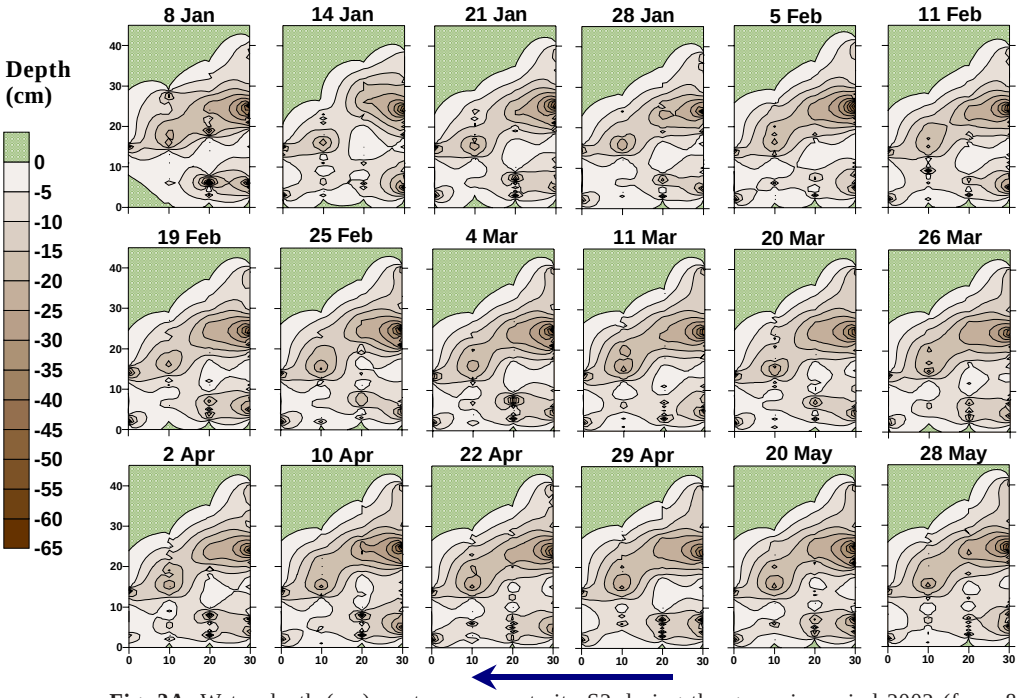

Fig. 3A. Water depth (cm) contour maps at site S2 during the geosmin period 2002 (from 8 January to 28 May). Axis scale are meters. The arrow indicates the direction of the water flow. Green part represents the river outside. Maximum depth was 65 cm.

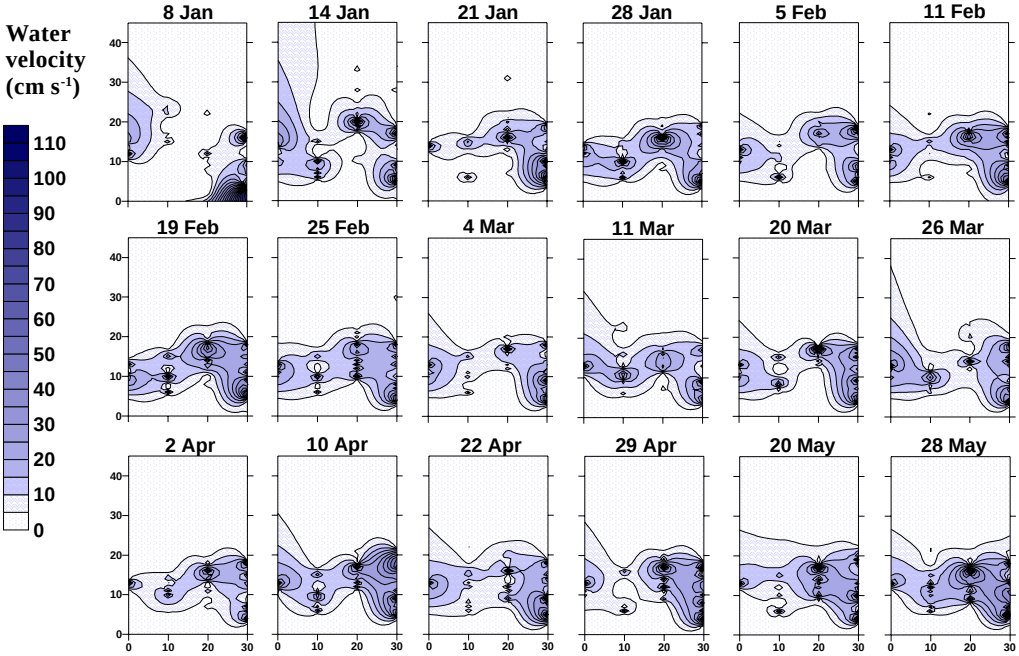

Fig. 3B. Water velocity (cm s^{-1}) contour maps, representing the littoral and riffles zones. The riffles zones were comprised between 5m to 20m of the river widht, while littoral and pools covered and extended region in the upper part of the map.

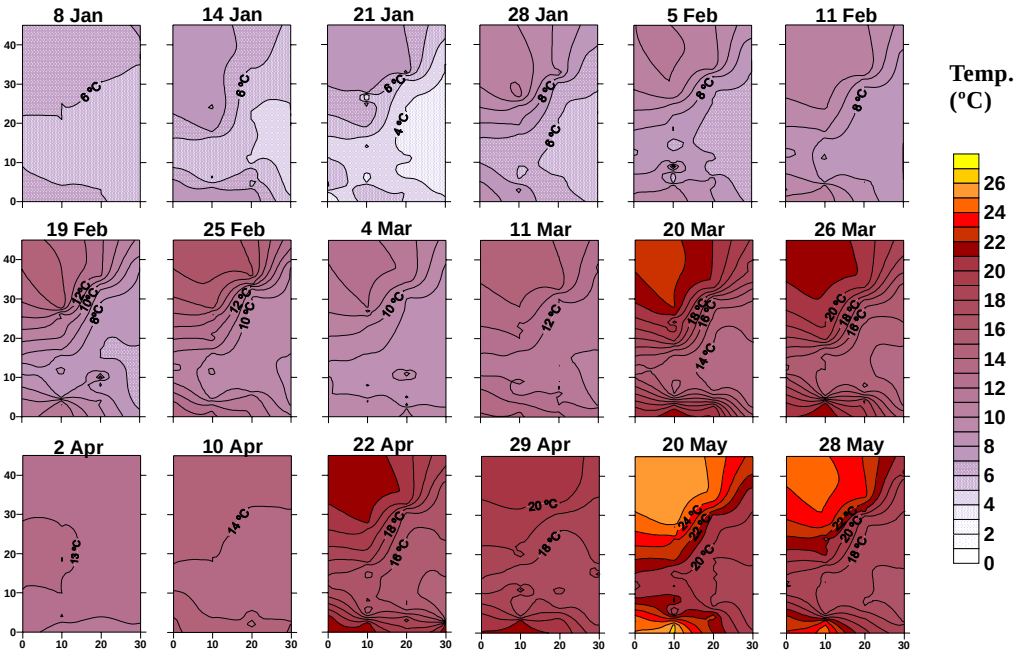

Fig. 3C. Water temperature (ºC) contour maps.

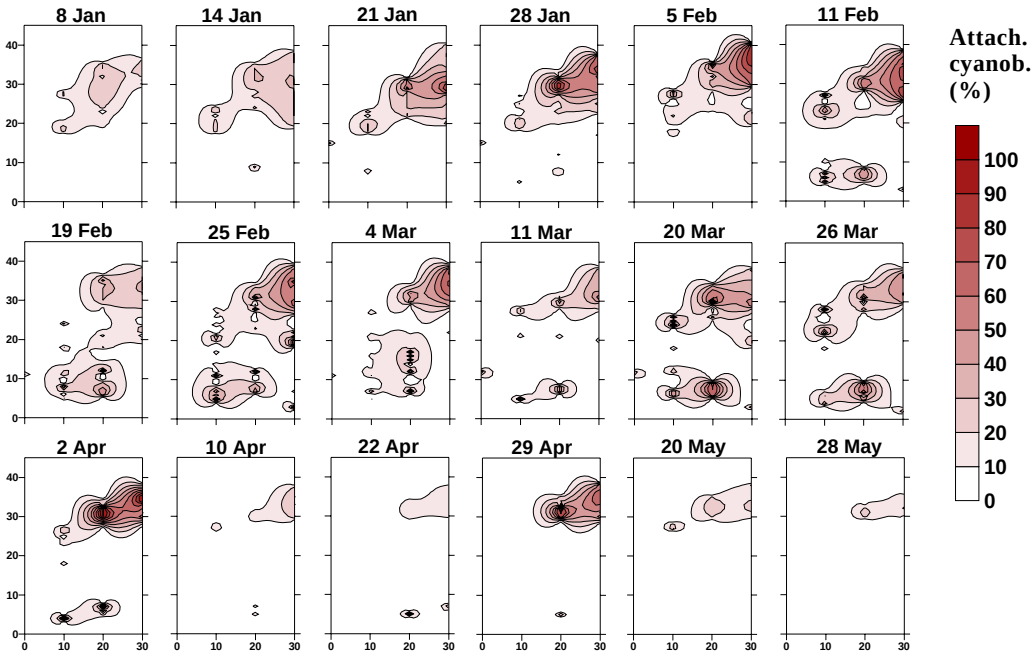

Fig. 3D. Attached cyanobacteria distribution (in % of cover) contour maps.

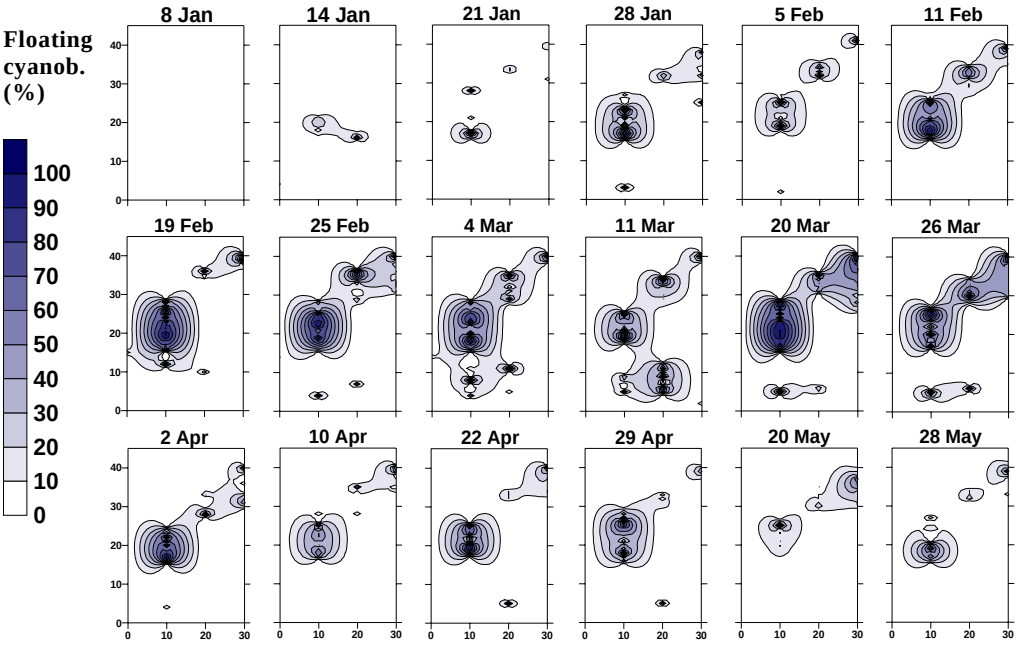

Fig. 3E. Free-floating cyanobacterial mats distribution (in % of cover).

