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Selection Of Phototrophic Microorganisms For Use As Bioindicators Of Heavy Metals In Natural Environments. Optimization Of High-Resolution Microscopy Techniques.

Juan Maldonado Ortiz

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Memòria de Tesi presentada per obtenir el grau de Doctor en Microbiolgia per la Universitat Autònoma de Barcelona, per Juan Maldonado Ortiz.

Vist i plau dels Directors de la Tesi,

Dra. Isabel Esteve Martínez   Dr. Antonio Solé Cornellà

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Everything is everywhere, but the environment selects

Lourens G. M. Baas Becking

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investigar. (Del lat. *investigāre*). Realizar actividades intelectuales y experimentales de modo sistemático con el propósito de aumentar los conocimientos sobre una determinada materia.

Cuando me preguntaban de pequeño, que quería ser de mayor o a que me gustaría dedicar, yo decía siempre: quiero ser biólogo, científico, investigador. Creo que algo he conseguido en parte, aunque se que todavía me queda mucho camino, todavía tengo muchas ganas de aprender pero sobre todo, de investigar. Llegar hasta aquí después de 4 años, no ha sido para nada una tarea fácil. Si miro hacia atrás han sido muchas horas de esfuerzo y dedicación, buenos y malos momentos (también los tiene que haber, no?), pero el final de ese viaje o camino, llega y aquí está la recompensa.

En primer lloc, vull agraïr a la meva directora de tesi, la Dra. Isabel Esteve ja que gràcies a ella he pogut realitzar aquest treball. Isabel, em quedo sense paraules per agraïr tot el que m'has ensenyat, aconsellat i el gran suport que m'has donat en tot aquest temps, moltes gràcies. Gràcies també al meu altre director de tesi, el Dr. Antoni Solé per haver-me ensenyat tant desde el primer dia i per recordar-me que pensant una mica en positiu, res, només una mica, les coses surten.

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Resum

En aquest treball s'ha determinat l'efecte i la capacitat de captació dels metalls pesants (Pb i Cu) per part de diferents microorganismes fotòtrofs i heteròtrofs, mitjançant tècniques microscòpiques d'alta resolució per tal de seleccionar microorganismes com a indicadors de contaminació per metalls.

Per a aquest objectiu, han estat seleccionats diferents microorganismes fotòtrofs aïllats dels tapets microbians del delta de l'Ebre (un ecosistema declarat Parc Natural al 1983) i cianobacteris de la col.lecció de cultius Pasteur (PCC). Aquests tapets on dominen els microorganismes fotosintètics com els cianobacteris i les algues (productors primaris en aquests ecosistemes) poden patir contaminació per compostos tòxics com ara el petroli o els metalls pesants. En aquest treball, els metalls assajats per a la selecció de bioindicadors, han estat el plom (Pb) i el coure (Cu) donat que tots dos s'han detectat en el riu Ebre, i també perquè el primer és molt tòxic i no presenta cap funció biològica i el segon és en canvi un element essencial a baixes dosis i tòxic a elevades dosis.

Per determinar la sensibilitat a un metall (tolerància-resistència), s'ha utilitzat la microscopia làser confocal acoblada a un detector espectrofluoromètric (CLSM-$\lambda scan$). Els resultats demostren que *Chroococcus* sp. PCC 9106 i la microalga DE2009 presenten una major tolerància a Pb (0.5 mM) que *Oscillatoria* sp. PCC 7515 i *Spirulina* sp. 6313 (0,1 mM).

Per determinar la biocaptació externa i interna del metall, s'ha utilitzat la microscòpia electrònica de rastreig (SEM), la microscòpia electrònica de transmissió (TEM) i el microanàlisi d'energia dispersiva per raigs X (EDX) acoblada a aquests dos microscopis. Els resultats indiquen que, tots els microorganismes utilitzats (tant els fotòtrofs com els heteròtrofs) tenen la capacitat d'acumular Pb i Cu externament en el seu EPS.

Finalment, l'anàlisi de les seccions ultrafines (TEM-EDX) mostra que tots els microorganismes fotòtrofs estudiats acumulen Pb en les inclusions de polifosfat (PP).

Per contra, els bacteris heteròtrofs tot i presentar aquestes inclusions, no acumulen cap dels dos metalls, ni en el citoplasma ni en les inclusions intracel·lulars.

Una vegada provada l'eficàcia d'aquestes tècniques microscòpiques tant en els microorganismes aïllats de l'ambient natural com en els de col.lecció, s'ha realitzat el mateix tipus d'assajos en microcosmos, amb l'objectiu d'obtenir bons bioindicadors de contaminació per metalls, tenint com a objectiu final la seva aplicació en l'ambient natu-

ral. En aquests experiments s'ha assajat únicament el Pb, i s'ha comprovat que igual que en els cultius, els microorganismes més abundants seleccionats en aquest sistema artificial, *Lyngbya*-like i *Phormidium*-like (cianobacteris) tenen la capacitat d'acumular aquest metall tant externa com internament i sempre en inclusions de PP.

Considerant tots els resultats obtinguts es podria concloure que tots els cianobacteris i la microalga DE2009 analitzats podrien ser considerats bons indicadors de contaminació per metalls. No obstant això, *Oscillatoria* sp. i *Microcoleus* sp. han estat seleccionats per ser: els més abundants en l'ambient natural, de major grandària, tolerants a elevades concentracions de metalls i finalment per la seva capacitat d'acumularlos extra i intracel·lularment.

Resumen

En este trabajo se ha determinado el efecto y la capacidad de captación de los metales pesados (Pb y Cu) por parte de diferentes microorganismos fotótrofos y heterótrofos, mediante técnicas microscópicas de alta resolución con el fin de seleccionar microorganismos como indicadores de contaminacion por metales.

Para este objetivo, han sido seleccionados diferentes microorganismos fotótrofos aislados de los tapetes microbianos del delta del Ebro (un ecosistema declarado Parque Natural en 1983) y cianobacterias de la colección de cultivos Pasteur (PCC). Estos tapetes en los que dominan los microorganismos fotosintéticos como las cianobacterias y las algas (productores primarios en estos ecosistemas) pueden sufrir contaminación por compuestos tóxicos tales como el petróleo o los metales pesados. En este trabajo, los metales ensayados para la selección de bioindicadores, han sido el plomo (Pb) y el cobre (Cu) ya que ambos se han detectado en el río Ebro, y también porqué el primero es muy tóxico y no presenta ninguna función biológica y el segundo es en cambio un elemento esencial a bajas dosis y tóxico a elevadas dosis.

Para determinar la sensibilidad a un metal (tolerancia-resistencia), se ha utilizado la microscopia láser confocal acoplada a un detector espectrofluorométrico (CLSM-λscan). Los resultados demuestran que *Chroococcus* sp. PCC 9106 y la microalga DE2009 presentan una mayor tolerancia al Pb (0.5 mM) que *Oscillatoria* sp. PCC 7515 y *Spirulina* sp. 6313 (0.1 mM).

Para determinar la biocaptación externa e interna del metal, se ha utilizado la microscopia electrónica de barrido (SEM), la microscopia electrónica de transmisión (TEM) y el microanálisis de energía dispersiva por rayos X (EDX) acoplada a estos dos microcoscopios. Los resultados indican que, todos los miroorganismos utilizados (tanto fototrófos como heterótrofos) tienen la capacidad de acumular Pb i Cu externamente en el EPS.

Finalmente, el análisis de las secciones ultrafinas (TEM-EDX) muestra que todos los microorganismos fotótrofos estudiados acumulan Pb en las inclusiones de polifosfato (PP).

Por el contrario las bacterias heterótrofas a pesar de presentar estas inclusiones, no acumulan ninguno de los dos metales, ni el en citoplasma ni en las inclusiones intracelulares.

Una vez probada la eficacia de estas técnicas microscópicas tanto en los microorganismos aislados del ambiente natural como en los de coleccion, se ha realizado el mismo tipo de ensayos en microcosmos, con el objetivo de obtener buenos bioindicadores de contaminación por metales, teniendo como objetivo final su aplicación en el ambiente natural. En estos experimentos se ha ensayado únicamente el Pb, y se ha comprobado que al igual que en los cultivos, los microorganismos más abundantes seleccionados en este sistema artificial, *Lyngbya*-like y *Phormidium*-like (cianobacterias) tienen la capacidad de acumular este metal tanto externa como internamente y siempre en inclusiones de PP.

Considerando todos los resultados obtenidos podría concluirse que todas las cianobacterias y la microalga DE2009 analizadas podrían ser consideradas buenos indicadores de contaminación por metales. No obstante, *Oscillatoria* sp. y *Microcoleus* sp. han sido seleccionados por ser: los mas abundates en el ambiente natural; de mayor tamaño; tolerantes a elevadas concentraciones de metales y finalmente por su capacidad de acumularlos extra e intracelularmente.

Summary

In this work, the effect and capacity to capture heavy metals (Pb and Cu) of different phototrophic and heterotrophic microorganisms have been studied through high-resolution microscopy techniques in order to select microorganisms as metal pollution indicators.

For this purpose, different phototrophic microorganisms isolated from Ebro Delta microbial mats (an ecosystem declared as Natural Park in 1983) and cyanobacteria from the Pasteur culture collection (PCC) have been selected. These mats, dominated by photosynthetic microorganisms like cyanobacteria and algae (primary producers in these habitats) can suffer pollution by toxic compounds such as oil or heavy metals. In this study, metals tested to select the bioindicators were lead (Pb) and copper (Cu), as both have been detected in the Ebro River, and also because the former is very toxic, showing no biological function, while the latter, by contrast, is an essential element at low doses, while at high doses it is toxic.

To determine the sensitivity to a metal (tolerance-resistance), confocal laser microscopy coupled to a spectrofluorometric detector (CLSM-λscan) has been used. The results show that *Chroococcus* sp. PCC 9106 and DE2009 microalgae show greater tolerance to lead Pb (0.5 mM) than *Oscillatoria* sp. PCC 7515 and *Spirulina* sp. 6313 (0.1 mM).

To determine the external and intracellular metal biocapture, scanning electron microscopy (SEM), transmission electron microscopy (TEM) and energy dispersive X-ray microanalysis (EDX) coupled with these microscopes have been used, respectively. The results indicated that, all the microorganisms tested (both phototrophic and heterotrophic) have capacity to accumulate Pb and Cu externally in the EPS.

Finally, the analysis of ultrathin sections (TEM-EDX) shows that all the phototrophic microorganisms studied accumulate Pb in the polyphosphate inclusions (PP).

By contrast, although the heterotrophic bacteria show these inclusions, they do not accumulate either of the two metals either in cytoplasm or in intracellular inclusions.

Once the effectiveness of these microscopy techniques has been shown both in microorganisms isolated from the natural environment and in those from the culture collection, the same tests have been carried out in microcosms in order to obtain good bioindicators of metal pollution, with the ultimate aim of applying them in the natural

environment. In these experiments it has been shown that, as in cultures, the most abundant microorganisms selected in this artificial ecosystem, *Lyngbya*-like and *Phormidium*-like (cyanobacteria), have the capacity to accumulate this metal both externally and internally, always in PP inclusions.

Considering all the results obtained it can be concluded that all cyanobacteria and DE2009 microalga analysed could be considered good metal pollution indicators. However, *Oscillatoria* sp. and *Microcoleus* sp. have been selected as the best indicators of metal pollution in the natural environment because: they are the most abundant, largest in size, tolerant to high concentrations of metals and, finally, capable of accumulating metal extra- and intracellularly.

Contents

Tables and figures

Structure of the Thesis

Introduction: This chapter introduces the natural environment studied, the Ebro Delta microbial mats, and the effect of metal pollution in natural ecosystems. Finally it emphasizes the importance of searching photosynthetic microorganisms as indicators for metal pollution in natural habitats.

Material and methods: In this chapter is described the methodology used in this work in addition, the microorganisms used and their culture conditions.

Isolation and identification of a bacterium with high tolerance to lead and copper from a marine microbial mat in Spain: In this chapter is described the identification of a heterotrophic bacterium (DE2008) isolated from Ebro Delta microbial mats. At the same time, is determined the strain's ability to tolerate high concentrations of heavy metls and accumulate these externally in sheaths or internally in intracytoplasmic inclusions. This work has been published in the journal *Annals of Microbiology*. The molecular identification of the strain DE2008 has been made in collaboration with the *Equipe Environnement et Microbiologie, IPREM UMR CNRS 5254, Université de Pau et des Pays de l'Adour, France.*

Electron microscopy techniques and energy dispersive X-ray applied to determine the sorption of lead in *Paracoccus* sp. DE2007. This chapter details the ability to bioaccumulate Pb (externally and internally) by the heterotrophic bacterium *Paracoccus* sp. DE2007 using electron microscopy techniques and energy dispersive X-ray analysis. This work has been published as a chapter book in *Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology.*

Sequestration and *in vivo* effect of lead on DE2009 microalga, using high-resolution microscopic techniques. This chapter describes the tolerance-resistance of DE2009 microalga (isolated from microbial mats of the Ebro Delta) to Pb by means CLSM-$\lambda scan$ and to determine their total and individual biomass (CLSM-IA). Complementary studies using SEM, TEM and energy dispersive X-ray microanalysis coupled to both microscopes were also performed to test the capacity of DE2009 microalga for extra- and intracellular uptake of Pb. This work has been published in the *Journal of Hazardous Materials*. The molecular indentification of DE2009 microalga has been made in collabora-

tion the group *Instituto de Recursos Naturales, Centro de Ciencias Medioambientales (CSIC) Madrid, Spain.*

Selection of bioindicators to detect lead pollution in Ebro Delta microbial mats, using high-resolution microscopic techniques: In this chapter is described the potential of different cyanobacteria to be considered bioindicators of lead pollution in coastal microbial mats, using CLSM-$\lambda scan$ to determine the sensitivity to lead and SEM-EDX and TEM-EDX to determine the capacity to accumulate this metal extra and/or intracelularlly. This work has been published in the journal *Aquatic Toxicology.*

Results and General discussion: In this chapter are summarized the results presented in Chapters 3-6 and widely discussed.

Conclusions and Future prospects: Finally, the conclusions are listed and the future prospects opened up by this work.

1. Introduction

The Ebro Delta, located at the outfall of the Ebro River (Spain), is the second largest wetland in Spain after the Guadalquivir River marshes and the second biggest in the Mediterranean after the Camargue (France). In 1983, some of the most outstanding natural areas of the Delta were included in the Ebro Delta Natural Park (*Parc Natural del Delta de l'Ebre*) for their biological and cultural significance. The Ebro River is 928 km long, flows from the north of the Iberian Peninsula to the Mediterranean Sea and drains an area approximately of 85,000 km^2. Its basin is the most economically important area of northern of Spain (Ramos et al., 2004). The Ebro Delta is the third largest delta in the Mediterranean: a triangular area of 320 km^2 and located on the northeastern coastline of the Iberian Peninsula (0°35'E–0°56'E; 40°33'N–40°47'N) (Guerrero et al., 2002). Of its total area, 78 km^2 corresponds to the Natural Park (25%), 160 km^2 to rice farming (50%) and 85 km^2 to orchards and fruit (25%). The formation of the Ebro Delta began at the end of the last Ice Age about 50,000 years ago (Quaternary period). As a consequence, the sea level rose flooding the mainland and the continental shelf served as a base for sediment transported from the Ebro River. The Delta's geology has varied over the centuries, but its crucial formation period was established around the 17th Century. Different ecosystems such as microbial mats, marshes, salt ponds, dunes and sandy beaches provide habitat for a large number flora and fauna species forming part of the Ebro Delta.

Since 1990, our research team has studied the Ebro Delta microbial mats (Fig. 1.1). These ecosystems, developed in water-sediment interfaces, are formed by multi-layered benthic microbial communities distributed along vertical micro-gradients of different physical-chemical parameters. Microbial mats are widely distributed around the world in different environments, such as marine waters (Esteve et al., 1992; Otte et al., 1999), fresh waters (Brunberg et al., 2002), hypersaline ponds (Hoehler et al., 2001), estuaries (Olendzenski, 1999), hot deserts (Campbell, 1979), hot springs (Nakagawa and Fukui, 2002; Petroff et al., 2010), soils (Watanabe et al., 2000) in Antarctic ice ponds (Jungblut et al., 2011) and hydrothermal vents in deep oceans (Kato et al., 2009). Ebro Delta microbial mats are formed by different microorganisms mainly: cyanobacteria, algae, colorless sulfur bacteria, purple sulfur bacteria an sulfate-reducing bacteria. Among these, cyanobacteria are the most abundant and they are located mainly in the upper layers (green layer) of microbial mats. These microorganisms are phototrophic

gram-negative oxygenic bacteria and they have the capacity to assimilate CO_2. Their major photosynthetic pigment is chlorophyll *a* (chl *a*), as in algae and plants. It is thought that during the Proterozoic period, cyanobacteria contributed to the formation of an oxygenated atmosphere (Hoehler et al., 2001).
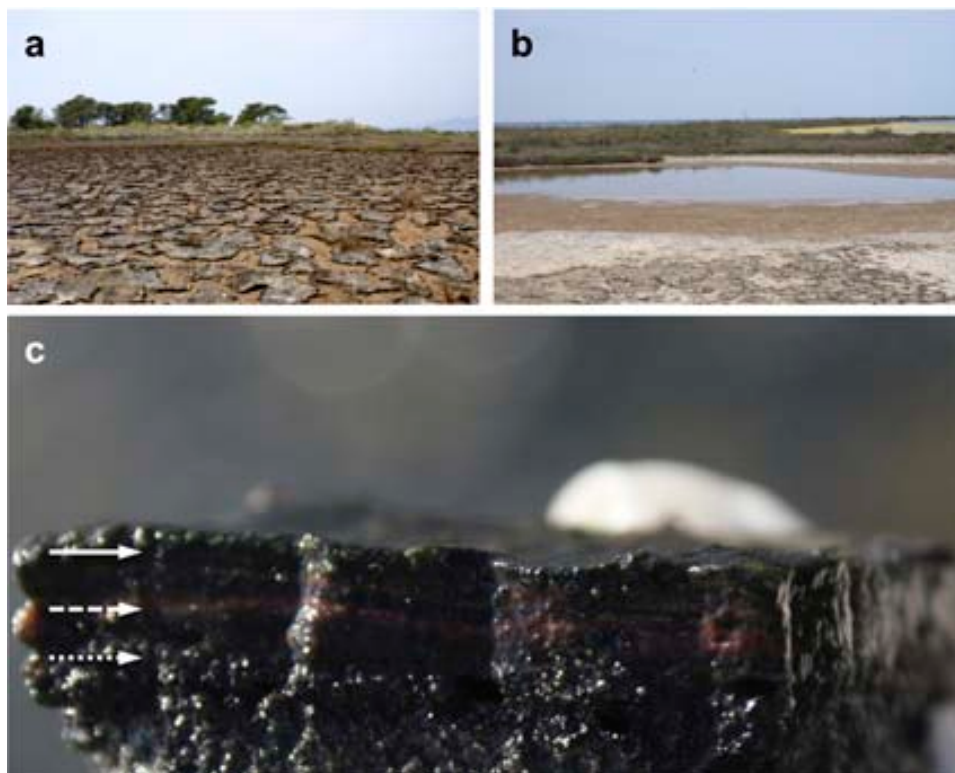
Fig. 1.1. Ebro Delta microbial mats. Microbial mats in the dry season **(a)** and covered by water **(b)**. Microbial mat structure. Phototrophic microorganisms, green layer (⸺); purple reducing bacteria, red layer (----) and sulfate reducing bacteria, black layer (·····) **(c)**.

In recent years, microbial mats have also become particularly interesting ecosystems due to their bioremediation capacities. Different authors have demonstrated the significant role of these ecosystems in the bioremediation of oil-polluted coastal areas of the Arabian Gulf, during the 1991 Gulf War, achieving hydrocarbon degradation in few months (Al-Hassan et al., 1998). The ability of these ecosystems to sequester heavy metals has also been demonstrated (Bender et al., 1994). As mentioned above, the Ebro Delta has been declared a protected area for more than 25 years. Despite this, both the River and the Delta are nowadays subjected to anthropogenic pollution, mainly as a consequence of industrial activities that discharge their waste into the river and the agricul-

tural crops (rice and fruit), which increase the amount of pesticides in the Delta. All these circumstances cause serious environmental effects attributable to the accumulation of the contaminants in sediments, water, soil and biota. The predominant pollutants in these habitats are: herbicides, insecticides, hydrocarbons and heavy metals (Mañosa et al., 2000). Another important source of pollution has been the use of lead (Pb) pellets in hunting waterfowl (Mateo et al., 1997). Nowadays, metals are released from natural and anthropogenic sources (e.g. industry, transport, fossil fuel combustion, the mining industry and agriculture) into natural aquatic environments (Nogales et al., 2011). These are accumulated in the sediments and biota, generating resistance in microorganisms leading to environmental and public health problems. The toxicity of metals in these biological systems depends on their bioavailable form and on different biotic and abiotic factors (Roane and Pepper, 2000).