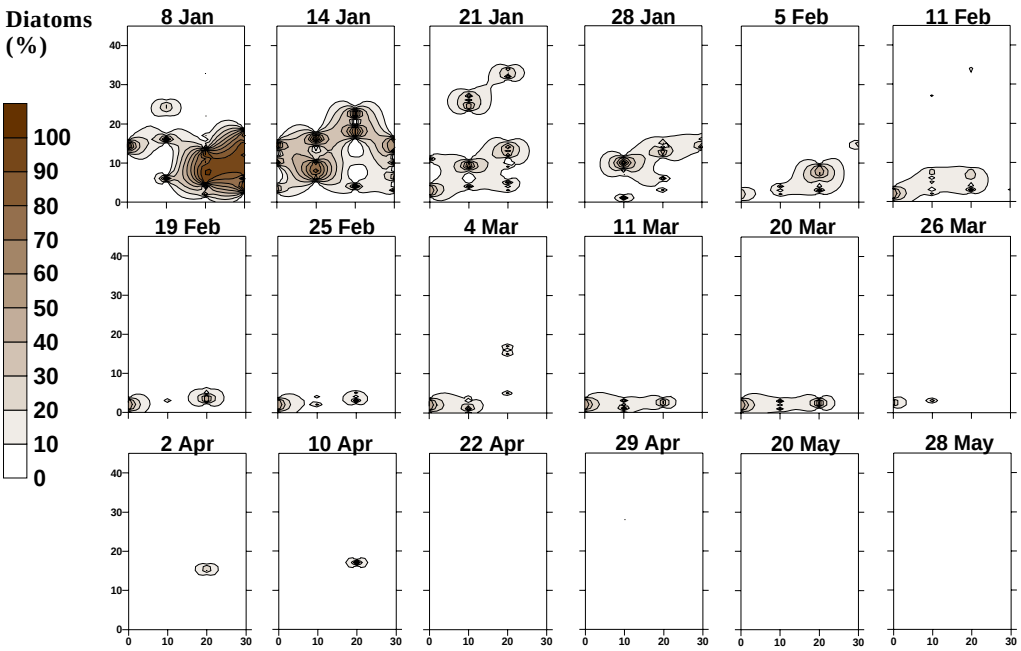

Fig. 3F. Diatom patches distribution (in % of cover).

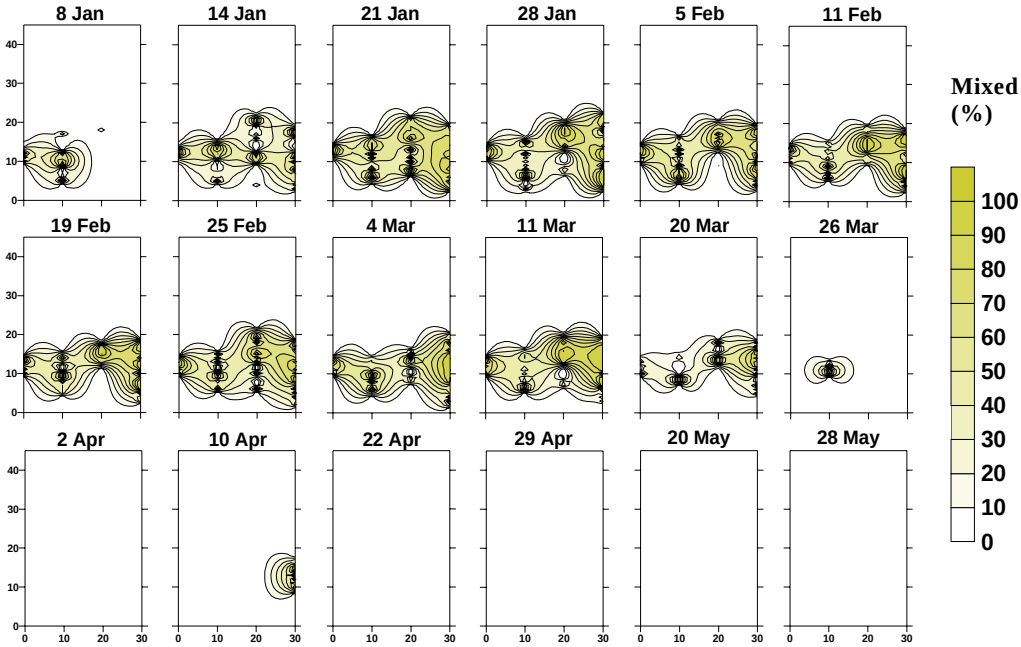

Fig. 3G. Mixed patches (green filamentous algae + diatoms) distribution (in % of cover).

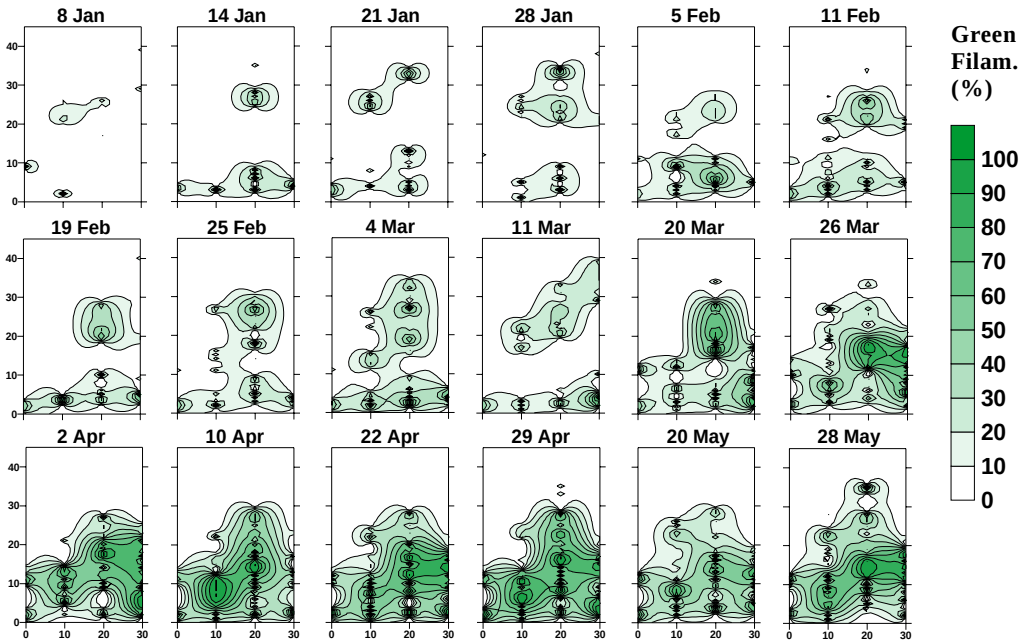

Fig. 3H. Green filamentous algae distribution (in % of cover).

Relevance of the habitat on the development of cyanobacterial masses

Ordination analysis. A multivariate analysis (RDA) sought the direct relationships between the algal and cyanobacterial occurrence and the most relevant environmental factors. The RDA accounted for 39.3 percent of the algal community variance (Fig.4) and showed the association between the geosmin-producing communities (attached biofilms and free-floating biofilms, both composed mainly of *Oscillatoria limosa*) with a low N/P ratio, low water velocities and high production conditions (as indicated by the close association of dissolved oxygen and the cyanobacterial masses). Other algal communities dominated by diatoms, *Cladophora*, 'mixed' diatoms with *Cladophora*, or *Vaucheria* developed mainly in riffles zones. Another algal community, dominated by Zygnematales (green algae), grew in similar conditions as the cyanobacteria.

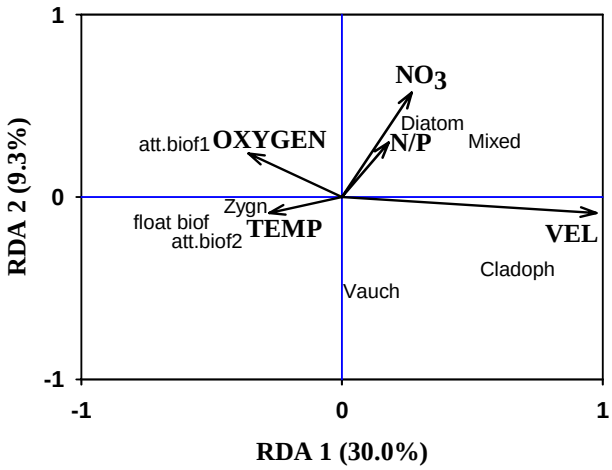

Fig. 4. Redundancy analysis based on algal communities at site S2 during geosmin period of 2002, with environmental variables (Oxygen, Temperature, Nitrate, N/P molar ratio, Water Velocity).
(att.biof1 and att.biof2 = attached biofilm in different phases of growth; float biof = free-floating biofilm; diatoms; mixed = mixed biofilm of green algae and diatoms; Cladoph = Cladophora glomerata; Vauch = Vaucheria sp; Zygn = Zygnematales).

DISCUSSION

Causes for the cyanobacterial abundance

The cyanobacterial mass growth and the subsequent geosmin production were related to several environmental factors in the Llobregat River. Cyanobacterial growth period occurred when river waters recorded high abundance of nutrients, phosphorus and nitrogen. Moreover, moderate temperature conditions, as well as low flow situations (or slow current velocity areas), enabled cyanobacterial mats to grow and accumulate. Low turbulence and nutrient-rich conditions have been already described as favourable to cyanobacterial growth (Paerl 1996). Low N:P ratios (Reynolds 1999, Smith & Bennett 1999) or high nutrient concentrations in general (Dowing & Watson 2001) are believed to cause massive growths of planktonic cyanobacteria. The mass growth of *Oscillatoria* mats in the Llobregat River occurred when there was a high phosphorus content in the water, combined with a low availability of nitrogen (low N:P ratios) (Table 1). Low flow and full light availability, in spite of low water temperature (Table 2), completed a favourable environmental scenario for the mass development of benthic cyanobacteria in the Llobregat River. Cyanobacterial masses avoided periods of extreme irradiance and heat, but were encouraged by open light regimes (Table 1). Cyanobacteria mats therefore developed in winter and spring, either in littoral areas (never in riffle sections) or in other protected areas of the river (such as downstream from dams). When the mats grew too thick they detached from the river bottom and drifted to areas with higher a water flow, from where they could be transported very far downstream. The cyanobacterial mass dominance came to an end when green algae (*Cladophora*) replaced them in conditions of nitrogen abundance, higher light and warmer waters (late spring, summer and fall).

Geosmin production and dispersion

Within the period described of cyanobacterial production, the unbalanced proportion between nitrogen and phosphorus (N/P ratio ca. 10) coincided with the geosmin water peak (Fig. 1). There was therefore an indication that nitrogen deficiency could favour the mass production of cyanobacteria and the subsequent geosmin production. The link between the producers and their manifestation in the water cannot be separated from the particular dynamics of the cyanobacterial mats. In the Llobregat, the mats collapsed and detached from the river bottom, apparently because of their own dynamics of growth and decay. The ecological meaning of the two types of biofilms is essential for two major reasons. First, the concentration of geosmin

was higher in the free-floating than in the attached biofilm. Second, while the attached mats could be responsible for the local occurrence of geosmin at a given site, the free-floating mats become a significant agent in the dispersion of the metabolite downstream. Accumulations of the free-floating mats have been observed as far as 40 km from the production areas. This transport likely reinforces the spreading of the molecule with the water downstream. A suggestion that this could be the case is the difference in the correlation between the geosmin in the water and in the biofilm. While it was not significant for the attached compartment, it was extremely high among the geosmin content in the free-floating biofilm.

Management implications

Even if it is true that geosmin occurrence may be partly controlled during the water treatment (e.g. Kim et al. 1997), improving the resource as opposed to updating the water treatment may be the most appropriate. Improving water quality has to be tackled as part of a river basin approach, therefore contributing to its ecosystem health (Karr 1999). Such an approach fulfils the Water Framework Directive, and is economically sound. Since geosmin dynamics in the river are related to nutrient availability and low flow, control at its source has to consider necessarily these two aspects. Firstly, reduction of nutrients may cause the corresponding decrease in biomass, and create the conditions for a decrease in geosmin. Secondly, biomass accumulation is related to reduced flow in shallow areas. Removal of unnecessary obstacles in the river (dams, channels) could contribute to returning the water flow to natural conditions, making it more difficult for mass accumulation to occur. These two aspects could be the basis for an ecological approach in order to control high geosmin concentration in nutrient-rich shallow water rivers.