The term *heavy metal* has been often used as a group named for metals and semimetals (metalloids) associated with pollution and potential ecotoxicity. Heavy metals are a group of elements, characterized by their high density and they are member of a subgroup of elements with metallic properties. They mainly include the transition metals, some metalloids, lanthanides, and actinides. Among these metals are arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), lead (Pb), mercury (Hg), nickel (Ni), selenium (Se) and zinc (Zn) (Duffus, 2002). From a physiological point of view, metals may fall into three main categories: (i) those essential and basically non-toxic ones e.g. calcium (Ca) and magnesium (Mg), (ii) essential ones, but harmful at high concentrations e.g. iron (Fe), manganese (Mn), zinc (Zn), copper (Cu), cobalt (Co), nickel (Ni) and molybdenum (Mo), and (iii) toxic ones e.g. mercury (Hg) or cadmium (Cd). Moreover, the interactions with metals depend not only on the particular element but also on its chemical speciation (Valls and de Lorenzo, 2002). For many microorganisms some metals are considered essential because they form part of metabolic processes or the active centers in some proteins. At low concentrations these might not be harmful for cells but at high concentrations they could cause cellular damage. For instance, Cu is an essential metal for cyanobacteria because it forms part of the plastocyanin and a *c*-type cytochrome oxidase (necessary proteins for the photosynthesis). However, at high concentrations it causes serious toxic effects. For example, Surosz and Palinska, (2004) demonstrated the reduction of chl *a* concentration in *Anabaena flos-aquae* while this cyano-

bacterium was growing up to 6 mg L^{-1} of Cu. On the other hand, metals such as Pb, Hg or Cd are toxic at any concentration and do not have any kind of biological function.

Metal pollution is particulary serious, which is why various techniques have been employed to treat of metal industrial effluents, usually falling into two broad divisions: abiotic and biotic methods. Abiotic methods include precipitation, adsorption, ion exchange, and membrane and electrochemical technologies. Regarding biotic methods, there are three main advantages of biological technologies for removing pollutants: biological processes can be carried out *in situ* at the polluted site; bioprocesses are environmentally benign (no secondary pollution is generated) and thirdly, they are cost-effective (Vijayaraghavan and Yun, 2008). Biotic processes are based on the ability of organisms to interact with metals by biosorption, bioaccumulation, biomineralization, bioleaching and enzymatic transformation of metals (Fig. 1.2).
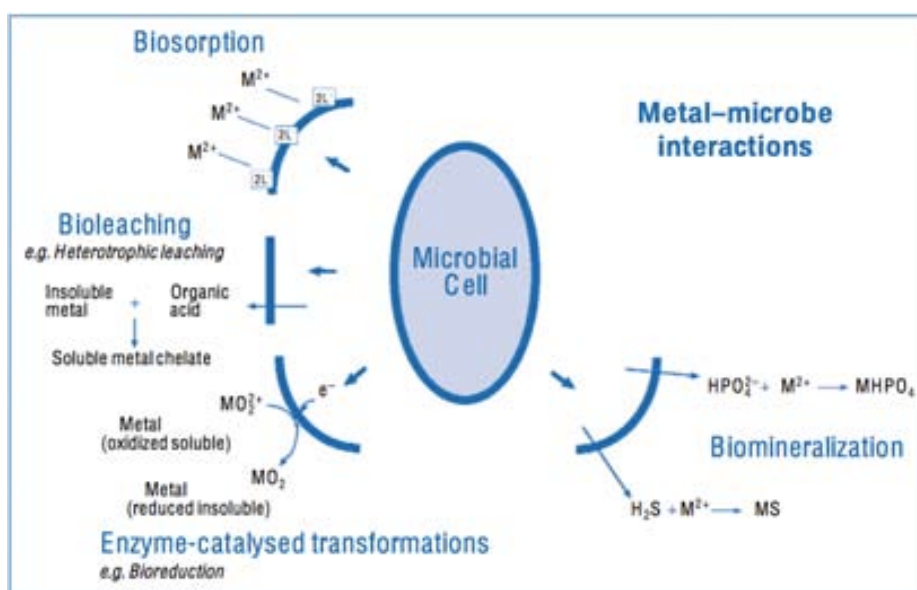
Fig. 1.2. Metal–microbe interaction mechanisms that can be harnessed for bioremediation applications (Lloyd et al., 2002).

In the face of this problem, studies are being conducted on how heavy metals affect the microbial communities of natural environments (including environments such as microbial mats) at the level of their activity, structure, biodiversity and biomass.

In Table 1.1., data are collected about the effects of metals on the diversity and biomass of microbial populations in natural samples and techniques used for that purpose.

Table 1.1. Different methodologies used to determine the effects of metals on microbial diversity and biomass in different natural environments.

Methodology	Metal	Environment	Reference
DGGE	Cu	biofilms	Boivin et al., 2005
DGGE	Cu	biofilms	Massieux et al., 2004
CLSM	Cu, Pb	microbial mat	Burnat et al., 2009
B-ARISA	Cu, Cd, Hg	soil	Ranjard et al., 1994
Timidine incorporation technique and plate counts	Cu, Cd, Zn, Ni, Pb	soil	Díaz-Raviña et al., 1994
TGGE	Cr, Pb	biofilms	Vílchez et al., 2011
DGGE	Cd, Cu	marine sediments	Toes et al. 2008
DGGE, FISH and Cloning libraries	Cd, Cu, Pb, Zn	marine sediments	Gilland et al., 2005
DGGE	As, Cd, Cr, Cu, Hg, Ni, Pb, Zn	soil	Ellis et al., 2003
DGGE	As, Cd	soil	Lorenz et al., 2006
DGGE	Cu, Pb, Cd	soil	Lin et al., 2006
HPLC	Co, Ni, Cu, Zn	river	Chakraborty et al., 2010

DGGE: Denaturing gradient gel electrophoresis
B-ARISA: Bacterial automated ribosomal intergenic spacer analysis
TGGE: Temperature gradient gel electrophoresis
FISH: Fluorescense in situ hybridation
HPLC: High performance liquid chromatography

On the other hand, many studies have been carried out to demonstrate the capacity of microorganisms to sequester metals (Table 1.2). All these studies have been conducted to investigate the bioremeditaion capacity present in some microorganisms with respect to the metals, although there are few studies aimed at the rapid detection of the presence of these as bioindicators of pollution and these studies are usually performed in high-polluted areas. The term *bioindicator* is used to refer to an organism whose status in an ecosystem is analyzed as an indication of the ecosystem's quality. Several species of macroorganisms have been used as bioindicators of metal pollution in natural habitats (in water and soil quality). Plants species and their organs have been proved to accumulate toxic metals (Gjorgieva et al., 2011). In aquatic environments the fish *Cathorops spixii* has been considered an effective bioindicator to evaluate the contamination of trace metals (Co, Fe, Se, Zn and Hg) in Santos Bay (Brazil) (Azevedo et al., 2009). Invertebrates such as freshwater bivalve species were proved to be tolerant to Hg in the Ebro River (Faria et al., 2010).

Table 1.2. Capacity of different microorganisms to tolerate and/or sequestrate metals.

Microorganisms	Sample's origin	Metals	References
Heterotrophic bacteria			
Bacillus sp.	environment	Cu, Pb	Tunali et al., 2006
Chlorobium spp.	environment	Mn, Fe, Cu, Zn, Cd	Garcia-Gil and Borrego, 1997
Corynebacterium glutamicum	industry	Pb	Choi and Yun, 2004
Micrococcus luteus	culture collection	Cu	Nakajima et al., 2001
Pseudomonas aeruginosa	laboratory strain	Zn, Cu	Teiztel and Parsek, 2003
Pseudomonas sp.	enviroment	Cd, Cr	Ziagova et al., 2007
Staphylococcus xylosus	enviroment	Cd, Cr	Ziagova et al., 2007
Phototrophic microorganisms			
Cyanobacteria			
Anabaena flos-aquae	culture collection	Pb, Cd	Heng et al., 2004
Aphanothece halophytica	environment	Zn	Incharoensakdi and Kitjaharn, 2002
Cyanospira capsulata	culture collection	Cu	De Philipis et al., 2003
Gloeothece sp. PCC 6909	culture collection	Cu, Pb	Pereira et al. 2011
Microcoleus sp.	environment	Pb, Cu	Burnat et al., 2009
Microcystis areugionsa	environmental	Cu, Cd, Ni	Parker et al., 1998
Microcystis areugionsa	environmental	U	Li et al., 2004
Microcystis sp.	industry	Cu, Cd, Zn	Pradhan and Rai, 2001
Nostoc PCC 7936	culture collection	Cu	De Philipis et al., 2003
Oscillatoria anguistissima	culture collection	Co	Ahuja et al., 1999
Spirulina maxima	industry	Pb	Gong et al., 2005
Synechococcus elongatus	culture collection	U	Acharya et al., 2009
Synechocystis sp. PCC 6803	culture collection	Pb	Roy et al., 2008
Algae			
Chlorella vulgaris	culture collection	Ni, Cu	Mehta and Gaur, 2001
Geminella terricola	environment	Cu	Kalinowska and Pawlik-Skowronska, 2010
Stichococcus minor	environment	Cu	Kalinowska and Pawlik-Skowronska, 2010
Fungi			
Aspergillus japonicus	industry	Fe, Ni, Cr, Hg	Binupriya et al., 2006
Aspergillus niger	culture collection	Pb	Lo et al., 1999
Mucor rouxii	culture collection	Pb	Lo et al., 1999
Penicilum purpurogenum	environment	Cd, Pb, Hg, As	Say et al., 2003

Finally, birds such as the ornate tinamou (*Nothoprocta ornata*) and mammals like the greater white-toothed shrew (*Crocidura russula*) have been used as bioindicators of metal pollution by mean of tissues analysis at Bolivian mining sites and the Ebro Delta, respectively (Garitano-Zavala et al., 2010; Sánchez-Chardi et al., 2007).

Despite the extensive information mentioned above, nothing is known about the use of phototrophic microorganisms (algae and cyanobacteria) in microbial mats although they are the most abundant microorganisms in these habitats. **The aim of this work is to select the best phototrophic microorganisms as indicators of metal pollution by Pb and Cu using high-resolution microscopic techniques.**

For this purpose, we will consider the following characteristics in selecting the appropriate bioindicators: ubiquity and abundance of the selected microorganisms; ease of growth under laboratory conditions; sensitivity to the chosen metal and the ability to accumulate it. Among the methods used for this goal, in recent years our working group has optimized different high-resolution microscopy techniques: confocal laser scanning microscopy (CLSM), transmission electron microscopy (TEM) and scanning electron microscopy (SEM) both coupled to an electron dispersive X-ray detector (EDX). The first, technique determines the *in vivo* effect of different metals on phototrophic microorganisms (sensitivity), while the others, to detect the ability of these microorganisms to capture metals extra- and/or intracellularly (Burnat et al., 2009, 2010).

For this work, different microorganisms (phototrophic and heterotrophic) have been selected: a) two heterotrophic bacteria (*Paracoccus* sp. DE2007 and *Micrococcus luteus* sp. DE2008) and a microalga (DE2009) isolated from a consortium of microorganisms of Ebro Delta microbial mats, b) three cyanobacteria (*Oscillatoria* sp. PCC 7515, *Chroococcus* sp. 9106 and *Spirulina* sp. PCC 6313) chosen from the Pasteur culture collection of cyanobacteria (PCC) and c) environmental samples from Ebro Delta microbial mats collected to perform pollution experiments in microcosms.

Finally, Cu and Pb were the metals chosen to be tested in this study. As mentioned above, Cu has biological functions at low concentrations, although at high concentrations it has a toxic effect. On the other hand, Pb is always a toxic metal with no biological functions.

The methodologies used are described in Chapter 2. The methods used and results obtained in this work are detailed in Chapters 3, 4, 5, and 6. Finally, all the results are summarized and discussed in Chapter 7.

2. Material and methods

In this chapter is described the sampling area and the methodologies used for processing the samples of microcosms and the microorganisms used to select microorganisms as metal pollution indicators.

2.1. Characterization and sampling site of the Ebro Delta microbial mats

The Ebro Delta is the third largest delta in the Mediterranean: a triangular area of 320 km^2 and located on the northeastern coastline of the Iberian Peninsula (Guerrero et al., 2002). Ebro Delta is second most important wetland area in the Iberian Peninsula after the Guadalquivir River marshes (Spain), and the second of the Mediterranean after the Camargue (France). In the Delta exist numerous ecosystems; beaches, salt ponds, marshes, dunes and microbial mats.

Ebro Delta microbial mats develope 1 to 7 cm below the water surface. The temperature of water covering these mats range from 12°C to 30°C; conductivity from 59 to 105 mS cm^{-1}; salinity from 40‰ to 75‰, pH from 7.5 to 9.0, and being exposed to an annual rainfall of 500 L m^{-2} (Esteve et al., 1994).

2.2. Microcosms setup

To determine the ability of cyanobacteria from microbial mats to capture external and/or internally Pb, a laboratory microcosms experiments with samples from Ebro Delta microbial mats was designed. Microcosms are artificial laboratory systems that are used to simulate and predict changes in natural environments under controlled conditions.

Samples were collected from the Ebro Delta on October 2009 (Fig. 2.1). Sediment samples were taken taken carefully in 55 x 43 x 88 mm poly(methyl methacrylate) boxes preserving the structure of the microbial mat. Microcosms were transferred to the laboratory protected from light and refrigerated at 4ºC.

Fig. 2.1. Ebro Delta location (**a**), (**b**). Microbial mats sampling site (**c**), (**d**).

One microcosm was used as a control experiment (unpolluted) and the other microcosm was polluted with 25 mL of 10 mM [Pb(NO$_3$)$_2$] solution. In the control experiment the same amount of deionized water was added (Fig. 2.2). The microcosms were maintained in laboratory conditions for 9 days, after which the metal solution was removed by decanting from the polluted microcosm.

Samples for electron microscopy (SEM and TEM) and CLSM were taken with glass cores ⌀ (6 mm) from the upper layers of the microbial mat.
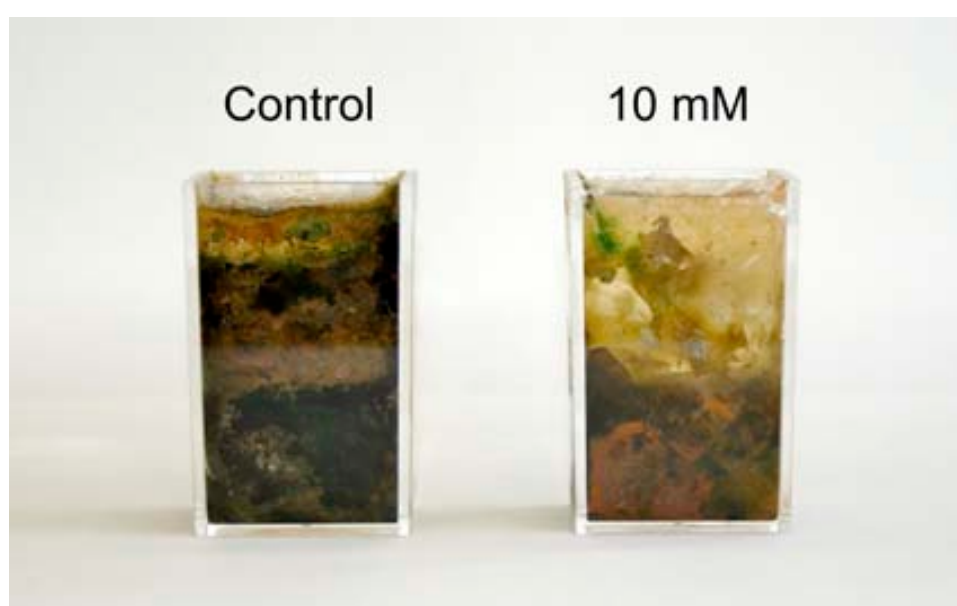
Fig. 2.2. Control and polluted microcosms.

2.3. Microorganisms and culture conditions

The different microorganisms (heterotrophic and phototrophic) and its culture conditions used in this work are following described.

2.3.1. Heterotrophic microorganisms

Paracoccus sp. DE2007 and *Micrococcus luteus* DE2008 both isolated from a *Microcoleus* consortium from Ebro Delta microbial mats (Diestra et al., 2007; Maldonado et al.,

2010a) were cultivated in Luria-Bertani rich medium (LB) (agar or broth) at 27°C in darkness (Fig. 2.3). (Composition of LB medium is described in Annex I).
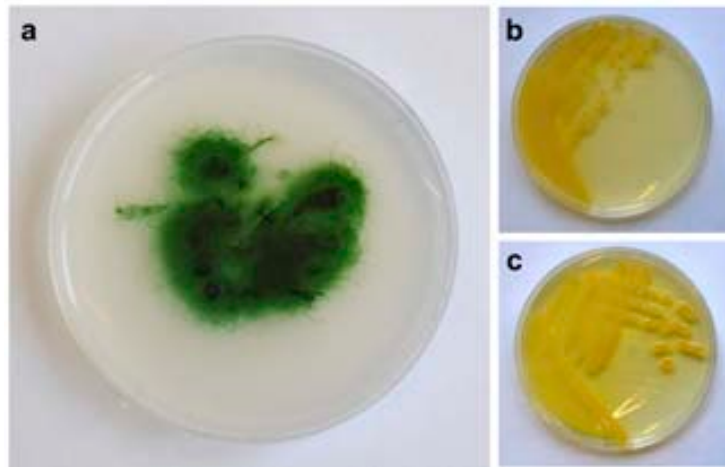
Fig. 2.3. *Microcoleus* sp. consortium **(a)**, *Micrococcus luteus* DE2008 **(b)** and *Paracoccus* sp. DE2007 **(c)**.

2.3.2 Phototrophic microorganisms

A microalga (DE2009) and three different cyanobacteria *Oscillatoria* sp. PCC 7515, *Chroococcus* sp. PCC 9106 and *Spirulina* sp. PCC 6313 from culture collection were used in this work (Fig 2.4).
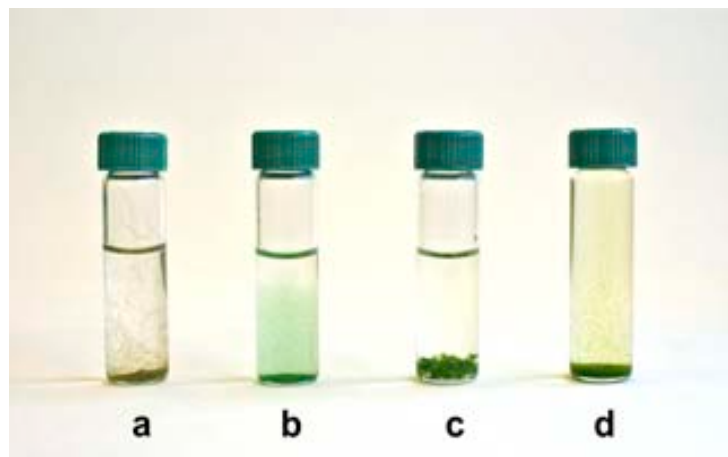
Fig. 2.4 Phototrophic microorganisms. *Oscillatoria* sp. PCC 7515 **(a)**, *Chroococcus* sp. PCC 9106 **(b)**, *Spirulina* sp. PCC 6313 **(c)** and DE2009 microalga **(d)**.

The DE2009 microalga was isolated from *Microcoleus* consortium cultures from from Ebro Delta microbial mats. It was cultivated and maintained in Pfennig mineral medium (Pfennig and Trüper, 1992) at 27ºC and under light conditions (15 µE m^{-2} s^{-1}). (Composition of Pfennig mineral medium is described in Annex I).

Different cyanobacteria were selected from the culture collection of Pasteur Culture Collection of cyanobacteria (PCC): *Oscillatoria* sp. PCC 7515, *Chroococcus* sp. PCC 9106 and *Spirulina* sp. PCC 6313, since they are always found in abundance in microbial mats. Besides, these microorganisms were chosen on the basis of their different morphologies, sizes, and photosynthetic pigment composition.