CHAPTER 5
BIOFILM STRUCTURE AND FUNCTION AND ITS IMPLICATION TO WATER GEOSMIN DYNAMICS

INTRODUCTION

The production of unpleasant (e.g. odorous) or toxic metabolites has been placed in the ecological context of competition and uncoupling between nutrient availability and biomass accumulation (Paerl & Millie 1996). However, to link the occurrence of nuisance metabolites with the scarcity of resources may be difficult in microbial mats, where a tight package of cells and associated diffusion problems may complicate the observations (Sabater et al. 2002). This may be particularly true for cyanobacterial mats, which are characterised by steep and fluctuating light and oxygen gradients (Stal 1995, Pringault & Garcia Pichel 2000).

In the Llobregat River, cyanobacterial masses experiences a seasonal growth, the occupation of the river surface area with a subsequent detachment and drift, and further collapse. The dynamic changes from attached to free-floating compartments are accompanied by a high production of geosmin by the cyanobacterial mats and may be related to the fate of the metabolites in a river context (see Chapter 3 and 4). Cyanobacterial masses grow under high abundance of nutrients, phosphorous and nitrogen, moderate water temperatures, full light availability and slow water current. Furthermore, geosmin water peak coincided with an unbalanced proportion between nitrogen and phosphorus (N/P ratio ca. 10), suggesting an apparently nutrient limitation.

To determine the factors related to the dynamics of geosmin production, the community dynamics of algae and cyanobacteria were studied from both structural and functional perspectives. The aim of this study was to determine the relationship between the dynamics of cyanobacterial masses and the occurrence of geosmin production. In addition, the causes for the occurrence of geosmin were explored, with special emphasis in the possible nutrient limitation affecting the mats.

MATERIALS AND METHODS

Sampling strategy

During winter and spring 2002 (January to May 2002) algal mat samples were collected from the attached and the free-floating mats weekly at site S2 in the Llobregat River.

Algal composition and abundance, chlorophyll-a, geosmin content and SEM observations

Algal mat samples (3 replicates) were collected from the attached and the free-floating mats with a small PVC corer (3.1 cm^2) which was introduced into each mass. Samples for algal composition and abundance were fixed with 4% formaldehyde. Algal mat samples for chlorophyll-a concentration, geosmin content and scanning electron microscope (SEM) were frozen in the field in liquid nitrogen.

Structural measurements

Samples (3 replicates) were frozen in the field in liquid nitrogen and kept at –20ºC until analysis. Total carbohydrates, phosphorus, carbon and nitrogen content were measured for the attached, free-floating and brown mats collected on 2 sampling dates: before the geosmin peak (14 January 2002) and during the peak (20 March 2002).

Extracellular enzymatic activity

The potential extracellular enzyme activity of β-glucosidase, phosphatase (APA) and leucine-aminopeptidase (AMA) were measured from the different algal patches (attached, free-floating and brown). Incubations were performed at saturation conditions (300 μM). The enzymatic activities were expressed by μmol (of MUF or AMC) per unit organic matter [OM] and per hour (see section III. Materials and Methods).

PAM fluorescence

The maximum photosynthetic capacity (photon yield) in the dark for the different mats (attached, free-floating and brown growth) was estimated in the field using Pulse Amplitude Modulation (PAM) fluorescence. Estimates were performed by taking cyanobacterial mats collected in full sunlight (i.e. light saturated at 12:00 h) and placing them in the dark for 30 min. Five replicates were considered for each sampling date. The canobacterial mats were placed in the bottom of 20 ml glass vials with the top side facing up and immersed in 10 ml of river water. Glass vials were placed directly on top of the optical fiberoptics. Photon yield was calculated as the average of the 5 measurements.

Statistical analyses

A correlation analysis (Pearson coefficient) was performed to determine possible relationship between the variables. A 1-way ANOVA with repeated measures (using the attached vs. free-floating mats as a factor) was used to detect significant differences of environmental and biological variables with time (Winer 1971).

RESULTS

Biofilm structure

Composition and abundance of benthic algal and cyanobacterial communities. The community composition of the cyanobacterial mats was fairly similar in the attached and the free-floating fractions (Table 1). In both, the dominant components were the cyanobacteria *Oscillatoria limosa* and *Oscillatoria tenuis*. However, *Vaucheria sp* was also abundant in the attached mat, while it was less frequent in the free-floating one. A few diatoms formed part of the cyanobacterial mats throughout the whole period, but to a much lesser extent in the free-floating mat. SEM observations and ash content determined that inorganic particles were less abundant in the free-floating mat (Table 2), and its porosity was higher (Fig.1).

Structural components. The structural differences between attached and free-floating mats were obvious when the polysaccharides, nitrogen and phosphorus in the two fractions were analysed (Table 2). All of these structural components were consistently higher in the free-floating mat ($p=0.05$, $p=0.027$, $p=0.046$, respectively). The percent of inorganic particles was lower in the free-floating mat, but differences were not significant.

Table 1. Community composition and total cell density of the algal and cyanobacterial community in the attached and free-floating mats developing in the Llobregat River at site S2 during the studied period (January to May 2002). Values expressed as the percentage contribution of the different taxa or groups to the total density (with the exception of Vaucheria, where abundance is expressed as mm filament cm^{-2}). Presence is expressed by +.

	Attached mat						Free-floating mat					
	14 Jan	11 Feb	4 Mar	20 Mar	22 Apr	20 May	14 Jan	11 Feb	4 Mar	20 Mar	22 Apr	20 May
Total density [cells cm^{-2}] x10^6	7.1	14.0	2.2	4.2	4.1	1.3	28.6	38.6	110.6	20.3	42.3	5.3
Chl-*a* [µg cm^{-2}]	85.4	19.0	2.0	2.9	19.2	5.4	203.7	67.7	213.9	16.1	31.2	11.2
CYANOBACTERIA												
Geitlerinema sp								3.0	0.4		0.2	
Merismopedia glauca ((Ehrenb.) Näg.)		1.7					0.1	0.1	0.1			1.7
Microcoleus sp	1.3											
Oscillatoria limosa (Ag. ex. Gom.)	37.3	69.3	51.1	61.0	42.9	11.1	55.7	44.9	76.0	90.7	79.8	32.7
O. aff tenuis (morph.1) (Ag. ex. Gom.)	8.8	0.7		6.5	19.7	26.7	13.4	0.7	0.5		6.5	18.3
O. aff tenuis (morph.2) (Ag. ex. Gom.)				1.5	1.5	2.4	2.2	9.9	1.5	0.3	0.3	10.8
Phormidium sp	7.6				9.6	2.9	6.1	0.2	0.1		1.0	
Pseudoanabaena catenata (Lauterb.)	14.0	16.2				13.0	15.0	35.4	17.8	1.5	5.6	1.9
Spirulina maior (Kütz.)								0.4			1.1	1.0
ALGAE (non diatoms)												
Cladophora glomerata ((Linn.) Kütz.)									0.1		+	
Closterium sp	+	+				+	+	+	+		+	+
Euglena sp			2.6	0.1					+	+	+	+
Klebsormidium sp					2.3			3.3	1.1	0.4		
Oedogonium sp					1.3	0.1			0.1	2.0	+	
Scenedesmus sp		0.1				0.5		+		+		
Spirogyra sp					0.6			+	+	+	0.1	
Vaucheria sp (mm cm^{-2})	1150	2265	10151	4807	71752		3225	657	7790	796	352	
DIATOMS	31.1	12.1	46.2	31.0	21.9	43.2	7.4	2.1	2.4	4.9	5.2	33.4

Table 2. Values of C:N (molar ratio), carbon, nitrogen, phosphorus and glucose (carbohydrate estimate) as well as ash content from the different mat compartments studied in the Llobregat River at site S2 for selected dates. Values are means and standard deviations (in parentheses).

	14/01/2002			20/03/2002		
	Attached	**Free-floating**	**Brown**	**Attached**	**Free-floating**	**Brown**
C:N	**30.2 (2.6)**	**13.8 (2.5)**	**23.4 (1.2)**	**32.5 (2.8)**	**10.1 (3.3)**	**9.2 (2.4)**
C [μg $mgDW^{-1}$]	**61.1 (1.4)**	**80.7 (8.2)**	**91.6 (16.1)**	**61.4 (1.7)**	**110.8 (37.2)**	**143.1 (38.7)**
N [μg $mgDW^{-1}$]	**2.0 (0.2)**	**6.1 (1.7)**	**3.9 (0.8)**	**1.9 (0.2)**	**12.9 (9.5)**	**16.8 (7.9)**
P [μg $mgDW^{-1}$]	**0.4 (0.1)**	**1.2 (0.4)**	**0.9 (0.2)**	**0.6 (0.1)**	**2.2 (1.5)**	**3.1 (0.5)**
Glucose [μg $mgDW^{-1}$]	**6.6 (0.1)**	**11.2 (2.5)**	**10.3 (0.7)**	**5.9 (0.6)**	**27.4 (18.9)**	**35.8 (13.0)**
% ash	**95.0 (0.5)**	**91.3 (0.5)**	**91.9 (1.4)**	**95.1 (0.5)**	**84.7 (7.1)**	**75.3 (15.3)**

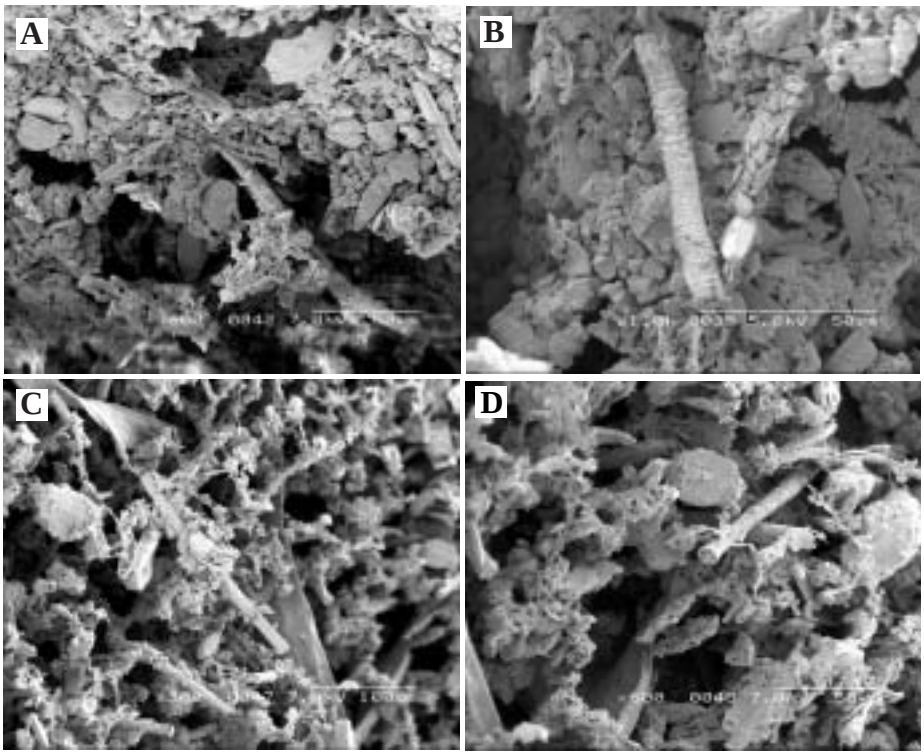

Fig. 1. SEM (Scanning Electron Microscope) photographs of freeze-dried samples of attached cyanobacterial mat (A,B) and free-floating cyanobacterial mat (C,D) in the Llobregat River.

Chlorophyll-a content. Chlorophyll-*a* in the attached and free-floating cyanobacterial mats ranged from 30 to 50 μg cm^{-2} for most of the period (Fig.2). The differences described above in structure and composition resulted in a slightly higher (not statistically significant) chl-*a* content for the free-floating mat, both with reference to surface area (34.6 ± 19.0 and 24.9 ± 19.5 sd μg cm^{-2}, respectively) or to DW (5.3 ± 2.5 and 2.6 ± 1.6 μg $mgDW^{-1}$, respectively). Chl-*a* concentration of the attached fraction was positively correlated with water nutrient content (nitrate: r=0.501, p=0.004; ammonia: r=0.502, p=0.04; reactive phosphorus: r=0.520, p=0.029, n=17). Similar relationships were not observed between chl-*a* and water nutrient for the free-floating fraction.

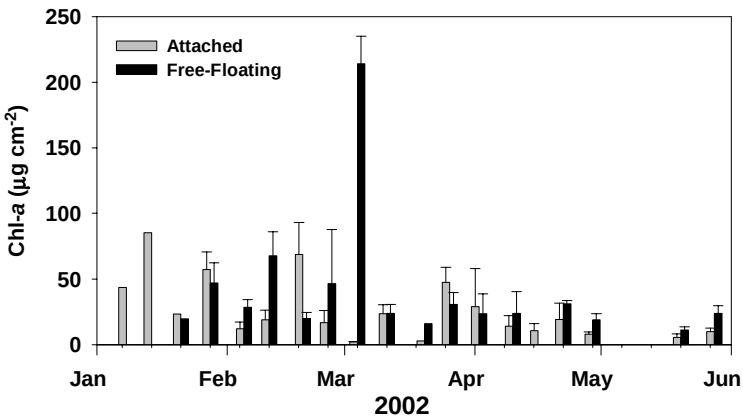

Fig. 2. Chlorophyll-*a* dynamics (μg Chl-*a* cm^{-2}) in the attached and free-floating compartments of the cyanobacterial mats in the Llobregat River.

Geosmin concentration. There was a notable difference in geosmin concentration between the attached mat (0.11 ± 0.19 sd ng geosmin $mgDW^{-1}$) and the free floating mat (1.05 ± 0.99 sd ng geosmin $mgDW^{-1}$). This difference was statistically significant (one-way ANOVA, F=14.67, p=0.0005). Geosmin concentration in the free-floating mat was correlated to the cell density (r=0.884, p=0.019), but this was not so in the attached mat.

Biofilms function

Enzymatic activities. Enzymatic activities showed distinct patterns throughout the period of cyanobacterial mass growth (Fig.3). B-glucosidase was low during the period of maximum geosmin production in 2002 (from 19 February to 2 April) and increased during the initial and final periods of cyanobacterial growth. B-glucosidase activity was higher (repeated measures ANOVA, p=0.012) in the free-floating mats (average of 2.4 ± 2.2 sd μmol MUF g^{-1} organic matter [OM] h^{-1}) than in the attached ones (2.2 ± 2.5 sd μmol MUF gOM^{-1} h^{-1}). APA was very high at the onset of the cyanobacterial episode and progressively decreased. APA was higher in the free-floating mat (average of 3.8 ± 2.2 sd μmol MUF gOM^{-1} h^{-1}) than in the attached mat (average of 3.1 ± 2.3 sd μmol MUF gOM^{-1} h^{-1}), the differences being significant (repeated measures ANOVA, p=0.033). APA was correlated with chl-*a* concentration (r=0.657, p=0.003) in the attached mat, but not in the free-floating mat. Finally, AMA was 3 times higher in the free-floating mat (average of 35.3 ± 8.6 sd μmol AMC gOM^{-1} h^{-1}) than in the attached mat (average of 17.9 ± 13.4 sd μmol AMC gOM^{-1} h^{-1}). These differences were highly significant (repeated measures ANOVA, p=0.0001). Other algal communities that co-occurred with the cyanobacterial masses, called 'brown mats' and composed mainly by diatoms or 'mixed' (diatom + green algae) communities (see Chapter 4), had a similar β-glucosidase activity (2.3 ± 1.5 sd μmol MUF gOM^{-1} h^{-1}), but a much higher APA (6.2 ± 2.2 sd μmol MUF gOM^{-1} h^{-1} on average) than the free-floating mat. In contrast, AMA in the free-floating mat exceeded that of the 'brown mats' (26.1 ± 8.1 sd μmol AMC gOM^{-1} h^{-1}).

The APA:AMA ratio was lower in the free-floating mat than in the attached fraction (t-test for independent samples: p=0.011 and p=0.00003, respectively) and in the 'brown mats' (t-test for independent samples: p=0.00003; Table 3).

Fluorescence measurements (photon yield) were not different between the free-floating and attached mats (Table 4; repeated measures ANOVA, p=0.883). The photon yield was significantly higher in the 'brown mats' than in the two others (p=0.042). In general, the photon yield were maximal during March (Table 4).

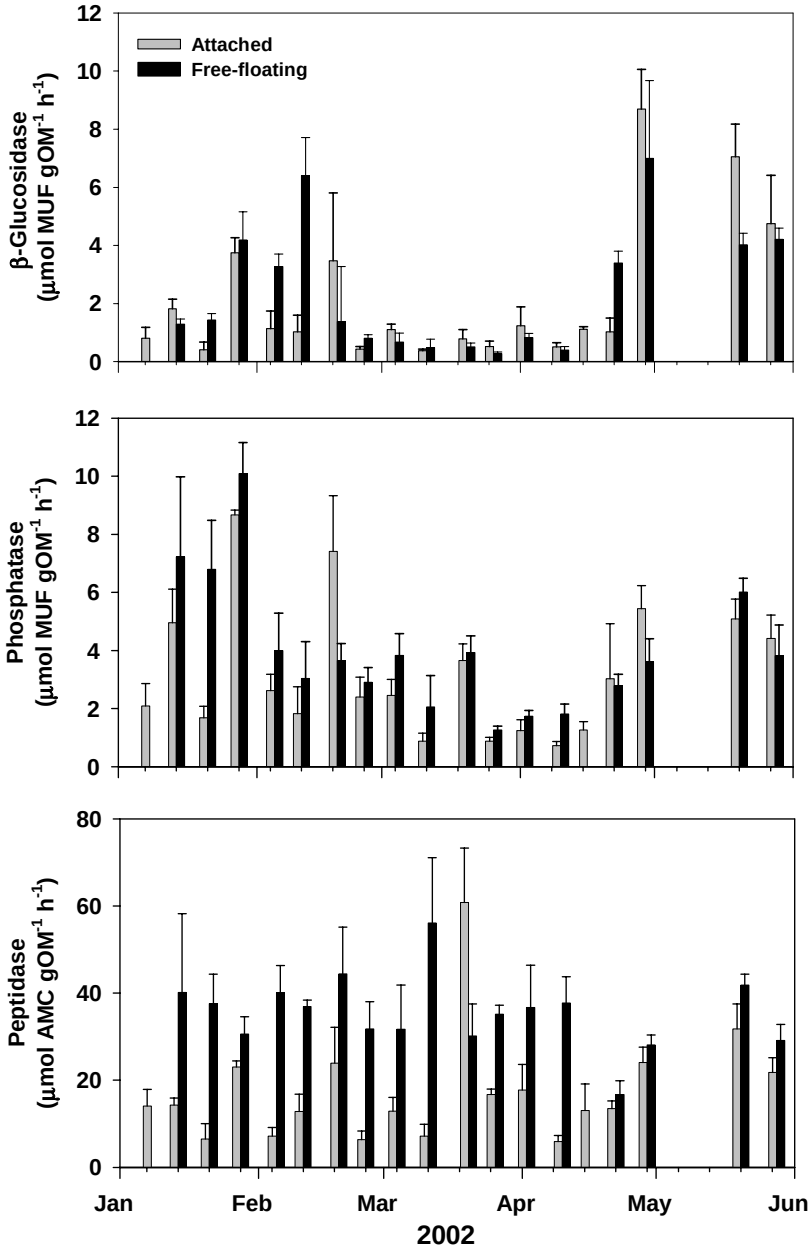

Fig. 3. Exoenzyme activities (β-glucosidase, alkaline phosphatase and peptidase) using the studied period in the Llobregat River for the attached and the free-floating cyanobacterial mats. OM:organic matter.

Table 3. Values of the phosphatase (APA) and aminopeptidase activity (AMA) ratio in the attached, free-floating and brown (diatom-dominated) mats, derived form the corresponding enzymatic activities during the studied period (January to May 2002) in the Llobregat River at site S2.

Date (d/mo/yr)	Attached mat	Free-floating mat	Brown mat
08/01/02	**0.149**	------	**0.264**
14/01/02	**0.347**	**0.180**	**0.259**
21/01/02	**0.262**	**0.181**	**0.218**
28/01/02	**0.377**	**0.330**	**0.405**
05/02/02	**0.368**	**0.100**	**0.280**
11/02/02	**0.143**	**0.082**	**0.254**
19/02/02	**0.310**	**0.082**	**0.501**
25/02/02	**0.378**	**0.092**	**0.169**
04/03/02	**0.191**	**0.121**	**0.254**
11/03/02	**0.124**	**0.037**	**0.130**
20/03/02	**0.060**	**0.130**	**0.205**
26/03/02	**0.053**	**0.036**	**0.197**
02/04/02	**0.070**	**0.047**	**0.306**
10/04/02	**0.121**	**0.048**	**0.232**
16/04/02	**0.097**	------	**0.223**
22/04/02	**0.225**	**0.167**	**0.117**
29/04/02	**0.226**	**0.129**	**0.249**
20/05/02	**0.160**	**0.144**	**0.213**
28/05/02	**0.203**	**0.131**	**0.292**

Table 4. Values of photon yield (relative units of fluorescence) estimated for the attached, free-floating and brown (diatom dominated) mats, estimated in the field during the studied period (January to May 2002) in the Llobregat River at site S2.

Date (d/mo/yr)	Attached mat	Free-floating mat	Brown mat
14/01/02	**0.5340**	**0.4376**	------
21/01/02	**0.5137**	**0.5987**	**0.5273**
28/01/02	**0.5410**	**0.5100**	**0.5350**
05/02/02	**0.5012**	**0.5269**	**0.5402**
11/02/02	**0.4638**	**0.4963**	**0.5676**
19/02/02	**0.2451**	**0.3428**	**0.5726**
25/02/02	**0.3651**	**0.3924**	**0.5758**
04/03/02	**0.4272**	**0.4870**	**0.5947**
11/03/02	**0.3103**	**0.4344**	**0.5128**
20/03/02	**0.2957**	**0.4022**	**0.3591**
26/03/02	**0.3526**	**0.4420**	**0.4864**
02/04/02	**0.4673**	**0.5189**	**0.5057**
10/04/02	**0.4547**	**0.4563**	**0.5392**
16/04/02	**0.4518**	------	**0.4996**
22/04/02	**0.3663**	**0.3644**	**0.5036**
29/04/02	**0.4674**	**0.5070**	**0.4316**

DISCUSSION

The mass growth of Oscillatoria mats in the Llobregat River occurred in nutrient-rich conditions, with a low N:P ratio, and furthermore, with low turbulence and full light availability conditions (see Chapter 4). Chlorophyll-*a* concentrations (200 to 500 mg m^{-2}) attained by the cyanobacterial masses in the Llobregat River were among those commonly found in eutrophic situations elsewhere (Dodds et al. 1997, Romaní & Sabater 2000).