Chroococcus sp. PCC 9106 and *Spirulina* sp. PCC 6313 strains were grown in BG-11 and ASN III (1:1 v/v) medium (Rippka et al., 1979) and mantained at 27ºC and under light conditions (15 µE m^{-2} s^{-1}). *Oscillatoria* sp. PCC 7515 strain was cultivated and maintained in MN medium with nitrate ommited (Rippka et al., 1979) and mantained at 27ºC and under light conditions (15 µE m^{-2} s^{-1}). (Composition of BG-11, ASN III and MN media are described in Annex I).

2.4. Isolation and identification procedures of the strains DE2007, DE2008 and DE2009 from natural habitats

2.4.1. DE2007

The heterotrophic bacterium *Paracoccus* sp. DE2007 was previously isolated from the *Microcoleus* consortium, and biochemical and physiologically characterized by Diestra et al., (2005).

2.4.2. DE2008

Isolation, biochemical and physiological characterization

The strain DE2008 was isolated from a *Microcoleus* consortium (Diestra et al., 2005) Inoculums from this consortium were transferred to LB. The cultures were incubated in darkness at 27°C. Different colonies were obtained, one of which was streaked on LB

agar and isolated in pure culture. The morphological characteristics of strain DE2008 were examined with an Olympus BH2 conventional light microscope. Different stain methods were used to characterize the DE2008 bacterium (Gram, Toluidine Blue, Wirtz-Conklin, and Negative stains). Motility was determined with an optical microscope using the hanging-drop technique. The biochemical assays were made using the API 20 NE strip-identification system (BioMerieux, Marcy l'Étoile, France). Oxidase activity was analysed by oxidation of 1% p-aminodimethylaniline oxalate (Cowan and Steel, 1965). Catalase activity was determined by the presence of bubbles with a 3% H_2O_2 solution (Takeuchi et al., 1996). Starch hydrolysis was analyzed as described by Cowan and Steel (1965).

The effect of different physical–chemical parameters were also tested on bacterial strain DE2008. The pH range for growth was determined by incubating cells in LB medium at 27°C for 4 days at the following pH: 2, 3, 4 (LB broth medium), 5, 6, 7, 8, 9, 10, and 11 (LB agar medium). NaCl tolerance was measured in LB agar medium at concentrations of 0, 1, 2, 3, 4, 5, 6, 7, 8, and 10% (w/v). To determine the temperature range, the growth of the strain at different temperatures (8–43°C) was tested.

Molecular identification of the strain DE2008

Genomic DNA was extracted from 2 mL of an overnight DE2008 culture in liquid LB using UltraClean™ Soil DNA Isolation Kit (Mobio Laboratories, Carlsbad, USA) according to manufacturer's instructions. The 16S rRNA gene fragment was obtained by PCR amplification using primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1489R (5′-TACCTTGTTACGACTTCA-3′), according to the procedure previously described (Bordenave et al., 2004). The following PCR conditions were applied: 35 cycles of 10 min at 95°C, 45 s at 95°C, 45 s at 51°C, and 1.5 min at 72°C, with a final step of 10 min at 72°C. PCR product was then purified using the GFXTM PCR DNA and Gel Band Purification Kit as directed by the supplier (GE Heathcare, UK). The sequence of the purified DNA fragment was obtained using the BigDye Terminator Cycle Sequence Kit (versions 1.1; Applied Biosystems, Foster City, USA) on an ABI PRISM310 Genetic Analyzer (Applied Biosystems).

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The sequence was compared to those present in the NCBI database (http://www.ncbi.nlm.nih.gov) using the BLAST program. For phylogenetic analysis, a dataset containing GenBank 16S rRNA gene sequences was used. Pairwise evolutionary distances based on 1,455 unambiguous nucleotides were computed by the method of Jukes and Cantor (1969) with MEGA version 3.0 software (Kumar et al., 2004). The phylogenetic tree was constructed using neighbor-joining (Saitou and Nei, 1987). Confidence in the tree topology was determined by bootstrap analysis using 1,000 resampling of the sequences.

2.4.3. DE2009

The DE2009 microalga was isolated from *Microcoleus* consortium cultures from Ebro Delta microbial mats.

Molecular identification of the strain DE2009

Genomic DNA was extracted from an DE2009 overnight culture in Pfennig medium using UltraClean™ Microbial DNA Isolation Kit (Mobio Laboratories, Carlsbad, USA) according to manufacturer's instructions. The 18S rRNA gene fragment was obtained by PCR amplification using SR1 (5′ -TACCTGGTTGATCCTGCCAG-3′) and Euk516 (5′-ACCAGACTTGCCCTCC-3′) primers (Diez et al., 2001), using PureTq™ Ready-To-Go™ PCR (GE Healthcare). The PCR conditions were those described in (Diez et al., 2001). The PCR product was then purified using the QIAquick PCR purification Kit (Quiagen) as directed by the supplier. Both complementary strands were sequenced separately at the SECUGEN sequencing company (S.L. Madrid, Spain).

2.5. Preparation of metal stock solutions and pollution conditions in cultures and microcosms

Lead and copper stock solutions were prepared with $Pb(NO_3)_2$ and $CuSO_4$, respectively (Merck KGaA, Darmstadt, Germany) in deionized water and sterilized by filtration in Millex-GP 0.22 μm filters (Millipore, USA).

Micrococcus luteus DE2008

The concentrations used for both Pb and Cu to determine the growth of *Micrococcus luteus* DE2008 with metals and the ability to accumulate them external and/or internally were: 0, 0.1, 0.5, 1, 1.5, 2, 5 and 25 mM for period of 12-24 h.

Paracoccus sp. DE2007

The concentrations used to determine the ability of *Paracoccus* sp. DE2007 to accumulate Pb external and/or internally were: 0 and 5 mM for a period of 12-24 h.

DE2009 microalga

The concentrations used to determine the sensitivity of DE2009 microalga to Pb and the ability to accumulate the metal external and/or internally were: 0, 0.1, 0.5, 0.75, 1, 5 and 10 mM at two pHs (7 and 4) for a period of 9 days.

Oscillatoria sp. PCC 7515, *Chroococcus* sp. PCC 9106 and *Spirulina* sp. PCC 6313.

The concentrations used to determine the sensitivity of cyanobacterial strains to Pb and the ability to accumulate the metal external and/or internally were: 0, 0.1, 0.5, 0.75, 1, 2 and 10 mM for a period of 9 days.

Microcosms

The concentrations used to determine the ability of cyanobacteria from polluted micro-cosms to accumulate Pb external and/or internally were: 0.5 and 10 mM for a period of 9 days.

2.6. Methods to determine the effect of metals on heterotrophic and phototrophic microorganisms

Growth rates and minimum inhibitory concentration methods were used to determine the effect of Pb and Cu in *Micrococcus luteus* DE2008 and CLSM-*λscan* was used to determine the Pb sensitivity in microalga DE2009, *Oscillatoria* sp. PCC 7515, *Chroococcus* sp. PCC 6313 and *Spirulina* sp. PCC 9106.

This section only describes the protocols for the heterotrophic microorganisms, while CLSM-*λscan* technique is described in section 2.7.1.

2.6.1 Growth rates

Overnight cultures of DE2008 strain grown on LB medium were inoculated into LB medium supplemented with different $Pb(NO_3)_2$ or $CuSO_4$ concentrations: 0.1, 0.5, 1, 1.5, and 2 mM. The cultures were incubated in an orbital shaker (180 rpm) at 27°C for 12 h. Growth rate was determined by measuring the increase in turbidity (absorbance at 550 nm; Greenblatt et al., 2004) every 30 min with a Beckman Coulter DU 730 Life/Science UV/ Vis spectrophotometer.

The specific growth rate (μ) was calculated for each Pb and Cu concentration using the following formula (Wang, 2005).

$$\mu = \frac{1}{OD_0} \times \frac{(OD_t - OD_0)}{(T_t - T_0)}$$

OD_t and OD_0 represent the optical density (550 nm) of the cultures at the time t (final time) and 0 (initial time), and T_t and T_0 represent homologous times (h).

2.6.2 Minimum inhibitory concentration

In order to determine the minimum inhibitory concentration (MIC) for lead and copper, two techniques were used: the agar diffusion method and an assay by tube dilution.

For the agar diffusion method, Petri plates containing LB agar medium evenly inoculated with the test microorganism were prepared. Known amounts (20 µL) at different concentrations of metals were placed on the surface of the agar. Milli-Q water solutions without metals were used as a control experiment. Plates were incubated at 27°C for 48 h to determine zones of inhibition of bacterial growth.

For the assay by tube dilution, series of culture were prepared, each one containing medium with a different concentration of the metal. All tubes of the series were inoculated with the test microorganism. Cultures without metals were used as a control experiment. The tubes were incubated at the same conditions mentioned above.

2.7. High-resolution microscopy techniques

In this section are described the high-resolution microscopy techniques used in this work for the selection of metal bioindicators among the microorganisms tested, to determine the sensitivity and the capacity to accumulate the metal externally and/or internally.

2.7.1 Confocal laser scanning microscopy

This microscopic technique was used in order to determine: i) the sensitivity of DE2009 microalga and cyanobacteria strains (*Oscillatoria* sp. PCC 7515, *Chroococcus* sp. PCC 9106 and *Spirulina* sp. PCC 6313) to lead by using the *λscan* function (CLSM-*λscan*), and ii) to determine the biomass of DE2009 microalga to different lead concentrations using the CLSM and the image analysis software *ImageJ* (CLSM-IA).

CLSM-*λscan*

Pigment analysis of DE2009 microalga and *Oscillatoria* sp. PCC 7515, *Chroococcus* sp. PCC 9106 and *Spirulina* sp. PCC 6313, was determined by *λscan* function of CLSM. This technique provides information on the state of the photosynthetic pigments of phototrophic microorganisms on the basis of the emission wavelength region and the fluorescence intensity emitted (autofluorescence).

Each image sequence was obtained by scanning the same *xy* optical section throughout the visible spectrum. Images were acquired at the *z* position at which the fluorescence was maximal, and acquisition settings were constant throughout the experiment. The sample excitation was carried out with an Argon Laser at 488 nm (λ_{exe} 488) with a λstep size of between 3 and 4.74 nm for an emission wavelength between 510 and 752 nm. In order to measure the mean fluorescence intensity (MFI) of the *xyλ*, CLSM data sets obtained by means of the Leica Confocal Software (Leica Microsystems CMS GmbH) were used. The regions-of-interest (ROIs) function of the software was used to measure the spectral signature. For each sample, 70 ROIs of 1 μm^2 taken from cyanobacteria and DE2009 microalga cells were analysed.

CLSM-IA

This technique was applied to estimate the microalga DE2009 biomass (total and individual).

Two types of fluorescence at cell level from the same DE2009 microalga were observed in images obtained in all cultures analysed. Red (red cells) and green (green cells) were distinguished on screen as pseudo-colours. For that reason, a *sequential scan* in two channels was carried out from each same *xy* optical section. On the first channel, samples were excited with a diode 561 nm (λ_{exe} 561) and the emission of fluorescence was captured between 670 and 794 nm (red pseudo-colour). On the second channel, samples were excited with an Argon Laser at 488 nm (λ_{exe} 488) and the emission of fluorescence was captured between 550 and 575 nm (green pseudo-colour). Finally, 10 red and 10 green confocal images were obtained from all cultures studied. Total biomass estimations from the red and green algal cells were obtained separately. Moreover, individual biomass for both types of cells was studied. Finally, total and individual biomass was estimated for each metal concentration.

In this work, we have used a modification of the method described by Solé et al. (2007) using CLSM and a free image-processing analysis software, *ImageJ* v1.41. (CLSM-IA). This method was used to determine the percentage between red and green pixels of DE2009 microalga and their biomass from the different cultures studied in this work.

The method used in this paper is as follows: for total biomass each pair of images (red and green) from an identical *xy* optical section were opened in their original format (8-bit, 1024 × 1024 pixels) as tiff images and the corresponding overlay image was obtained. These three images were transformed to binary images (black/white) using different thresholds. Values of 70 and 25 were applied respectively to red and green images from 0.1, 0.5, 0.75, 1 and 5 mM metal concentrations. Conversely, threshold values of 50 and 60 were applied respectively to red and green images from the 10 mM metal concentration.

In order to determine the percentage between the red and green fluorescences at pixel level the image calculator function of the *ImageJ* was used. To obtain images with cells showing up only as red fluorescence, all green fluorescence was subtracted from the image. In the same way, red fluorescence is subtracted from the image when greens only are obtained. In both cases to clean the images it was necessary to filter out the red and green pixels. A smoothing filter (median filter with a radius of 2.0 pixels) was then applied to the images. To obtain biovolume values, the Voxel Counter plug-in was applied to these filtered images (Rasband, 2010). This specific application calculates the ratio of the thresholded voxels (the red or green microalga volume), to all voxels from the binary image determined. The biovolume value obtained (Volume Fraction) was finally multiplied by a conversion factor of 310 fgC μm^{-3} to convert it to biomass (Bloem et al., 1995; Fry, 1990). To calculate the individual biomass, 30 red and 30 green cells were selected using *ImageJ* software and then the cells were analysed following the same protocol described above.

2.7.2 Scanning electron microscopy

This microscopic technique was used in order to observe the external morphology and the EPS formation on heterotrophic bacteria (*Paracoccus* sp. DE2007 and *Micrococcus*

luteus DE2008), DE2009 microalga, cyanobacteria strains (*Oscillatoria* sp. PCC 7515, *Chroococcus* sp. PCC 9106 and *Spirulina* sp. PCC 6313) and cyanobacteria from microcosms experiments.

Samples were filtrated in Nucleopore™ polycarbonate membranes (Whatman, Ltd.) and then were fixed in 2.5% glutaraldehyde Millonig's buffer phosphate (Millonig, 1967) (0.1M pH 4) at 4ºC for 2 hours washed four times (15 min) in the same buffer at 4ºC to remove excess of fixative. They were then dehydrated in a graded series (30%, 50%, 70%, 90%, and 100%) of ethanol (5 min) and dried by critical-point (CPD 030 Critical Point Drier, BAL-TEC GmbH D - 58579 Schalksmühle). Samples were mounted on aluminum metal stubs using a electrically-conductive double-sided adhesive tape and then coated with a 5 μm gold layer (K550 Sputter Coater, Emitech, Ashford, UK) Finally, samples were viewed in a Jeol JSM-6300 (Jeol Ltd., Tokyo, Japan) and in a Zeiss EVO® MA 10 (Carl Zeiss NTS GmbH, Oberkochen, Germany) scanning electron microscopes.

2.7.3 Transmission electron microscopy

This microscopic technique was used in order to observe the ultrastructure of heterotrophic bacteria (*Paracoccus* sp. DE2007 and *Micrococcus luteus* DE2008), DE2009 microalga, cyanobacteria strains (*Oscillatoria* sp. PCC 7515, *Chroococcus* sp. PCC 9106 and *Spirulina* sp. PCC 6313) and cyanobacteria from microcosms experiments.

Samples were fixed in 2.5% glutaraldehyde Millonig's buffer phosphate (Millonig, 1967) (0.1M pH 4) at 4ºC for 2 hours washed four times (15 min) in the same buffer at 4ºC. Then samples were post-fixed in 1% osmium tetroxide (OsO$_4$) at 4ºC for 2 hours, and washed four times in the same buffer. Then samples were centrifuged in order to obtain a pellet. They were then dehydrated in graded series (30%, 50%, 70%, 90%, 100%) of acetone and embedded in Spurr resin. Once the samples were included in the resin, a piramidotome was used (TM 60, C. Reichert AG. Wien, Austria) for pyraminding samples and an ultramicrotome (Leica EM UC6 ULTRACUT, Leica Microsystems, GmbH, Heidelberg, Germany) for the ultrathin sections. To show a better quality image, sections of 70 nm of the samples were mounted on carbon-coated copper grids and stained with uranil acetate and lead citrate according to the method described by Reynolds et al.,

(1963). Samples were viewed in a Hitachi H-7000 electron microscope (Hitachi Ltd., Tokyo, Japan).

2.7.4 Energy dispersive X-ray microanalysis

EDX is a technique used for the elemental analysis of a sample. The technique is based on the analysis of X-rays emitted by matter in response to collisions with charged particles. To stimulate the emission of X-rays, a high energy beam of charged particles such as electrons, protons or an X-ray beam is focused in the area of the study sample. This charged particle collides with internal shell of the element and then an electron from an outer shell moves into empty shell. Since there are a series of jumps from higher to smaller shells, at this time X-rays are generated. This phenomenon is due to the fundamental principle that every element has a unique atomic structure that allows X-rays that are characteristic of the atomic structure of an element to be identified only from each other. The number and energy of emitted X-rays in a sample can be measured semiquantitatively by an energy dispersive spectrometer.

This detector that may be coupled to both a SEM and a TEM, is connected to a computer with the software INCA v.4.13 (Oxford Instruments, Bucks, England) that generates graphics with different peaks corresponding to each of the chemical elements present in the analized area. Usually the analysis of a sample area takes 60 s. The great advantage of this approach is that the area to be analyzed can be selected by the user.

SEM-EDX

In order to determine the capacity of heterotrophic bacteria (*Paracoccus* sp. DE2007 and *Micrococcus luteus* DE2008), DE2009 microalga, cyanobacteria strains (*Oscillatoria* sp. PCC 7515, *Chroococcus* sp. PCC 9106 and *Spirulina* sp. PCC 6313) and cyanobacteria from microcosms experiments to accumulate (adsorbe) Pb and/or Cu extracellularly, SEM coupled to EDX was used.

For this analysis, samples followed the same protocol as for conventional SEM. A Jeol JSM-6300 (Jeol Ltd., Tokyo, Japan) and a Zeiss EVO® MA 10 (Carl Zeiss NTS GmbH, Oberkochen, Germany) scanning electron microscopes with an EDX Link Isis-

200 (Oxford Instruments, Bucks, England) operated at 20kV coupled to the microscopes was used for the analysis of the samples.

TEM-EDX

In order to determine the capacity of heterotrophic bacteria (*Micrococcus luteus* DE2008 and *Paracoccus* sp. DE2007), DE2009 microalga, cyanobacteria strains (*Oscillatoria* sp. PCC 7515, *Chroococcus* sp. PCC 9106 and *Spirulina* sp. PCC 6313) and cyanobacteria from microcosms experiments to bioaccumulate Pb and/or Cu intracellularly in the cytoplasm or in the polyphosphate (PP) inclussions, TEM coupled to EDX was used.

For the analysis, the above-mentioned protocol was used, but in this particular case, the sections were about 200 nm thick. These sections were not stained with lead citrate in order to avoid elemental substitution during the analysis. Then were mounted on carbon-coated titanium and/or gold grids. All samples were analyzed with a Jeol Jem-2011 (Jeol LTD, Tokyo, Japan) transmission microscope with an EDX Link Isis-200 (Oxford Instruments, Bucks, England) operated at 20kV coupled to this microscope.

2.8. Statistical analysis

Data obtained from from growth cultures, percentages, biomass, MFI data, were compared using a Student's t test, one-way analysis of variance (ANOVA) and Tukey and Bonferroni's comparison *post hoc* tests. Significant differences were accepted at $p < 0.05$. The analyses were performed using SPSS software (version 19.0 for Mac OS X).

Results

3. Isolation and identification of a bacterium with high tolerance to lead and copper from a marine microbial mat in Spain

Isolation and identification of a bacterium with high tolerance to lead and copper from a marine microbial mat in Spain

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Abstract

A bacterial strain (DE2008) was isolated from a consortium of microorganisms living in the microbial mats of the Ebro Delta with the ability to grow under conditions of high concentrations of lead and copper. Strain DE2008 has been characterized by microscopic and metabolic techniques and identified by sequencing of PCR-amplified 16S rRNA gene fragments as *Micrococcus luteus*. The strain is highly resistant to lead and copper. *Micrococcus luteus* DE2008 grew optimally until levels of 1 mM of Pb $(NO_3)_2$ and $CuSO_4$, respectively, and was completely inhibited at 3 mM $Pb(NO_3)_2$ and at 1.5 mM $CuSO_4$. Elemental analysis determined by energy-dispersive X-ray spectroscopy (EDX) coupled with scanning electron microscopy and transmission electron microscopy from polluted cultures of DE2008 show that this microorganism can biosorpt Pb and Cu in exopolysaccharide envelopes, without accumulating them inside the cells.

Introduction

Contaminated environments such as soils and sediments polluted by crude oil, heavy metals or pesticides can be considered as extreme conditions for microbial growth. The effects of these contaminants in different populations have been reported (Al-Hasan et al., 1998; Radwan and Sorkhoh, 1993). The bioremediation capacity of phototrophic microorganisms (cyanobacteria and algae), heterotrophic bacteria, yeast and fungi have been investigated (Binupriya et al., 2006; Gong et al., 2005).