Nitrogen limitation inside the mat

Even though the low N:P ratio in the Llobregat River water was coincided with the mass growth of cyanobacteria, clearer evidence of nitrogen limitation by benthic cyanobacteria is required. The use of enzymatic activities as a proxy for nutrient limitation has been proposed for several environments and situations (Whitton 1991, Vrba et al. 1995, Cotner et al. 1997, Hoppe et al. 1998). AMA hydrolyses peptides and proteins, which comprise the largest part of the organic N pool (Halemejko & Chróst 1986). High AMA levels indicate that inorganic nitrogen is being obtained from organic sources, which is what occurs when inorganic nitrogen availability is low (Patel et al. 2000, Sala et al. 2001). In the Llobregat River, there is a high concentration of nitrate but lower values of ammonia (Chapter 4), which could result in a disadvantage for cyanobacteria in the light-limited conditions inside the mat (Oliver & Ganf 2000). APA converts phosphorus from organic molecules into inorganic phosphorus, and low values suggest that phosphorus is not limiting the metabolism of bacteria or primary producers (Chróst 1990). The ratio APA:AMA may be a useful summary of whether nutrient limitation is due to phosphorus or nitrogen availability (Sala et al. 2001). Different APA:AMA ratios were observed for the different cyanobacterial communities (free-floating, attached) as well as for the diatom brown mats. However, nutrient content was not significantly different between riffle zones and pool areas. This suggests that the specific enzymatic behaviour in the cyanobacterial mats, especially in the free-floating mats (where AMA levels were higher) might be related to their microhabitat conditions or specific mat physiology. Nutrient depletion in the mats is related to thickness and associated difficulties in diffusion of resources (Sabater et al. 2002). This might be higher in drifting masses, where replacement of overflowing waters can be much limitation by the Oscillatoria mats would be related to its low enzymatic APA:AMA ratio. The observation of suspected nitrogen limitation is consistent with the non-heterocystous nitrogen-fixer character described for this cyanobacteria (Villbrandt et al. 1990).

Cyanobacterial toxins and odour/taste metabolites may be regulated by complex environmental factors affecting the physiological state and growth stage (Paerl & Millie 1996). The synthesis of these metabolites may be interpreted as a mechanism for dissipating excess carbon during changes in physiological state (Naes et al. 1985) which may arise during growth. In fact, in the Llobregat River, cyanobacteria mass production was coincident with a peak of the odorous metabolite, geosmin, suggesting that the ecological mechanisms behind its production were linked to cyanobacteria mass growth. The lower quantum yield characteristic of the cyanobacterial mats (free-floating and attached) could not be related to geosmin occurrence. The attached mat possibly plays an important role in geosmin production and, once developed into the free-floating form, may become important in geosmin release and dispersion following cell lysis processes. Synthesis of geosmin in culture has been linked to the alteration of cell growth caused by nutrient deficiency (Wu et al. 1991). Bafford et al. (1993) confirmed that geosmin was present even in young cells of Oscillatoria limosa, but Wu et al. (1991) found that geosmin increased during the lag-phase of growth, when the population was not at its optimum. Naes & Post (1988) observed that transient changes of light and nitrogen could only explain a fraction of geosmin occurrence by cyanobacterial populations, and concluded that biomass level could be a critical factor. Therefore, geosmin synthesis may be controlled by the growth, which in the Llobregat River may be related mainly to nitrogen scarcity, might result in an increase in geosmin.

While nitrogen limitation may act as a trigger for the increase of geosmin within the cyanobacterial mats, physiological differences between the attached and the free-floating compartments indicate that mechanisms at work diverge. The specific high AMA observed in the free-floating mat may be related to the decay dynamics of the mat, where cell lysis processes could be relevant. Lower APA:AMA ratios were recorded form March to early April both in the attached and free-floating mats (Table 3), when geosmin production was maximum. A further indication of decay is the joint increase of AMA and β-glucosidase that characterises the evolution of this compartment. Similar patterns have been observed following the spring phytoplankton bloom in lakes (Halemejko & Chróst 1986, Chróst 1989), since degradation and lysis of senescent algal cells leads to a release of proteinaceous and polysaccharidic compounds (Middelboe et al. 1995). Furthermore, these patterns in the Llobregat River underlie the possible relationship between the degradation processes occurring within the mat and the release of geosmin into the water.

Excretion of geosmin from cell structure

Geosmin is bound to cell structures, such as chloroplast lamellae and lipophylic cell materials (Wu & Jüttner 1987). Therefore, this metabolite does not leave the cell except by cell lysis. In support of this view, it has been shown that treatment with chlorine, copper sulphate or potassium permanganate during water purification can cause geosmin increase in the water (Peterson et al. 1995, Chow et al. 1988). The suggested lysis process in the free-floating compartment is in agreement with the higher geosmin content found there. The meiofauna is not excluded from this process, since high density of Nematoda were found in the cyanobacterial mats (Gaudes et al. unpublished), with especial emphasis in the free-floating compartment, and therefore, they may have a relevant role in enhancing cell lysis, and perhaps in stimulating geosmin synthesis. Meiofauna density was much higher in the free-floating mat compared to the attached mat, possibly because of the higher quality of this material. Lower C:N ratio and higher polysaccharide content in the free-floating mat compared to the attached mat (Table 2) may favour greater development of meiofauna in that compartment, both directly and via bacterial growth. The availability and the quality of organic matter strongly influence survival, development time, growth and reproduction of invertebrates (Cummins & Klug 1979). Lenting et al. (1997) and Palmer et al. (2000) observed that meiofauna abundance was greater in substrates with high microbial biomass or low C:N content. Apart from the nutritional value, lower sediment particles could result in a better habitat for meiofauna in the cyanobacterial mats, and especially in the free-floating ones, meiofauna can contribute both to the degradation activity and to the transport and diffusion of geosmin. Suspected grazing and the movement of meiofauna through interstitial spaces may increase permeability (Boulton et al. 2002) and, therefore, enhance dispersion of the mat in its travel down river. It has been observed elsewhere that the release of geosmin from planktonic cyanobacteria was determined by zooplankton grazing (Durrer et al. 1999), while ciliates were effective in grazing geosmin-producing cyanobacteria (Sudo et al. 1989). In the Llobregat River, because they are being transported passively downstream, the free-floating mats contribute enormously to the potential distribution of geosmin in the river. In this process, the link between the cyanobacterial dynamics and the meiofauna inhabiting the mats may be essential in determining the waning of the mats and the related geosmin diffusion into the river water.

CHAPTER 6
STRUCTURAL HETEROGENEITY AND ASSOCIATED DYNAMICS OF CYANOBACTERIAL MATS IN RIVERS

INTRODUCTION

In previous chapters it was observed that the adherence state of the cyanobacterial masses (attached or free-floating) as well as differences in the mat structure and composition seemed to be related with the geosmin concentration within the mat. The free-floating cyanobacterial masses had a higher proportion of geosmin than those attached. Moreover, it was noticed that a significant nitrogen limitation co-occurred in the free-floating mat with geosmin maximum. In order to uncover the possible relationship between the structure of the mat and the physiological condition which leads to the production of geosmin, the functional structure of the mats was approached by means of microelectrodes. Microelectrodes have been revealed to be useful in uncovering the intimate differences both in biofilms (Santegoeds et al. 1998, Yu & Bishop 1998) and cyanobacterial mats (Epping & Külh 2000, Külh & Fenchel 2000) as well as in sediments (Meijer & Avnimelech 1999, Stief et al. 2002). Oxygen profiles and redox potential profiles were measured in order to detect the temporal evolution and heterogeneities within the mats. A shift in these profiles within a narrow region indicates a well stratified mat where different metabolic processes could take place (Yu et al. 2002).

In the present study the physiological study derived from O_2 and redox microelectrodes was combined with structural and exoenzymatic measurements in order to ascertain in detail the causes for the dynamics of the masses as well as for the production of geosmin.

MATERIALS AND METHODS

Sampling strategy

Cyanobacterial mats were collected in the Llobregat River during the geosmin episode period in March and April 2003. Intact mats and accompanying river water were collected from the river using Plexiglas cores (inner diameter of 4.6 cm, 15 cm length), which were sealed with rubber stoppers in both ends after collection. Samples of attached and free-floating mats were collected separately. Typically, the attached mats were collected at 8-20 cm depth on the river bottom; the lower part of the collected sample consisted of sediment. The free-floating mats were analogously collected from the river water surface. The core samples were transported in cold and dark conditions to the laboratory within a maximum of 2h.

Experimental set-up

In the laboratory, the cores were untapped on the upper end and kept in a recirculating water bath which allowed the water temperature to be adjusted to that of the river. The cores and the water bath were placed outdoors which allowed natural light conditions. The cores received a continuous flow in their upper end, only interrupted during the micro-profile analyses (Fig. 1A,B). The flow was adjusted at 2 L h^{-1} (the one estimated as common in the natural conditions where the mats develop; Guasch et al. unpublished results). A peristaltic pump provided this flow from a water tank filled with fresh Llobregat River water; displaced water from the cores was discarded. Microelectrode measurements on the cyanobacterial mats in the cores started within a couple of hours of returning to the laboratory. The measurements extended for 24h but no further in order to avoid changing excessively the natural conditions. The oxygen microprofiles were carried out in three stages observed in the mats: attached (Fig.2A), free-floating (Fig.2B) and collapsed (described as being initially free-floating but subsequently becoming partially submerged after a few hours of the experimental conditions). Redox microprofiles were carried out only in the free-floating mat.

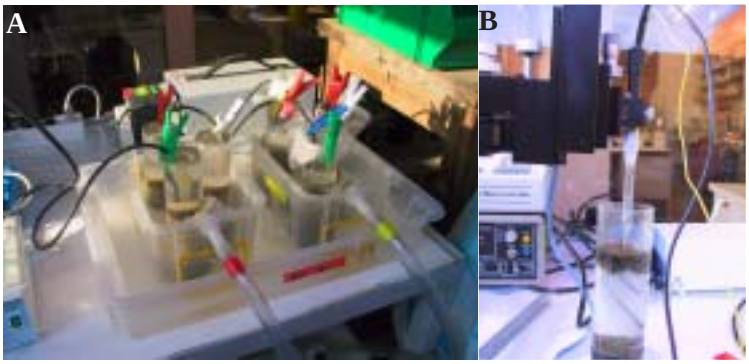

Fig. 1. (A) Experimetal set-up with Perspex cores in a continous flow of river water. (B) Setting of Perspex core with a free-floating mat during the microelectrode measurement.

Subsamples analyses

Subsamples (3 replicates) from the different mat types analysed in the microelectrodes measurements were taken with a small PVC core (3.1 cm^2) in the field to measure algal composition and abundance, chlorophyll-*a* concentration, geosmin content, C/N content and the potential extracellular enzyme activities of leucine-aminopeptidase (AMA) and alkaline phosphatase (APA).

Microelectrode measurements

Oxygen microprofiles. A miniaturized Clark-type oxygen sensor with an internal reference and a guard cathode (Unisense A/S, Denmark), with a tip diameter of 10µm for fine scale measurements, was used. The sensor was connected to a high-sensitivity picoammeter (Unisense PA2000) and was mounted on a motor-driven micromanipulator Oriel™ Encoder Mike Controller 18011 (Oriel, USA; Marzhauser, Germany). The data were recorded on the computer data acquisition software Profix 2.0 (Pyro-Imagination, Unisense A/S) after read data from the microsensor amplifier via the A/D converter ADC-101 (Unisense A/S). Profix software also controlled the micromanipulator. A pre-polarization of the oxygen microsensor was done immersing the tip of the microelectrode in continuously aerated water to consume the oxygen of the electrolyte by the sensing cathode and the guard cathode. After the sensor signal was stabilized during pre-polarization, calibration was performed in a calibration chamber (Fig.3). Signals were read from well-aerated water (after 5 minutes of vigorous bubbling in 100% air saturation) and from oxygen-free water (after bubbling with N_2 gas, 0% air saturation).

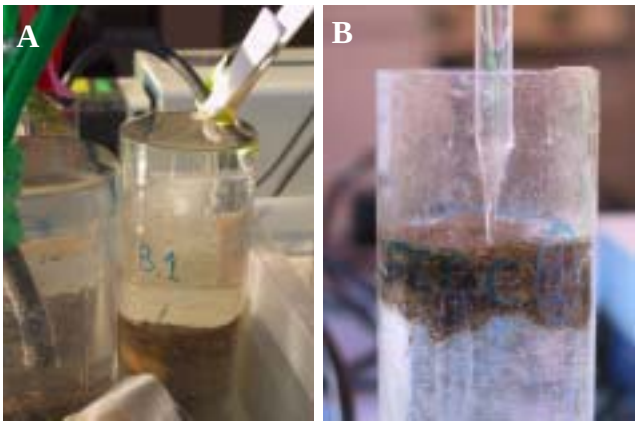

Fig. 2. Picture of attached cyanobacterial mat corer (A) and free-floating mat compartment (B).

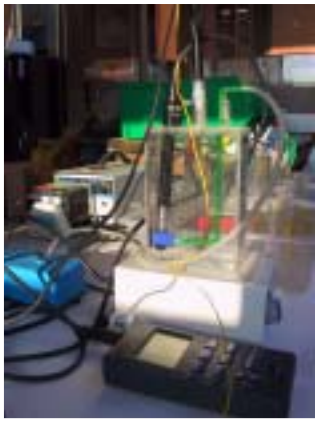

Fig. 3. Calibration chamber where the oxygen microelectrode was calibrated.

Steady-state O_2 microprofiles were measured in intervals of 300 µm vertical depth. Signals were read as partial pressure of oxygen (S) and they were converted to the equivalent concentration of oxygen (C):

$$C = \alpha (S-S_o) / (S_a-S_o)$$

where α is the atmospheric level solubility of oxygen, S_o is the partial pressure at zero reading and S_a the partial pressure at atmospheric reading.

Net photosynthesis (P_n) of the mats was calculated as the diffusive flux of O_2 across the mat-water interface, using the Fick's first law of (one-dimensional) diffusion:

$$J_o = -D_o (dC/dz)$$

where D_o is the free solution molecular diffusion coefficient of O_2, and (dC/dz) is the linear slope of the oxygen concentration profile in the Diffusive Boundary Layer (DBL), where transport of solutes is dominated by molecular diffusion (Jørgensen and Revsbech 1985). $D_o = 1.83 \times 10^{-5}$ cm^2 s^{-1} at salinity (0‰) and temperature (15ºC) (Li and Gregory, 1974). The net photosynthesis (P_n) at light saturation (defined as the irradiance >300 µmol photons m^{-2} s^{-1}; Boston & Hill 1991), was estimated as the relative O_2 export across the mat-water interface (600 µm of depth), using the upper parts of the mat in the attached and collapsed fractions and the bottom part for the free-floating one. Net photosynthesis was estimated both per area (P_n^{area}) and per chlorophyll-*a* (P_n^{chl}).

Redox microprofiles. A miniaturized redox platinum electrode (Unisense A/S, Denmark) was used in combination with a reference electrode (Unisense A/S, Denmark), a simple open-ended Ag-AgCl electrode with a gel-stabilized electrolyte, both with a tip diameter of 10 µm for fine scale measurements. They were connected to a high-impedance millivolt-meter (PHM210, Unisense A/S, Denmark) to measure potentiometrically the oxidation-reduction potentials. A calibration was done using 2 points of calibration immersing the redox and the reference microelectrodes tip to quinhydrone redox buffers (pH 4.0 and pH 7.0). The calibration was done at the same temperature that the measurements. The redox potential was standardized against a standard hydrogen reference electrode.

RESULTS

Composition and abundance of the communities

There were some differences between the different mat types, especially considering their cell density per surface area (Table 1). In the attached fraction, *Vaucheria* sp. and some diatom taxa were abundant and even *Cladophora glomerata* was present. *Oscillatoria limosa* and *Oscillatoria tenuis* accounted for the 30.6% of the total cells cm^{-2}. The total cell density in that fraction was 1.6 x 10^6 cell cm^{-2} on the average, and chlorophyll-*a* ranged from 27.71 to 33.37 µg cm^{-2} (Table 2). The collapsed mat had higher cell density (6.8 x 10^6 cell cm^{-2}). In this mat also *Vaucheria* sp. and diatoms were present but *Oscillatoria* spp. had a higher proportion (49.7%) than in the attached fraction (Table 1). Chlorophyll-*a* in this mat ranged from 13.51 to 40.78 µg cm^{-2}. Finally, the free-floating mat had the highest cell density (9.9 x 10^6 total cell density cm^{-2}) with the highest proportion of *Oscillatoria* spp (90.0%), where 76.1% was *O. limosa* (Table 1). *Vaucheria* sp. and diatoms had a much lower proportion (9.7%) than in the other mat types. Chlorophyll-*a* ranged from 7.46 to 53.33 µg cm^{-2}. Geosmin in the different mats was also analysed. While it was close to zero in the attached mat, concentration inside the collapsed mat was 0.76 ng geosmin mg DW^{-1} and 1.03 ng mg DW^{-1} on the free-floating mat. (Table 2).

Table 1. Community composition and total cell density of the algal and cyanobacterial community in the attached, collapsed and free-floating mats. Values expressed as the percentage contribution of the different taxa of groups to the total density (with the exception of *Vaucheria*, where abundance is expressed as mm filament cm^{-2}). Taxa presence is expressed by +.

	Attached	Collapsed	Free-floating
Total density [cells cm^{-2}] x10^6	1.6	6.8	9.9
CYANOBACTERIA			
Geitlerinema sp			0.4
Merismopedia sp		0.1	
Oscillatoria limosa (Ag. ex Gom.)	17.3	31.1	76.1
O. aff *tenuis* (morph.1) (Ag. ex Gom.)	11.9	12.4	5.2
O. aff *tenuis* (morph.2) (Ag. ex Gom.)	1.4	6.2	8.7
Phormidium sp	0.1		
Pseudoanabaena catenata (Lauterborn)	27.5	36.7	
ALGAE (non diatoms)			
Cladophora glomerata ((Linn.)Kütz.)	+		
Spirogyra sp		0.1	+
Zygnema sp		0.1	
Closterium sp	+		+
Vaucheria sp (mm cm^{-2})	1143	40	222
DIATOMS	41.7	13.4	9.7

Some micro-patches were distinct within the free-floating mat. Black and thick micro-patches were constituted by *O. limosa* and *O. tenuis*. Diatoms, sediment particles and a few *Oscillatoria* spp. filaments, formed a brownish micro-patch. Subsample analyses showed that chlorophyll-*a* concentration per mg DW was higher in the black than in the brown micro-patches: 1.02 µg and 0.55 µg respectively (Table 2). Geosmin concentration was significantly higher in the black fraction (4.03 ng $mgDW^{-1}$) than in the brown (1.64 ng $mgDW^{-1}$). The C/N ratio was higher in the brown part (17.34) than in the black (10.66), indicating the higher carbon fraction in the former (Table 2).

Table 2. Values of Chlorophyll-*a* per cm^2 and mg DW (dry weight), geosmin content per mg DW, percentage of ash content, C/N (molar ratio) and carbon and nitrogen content per mg DW fromg the attached, collapsed and free-floating mats as well as the black and brown fractions. Values are means and standard deviations (in parentheses). Not measured values are indicated by n.d.

	Attached	Collapsed	Free-floating	Black	Brown
Chl-*a* [µg cm^{-2}]	31.5 (3.3)	28.7 (13.9)	33.0 (16.0)	n.m	n.m
Chl-*a* [µg $mgDW^{-1}$]	0.40 (0.08)	0.99 (0.29)	0.63 (0.07)	1.02 (0.14)	0.55 ± 0.11
Geosmin [ng µgChl-*a* $^{-1}$]	n.d	0.91	1.06	2.00	2.28
Geosmin [ng $mgDW^{-1}$]	n.d	0.76	1.03	4.03	1.64
C:N	21.5 (0.6)	15.5 (1.2)	16.0 (3.0)	10.7 (0.1)	17.3 (0.2)
C [µg $mgDW^{-1}$]	83.9 (0.9)	102.7 (6.9)	95.6 (12.0)	111.2 (0.9)	97.7 (0.1)
N [µg $mgDW^{-1}$]	3.9 (0.1)	6.7 (0.9)	6.2 (1.7)	10.4 (0.2)	5.6 (0.1)
% ash	94.4 (0.4)	90.7 (1.5)	90.8 (2.0)	82.2 (3.4)	79.2 (11.0)

Oxygen fluxes in the attached, collapsed and free-floating mat

Differences between the attached, free-floating and collapsed mats were evidenced when the profiles of dissolved oxygen obtained at light saturation (>1000 µmol photons m^{-2} s^{-1}) were compared (Fig.2 A, B and C; n=3 in the three cases). Subsurface maximum of O_2 concentration occurred within the first 2 mm of depth both in the attached and collapsed mats. Bigger O_2 maximum occurred in the attached mat (1177-1316 µM; corresponding to oxygen saturation of 365-409%, Fig.2 A). Oxygen maximum in the collapsed mat ranged from 546 µM (170%) to 725 µM (225%) (Fig.2 B). The maximum O_2 concentration in the free-floating mat never occurred in the first few millimetres, but in the deepest parts of the mat reaching from 796 µM (247%) to 927 µM (290%) (Fig.2 C). Heterogeneous profiles inside the same mat were due to varying thickness in nearby zones of the mats.

The net photosynthesis at light saturation (P_n^{area}) was higher in the attached (4.876 ± 1.843 µmols O_2 cm^{-2} d^{-1}) than in the collapsed mat (2.959 ± 0.821 µmols O_2 cm^{-2} d^{-1}). Otherwise, the free-floating mat had the lowest P_n^{area} values (1.624 ± 0.636 µmols O_2 cm^{-2} d^{-1}). These differences were also obvious when the results were expressed per unit Chl-*a*. P_n^{Chl} of the attached community was higher (0.155 ± 0.059 µmols O_2 Chl-a^{-1} d^{-1}) than the collapsed (0.103 ± 0.029 µmols O_2 Chl-a^{-1} d^{-1}) or free-floating (0.040 ± 0.017 µmols O_2 Chl-a^{-1} d^{-1}).

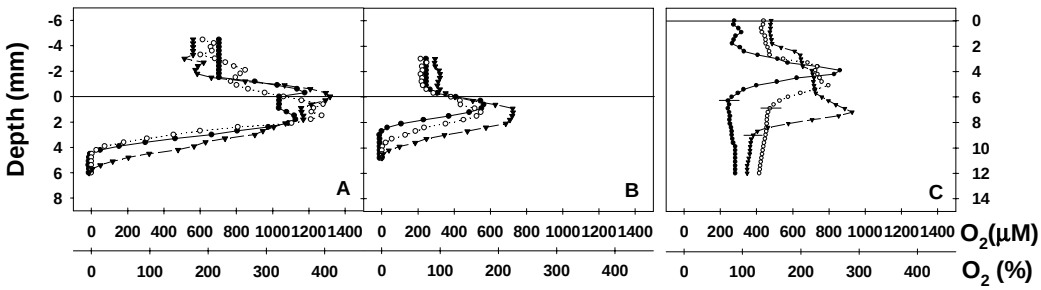

Fig. 2. Oxygen concentration profiles (n=3) expressed by µM and percentage of air saturation (%) from attached (A), collapsed (B) and free-floating (C) mats measured at light saturated contiditon (>1000 mmol photons m^{-2} s^{-1}) and temperature between 15ºC and 17ºC. Depth is in mm. Note that the horizontal black line indicated the surface of the mat in contact with the water (with the exception of the free-floating mat (C) where the surface is in contact with the air). Short horizontal solid lines indicate the position of the bottom part of the free-floating mat (C) being in contact with the water.

Oxygen dynamics in the free-floating mat

The dynamics in the oxygen profiles of the free-floating mat were associated with the sequence of addition or detachment of parts of the mat. Initially the free-floating mat was relatively thin (7mm) (Fig.3 A, B and C), but later become thicker (10-14mm) (Fig.3 D, E and F) due to the incorporation of a partly collapsed part which became free-floating. The thin free-floating mat reached a minimum O_2 concentration of 120 µM (35%) in the dark (Fig.3 B). At 800 µmol photon $m^{-2}s^{-1}$, O_2 production increased again and concentration reached a maximum of 860 µM (267%) at 4 mm in depth. The thicker free-floating mat, which was produced later, was completely supersaturated after midday (1300 µmol photon $m^{-2}s^{-1}$). Maximum O_2 concentration at that time was of 927 µM (290%) (Fig.3 D). This high oxygen concentration within the mat caused the increase in floatability in the collapsed fraction, which become incorporated to the already floating mat. During the early afternoon, at lower irradiance, some parts of free-floating mat partially sank and

the mat had a loose appearance but a higher thickness (14mm). In these conditions, some black fractions located in the lowest part of the mat became highly compacted and showed anoxic conditions, while the upper parts of the mat still had values over saturation (Fig.3 E and F).