Gram-positive bacteria are known to possess high metal sorption capacities. The ability of the cell wall of *Bacillus subitilis* to interact with different heavy metals has been largely studied (Tunali et al., 2006). Although several papers have shown that the cell walls of Gram-positive cocci such as those of *Staphylococcus xylosus* and *Micrococcus luteus* have an affinity for metal ions (Nakajima and Tsuruta, 2004; Ziagova et al., 2007), the metal sorption by the cell walls still to be characterized. Indigenous microbes in nature, tolerate high concentrations of heavy metals and may play an important role in restoring contaminated soils. For example, Wei et al. (2009) recently identified *Agrobacterium* (CCNWRS33-2), from the Taibai gold mining region in China as the bacterial strain most resistant to heavy metals. This bacterium was able to grow in up to 2 mM of copper and lead.

Stratified benthic marine ecosystems (microbial mats) are often exposed to heavy metals (Sánchez-Chardi et al., 2007). The microorganisms that compose these ecosystems are mainly cyanobacteria, oxygenic phototrophic microorganisms, which are primary producers on these habitats (Stal, 2000). Our group of work has studied the diversity and biomass of cyanobacteria in oil-polluted and unpolluted microbial mats around Europe (Diestra et al., 2004; Solé et al., 2009). Over the last few years, we have also isolated a consortium, formed mainly by a single cyanobacterium, *Microcoleus chthonoplastes*, and by different heterotrophic bacteria (Diestra et al., 2005; Sánchez et al., 2005). In addition, this consortium was able to degrade crude oil (García de Oteyza et al., 2004) and tolerate heavy metals (Burnat et al., 2010).

Recently, we have isolated a Gram-positive heterotrophic bacterium (DE2008) from the above-mentioned consortium, with the ability to grow in high concentrations of heavy metals. Two metals were selected for this work: Pb and Cu. The first one is a

toxic metal, whilst the second one is an essential metal with known biological functions in all organisms (Roane and Pepper, 2000).

The goals of this work are to identify this bacterium and to determine the maximum concentration levels of both metals in which the bacterium can grow. At the same time, we are trying to determine whether the strain's ability to tolerate such high concentrations of heavy metals is from an ability to accumulate Pb and/or Cu externally in sheaths or internally in intracytoplasmic inclusions.

Materials and methods

Isolation, biochemical and physiological characterization of the strain DE2008

Inoculums from a *Microcoleus* consortium (Diestra et al., 2005) were transferred to Luria-Bertani (LB) agar medium containing tryptone (10.0 g L^{-1}), yeast extract (5.0 g L^{-1}), and NaCl (10.0 g L^{-1}), pH 7.0. The cultures were incubated in darkness at 27°C. Different colonies were obtained, one of which was streaked on LB agar and isolated in pure culture. The morphological characteristics of strain DE2008 were examined with an Olympus BH2 conventional light microscope. Different stain methods were used to characterize the DE2008 bacterium (Gram, Toluidine Blue, Wirtz-Conklin, and Negative stains). Motility was determined with an optical microscope using the hanging-drop technique. The biochemical assays were made using the API 20 NE strip identification system (BioMerieux, Marcy l'Étoile, France). Oxidase activity was analysed by oxidation of 1% p-aminodimethylaniline oxalate (Cowan and Steel, 1965). Catalase activity was determined by the presence of bubbles with a 3% H_2O_2 solution (Takeuchi et al., 1996). Starch hydrolysis was analyzed as described by Cowan and Steel (1965).

The effect of different physical–chemical parameters were also tested on bacterial strain DE2008. The pH range for growth was determined by incubating cells in LB medium at 27°C for 4 days at the following pH: 2, 3, 4 (LB broth medium), 5, 6, 7, 8, 9, 10, and 11 (LB agar medium). NaCl tolerance was measured in LB agar medium at concentrations of 0, 1, 2, 3, 4, 5, 6, 7, 8, and 10% (w/v). To determine the temperature range, the growth of the strain at different temperatures (8–43°C) was tested.

Phylogenetic characterization

Genomic DNA was extracted from 2 mL of an overnight DE2008 culture in liquid LB using UltraClean™ Soil DNA Isolation Kit (Mobio Laboratories, Carlsbad, USA) according to manufacturer's instructions. The 16S rRNA gene fragment was obtained by PCR amplification using primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1489R (5′-TACCTTGTTACGACTTCA-3′), according to the procedure previously described (Bordenave et al., 2004). The following PCR conditions were applied: 35 cycles of 10 min at 95°C, 45 s at 95°C, 45 s at 51°C, and 1.5 min at 72°C, with a final step of 10 min at 72°C. PCR product was then purified using the GFX™ PCR DNA and Gel Band Purification Kit as directed by the supplier (GE Heathcare, UK). The sequence of the purified DNA fragment was obtained using the BigDye Terminator Cycle Sequence Kit (versions 1.1; Applied Biosystems, Foster City, USA) on an ABI PRISM310 Genetic Analyzer (Applied Biosystems).

The sequence was compared to those present in the NCBI database (http://www.ncbi.nlm.nih.gov) using the BLAST program. For phylogenetic analysis, a dataset containing GenBank 16S rRNA gene sequences was used. Pairwise evolutionary distances based on 1,455 unambiguous nucleotides were computed by the method of Jukes and Cantor (1969) with MEGA version 3.0 software (Kumar et al., 2004). The phylogenetic tree was constructed using neighbor-joining (Saitou and Nei, 1987). Confidence in the tree topology was determined by bootstrap analysis using 1,000 resampling of the sequences.

Effects of Pb and Cu on the growth of the strain DE2008

Overnight cultures of DE2008 strain grown on LB medium were inoculated into LB medium supplemented with different $Pb(NO_3)_2$ or $CuSO_4$ concentrations: 0.1, 0.5, 1, 1.5, and 2 mM. The cultures were incubated in an orbital shaker (180 rpm) at 27°C for 12 h. Growth rate was determined by measuring the increase in turbidity (absorbance at 550 nm; Greenblatt et al., 2004) every 30 min with a Beckman Coulter DU 730 Life/Science UV/ Vis spectrophotometer.

The specific growth rate (μ) was calculated for each Pb and Cu concentration using the following formula (Wang, 2005).

$$\mu = \frac{1}{OD_0} \times \frac{\left(OD_t - OD_0\right)}{\left(T_t - T_0\right)}$$

OD_t and OD_0 represent the optical density (550 nm) of the cultures at the time t (final time) and 0 (initial time), and T_t and T_0 represent homologous times (h).

In order to determine the minimum inhibitory concentration (MIC) for lead and copper, two techniques were used: the agar diffusion method and an assay by tube dilution.

For the agar diffusion method, Petri plates containing LB agar medium evenly inoculated with the test microorganism were prepared. Known amounts (20 μL) at different concentrations of metals were placed on the surface of the agar. Milli-Q water solutions without metals were used as a control experiment. Plates were incubated at 27°C for 48 h to determine zones of inhibition of bacterial growth.

For the assay by tube dilution, series of culture were prepared, each one containing medium with a different concentration of the metal. All tubes of the series were inoculated with the test microorganism. Cultures without metals were used as a control experiment. The tubes were incubated at the same conditions mentioned above.

Heavy metal accumulation capacity of DE2008

To test the ability of the strain to capture heavy metals (intra- or/and extra-cellularly), polluted cultures with the highest concentrations of $Pb(NO_3)_2$ and $CuSO_4$ (5 and 25 mM) were assayed to obtain a rapid response to both metals (Burnat et al. 2010). To determine structural characteristics and for the heavy metals localization in DE2008 cells, scanning electron microscopy (SEM), transmission electron microscopy (TEM) and energy-dispersive X-ray spectroscopy (EDX) were used.

For SEM analysis, samples of DE2008 cultures were fixed in 2.5% glutaraldehyde Millonig buffer phosphate for 2 h and washed four times in the same buffer. They were then dehydrated in successively increasing gradient con- centrations of acetone (30, 50, 70, 90, and 100%) and dried by critical-point drying. Finally, all samples were mounted on metal stubs and coated with gold. A Jeol JSM-6300 scanning electron microscope (Jeol Ltd., Tokyo, Japan) was used to view the images.

For X-ray analysis, cultures were homogenously distributed and filtered on poly-carbonate membrane filters. These filters were then dehydrated and dried by the same procedure as culture samples. An EDX Link Isis-200 (Oxford Instruments, Bucks, England) operated at 20 kV coupled to SEM was used.

For TEM analysis, samples of DE2008 cultures were fixed in 2.5% glutaraldehyde Millonig buffer phosphate for 2 h and washed four times in the same buffer. Samples were post-fixed in 1% OsO_4 at 4°C for 2 h, and washed four times in the same buffer. They were then dehydrated in a graded series (50, 70, 90, 95, and 100%) of acetone and embedded in Spurr resin. Ultrathin sections (70 nm) were mounted on carbon-coated copper grids and stained with uranyl acetate and lead citrate. Samples were viewed in a Hitachi H-7000 transmission electron microscope (Hitachi Ltd., Tokyo, Japan). Sections of 200–300 nm thickness were not stained and then mounted on titanium grids for EDX. Samples were analyzed with a Jeol Jem-2011 transmission electron microscope (Jeol Ltd., Tokyo, Japan).

Data analysis

Data obtained from growth cultures (control and different metal concentrations) were compared with a Student's t test with 95% significance ($p < 0.05$). Statistical analyses were performed with SPSS software (version 15.0 for Windows).

Results and discussion

Identification of the strain DE2008

Cells of strain DE2008 are facultative aerobic, Gram-positive cocci, (1.5 μm in size), non-motile, encapsulated and non-spore-forming. Colonies grow in LB agar are cream coloured, low convex, smooth and circular. Growth occurs between 8 and 43 ºC (optimum 27ºC) and at pH between 3 and 11. No growth occurs in the presence of more than 8% of NaCl.

The biochemical assays indicated that strain DE2008 hydrolyzes glucose, arabinose, mannose, maltose, adipic acid, malic acid, tributirine and trisodium citrate, even after 8 days of growth. Starch cannot be hydrolyzed. The strain can reduce the nitrates to nitrites, but denitrification does not occur.

In addition, electron microscopy techniques (SEM and TEM) were used to determine morphological parameters. The images obtained by SEM indicate that the bacterium occurs in tetrads, irregular clusters or regular packets, and without flagella (Fig. 3.1a). Ultrathin sections show the characteristic cell wall of Gram-positive bacteria, and inside the cytoplasm few high electron-dense (HE) inclusions were detected (Fig. 3.1b).

16S rRNA gene sequence analysis (Fig. 3.2) revealed that the isolate belonged to the Actinobacteria class and was closely related to representatives of the genus *Micrococcus*. According to 16S rRNA gene sequence comparison, the isolate showed 99% similarity with the type strain *M. luteus* DSM 20030^T (AJ536198) and *M. luteus* strain Ballarat (AJ409096). This value was over the 97% similarity indicating that the isolate belongs to the same species. We thus indentified the isolate DE2008 as *M. luteus*.

Effects of Pb and Cu at different concentrations on M. luteus DE2008 growth

The Pb effect on the strain varied significantly depending on the concentrations. The differences were not statistically significant ($p < 0.05$) between the control experiment and 0.1, 0.5 and 1 mM Pb(NO$_3$)$_2$. Thus, these concentrations had only a small effect on the growth of the strain. The differences were statistically significant ($p < 0.05$) between the control experiment and 1.5 and 2 mM Pb(NO$_3$)$_2$ (Fig. 3.3a). The Pb MIC was 3 mM.

Similar results were obtained with cultures containing Cu. However, differences in toxicity were observed from 1 mM indicating that copper had stronger effect than lead (Fig. 3.3b). The Cu MIC was 1.5 mM. Although Wei et al. (2009) verified that Cu increased the growth rate of the strain CCNWRS33-2 at this concentration, our results are more in accordance with those obtained by Chaudri et al. (2000), which demonstrated a negative effect of Cu on rhizobial species in soils.

M. luteus growing at 0, 0.1, 0.5 and 1 mM of $Pb(NO_3)_2$ had a specific growth rate of 0.5; 0.49; 0.44 and 0.32 respectively. The same microorganism growing at the same concentrations of $CuSO_4$ had a specific growth rate of 0.49; 0.25; 0.27 and 0.18 respectively. These results demonstrated the high tolerance of *M. luteus* DE2008 to heavy metals. The genus *Micrococcus* seems to be well suited to grow under extreme conditions such as in amber (Greenblatt et al., 2004), in the upper stratosphere at very low atmospheric pressures (Bamji and Krinsky, 1966), and in unusual metabolic sources for nutrition (Doddamani and Ninnekar, 2001; Sims et al., 1986; Tallur et al., 2008; Zhuang et al., 2003).

M. luteus DE2008 grows at higher metal concentrations than those described for other strains. For example, Roy et al. (2008) proved that the *Synechocystis* sp. growth was completely inhibited at 400 μg mL^{-1} Pb^{2+}. Although *M. luteus* DE2008 is unable to grow at the highest concentrations assayed, it maintains its capacity to bioaccumulate Pb and Cu.

Heavy metal accumulation in M. luteus DE2008

With the aim of proving whether *M. luteus* DE2008 could capture metals, cells from cultures with and without metals were analysed by EDX coupled to SEM and TEM.

Cells from cultures without metals show thick envelopes of EPS surrounding the cells when they are analysed by SEM and TEM. Few HE inclusions inside the cells were observed. The EDX images obtained coupled to SEM and TEM and used as a control experiment corroborated that neither of the two contaminants metals were detected neither outside nor inside the cells.

M. luteus DE2008 from cultures containing Pb was analysed by the same above-mentioned procedures. No important differences in the structure of *M. luteus* DE2008 were observed in the cells from Pb (Fig. 3.4c). The analysis of the energy-dispersion spectra coupled to SEM demonstrated that Pb was found in EPS (Fig. 3.4b). The ultrathin sections of the cells also exhibited discernible changes (distortion of the cells) after exposure to Pb. A high number of HE inclusions, which can be observed in the ultrathin sections of cells grown in the presence of Pb, were evident (Fig. 3.4c). These inclusions were identified as polyphosphate granules (PPG) (see peak of P, Fig. 3.4d). In many cases, similar inclusions have been found when cells are grown in in adverse culture conditions (Jensen and Sicko, 1974; Sicko, 1972; Stevens et al., 1985). However their role in other functions such as a detoxification by sequestering heavy metals has been suggested (Goldberg et al., 2001). The analysis of the energy-dispersion spectra of the inclusions confirmed that Pb was not accumulated in PPG inside the cells. No significant peaks of Pb were visible.

Equivalent experiments were made in *M. luteus* DE2008 cultures containing Cu. When the structure of the cell from Cu cultures was compared with that of cells from Pb cultures, we could not observe effect of Cu on the cell structure. The analysis of the energy-dispersion spectra coupled to SEM proved that this microorganism was able to accumulate Cu outside, but not inside the cells (Fig. 3.5). Cu peaks in Fig. 3.5d are not relevant because they belong to the TEM holder support, which is made of Cu.

These results indicated that the EPS envelopes have a great affinity for both metals. It is well known that the EPS matrix can protect cells against toxic compounds such as metals that can be toxic to cells and further, and that the presence of these metals can overproduce exopolymer secretion (Decho, 1994). Specifically, uronic acids and sulphate groups present in EPS may interact with various metals thereby immobilizing them (Stal, 2000).

From these results it can be concluded that: A. *M. luteus* DE2008 grew optimally until 1 mM of $Pb(NO_3)_2$ and $CuSO_4$ respectively and was completely inhibited at 3 mM $Pb(NO_3)_2$ and at 1.5 mM $CuSO_4$. B. The strain can tolerate higher concentrations of both metals by extracellular biosorption.

On the other hand, *M. luteus* can be considered an indigenous microorganism, which probably play a very important role in removing Pb and Cu from polluted natural marine ecosystems. In future studies this microorganism could be considered a promising candidate of the removal of heavy metals from wastewaters.

Acknowledgments
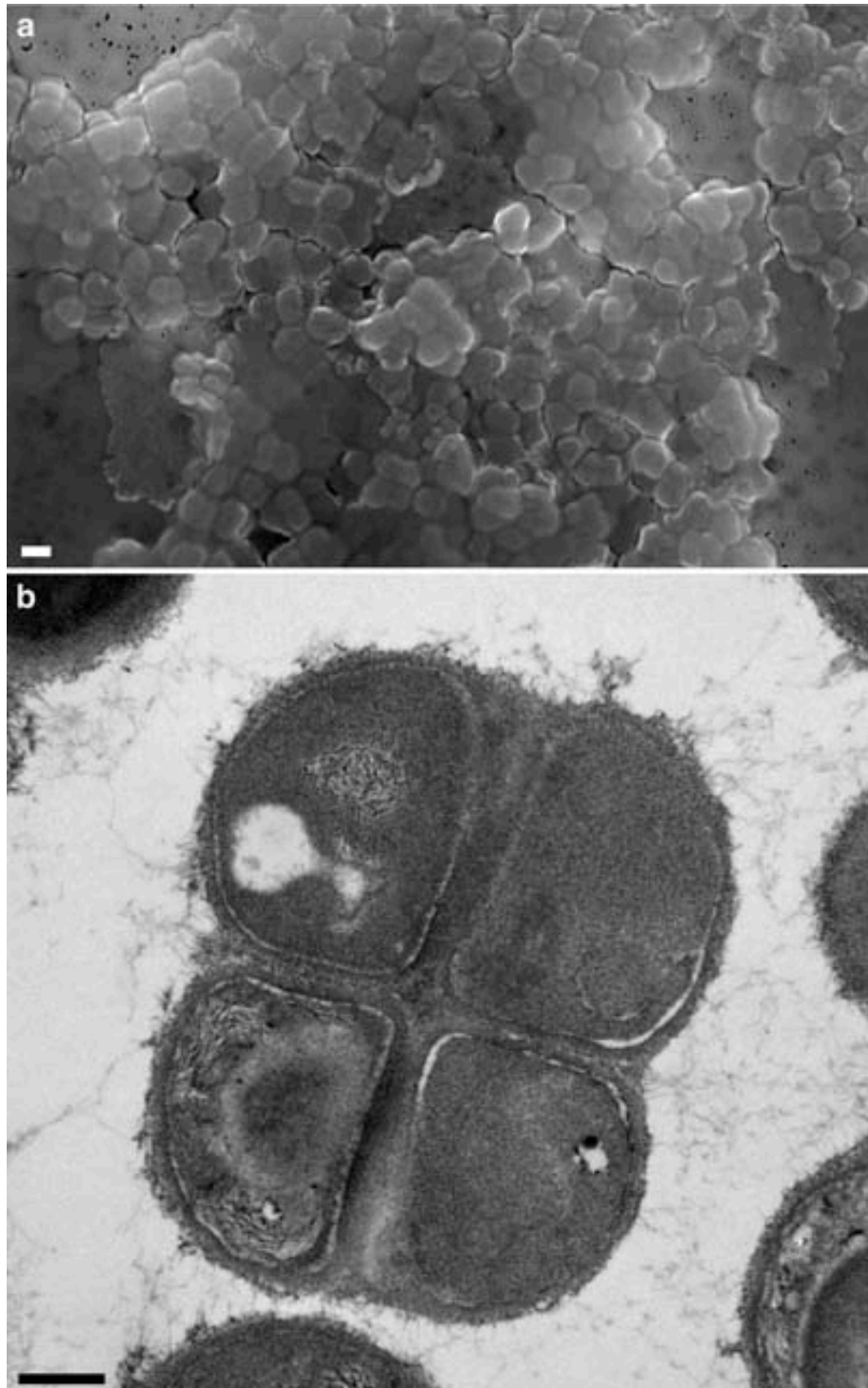
Fig. 3.1. Electron microscopy images of *Micrococcus luteus* DE2008 **(a)**. SEM image showing clusters. Scale bar 1 μm. TEM image showing a single tetrad surrounded by EPS. Scale bar 0.2 μm **(b)**.