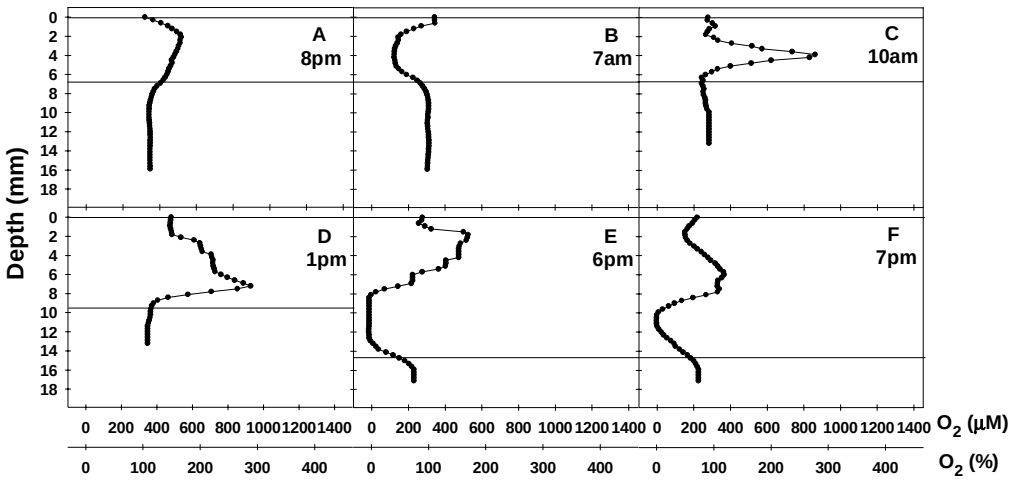

Fig. 3. Oxygen concentration profiles expressed by µM and percentage of air saturation (%) inside the free-floating mat at different times (from 8pm to 7pm the day after) and different water temperature and water surface irradiances: (A) 12ºC, dark; (B) 10ºC, dark; (C) 13ºC, 800 µmol photons m^{-2} s^{-1}; (D) 17ºC, 1300 µmol photons m^{-2} s^{-1}; (E) 15ºC, 10 µmol photons m^{-2} s^{-1} and (F) 14ºC, dark conditions. The horizontal solid lines indicate the upper and lowest part of the mat.

Redox potential profiles in the free-floating mat

Redox profiles were measured in a thick (12mm) free-floating mat (Fig.4). During the early afternoon the cyanobacterial mat presented a positive redox potential (about +500mV) (Fig.4 A). At that time the mat had some loose fraction in the lowest part while the upper was more compacted. These conditions remained during the late afternoon and some hours of darkness. However, after 8h in the dark, a negative redox potential (–400mV) was detected inside the mat in a black compacted fraction (Fig.4 B,C, dark circles). However, redox potential was always positive in the brownish micro-patches (Fig.4, white circles). The negative redox potential inside the black fraction remained after midday, when coinciding with the highest irradiances, the redox potential became positive again (Fig.3 D black circles). Negative redox potential occurred again when light irradiance decreased in the afternoon (Fig.4 E,F black circles).

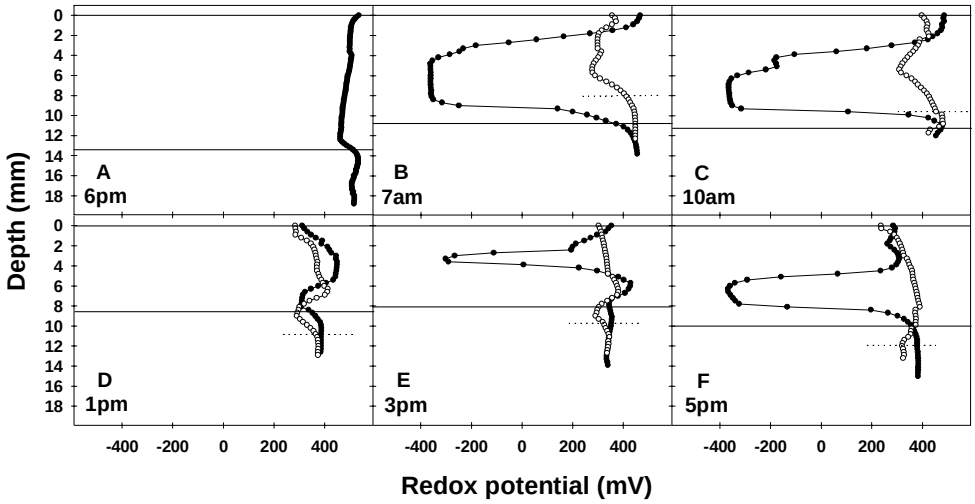

Fig. 4. Redox potential profiles expressed by mV inside the free-floating mat at different times (from 6 pm to 5 pm the day after) and different water temperature and water surface irradiance: (A) 19ºC, 60 µmol photons m^{-2} s^{-1}; (B) 16ºC, dark; (C) 17ºC, 50 µmol photons m^{-2} s^{-1}; (D) 20ºC, 1100 µmol photons m^{-2} s^{-1}; (E) 21ºC, 1100 µmol photons m^{-2} s^{-1} and (F) 19ºC, 100 µmol photons m^{-2} s^{-1}. Black circles indicate the blak fraction profiles, and white circles the brown fraction profiles (see text). Note that the solid lines indicate the upper and lowest part of the mat, while short dotted lines indicate the lowest part of the brown fraction.

Metabolic activities inside the free-floating mat

Exoenzymatic activities were separately measured in the black and brownish micro-patches. The black patch had higher peptidase (107.37 µmol AMC gOM^{-1} h^{-1}) and phosphatase (13.13 µmol MUF gOM^{-1} h^{-1}) activities than the brown patch (44.22 µmol AMC gOM^{-1} h^{-1} and 5.91 µmol MUF gOM^{-1} h^{-1} respectively). The phosphatase/ peptidase ratio (Table 3) was low in the two micro-patches (0.122 ± 0.120 and 0.134 ± 0.048, black and brown respectively).

Table 3. Exoenzyme activities (Peptidase and alkaline Phosphatase) as well as Phosphatase/Peptidase ratio from the black and brown fractions. OM: organic matter. Values are means and standard deviations (in parentheses).

	Peptidase [µmol AMC g OM^{-1} h^{-1}]	Phosphatase [µmol MUF g OM^{-1} h^{-1}]	Phosph/Peptidase
Black	**107.37 (18.81)**	**13.13 (2.26)**	**0.122 (0.120)**
Brown	**44.22 (11.24)**	**5.91 (0.54)**	**0.134 (0.048)**

DISCUSSION

The evolution of the oxygen profiles shows that the dynamics of detachment and flotation of the algal and cyanobacterial mats in the Llobregat is a result of the photosynthesis and respiration dynamics. The algal (*Vaucheria*, diatoms) and cyanobacterial components (*Oscillatoria* spp.) constitute a loosely attached mat which may float or sink through variations in the oxygen dynamics of the mat, provided they develop in quiet waters. In the process of detachment, the mat experiences large changes in its structure and composition, favouring that cyanobacteria become dominant in the unattached, potentially drifting forms. In the free-floating fraction there was a lower oxygen efflux from the mat to the water, while oxygen production inside the mat was still high. This lead to oxygen supersaturation inside the mat and formation of bubbles, which could perform as a floating mechanism. The diel oxygen dynamics showed that the floating mats could collapse during the hours of darkness and float again when there was high oxygen production and bubble formation. In the river, the maximum occurrence of free-floating mats was at noon, when high irradiances leads to high productivity. Burlingame et al. (1986) already reported that maximum concentration of free-floating masses of *Oscillatoria* occurred after midday, and that their occurrence coincided with the highest levels of geosmin in water.

Once the mat becomes detached it remains in direct contact with the air, like a planktonic bloom of cyanobacteria, profiting from being closer to the light. It might be assumed that the high light irradiances could cause photoinhibition (Guasch & Sabater 1995, Ibelings & Maberly 1998) in the mat. Even though Production-Irradiance relationships (Guasch & Sabater 1998, Guasch et al. 1998) were not measured in the different mat fractions, the sustained Pn during the highest irradiances indicate that this was not the case for the mats in the Llobregat. During high irradiances (e.g. during midday) the peak of oxygen production inside the mat always occurred at some millimetres of depth, never at the surface of the mat. The cyanobacteria may use the detritus and inorganic particles entrapped in the mucchopolysaccaridic matrix of the mat (see chapter 5) as a protection against the light. The dominating *Oscillatoria* filaments could be positioned at some depth to avoid photoinhibition, since photosynthesis in cyanobacteria saturates at low light intensity (Stal 2000). Additionally, the own thickness of the microbial mat could protect the cyanobacteria against photooxidative effects, as it has been observed on dense planktonic algal blooms elsewhere (Eloff et al. 1976).

Dynamics of the free-Floating mats and geosmin production.

Geosmin is a secondary metabolite derived from monoterpene and sesquiterpene precursors within the isoprenoid biosynthetic pathway, where carotenoids and the phytol chain of chlorophyll-*a* are synthesized (Naes & Post 1988). Geosmin is produced when the demand for pigment precursors is low. Therefore its production is related with changes in the physiological state of the cyanobacteria, which may be induced by altered light regimes (Paerl et al. 1996, Naes et al. 1988) or resource limitation. Nitrogen limitation may affect geosmin production (Naes & Post 1988). Wu et al. (1991) found a negative correlation between the amount of geosmin produced and the growth rate of cells in *Anabaena* sp. Saadoun et al. (2001) observed that high nitrate suppressed, while ammonium enhanced geosmin production. A correlation between nitrogen imbalance in the water and the growth of cyanobacteria mats and geosmin production was observed in the Llobregat River (see chapter 4). In the present study, the remarkable peptidase and phosphatase activities observed in the mats indicated a major limitation of inorganic nitrogen and phosphorus.

The oxygen and redox profiles indicate that potential conditions for the production of geosmin could occur in the Llobregat mats related with diffusion constraints within the mat which could be associated with resource depletion. In the ecological scale of the river the low water turbulence characteristic of slow moving areas, where the mats accumulated, could favour low oxygen and limited nutrient diffusion (Glud et al. 1994). However, the relevance of this effect seems heterogeneous in the Llobregat mats. The black micro-patch reached anoxia during the night which could persist until midday, but this did not occur in the brown micro-patch. While in the former the high oxygen concentration produced during the day was consumed by respiration during the night, in the latter oxygen diffusion between the mat and the bulk water was still possible. The difference on the redox profiles between the two micro-patches is most probably related to the much higher cell density in the black micro-patch.

Oscillatoria limosa has been described as a potential non-heterocystous nitrogen-fixer (Villbrandt et al. 1990), in response of nitrogen deficiency. The oxygen depletion in the black micropatches of *Oscillatoria* produced a strongly negative redox potential which indicate the appropriate conditions for nitrogenase activity (Stal & Krumbein 1987). Nitrogenase requires of a very high energy consumption and low-potential reducing equivalents, since oxygen exerts a negative effect on this enzyme (Stal 2000). Moreover, the extreme values in redox potential within the mat suggest an

extreme metabolic plasticity in *Oscillatoria*. When oxygen is present inside the mat during the dark, these cyanobacteria can obtain energy by respiring endogenous storage carbohydrate as glycogen (Stal 2000). However, in anaerobic conditions in the dark, *Oscillatoria limosa* is able to cover its energy demands by fermentation (Heyer et al. 1989). Production of energetically efficient fermentation products (Stal 2000) may be associated to the highly negative redox potentials found inside the free-floating mat.