Fig. 3.2. Phylogenetic position of the isolate DE2008 based on the 16S rRNA gene sequence analysis. Phylogenetic distances and tree construction were carried by the neighbor-joining method with MEGA (Phylogenetic Tree-Mega Software). The tree was rooted with the sequence of *Kytococcus sedentarius* DSM20547T. Values at nodes represent percentage occurrence based on 1,000 bootstrap replications. Scale bar corresponds to 0.5 substitutions per 100 nucleotide positions. Accession numbers are given in parentheses.
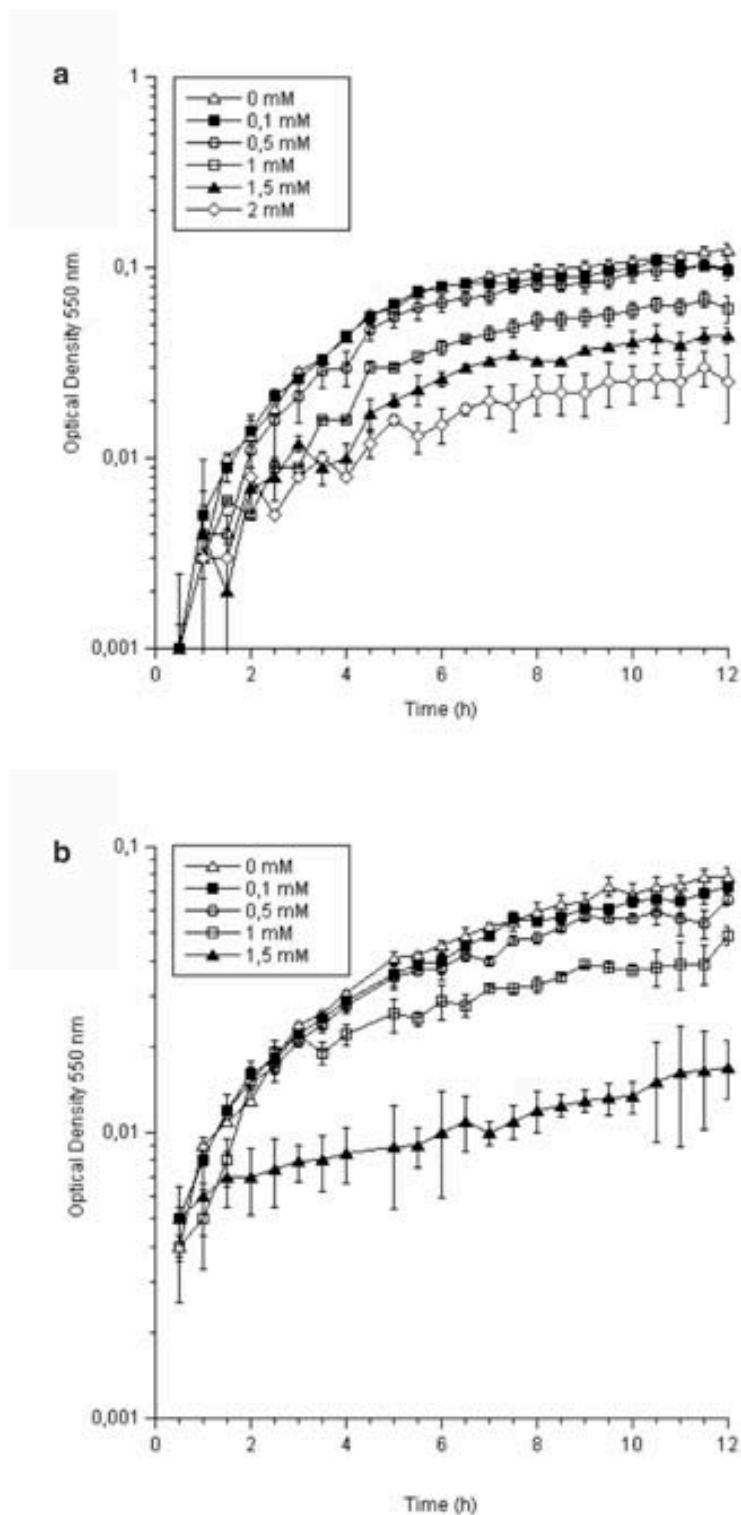
Fig. 3.3. Growth rates of *Micrococcus luteus* DE2008 growing between 0 and 2 mM Pb(NO$_3$)$_2$ **(a)** and between 0 and 1.5 mM CuSO$_4$ **(b)**.
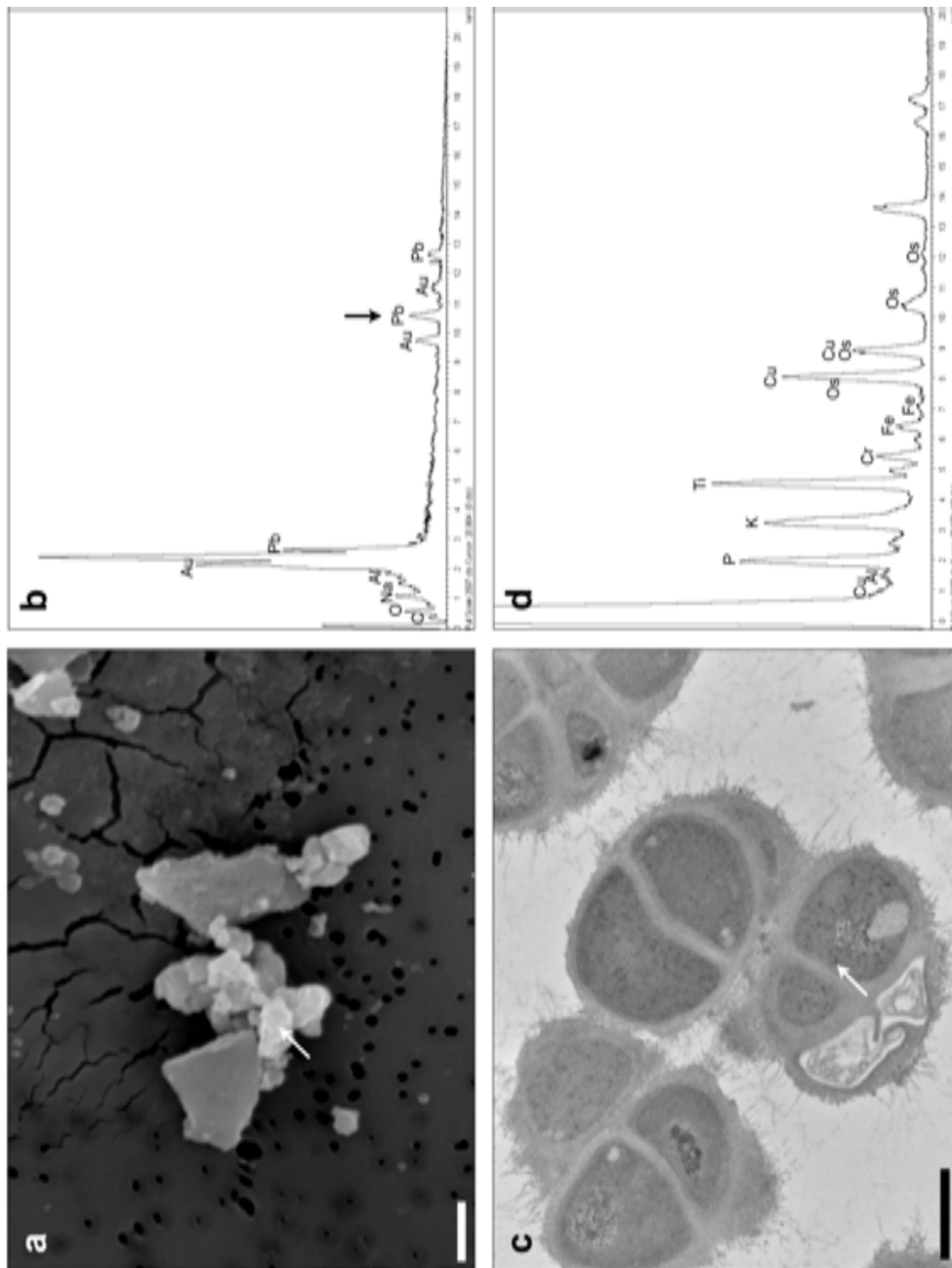
Fig. 3.4. SEM image of *Micrococcus luteus* DE2008 culture treated with 25 mM Pb (NO₃)₂ forming aggregates (arrow). Scale bar 1 μm (**a**). X-ray analysis spectra coupled to SEM, see the main Pb peak at 10.5 keV (arrow) (**b**). Ultrathin sections of the same bacteria showing high electron-dense (HE) inclusions (arrow). Scale bar 500 μm (**c**). X-ray analysis spectra coupled to TEM from HE inclusions. No peaks of Pb were detected (**d**).
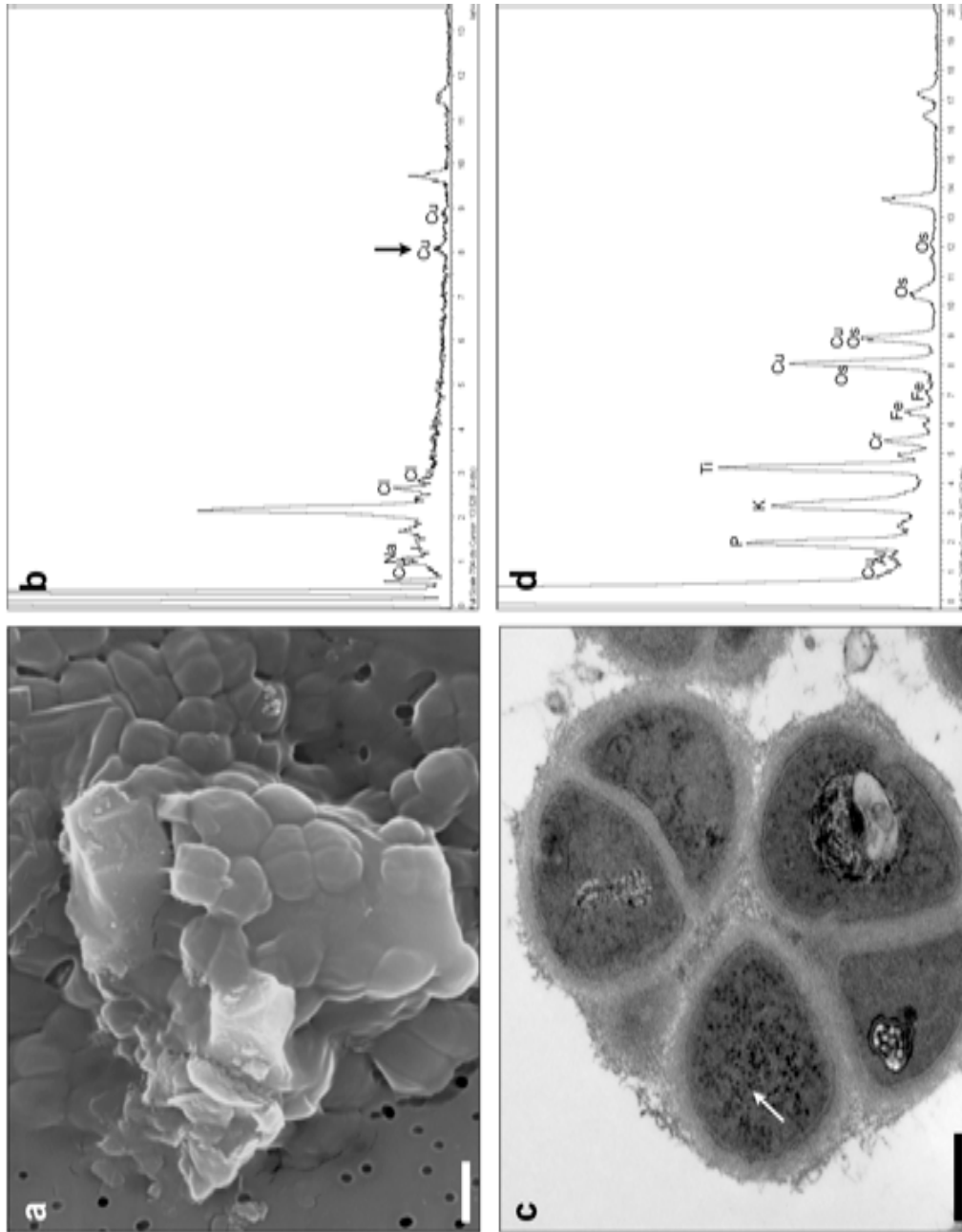
Fig. 3.5. SEM image of *Micrococcus luteus* DE2008 culture treated with 25 mM CuSO$_4$ **(a)**. X-ray analysis spectra coupled to SEM. Note the main Cu peak at 8 keV (arrow) **(b)**. Ultrathin sections of the same bacteria showing high electron-dense (HE) inclusions (arrow). Scale bar 200 µm **(c)**. X-ray analysis spectra coupled to TEM from HE inclusions. Cu peaks are irrelevant because they belong to the TEM holder **(d)**.

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4. Electron microscopy techniques and energy dispersive X-ray applied to determine the sorption of lead in *Paracoccus* sp. DE2007

Electron microscopy techniques and energy dispersive X-ray applied to determine the sorption of lead in *Paracoccus* sp. DE2007

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Abstract

Paracoccus sp. DE2007 was isolated from a consortium of microorganisms in Ebro Delta microbial mats. The isolated strain was identified according to its biochemical and molecular characteristics as *Paracoccus* sp. DE2007 (Diestra et al., 2007).

The strain was able to grow in solid medium in the presence of heavy metals and crude oil. In the present work electron microscopy techniques coupled to Energy Dispersive X-ray were used to determine if this strain is able to capture lead extra- and/or intra-cellularly because lead has been detected in Ebro delta as a toxic pollutant.

The results indicate that *Paraccoccus* sp. DE2007 has a great affinity for lead, which is absorbed into the external envelope but not into the cytoplasmic space.

Introduction

Among the possible applications of the capacity of microorganisms to interact with metals, biosorption and bioaccumulation are potential options for replacing abiotic techniques in the removal of toxic metals (Vijayaraghavan and Yun, 2008). The biosorption process takes place mainly at the cell surface, where there are a large variety of cellular compounds that can bind metals, such as the peptidoglycan, teichoic acids and lipoteichoic acids and several polysaccharides and proteins (Wang and Chen, 2009). Functional groups containing O-, N-, S- or P- are directly involved in the binding of metals. The most important of these groups are: carbonyl (ketone), carboxyl, sulfhydryl (thiol), sulfonate, thioether, amine, secondary amine, amide, imine, imidazole, phosphonate and phosphodiester (Wang and Chen, 2009). The mechanisms involved in metals binding to the cell surface are metabolically passive and include electrostatic interactions, Van der Waals forces, covalent bonds, redox interactions, extracellular precipitation, mineral nucleation and combinations of these (Gavrilescu, 2004) The extracellular polymeric substance (EPS) is currently receiving much attention as it is able to flocculate and absorb metal ions and enhance colonization and microbial growth through the formation of biofilms (Vu et al., 2009). Many microorganisms produce EPS, which is a mixture of biopolymers composed mainly of polysaccharides, as well as proteins, nucleic acids, lipids and humic substances. The EPS is also located in the intercellular space of microbial aggregates and forms the structure and architecture of the biofilm matrix. Its secretion and features depend on the species and growing conditions. It has been observed that some bacteria can increase their EPS production in the presence of toxic compounds (e.g. metals) as a defense mechanism (Decho, 1994).

On the other hand, bioaccumulation is defined as the intracellular accumulation of metals. It consists first in biosorption to the cell surface, followed by transport and concentration in intracytoplasm compartments. The extracellular heavy metals can move in the cytoplasm through two processes: the first is rapid, nonspecific, and usually based on the chemiosmotic gradient (passive transport), and since it is used by a wide variety of substrates it is expressed constitutively; the second transport system has a high specificity for the substrate, is slower and often uses adeninatriphosphate hydrolysis (ATP) as the energy source (active transport), but can also function by osmotic gradient.

The latter system is inducible and is produced by the cell during stress conditions or cell starvation (Nies, 1999). The metals released into the cell bind or chelate different cellular components before being incorporated into cellular compartments, if they are suitable. The captured metal can form complexes with compounds such as proteins rich in thiol groups physiologically present in cells (enzymes of metabolic pathways) or synthesized in the presence of metals (metallothioneins and phytochelatins) and glutathione (a compound that determines oxidative stress in cells) (Wang and Chen, 2009).

The need for more new products to bioremediate polluted ecosystems makes it necessary to find microorganisms with unique capabilities to capture metals. These microorganisms are often found and isolated in polluted environments where there is high selective pressure.

In recent years a *Microcoleus* consortium that is capable of degrading crude oil (García de Oteyza et al., 2004) was isolated in our laboratory by Diestra et al. (2005) from Ebro Delta microbial mats (Tarragona-Spain). The *Microcoleus* consortium was formed by a phototrophic cyanobacterium and different heterotrophic microorganisms. *Microcoleus* sp. DE2005 (Diestra et al. 2005) and *Micrococcus* sp. DE2008 (Maldonado et al., 2010a) were isolated and their capacities to tolerate or biocapture heavy metals were determined. Although *Paracoccus* sp. DE2007 isolated from the same consortium was also identified by Diestra et al. (2007), its capacity to biosorption and/or bioaccumulate heavy metals was not determined.

Despite the extensive information available on the genus *Paracoccus*, very little is known about its ability to biocapture heavy metals. Since both the *Microcoleus* consortium and some of the isolated microorganisms mentioned above are able to biosorption or bioaccumulate heavy metals, the objective of this study was to determine whether *Paracoccus* DE2007 has this ability. For this purpose we used electron microscopy techniques in combination with an electron dispersive X-ray detector. We select lead among heavy metal because it is very toxic and has no biological functions and because it has been detected in Ebro Delta.

Material and Methods

Bacterial strain and culture conditions

Paracoccus sp. DE2007 was isolated from a *Microcoleus* consortium (Diestra et al., 2007). Cells were grown in Luria Bertani (LB) liquid medium containing tryptone (10.0 g L^{-1}), yeast extract (5.0 g L^{-1}) and NaCl (10.0 g L^{-1}) at pH 7 and 27ºC for 24 h. Lead stock solution was prepared with Pb(NO$_3$)$_2$ (Merck KGaA, Darmstadt, Germany) in deionized water and was sterilized by filtration with polycarbonate membrane filters. To test whether *Paracoccus* sp. DE2007 could grow in the presence of lead. Pb stock solution was added to LB liquid medium to reach a final concentration of 5 mM. Cultures were incubated at 27ºC for 24 h.

Scanning electron microscopy (SEM) and energy dispersive X-ray (EDX) microanalysis:

For SEM analysis, samples of Paracoccus sp. cultures were fixed in 2.5% glutaraldehyde (Millonig's buffer) (Millonig, 1961) (0.1M and pH7) for 2 h and washed four times in the same buffer. They were then dehydrated in successively increasing gradient concentrations of ethanol (30, 50, 70, 90 and 100%) and dried by critical point. Finally, all samples were mounted on metal stubs and coated with gold. A Jeol JSM-6300 scanning electron microscope (Jeol Ltd., Tokyo, Japan) was used to view the images.

For energy dispersive X-ray microanalysis, cells were homogenously distributed and filtered with polycarbonate membrane filters. These filters were dehydrated and dried by critical point and coated with gold. An energy dispersive X-ray spectrophotometer (EDX) Link Isis-200 (Oxford Instruments, Bucks, England) operated at 20 kV coupled to SEM was used.

Transmission electron microscopy (TEM) and energy dispersive X-ray (EDX) microanalysis:

For TEM analysis, samples of *Paracoccus* sp. DE2007 cultures were fixed in 2.5% glutaraldehyde (Millonig's buffer) 0.1 M, pH 7 for 2 h and washed four times in the same buffer. Samples were post-fixed in 1% OsO4 at 4°C for 2 h, and washed four times in the same buffer. They were then dehydrated in a graded series (50, 70, 90, 95 and 100%) of acetone and embedded in Spurr's resin. Ultrathin sections (70 nm) were mounted on carbon coated copper grids and stained with uranyl acetate and lead citrate. Samples were viewed with a Hitachi H-7000 transmission electron microscope (Hitachi Ltd., Tokyo, Japan). Sections with a thickness of 200 nm were not stained with lead citrate in order to avoid elemental substitution during the analysis. They were mounted on gold and copper grids for energy dispersive X-ray microanalysis. The samples were analyzed with a Jeol JEM-2011 transmission electron microscope (Jeol Ltd., Tokyo, Japan).

Results

We used SEM and TEM coupled to EDX to study the effect of lead on *Paracoccus* sp. DE2007 in order to determine the capacity of this microorganism to capture heavy metals.

Paracoccus sp. DE2007, as mentioned above, was isolated from a *Microcoleus* consortium formed by this cyanobacterium and different heterotrophic bacteria encased in a dense exopolysaccharide (EPS) envelope (Diestra et al., 2005; Sánchez et al., 2005). In liquid medium this bacterial aggregate formed a dense mucilaginous mass, and in solid medium it formed green filamentous colonies.

In this work *Paracoccus* sp. DE2007 cultures were prepared with a 5 mM lead concentration with the aim of determining whether *Paracoccus* sp. DE2007 can capture metals. Unpolluted and polluted cultures were prepared and analyzed by EDX coupled to SEM and TEM (Fig. 4.1 and 4.2).

Individual cells and groups of 2 to 4 cells are view in SEM image (Fig. 4.1a). The EDX analysis of the cells shows that lead is not detected in the cells (Fig. 4.1b) or in the filter (Fig. 4.1c). The ultrathin sections show cells with a wall and membrane structure

characteristic of Gram-negative cells. In the cytoplasm some vesicles that are only very slightly electrodense can be observed (Fig. 4.2a, indicated by arrows), similar to those found by other authors (Osanjo et al., 2009). We assumed that these vesicles have lipid content as they were positively stained with Sudan Black (data not shown). The results obtained in the TEM-EDX analysis (Fig. 4.2b) evidence the absence of lead in: the cytoplasm, the vesicles and the resin (Figs. 4.2c, 2d and 2e).

Cells from Pb-polluted cultures exhibit morphological and ultrastructural changes (Figs. 4.3 and 4.4). The SEM image shows cells that are more deformed and which have a higher tendency to aggregate (Fig. 4.3a). The EDX analysis evidences the presence of lead in the cells (Fig. 4.3b, indicated by an arrow); however, no lead was found in the filters in which the cells are retained (Fig. 4.3c).

The TEM analysis of the same cells shows an increase in the intracytoplasmic vesicles at the ultrastructural level (Fig. 4.4a, also indicated by arrows). The TEM-EDX analysis (Fig. 4.4b) gives evidence that lead is not retained in either the vesicles (Fig. 4.4c) or the cytoplasm (Fig. 4.4d). The resin in which the samples were embedded was also analyzed as a control, and as expected it gave a negative result (Fig. 4.4e).

Copper and gold were detected in the spectra of the unpolluted cells because the gold grids are made of Cu covered with Au.

Discussion

The phylum *Proteobacteria* is composed of an extensive and diverse group of bacteria, some of which, especially some *Pseudomonas* species, have been demonstrated to have the ability to absorb lead (Gavrilescu, 1994; Malik, 2004). The members of the genus *Paracoccus* are metabolically versatile and are widely distributed, both in land and marine environments. The isolation of some strains in polluted environments evidences the capacity of these organisms to tolerate the presence of toxic compounds. Moreover, their ability to grow heterotrophically on a large number of organic compounds allows them to degrade some organic pollutants (Liu et al., 2008, López-Cortés et al., 2008, Zhang et al., 2004).

In the present work we studied the ability of *Paracoccus* sp. DE2007 to biocapture lead. As mentioned above, lead was chosen as it is a very toxic metal, even at low concentrations, has no biological function in the cell and because it has been found to be a pollutant in the Ebro Delta microbial mats (Sánchez-Chardi et al., 2007). The *Paracoccus* sp. DE2007 strain that we studied was isolated from a microorganism consortium from a sample from the Ebro Delta (García de Oteyza et al., 2004). This strain also grew in the presence of crude oil and heavy metals (lead and copper) (Diestra et al., 2007), which indicates its capacity to tolerate these toxic compounds.

To determine whether the *Paracoccus* sp. DE2007 strain could capture or bioaccumulate lead, two high-resolution microscopy techniques, SEM and TEM, were employed coupled with an energy dispersive X-ray technique. Using these techniques together allowed us to analyze the microorganisms morphostructurally and evaluate the presence and also the location of the lead.

The energy dispersive X-ray spectra obtained with SEM detected the presence of lead extracellularly (Fig. 4.3b), which shows that the *Paracoccus* sp. DE2007 strain captures the metal in the EPS envelope.

Paracoccus sp. DE2007 produces a large amount of EPS in response to the presence of lead in the polluted cultures, which was observed with optical microscopy by means negative staining (data not shown). The increase in the EPS production is in accordance with the results obtained by other authors for other strains of the same genus, which can even eventually produce biofilms in particular culture conditions (Srinandana et al., 2010).

The presence of the extracellular polymeric substance in the cell is considered promising for bioremediation applications to eliminate heavy metals (De Philippis et al., 2003).

However, lead was not detected in either the cytoplasm or in any of the intracellular structures using TEM-EDX analysis of the *Paracoccus* sp. DE2007 cultures grown in the presence of lead (Fig. 4.4a). Therefore, *Paracoccus* sp. DE2007 does not bioaccumulate lead.

Conclusions

1. SEM and TEM coupled with EDX constitute a set of methodologies that allow a quick diagnosis of whether a microorganism can capture a metal extra- or intra-cellularly. Although in the present work we only assayed one metal (lead), it is possible to obtain information about any metal and in different types of microorganisms.

2. The *Paracoccus* sp. DE2007 strain is able to capture lead extracellularly in its EPS envelopes, but it does not bioaccumulate lead intracellularly. However, the number of intracellular vesicles increases considerably in presence of lead. This could be related to an increase in the synthesis of lipid compounds that usually do not have metal affinity but which play an important role as a carbon reserve in microorganisms under stress conditions, such as the exposure to heavy metals.

Acknowledgements
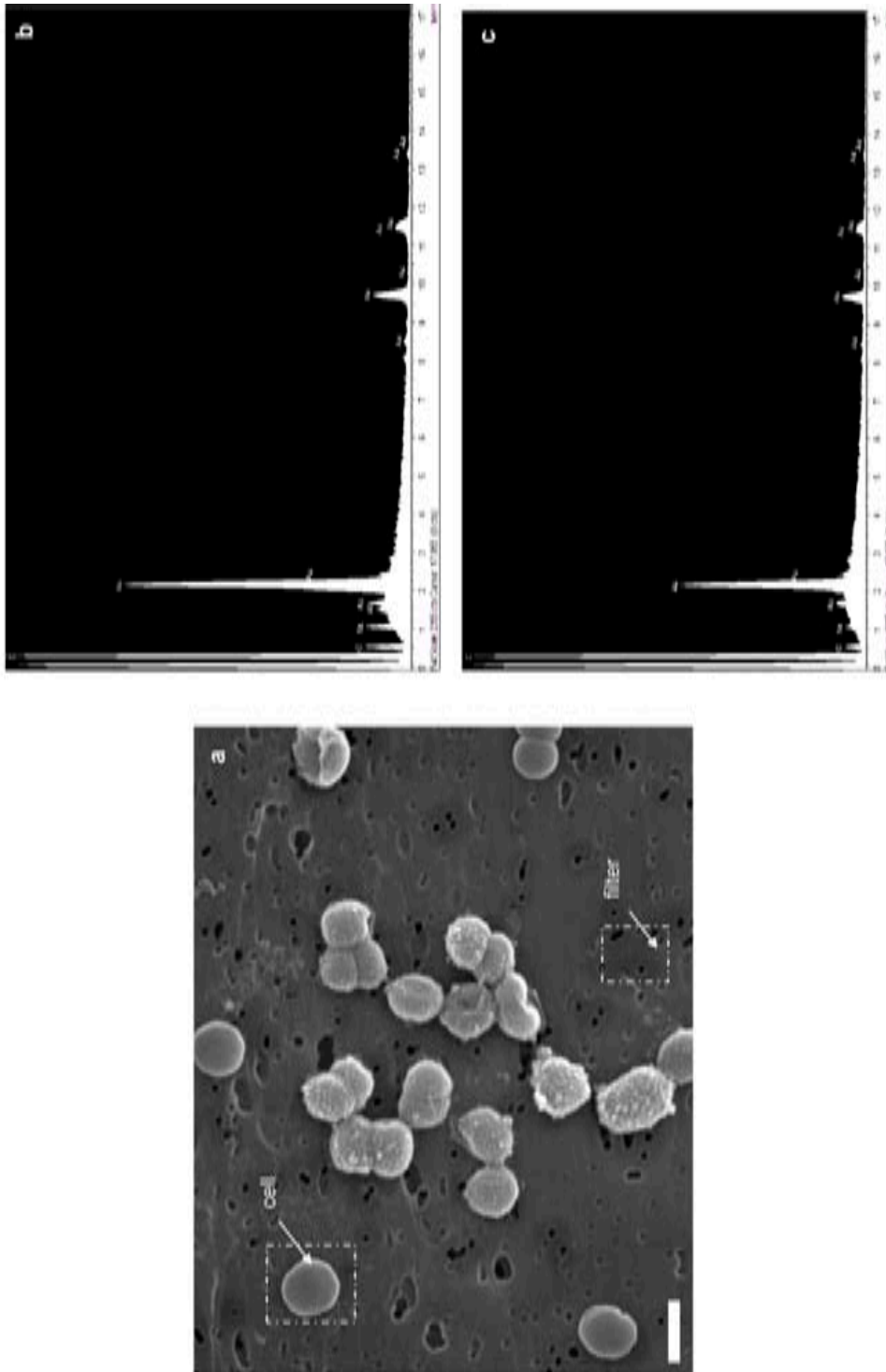
Fig. 4.1. Unpolluted cultures of *Paracoccus* sp. DE2007 cells are analyzed by SEM-EDX (**a**). Lead is not detected in either the cells (**b**) or the filter (**c**). Scale bar = 1μm in **1a**.
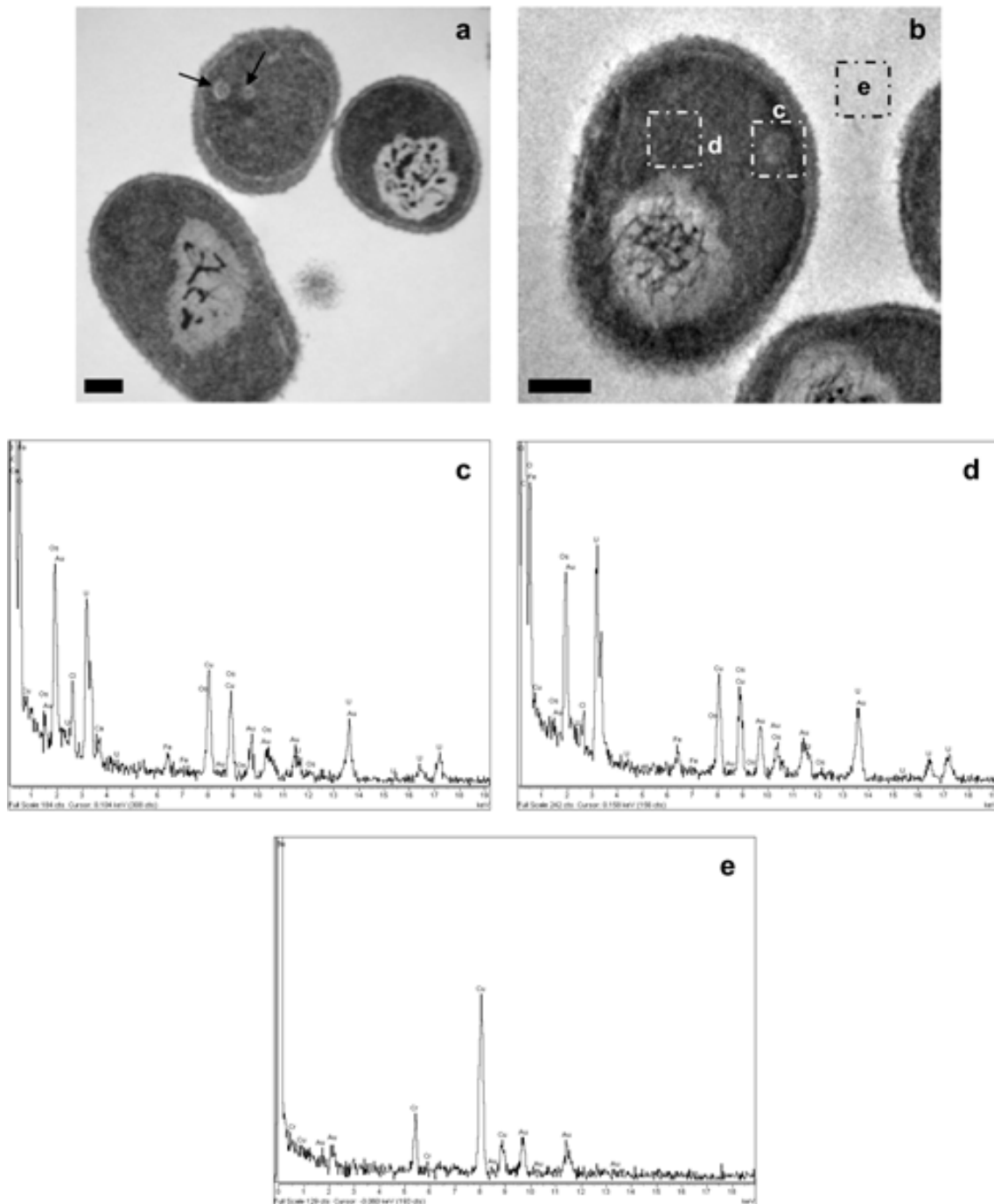
Fig. 4.2. Unpolluted culture of *Paracoccus* sp. DE2007 cells showing vesicles in the cytoplasm (arrows) **(a)**. Cells were analyzed by TEM-EDX **(b)**. EDX microanalysis spectra show the absence of lead in: the vesicles **(c)**, the cytoplasm **(d)** and the Spurr's resin. **(e)**. Au and Cu peaks are due to the gold grids. Scale bars = 200nm in **a** and **b**.
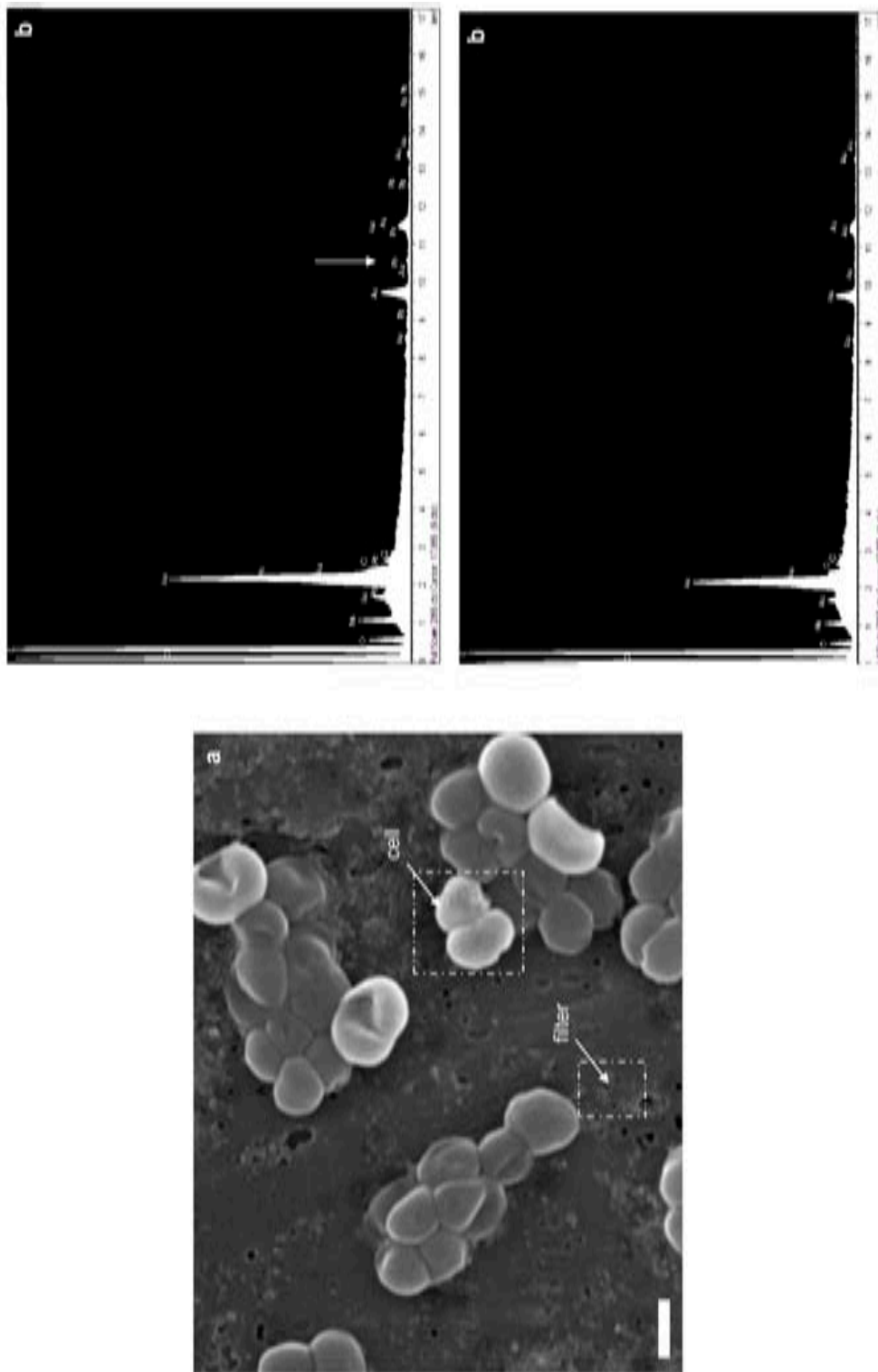
Fig. 4.3. Cells of *Paracoccus* sp. DE2007 grown in the presence of 5 mM of lead **(a)** Scale bar = 1µm. X-ray analysis spectra coupled to SEM show a Pb peak (arrow) **(b)** and the absence of lead in the filter **(c)**.
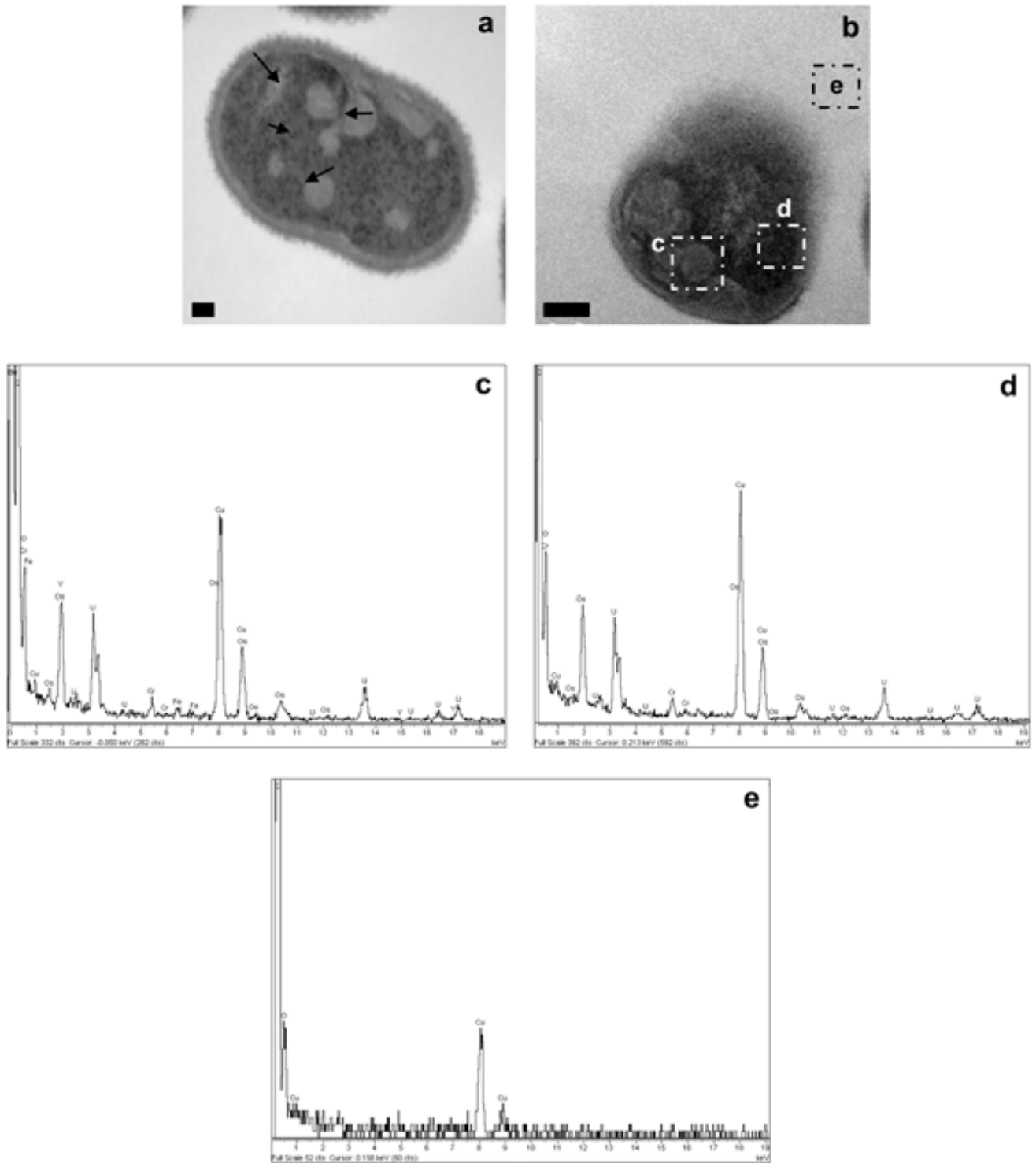
Fig. 4.4. *Paracoccus* sp. DE2007 cells grown in 5 mM lead salts show an increase in the number of intracitoplasmic vesicles (arrows) **(a)**. Cells were analyzed by TEM-EDX **(b)**. EDX microanalysis spectra show the absence of lead in: the vesicles **(c)**, the cytoplasm **(d)** and the Spurr's resin **(e)**. Scale bars = 100nm in **a** and 200 nm in **b**.