Altogether, the micro-profiles carried out with the different mat types in the Llobregat show that conditions inside the mat were those of limited diffusion, especially in the fractions where *Oscillatoria* was prevalent. The cyanobacterial mats in the Llobregat were highly dynamic both in their structure and relative composition, and showed a remarkable heterogeneity within the mat. The mats with prevalence of *Oscillatoria* functioned as real "hot spots" within the mat, where low nutrient availability related with diffusion constraints may be the appropriate for the production of metabolites such as geosmin. This could justify why geosmin is seasonally produced by the cyanobacterial mats, coinciding with an imbalance of nitrogen with respect to phosphorus. The cyanobacterial growth may attain a critical phase where density and agglomeration impedes diffusion of gases and resources, this being the starting point for the mass production of the metabolite in the river.

IV. CONCLUSIONS

The role of natural biofilms affecting the water quality in rivers has been the main theme of this study. Firstly, the study developed the capacity of biofilms in retention and/or production of DOC. Secondly, the study also approached the production of the geosmin metabolite by benthic cyanobacteria mats. In the two developed aspects, the structure and function of the biofilms showed their relevance in evaluating the capacity of biofilms on the amelioration of the water quality.

In particular:

I) The production and retention of water DOC is related to biofilms growth. Biofilm metabolism (extracellular enzymatic activities) were related to water DOC and BDOC concentration. Biofilms growing in light conditions, although presenting monthly variability of their DOC uptake/release rates, showed greater annual DOC uptake rate than that of the dark-grown biofilm. The light-grown biofilms were therefore net DOC consumers. This could be caused by the higher biomass and more complicated structure in this biofilm. The relevant growth of the algal component could enhance the development of bacterial community as well as the microbial heterotrophic activity. Further on, the higher development of a polysaccharide matrix could promote the abiotic adsorption by the biofilm. In contrast, the dark-grown biofilms were highly dependent on the amount and quality of organic matter that enters the system. The dark-grown biofilms presented a constant DOC consumption along the year, being key sites in the reduction of water DOC concentration.

II) The biofilm structure (algal composition, bacterial density, C/N content) had a relevant implication in the carbon recycling, since metabolic (extracellular enzymatic) activities were affected by variations in this structure. Autotrophic biomass growing in light conditions released high-quality DOC compounds from algal exudates, which could enhance the internal carbon recycling. Therefore, algal biomass and thickness could affect the capacity of DOC retention, indicating that thick biofilms could have lower efficiency in the water DOC uptake.

III) The study of the temporal and spatial dynamics of geosmin production at river and habitat scale, gave us an approximation of the physical factors potentially related with the growth of benthic cyanobacterial masses. Littoral zones, with low water velocity, warmer temperatures and higher nutrient concentration and low N/P ratios, favoured the mass development, and therefore the geosmin production. The progressively growth of the cyanobacterial masses and the further detachment and drifting downstream, were responsible for the dispersion of geosmin along the river. The massive growth of cyanobacterial mats, accumulating high biomass per surface unit, may cause the condition for resource depletion inside the mat, e.g. nitrogen limitation. The capacity of nitrogen fixation by some cyanobacteria could favour the extensive growth of these masses. Even though, this capacity have not been measured directly in the masses, there are some reports indicating that *Oscillatoria limosa* is a non-heterocystous atmospheric nitrogen fixer. Furthermore, since all toxicity responses detected in the biofilms were weak and not related to the occurrence of geosmin, we can conclude that the only relevance of this metabolite on water quality is the nuisance earthy and musty odour in drinking water.

IV) Structural and functional differences were found in the different fractions of the cyanobacterial mat, attached and free-floating. Free-floating mats presented higher biomass and exoenzymatic activities. Otherwise, the low Phosphatase/Aminopeptidase ratio found in both compartments, indicated that cyanobacterial mats were affected by nitrogen limitation. The structural heterogeneity between compartments was evidenced by oxygen and redox micro-profiles inside the mats. Microstructural analyses inside the mat have been useful to understand the function of the different cyanobacterial patches inside the mat. Oxygen supersaturation inside the mat favoured its flotation, retaining oxygen bubbles between layers of *Oscillatoria* and other algal micro-patches, such as diatoms. During the dark, oxygen was depleted inside the *Oscillatoria* micro-patches, which have the tendency to aggregate and accumulate in micro-patches, giving a very low redox potential conditions. This low diffusion could be associated with resource depletion, limiting the nutrient availability and defining the appropriate conditions for the geosmin production. However, the geosmin

release and diffusion into the water is still not clear. This process could be favoured by the degradation of the mat, due to the cell lyses process, or the contribution of meiofauna dispersing the aggregated patches.

Implications for water management

River DOC dynamics

The first conclusion of this study is that biofilms may play an important role in the retention of water DOC. In the two situations of open light and dark growth regime, biofilms respond to an entrance of water DOC concentration. Although light-grown biofilms presented monthly variation in DOC uptake/release rates, annual average presented a higher DOC uptake than dark-growth biofilms. However, dark-growth biofilms had a constant DOC consumption along the year, permitting a maintenance of low water DOC levels. From these results, we may suggest that the utilization of covered conduits may be advisable for water management as a tool to diminish the DOC concentration in the water before arriving to the water treatment process. This conclusion is based on the results found in the Ebre River, which during the studied period was characterized by relatively low water DOC concentrations. As a consequence, low water DOC levels could be determinant of the relationship between the biofilms and the flowing water.

One question still open is which will be the implication of biofilms in the retention of higher water DOC concentrations. Not only DOC concentration will affect the biofilm uptake, but also the quality of its composition. A higher fraction of biodegradable DOC (BDOC) will be more rapidly uptaken by biofilms than refractory DOC (e.g. humic components). Biofilms would be affected by the entrance of high DOC levels in the system, enhancing the heterotrophic metabolism and therefore an immediate DOC uptake. On the other hand, continuously high water DOC concentrations could affect a possible growth in biomass and thickness of the biofilms, affecting its internal carbon recycling, and therefore, the retention of external DOC should be reduced per unit of biofilm biomass. In those conditions it might be expected that DOC consumption along the channel and pipe systems will be irrelevant .

In the above situations, it should be considered the importance of natural streambed conditions, where biofilms growth covering heterogeneous substrates under different physical and chemical factors could improve the microorganisms interactions (and consequently enhancing the natural process of organic matter recycling). Otherwise,

in the use of artificial systems such as the channel and pipe devices, the high water velocity was one of the main physical factors affecting the growth of the biofilms. Since high water velocity does not allow large accumulation of biofilm biomass and therefore control its thickness, allocththonous DOC content from the water column would support the heterotrophic metabolism of the biofilm rather than the internal DOC, and therefore, a net DOC consumption will occur along those systems.

Geosmin production

Oscillatoria limosa has been the main responsible of the geosmin production, and its wax and wane coincide with a seasonal behaviour. Growing and accumulating by the end of January and decaying at the beginning of May. These peaks have been repeated every year, which means that some specific environmental factors are related to them. Although cyanobacterial masses occupy light open zones with warm temperatures, they were substituted by green algae when high irradiances and higher temperatures conditions occurred, since green algae tolerate higher light environments. Therefore, there is a natural cycle governing the occurrence of geosmin in the Llobregat waters.

The geosmin production in the Llobregat River was associated to the growth of these benthic cyanobacteria in littoral zones, since no planktonic cyanobacteria were present in the principal reservoir, La Baells, and other small dams located along the river. Cyanobacterial masses have the peculiarity of growing and occupying a wide range of environmental situations, and among those, nutrient rich waters favour their growth. Therefore, the first point to be considered in the management of the Llobregat River basin is the decrease in the amount of nutrients. A second point should be the removal of unnecessary dams in the river, permitting a natural conditions of water flow, reducing the possibility of mass accumulation. Accordingly to this scenario, during the last year (2003-2004) which has been exceptionally humid, the geosmin occurrence has decreased enormously. However, under normal situations, it is foreseeable that geosmin production restart unless management corrections are applied to the overall water basin.

The fluvial biofilms in their ecological context

Fluvial biofilms were described as a complex community characterized by the interaction of its biotic elements, and by its high structural and functional compartmentalization (Lock et al. 1984). A structural-functional model was proposed

by Lock et al. (1984), in which bacteria, algae and fungi are embedded in a polysaccharidic matrix, inhabited by protozoans and micro-metazoans which graze on this material. Overall, they are responsible of energy transformations such as the conversion of light to chemical energy by algal photosynthesis, adsorption and microbial uptake of heterotrophic carbon, and internal transfers due to extracellular release and cell lysis. As a consequence, biofilms are considered highly efficient in removing inorganic and organic compounds from the water, contributing to the natural self-purification process in rivers.

Biofilm function will be affected both by abiotic and biotic factors. Among abiotic conditions, there are physical (e.g. temperature, light penetration, water current, substrate heterogeneity) and chemical (pH, nutrient availability) factors. Otherwise, biological factors include the relative contribution of autotrophs and heterotrophs, community composition, polysaccharide matrix, biomass thickness and grazing.

Depending on the colonized substratum, the growth and occupation of the biofilms will be different, affecting the interaction between microorganisms. Moreover, water velocity will affect their growth, enhancing biofilm metabolism, since facilitate the diffusion of solutes through the biofilm. Responses to temperature changes will be both physiological (e.g. respiration and photosynthesis rates) and affecting the community structure. Light penetration will also affect the photosynthesis of the autotrophic compartment, determining the respective relevance of the autotrophs and heterotrophs compartments. Nutrient composition and availability will also affect the growth of the biofilm. Therefore, the careful determination of the environmental factors affecting the growth of biofilm would be the first step for evaluating later on the structural and functional parameters

Biofilms may achieve a high structural complexity. Algal taxonomic composition could affect the biofilm efficiency. For instance, green algae have a higher photosynthetic (and nutrient uptake) efficiency than diatoms and cyanobacteria community. However, not only the algal and bacterial composition will affect the biofilm functioning, since the tightly relationship between autotrophs and heterotrophs is also very affecting the functioning. The algal community may affect the heterotrophic capacity of degradation, and therefore, the biofilm capacity of ameliorating water quality. The polysaccharide matrix could also influence in the efficiency of organic matter and nutrient immobilization, enhancing the microbial metabolism. Moreover, extracellular enzymes are retained in the polysaccharide matrix, facilitating the degradation and uptake of high-molecular-weight DOM.

Biofilm thickness reduce diffusion, affecting, among others, the concentration of dissolved oxygen and nutrients. This may create marked gradients through the biofilm, leading to the creation of compartments characterized by different metabolic processes. The internal recycling of nutrients could increase as biofilms became thicker and biologically more complex, and therefore the community could develop largely independent of the nutrient concentration from the water column.

Grazing affects the functioning of river biofilms in several ways, since grazers simplify the composition of the biofilm community, favouring some taxa and favouring the occurrence of others. Moreover, grazers maintain low levels of biofilm biomass, decreasing the amount of nutrient uptake from the system. Otherwise, the effect of grazers inside the biofilm could enhance the formation of microchannels, improving the diffusion of organic and inorganic nutrients through the polysaccharide matrix.

A better understanding of the biofilm function, and a possible evaluation of its implication in river systems, require that all of the parameters mentioned above should be considered.

On the other hand, new methods are required for a better approximation of the biofilms functioning. For example, the possible evaluation of the tight relationship between algae, bacteria, fungi, meiofauna and the polysaccharide matrix. Revealing this could include the determination of its spatial configuration (e.g. by CLSM), or of its chemical composition (C/N/P ratios, exopolysaccharide concentration), or even their taxonomic composition. In particular, the taxonomical identification is still difficult for bacteria or fungi. Moreover, the use of techniques determining the active and inactive organisms in the biofilm need to be considered. The quantification of different pigments, such as phycobilines, which would relate more directly the cyanobacterial biomass. And finally, the quantification and determination of the grazers affecting directly the biofilms.

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