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5. Sequestration and *in vivo* effect of lead on DE2009 microalga, using high-resolution microscopic techniques

Sequestration and *in vivo* effect of lead on DE2009 microalga, using high-resolution microscopic techniques

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Abstract

Algae are primary producers in a wide variety of natural ecosystems, and these microorganisms have been used in bioremediation studies. Nevertheless, very little is known about the *in vivo* effect of heavy metals on individual living cells. In this paper, we have applied a method based on confocal laser scanning microscopy and lambda scan function (CLSM-$\lambda scan$) to determine the effect of lead (Pb), at different concentrations, on the DE2009 microalga. At the same time, we have optimized a method based on CLSM and image-analysis software (CLSM-IA) to determine *in vivo* biomass of this microorganism. The results obtained by lambda scan function indicated that the pigment peak decreases while the concentration of metal increases at pH 7. On the other hand at pH 4 there is no good correlation between the concentration of metal and the intensity of the emission of fluorescence of the pigment. Also, in some cases a displacement of the chl *a* peak towards 680 nm is produced. Total and individual biomass determined by CLSM-IA shows statistically significant differences between unpolluted and 10 mM polluted cultures.

Complementary studies using electron microscopy techniques coupled to energy dispersive X-ray microanalysis (EDX) demonstrate that the microalga can sequestrate Pb extra- and intracellularly.

Introduction

Microalgae and cyanobacteria are the most important primary producers in stratified laminated ecosystems, such as microbial mats, which cover large extensions of marine coastal environments (Esteve et al., 1994, Fourçans et al., 2004, Guerrero et al., 1993; Nakagawa and Fukui, 2002; Wieland et al., 2003;). In the last few years, we have isolated a consortium of microorganisms, from Ebro Delta microbial mats, dominated by a single cyanobacterium, *Microcoleus* sp., and different heterotrophic bacteria (Diestra et al., 2005; Sánchez et al., 2005). Recently we have isolated a new phototrophic microorganism, a microalga (DE2009) from the same habitat. Given that *Microcoleus* sp. was able to tolerate lead and copper (Burnat et al., 2009) in this study we propose an analysis of whether DE2009 microalga is able to sequestrate heavy metals.

Phototrophic microorganisms have been frequently used in biosorption research (De Philippis et al., 2003; Gong et al., 2005; Heng et al., 2004; Solisio et al., 2006). Metals are one group of contaminants frequently involved in marine environmental pollution. It is known that some metals at low concentrations, participate in different metabolic routes (essentials), but at high concentrations they are toxic for many living organisms; whilst others metals always have a toxic effect (Valls and de Lorenzo, 2002). Different methods have been proposed to study the toxic effect of heavy metals on microalgae, but most authors conclude that the metal concentration that affects growth in microalgae is variable and depends of many different factors, including the ability to accumulate heavy metals (Luoma and Rainbow, 2005; Moreno-Garrido et al., 2002). Algal surfaces have been found that containing different chemical function groups that differ in affinity and specificity towards these metals (Kaplan et al., 1987; Tien, 2002; Tien, 2005).

Although the capacity of some microalgae to capture heavy metals has been described, little is known about the effect of these metals in individual living cells, which is needed to predict the impact of heavy metals on natural ecosystems. In this study we selected Pb as a toxic metal and because the microbial mats studied are located in a lead-polluted area of the Ebro Delta (Sánchez-Chardi et al., 2007).

Confocal laser scanning microscopy (CLSM) based on natural pigment fluorescence emitted by phototrophic microorganisms is proving to be an excellent methodology for different types of studies related to these microorganisms. This optical micros-

copy technique avoids the need for either manipulating or staining the samples and allows accurate and non-destructive optical sectioning that generates high-resolution images, where out-of-focus is eliminated. Due to its high-resolution, it is easy to differentiate morphotypes of phototrophic microorganisms living in mixed populations, because they emit natural fluorescence.

The CLSM coupled to a spectrofluorometric detector (*λscan* function), provides simultaneous three-dimensional information on photosynthetic microorganisms and their fluorescence spectra profiles in stratified ecosystems, such as microbial mats and biofilms. The most significant application is the discrimination of cells with specific fluorescence spectra profiles within a colony, and the correlation of morphology and individual cell states (Roldán et al., 2004).

In this paper, we have applied CLSM-*λscan*, to determine the *in vivo* effect of Pb (at different concentrations) on DE2009 microalga and CLSM-IA to determine their total and individual biomass.

Complementary studies using scanning electron microscopy (SEM), transmission electron microscopy (TEM) and energy dispersive X-ray microanalysis (EDX) coupled to SEM and TEM were also performed to test the capacity of DE2009 microalga for extra- and intracellular uptake of Pb.

Experimental

Culture conditions

Cultures of DE2009 microalga 15 µE m^{-2} s^{-1} in liquid mineral Pfennig medium at two pHs (7 and 4) and at different concentrations (0, 0.1, 0.5, 0.75, 1, 5 and 10 mM) of lead [Pb(NO$_3$)$_2$] for 9 days.

Confocal laser scanning microscopy

The confocal experiments were performed using a confocal laser scanning microscope (Leica TCS SP5; Leica Heidelberg, Germany).

λscan function

Pigment analysis of DE2009 microalga cultures was determined by *λscan* function of CLSM. This technique provides information on the state of the photosynthetic pigments of phototrophic microorganisms on the basis of the emission wavelength region and the fluorescence intensity emitted (autofluorescence). Each image sequence was obtained by scanning the same *xy* optical section throughout the visible spectrum. Images were acquired at the *z* position at which the fluorescence was maximal, and acquisition settings were constant throughout the experiment. The sample excitation was carried out with an Argon Laser at 488 nm (λ$_{exe}$ 488) with a λ step size of 3 nm for an emission wavelength between 510 and 752 nm.

In order to measure the mean fluorescence intensity (MFI) of the *xyλ*, CLSM data sets obtained by means of the Leica Confocal Software (Leica Microsystems CMS GmbH) were used. The regions-of-interest (ROIs) function of the software was used to measure the spectral signature. For each sample, 70 ROIs of 1 µm^2 taken from DE2009 microalga cells were analysed.

Biomass estimations

Confocal images were obtained using the CLSM mentioned above. Two types of fluorescence at cell level from the same DE2009 microalga were observed in images obtained in all cultures analysed. Red (red cells) and green (green cells) were distinguished

on screen as pseudo-colours. For that reason, a *sequential scan* in two channels was carried out from each same *xy* optical section. On the first channel, samples were excited with a diode 561 nm (λ_{exe} 561) and the emission of fluorescence was captured between 670 and 794 nm (red pseudo-colour). On the second channel, samples were excited with an Argon Laser at 488 nm (λ_{exe} 488) and the emission of fluorescence was captured between 550 and 575 nm (green pseudo-colour). Finally, 10 red and 10 green confocal images were obtained from all cultures studied. Total biomass estimations from the red and green algal cells were obtained separately. Moreover, individual biomass for both types of cells was studied. Finally, total and individual biomass was estimated for each metal concentration.

In this paper, we have used a modification of the method described by Solé et al. (2007) using CLSM and a free image-processing analysis software, *ImageJ* v1.41. (CLSM-IA). This method was used to determine the percentage between red and green pixels of DE2009 microalga and their biomass from the different cultures studied in this work.

The method used in this paper is as follows: for total biomass each pair of images (red and green) from an identical *xy* optical section were opened in their original format (8-bit, 1024 × 1024 pixels) as tiff images and the corresponding overlay image was obtained. These three images were transformed to binary images (black/white) using different thresholds. Values of 70 and 25 were applied respectively to red and green images from 0.1, 0.5, 0.75, 1 and 5 mM metal concentrations. Conversely, threshold values of 50 and 60 were applied respectively to red and green images from the 10 mM metal concentration.

In order to determine the percentage between the red and green fluorescences at pixel level the image calculator function of the *ImageJ* was used. To obtain images with cells showing up only as red fluorescence, all green fluorescence was subtracted from the image. In the same way, red fluorescence is subtracted from the image when greens only are obtained. In both cases to clean the images it was necessary to filter out the red and green pixels. A smoothing filter (median filter with a radius of 2.0 pixels) was then applied to the images.To obtain biovolume values, the Voxel Counter plug-in was applied to these filtered images (Rasband, 2010). This specific application calculates the ratio of

the thresholded voxels (the red or green microalga volume), to all voxels from the binary image determined. The biovolume value obtained (Volume Fraction) was finally multiplied by a conversion factor of 310 fgC μm^{-3} to convert it to biomass (Bloem et al., 1995; Fry, 1990). To calculate the individual biomass, 30 red and 30 green cells were selected using *ImageJ* software and then the cells were analysed following the same protocol described above.

Scanning electron microscopy

To determine whether DE2009 microalga was able to capture Pb extracellularly, cultures polluted with 10 mM Pb were incubated under the same conditions as previous experiments. The following procedure was used: cultures were fixed in 2.5% glutaraldehyde Millonig buffer phosphate (0.1 M pH 4) for 2 h and washed four times in the same buffer. They were then dehydrated in a graded series (30%, 50%, 70%, 90%, and 100%) of ethanol and dried by critical-point. The samples were mounted on metal stubs and coated with gold and then viewed in a Jeol JSM-6300 scanning electron microscope (Jeol Ltd., Tokyo, Japan). For X-ray analysis, cultures were filtered on polycarbonate membrane filters. These filters were then dehydrated and dried by the same procedure used for culture samples. An EDX Link Isis-200 (Oxford Instruments, Bucks, England) operated at 20kV coupled to SEM was used.

Transmission electron microscopy

In order to assess whether DE2009 microalga was able to capture the metal intracellularly, cultures polluted with 10 mM Pb were incubated under the same conditions as previous experiments. The following procedure was used: cultures were fixed in 2.5% glutaraldehyde Millonig buffer phosphate (0.1 M pH4) for 2 hand washed four times in the same buffer. Samples were post-fixed in 1% OsO_4 at 4ºC for 2 h, and washed four times in the same buffer. They were then dehydrated in a graded series (30%, 50%, 70%, 90%, and 100%) of acetone and embedded in Spurr resin. To show a better quality image, ultrathin sections of 70 nm were mounted on carbon coated copper grids and stained with uranil acetate and lead citrate. Samples were viewed in a Hitachi H-7000 elec-

tron microscope (Hitachi Ltd., Tokyo, Japan). To determine the capacity of polyphosphate inclusions for accumulating Pb, sections of 200 nm thick mounted on titanium grids were used for energy dispersive X-ray microanal ysis. Samples were analysed with a Jeol Jem-2011 (Jeol Ltd., Tokyo, Japan).

Statistical analysis

Means and standard errors for each sample parameter determined in this study were calculated using SPSS software (version 15.0 for Windows). Data obtained for λscan experiments were compared using a Student's t test with a 95% significance ($p < 0.05$). Data obtained from percentages, and biomass was compared separately in the same way. All the statistical analyses were performed with the same software.

Molecular characterisation

Genomic DNA was extracted from an DE2009 overnight culture in Pfennig medium using UltraClean™ Microbial DNA Isolation Kit (Mobio Laboratories, Carlsbad, USA) according to manufac- turer's instructions. The 18S rRNA gene fragment was obtained by PCR amplification using SR1 (5′ -TACCTGGTTGATCCTGCCAG-3′) and Euk516 (5′-ACCAGACTTGCCCTCC-3′) primers (Diez et al., 2001), using PureTq™ Ready-To-Go™ PCR (GE Healthcare). The PCR conditions were those described in Diez et al., 2001). The PCR product was then purified using the QIAquick PCR purification Kit (Quiagen) as directed by the supplier. Both complementary strands were sequenced separately at the SECUGEN sequencing company (S.L. Madrid, Spain).

Results and Discussion

Characterization of the DE2009 microalga

DE2009 microalga was isolated from the Ebro delta microbial mats. Cells are spherical, with a diameter of 7-9 μm. Ultrathin sections of cells show: the thylakoids grouped into bands (inside the chloroplast); the nucleus and the pyrenoid. High electron-dense inclusions (HE) inside the cytoplasm were identified as polyphosphate granules (PPG). In pristine cultures (without Pb) no exopolysaccharides (EPS) were detected surrounding the cell wall (Fig. 5.1).

According to 18S rRNA gene sequence comparison, the closest cultured relatives were representatives of the *Scenedesmus* genus: *Scenedesmus pectinatus* (AB037092), *Scenedesmus acutiformis* (AB037089) and *Scenedesmus vacuolatus* (X56104) with 99% similarity.

However, the lack of ultrastructural similarity and the low variability of this marker among different closely related green algae genera make it difficult to assign this isolate to the genus *Scenedesmus* until more informative markers are sequenced.

Effect of Pb on DE2009 microalga

Different concentrations of Pb were used to study its effect on DE2009 microalga by CLSM. Two different experiments were prepared:

(A) The first experiment, was performed to determine the *in vivo* effect of Pb on microalga pigments by means of the *λscan* function of CLSM.

This method, allowed us to evaluate the physiological state of the microalga at single-cell level, considering changes in chlorophyll *a* (chl *a*) (maximum absorption at 685 nm). Cultures of DE2009 microalga were grown at pHs 7 and 4 and at different Pb concentrations.

An *xyz* optical section corresponding to the autofluorescence detected in control cultures growing at pH 7 is shown in Fig. 5.2a. The results demonstrate that the pigment peak decreases while the concentration of metal increases from 0 mM Pb (control cul-

ture) to 10 mM Pb. The chl *a* peak at the different Pb concentrations followed the same pattern as to that the obtained for the control culture (Fig. 5.2b).

An *xyz* optical section corresponding to the autofluorescence detected in control cultures growing at pH 4 is shown in Fig. 5.2c. At pH 4 there is no good correlation between the concentration of metal and the pigment's intensity of the fluorescence emission. In some cases, a displacement of the chl *a* peak towards 680 nm is produced (Fig. 5.2d). The differences in the effect of the metal on the cultures grown at both pHs could be attributed to the greater toxicity of the metal at pH 4.

Nevertheless, in both cases the Pb effect varied significantly according to the metal concentration used. The differences were not statistically significant ($p < 0.05$) between the control experiments and 0.1 mM Pb. However, statistically significant differences were observed between control and 0.5, 0.75, 1, 5 and 10 mM Pb cultures as pH 7 as pH 4.

(B) The second experiment, was performed to determine changes in total and individual biomass.

Changes in DE2009 microalga biomass depending on different Pb concentrations were studied in cultures growing at pH 4 and in the same light and temperature conditions.

To determine total biomass, previously, the red and green fluorescence pixel counts were measured, as mentioned in experimental section. The former ranged from 91,365.80 ± 15,695.33 (control experiment) to 13,972.90 ± 3,083.46 (at 10 mM Pb) and the latter varied from 10,593.70 ± 1,687.01 (control experiment) to 30,529.40 ± 17,706.84 (at 10 mM). The conversion of this data into biomass makes it possible to observe that the red cell biomass was drastically decreased from 27.01 ± 4.64 (mgC cm^{-3}) in the control experiment to 3.82 ± 0.80 (mgC cm^{-3}) at 10 mM Pb. In Fig. 5.3 these results are expressed as percentages for each Pb concentration. On comparing the growth of DE2009 microalga in unpolluted and 10 mM polluted cultures it is observed in the former case, red cells represent 89.61% and green cells 10.38%. In the latter case however, red cells represent 48.83% and green cells 51.16%. This data probably indicates that red cells could be considered physiologically active and green cells inactive.

To determine changes in individual biomass, only the red cells were considered for applying the CLSM-IA. In this case the pixel counts ranged from 595.87 ± 30.08 (control experiment) to 432.87 ± 25.21 at 10 mM Pb.

The cellular biomass, obtained from this data, decreased from 0.173 ± 0.09 (mgC cm^{-3}) to 0.128 ± 0.007 (mgC cm^{-3}). Statistically significant differences between the control culture (without lead) and 10 mM Pb cultures were found.

The results obtained both for the total and in individual biomass indicate the toxic effect at the highest concentration tested (10 mM Pb).

These experiments demonstrated the high *in vivo* tolerance of DE2009 to Pb. This microalga grows in higher metal concentrations than those described for other cyanobacteria and microalgae. For example, Roy et al., (2008) demonstrated that the *Synechocystis* sp. growth was completely inhibited at 1.9 mM Pb^{2+}, whilst Debelius et al., (2009) found that the maximum concentration of Pb tolerated by different microalgae was 0.03 mM Pb^{2+}. In the first case, the cyanobacterium was unable to grow at the concentrations used in this work, and in the second case, the time used for growth was 72h, a shorter period of time when compared to the time used in our experiments (9 days).

The results obtained with DE2009 microalga also show a higher tolerance to Pb than that observed for the heterotrophic bacterium *Micrococcus luteus* DE2008 (Maldonado et al., 2010a) and the cyanobacterium *Microcoleus* sp. (Burnat et al., 2010), both microorganisms forming part of the same indigenous consortium.

Heavy metal accumulation in DE2009 microalga

With the aim of proving whether the DE2009 microalga could capture metals, cells from cultures with and without Pb were analysed by EDX coupled to SEM and TEM. In control cultures Pb was not detected either externally or internally.

Cultures containing Pb were also analysed using the same above-mentioned procedure. In this case, major differences in the structure of DE2009 microalga were observed. A higher excretion of EPS was found surrounding the cells (Fig. 5.4a) and the EDX coupled to SEM demonstrated that Pb was found in EPS (Fig. 5.4b). It has been proved that different microorganisms have an EPS matrix which can protect cells

against toxic compounds such as metals and that its presence can overproduce exopolymer secretion (Decho, 1994). Also, more specifically, uronic acids and sulphate groups present in EPS may interact with various metals thereby immobilizing them (Stal, 2000).

Moreover, the ultrathin sections of DE2009 microalga also exhibited discernible changes (distortion of the cells) after exposure to Pb. An increase in the HE inclusions was evident (Fig. 5.4c), when comparing the cellular ultrastructure of the microalga grown in unpolluted and polluted cultures. These inclusions were identified as polyphosphate granules (PPG) (see peak P indicated by an arrow, (Fig. 5.4d). In many cases, similar inclusions have been found when cells are grown in adverse culture conditions (Jensen and Sicko, 1974; Sicko, 1972; Stevens et al., 1985). The results obtained through the energy dispersive X-ray analysis of the inclusions, confirmed that Pb was also accumulated in PPG inside the cytoplasmic space. A significant Pb peak was detected (Fig. IV-4d). These results agree with studies of Goldberg and colleagues (Goldberg et al., 2001), which suggested that these kind of inclusions had a detoxifying effect by sequestering heavy metals.

Our results also suggested that the DE2009 microalga has a great affinity for Pb both extra- and intracellularly.

Conclusions

In conclusion, we consider that the CLSM-$\lambda scan$ could be a rapid technique for studying *in vivo* the cellular responses to heavy metal pollution. At pH 7 there is and inverse correlation between the intensity of pigment's fluorescence emission and the concentration of essayed metal. At pH 4 there is no good correlation between the concentration of metal and the pigment's intensity of the fluorescence emission.

Moreover, this method combined with the values obtained by means of CLSM-IA enables evaluation of the changes in total and individual biomass depending on the Pb concentration used. Total and individual biomass is also drastically reduced at 10 mM Pb in the experiments performed at pH 4.

On the other hand, the DE2009 microalga has the ability to remove Pb extra- and intracellularly. As DE2009 microalga is an indigenous microorganism in marine coastal stratified ecosystems, this microalga is probably involved in removing Pb from these habitats.

Acknowledgments

Fig. 5.1. Ultrathin section of the DE2009 microalga. Chloroplast showing thylakoids (chl), nucleus (n), pyrenoid (py) and HE inclusions (indicated by arrows). Scale bar represents 2 μm.

Fig. 5.2. CSLM images and *λscans* plots of DE2009 microalga grow ing at pH7 and pH4. **(a)** and **(c)** represent CLSM images from a non-Pb treated cultures of DE2009 growing at pH 7 and 4 respectively. In these confocal images the pseudo-colour palette 4 (Leica Aplication Suite, Leica Microsystems CMS GmbH) was used, where warm colours represent the maximum intensities and cold colours represent the low intensities of fluorescence. *λscans* of DE2009 microalga cultures treated with different Pb concentrations at pH 7 **(b)** and pH 4 **(d)**. 2D plots represent the MFI spectra: emission wavelength (650–730 nm), *x* axis; MFI, *y* axis.

Fig. 5.3. Relative abundance of red and green DE2009 microalga cells at different Pb concentrations (expressed as a percentage of biomass).
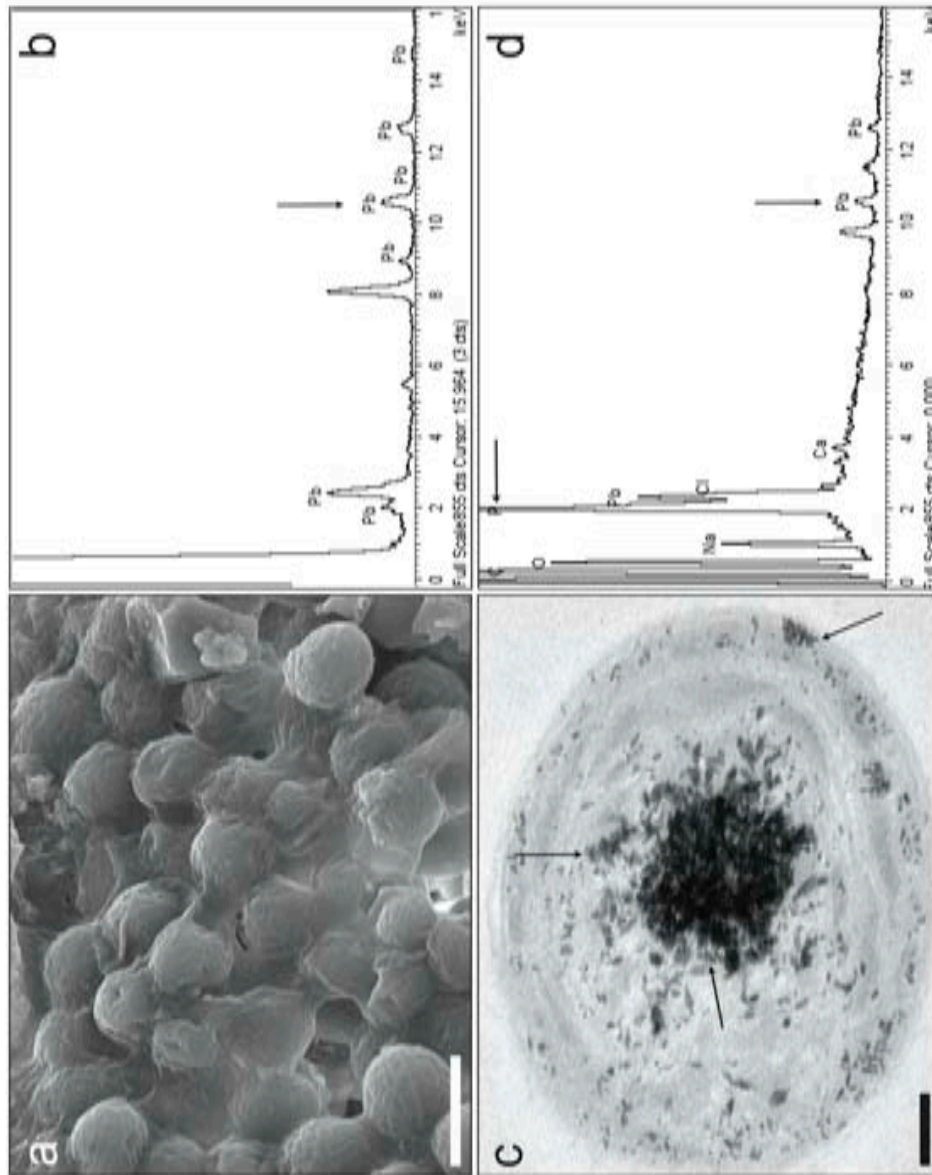
Fig. 5.4. DE2009 microalga culture treated with 10 mM Pb. SEM image. Scale bar represents 10 μm **(a)**. EDX spectrum coupled to SEM. Arrow indicates the main Pb peak at 10.5 keV **(b)**. Ultrathin section of DE2009 microalga. Arrows indicate the distribution of HE inclusions. Scale bar represents 0.2 μm **(c)**. EDX spectrum coupled to TEM from HE inclusions. Arrow indicates the main Pb peak at 10.5 keV **(d)**.

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6. Selection of bioindicators to detect lead pollution in Ebro Delta microbial mats, using high-resolution microscopic techniques

Selection of bioindicators to detect lead pollution in Ebro Delta microbial mats, using high-resolution microscopic techniques

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Abstract

Lead (Pb) is a metal that is non-essential to any metabolic process and, moreover, highly deleterious to life. In microbial mats – benthic stratified ecosystems – located in coastal areas, phototrophic microorganisms (algae and oxygenic phototrophic bacteria) are the primary producers and they are exposed to pollution by metals. In this paper we describe the search for bioindicators among phototrophic populations of Ebro delta microbial mats, using high-resolution microscopic techniques that we have optimized in previous studies. Confocal laser scanning microscopy coupled to a spectrofluorometric detector (CLSM-$\lambda scan$) to determine in vivo sensitivity of different cyanobacteria to lead, and scanning electron microscopy (SEM) and transmission electron microscopy (TEM), both coupled to energy dispersive X-ray microanalysis (EDX), to determine the extra- and intracellular sequestration of this metal in cells, were the techniques used for this purpose.

Oscillatoria sp. PCC 7515, *Chroococcus* sp. PCC 9106 and *Spirulina* sp. PCC 6313 tested in this paper could be considered bioindicators for lead pollution, because all of these microorganisms are indigenous, have high tolerance to high concentrations of lead and are able to accumulate this metal externally in extracellular polymeric substances (EPS) and intracellularly in polyphosphate (PP) inclusions.

Experiments made with microcosms demonstrated that *Phormidium*-like and *Lyngbya*-like organisms selected themselves at the highest concentrations of lead assayed.

In the present study it is shown that all cyanobacteria studied (both in culture and in microcosms) present PP inclusions in their cytoplasm and that these increase in

number in lead polluted cultures and microcosms. We believe that the application of these microscopic techniques open up broad prospects for future studies of metal ecotoxicity.

Introduction

Many metals are released from natural and anthropogenic sources into aquatic environments and can be bioaccumulated in sediments and in aquatic biota, agriculture and industry being the most important sources of this kind of pollution. The toxicity of metals in biological systems depends on their bioavailable form and on different biotic and abiotic factors (Roane and Pepper, 2000).

Faced with this problem, research has been undertaken on how metals affect the diversity, biomass, structure and activity of microbial communities in soil and sediments, including microbial mats (Boivin et al., 2005; Massieux et al., 2004; Ranjard et al., 2006).

In the past years our research group has studied the Ebro Delta microbial mats. These mats are coastal benthic stratified ecosystems formed by vertically laminated microbial communities, which develop coloured layers (Esteve et al., 1992; Guerrero et al., 1999). They are located in the NE of Spain (40º 40′ N, 0º 40′ E) and although the Ebro delta comprises an area of 320 km^2, the region occupied by these ecosystems covers 3 km^2. This area has been polluted by heavy metals, mainly the Ebro River (Mañosa et al., 2001).

Cyanobacteria are the most abundant prokaryotic microorganisms living in the upper layers of microbial mats. They are photoautotrophic bacteria, having chlorophyll *a* (chl *a*) as their major photosynthetic pigment. Among cyanobacteria, *Microcoleus chthonoplastes* is the most abundant cyanobacteria in Ebro delta microbial mats (Esteve et al., 1994; Mir et al., 1991; Solé et al., 2009). This cyanobacterium is also dominant in hypersaline environments (Garcia-Pichel et al., 1996) and in the hot deserts (Campbell, 1979).

In previous works, we have studied the cyanobacterial diversity in microbial mats being the most abundant *Microcoleus* sp., *Oscillatoria* sp., *Pseudanabaena* sp., *Phormidium* sp. and *Spirulina* sp. among the filamentous type and *Chroococcus* sp., *Gloeopcasa* sp., *Synechocystis* sp. and *Mixosarcina* sp. among the unicellular type (Diestra et al., 2004; Fourçans et al., 2004; Solé et al., 2003; Wieland et al., 2003).

Communities living in microbial mats are often subjected to strong environmental changes, including the presence of pollutants, among them heavy metals. Many studies have been carried out to demonstrate the capacity of microorganisms to sequestrate heavy metals: on bacteria (Borrego and Figueras, 1997), fungi (Lo et al., 1999); algae (Mehta and Gaur, 2001) and cyanobacteria (De Philippis et al., 2003; Incharoensakdi and Kitjaharn, 2002).

Although some species of plants, invertebrates, fish, birds and mammals, have been used as bioindicators of contamination and water quality (Azevedo et al., 2009; Faria et al., 2010) and soil quality (Garitano-Zavala, 2010, Gjorgieva et al., 2011; Sánchez-Chardi et al., 2007), nothing is known about the use of algae and cyanobacteria as metal bioindicators in polluted microbial mats.

As was mentioned above, given the ecological importance of heavy metal pollution and considering that no bioindicators have been described for this type of pollution in microbial mats; we propose, in this work to select bioindicators for lead.

We consider the following characteristics to select the bioindicators: ubiquity and abundance of the selected microorganisms; ease of growth under laboratory conditions; sensitivity to the chosen metal and the ability to accumulate it.

In the recent years, our working group has optimized high-resolution microscopy techniques (CLSM, SEM-EDX and TEM-EDX) to determine *in vivo* the effect of different heavy metals on phototrophic microorganisms (sensitivity) and to detect the ability of these microorganisms to capture metals extra- and/or intracellularly (Burnat et al., 2009, 2010; Maldonado et al., 2010b).

For this research, we chose three cyanobacteria from the Pasteur culture collection of cyanobacteria (PCC) that are abundant in the Ebro delta microbial mats. Lead was chosen because it is a non-essential toxic metal and in the past, lead pellets were reported to have been found in high densities in the Ebro River sediment (Mateo et al., 1997).

The aim of this work is therefore to assess the potential of different cyanobacteria to be considered bioindicators of lead pollution in coastal microbial mats, using high-resolution microscopy techniques.

Material and Methods

Cyanobacteria strains and culture conditions

Oscillatoria sp. PCC 7515, *Chroococcus* sp. PCC 9106 and *Spirulina* sp. PCC 6313, were chosen from the PCC (Herdman et al., 2005) because they are always present in Ebro delta microbial mats. The first strain was cultivated in Marine medium (MN) with nitrate omitted (Rippka et al., 1979) and the other two strains were grown in a 1:1 (v/v) BG11 and ASN III mixture medium (Rippka et al., 1979). All culture strains were grown at 27ºC and 15 μE m^{-2} s^{-1}.

Sampling site and microcosms setup

Microbial mat samples were collected from the Ebro Delta on October 2009. Samples were taken in 55 x 43 x 88 mm poly(methyl methacrylate) boxes and transferred to the laboratory. Two microcosm experiments were prepared, one microcosm was used as a control experiment (unpolluted) and the other microcosm was polluted with 10 mM [Pb(NO$_3$)$_2$] solution. The microcosms were maintained in laboratory conditions for 9 days, after which the metal solution was removed from the polluted microcosm. Samples for electron microscopy and CLSM were taken with glass cores $\varnothing$ (6 mm).

Confocal laser scanning microscopy

Cultures of the cyanobacteria strains were polluted at different Pb concentrations: 0 (unpolluted culture), 0.1, 0.5, 0.75, 1 and 2 mM (polluted cultures). All experiments were performed for a period of 9 days under the same culture conditions mentioned in culture conditions.

Pigment analysis was carried out by the *λscan* function of CLSM (Leica TCS SP5; Leica Heidelberg, Germany). This technique made it possible to obtain information about the state of pigments on the basis of the emission wavelength region and the fluorescence intensity emitted. Each image sequence was acquired by scanning the same *xy* optical section throughout the visible spectrum. Images were acquired at the *z* position at which the fluorescence was maximal, and acquisition settings were constant through-

out each experiment. The sample excitation was carried out with an Argon Laser at 488 nm (λ_{exe} 488) with a λ step size between 3 and 4.74 nm for an emission wavelength between 510 and 752 nm.

In order to measure the mean fluorescence intensity (MFI) of the *xyλ* data sets, the Leica Confocal Software (Leica Microsystems CMS GmbH) was used. The regions-of-interest (ROIs) function of the software was used to measure the spectral signature of the samples. 70 ROIs of 1 μm^2 taken from cells from each sample were analyzed.

Scanning electron microscopy

Scanning electron microscopy coupled to EDX (SEM-EDX) was used in order to assess whether the selected cyanobacteria strains and cyanobacteria from the polluted microcosm were able to capture Pb extracellularly. Cultures of the three strains and microcosms experiments were both polluted with 10 mM Pb for a period of 9 days. This concentration was used to obtain a rapid response to Pb from cyanobacteria.

The following procedure was used: samples were fixed in 2.5% glutaraldehyde Millonig buffer phosphate (0.1 M pH 4) for 2 hours and washed four times in the same buffer. They were then dehydrated in graded series (30%, 50%, 70%, 90%, and 100%) of ethanol and dried by critical point. The samples were mounted on metal stubs, coated with gold and viewed in a Zeiss EVO® MA 10 scanning electron microscope (Carl Zeiss NTS GmbH, Oberkochen, Germany). An EDX Link Isis-200 (Oxford Instruments, Bucks, England) operated at 20kV coupled to the microscope was used.

Transmission electron microscopy

TEM was used to determine the ultrastructure of cyanobacteria and TEM coupled to EDX (TEM-EDX) to assess whether the cyanobacteria strains and cyanobacteria from the polluted microcosm were able to bioaccumulate the heavy metal intracellularly. Culture conditions were the same as described for scanning electron microscopy.

The following procedure was used: samples were fixed in 2.5% glutaraldehyde Millonig buffer phosphate (0.1M pH 4) for 2 hours and washed four times in the same buffer. Samples were post-fixed in 1% OsO_4 at 4ºC for 2 h, and washed four times in the

same buffer. They were then dehydrated in graded series (30%, 50%, 70%, 90%, 100%) of acetone and embedded in Spurr resin. To show a better quality image, ultrathin sections of 70 nm were mounted on carbon-coated copper grids and stained with uranil acetate and lead citrate. Samples were viewed in a Hitachi H-7000 electron microscope (Hitachi Ltd., Tokyo, Japan). To analyze the samples with EDX, sections of 200 nm thick mounted on carbon-coated titanium grids were used. These sections were not stained with lead citrate in order to avoid elemental substitution during the analysis. Samples were analyzed with a Jeol Jem-2011 (Jeol Ltd., Tokyo, Japan). An EDX Link Isis-200 (Oxford Instruments, Bucks, England) operated at 20kV coupled to this microscope was used.

Statistical analysis

The MFI analysis of $\lambda scan$ experiments, were carried out by one-way analysis of variance (ANOVA) and Tukey and Bonferroni's comparison *post hoc* tests. Significant differences were accepted at $p < 0.05$. The analyses were performed using SPSS software (version 19.0 for Mac OS X).

Results and discussion

The bioindicators were chosen on the basis of whether the microorganisms being studied fulfilled the aforementioned conditions: (a) ubiquity and abundance of the chosen microorganisms; (b) ease of growth under laboratory conditions; (c) sensitivity to the chosen metal; (d) the ability to accumulate it.

Lead-sensitivity of cyanobacteria strains determined by means of the CLSM-λscan function

The three cyanobacteria studied in this work grew both in liquid medium and solid medium. These media and the culture conditions have been mentioned above in the section *cyanobacteria strains and culture conditions*. With the liquid medium cultures (unpolluted and polluted) as a starting-point, the corresponding experiments were performed using CLSM.

The CLSM-*λscan* experiments corresponding to each of the cyanobacteria tested were performed to determine sensitivity to Pb and its effect *in vivo*. To this end, the state of pigments was considered by means of the maximum fluorescence signal detected at: 575 nm [phycoerythrin (PE)] for *Oscillatoria* sp. PCC 7515 (Bryant, 1982); 680 nm [chlorophyll *a* (chl *a*)] for *Chroococcus* sp. PCC 9106 and 652 nm [chlorophyll *a* (chl *a*)] for *Spirulina* sp. PCC 6313.

(A) The autofluorescence detected in control cultures of *Oscillatoria* sp. PCC 7515 corresponding to an *xyz* optical section is shown in Fig. 6.1a. In the plot the maximum fluorescence peak is shown, which corresponds to 575 nm (Fig. 6.1b). The results demonstrate that the fluorescence peak decreases while the Pb concentration increases from 0 mM (control experiment) to 2 mM. The 0.1 mM fluorescence spectrum followed the same pattern as the control culture, although at the other concentrations (0.5, 0.75, 1 and 2 mM) there is a displacement of the fluorescence peak from 575 nm towards to 570 nm. In this study a blue-shift of the MFI from 0.5 mM has also been observed, which corresponds with the changes in the PE pigment. An analog effect has been demonstrated by Murthy and Mohanty (1991) in *Spirulina platensis* growing in the presence of mercury and at high tem-

peratures (Murthy et al., 2004). Statistically significant differences were found between all the conditions tested ($F = 236.980$) ($p < 0.05$). Using the Tukey and Bonferroni comparison tests, the only concentrations that were no significant between them were those of 0.5 mM and 0.75 mM, as these concentrations have the same effect on the MFI. The minimum metal concentration (when compared with the control) that affected the intensity of the pigment was 0.1 mM Pb.

(B) *Chroococcus* sp. PCC 9106 *xyz* optical section corresponding to the autofluorescence observed in control cultures is shown in Fig. 6.1c. The control spectrum shows the maximum fluorescence peak at 682 nm, which decreases, while the Pb concentration increases (from 0 to 2 mM). The 0.1 and 0.5 mM fluorescence spectra follow the same pattern as control cultures, but at 0.5 mM the fluorescence intensity is lower. From 0.75 mM there is a decrease in the MFI reaching the lowest levels and no fluorescence was detected at the highest concentration tested (2 mM). Statistically significant differences were found between all the conditions tested ($F = 1709.574$) ($p < 0.05$). The minimum metal concentration (when compared with the control) that affected the intensity of the pigment was 0.5 mM Pb.

(C) As in the two experiments mentioned above, a *Spirulina* sp. PCC 6313 *xyz* optical section corresponding to the autofluorescence in control cultures is shown in Fig. 6.1e. A maximum fluorescence peak is detected at 652 nm. Moreover, at the highest concentrations (1 and 2 mM) the MFI is drastically reduced (Fig. 6.1f). The 2 mM concentration was not included in this figure because no fluorescence signal was detected. Statistically significant differences were found between all the conditions tested ($F = 1109.582$) ($p < 0.05$). The minimum metal concentration (when compared with the control) that affected the intensity of the pigment was 0.1 mM Pb. In Fig. 6.1f, the fluorescence spectra obtained at 0.5 mM and 0.75 mM do not correspond with the expected trend, making this particular case difficult to interpret.

From these results, it can be concluded that *Oscillatoria* sp. PCC 7515 and *Spirulina* sp. PCC 6313 are sensitive at the concentration of 0.1 mM, while *Chroococcus* sp.

PCC 9106 is sensitive at that of 0.5 mM. In all three cases there is an inverse correlation between the MFI and the concentration of the metal (Figs. 6.1b, 6.1d and 6.1f). These results consistent with those obtained by Massieux et al. (2004), which showed that metals could affect the physiology and photosynthetic activity of the phototrophic community.

Lead sequestration in cyanobacteria strains by SEM-EDX and TEM-EDX

A rapid test by SEM-EDX was performed on each one of the cyanobacteria studied to determine the minimum concentration at which metal uptake occurs (considering the results obtained by CLSM-$\lambda scan$). Moreover, in polluted cultures analyzed we also tested different parts of the filters to be sure that Pb was retained only in cells.

The result was positive in all of them for the minimum metal concentration used, and negative for the control (Fig. 6.2).

With the purpose or ascertaining whether the three strains of cyanobacteria could accumulate metal internally, cultures of each strain were polluted with 10 mM, to increase the signal, and analyzed by TEM-EDX. In all cases, images by SEM-EDX were also obtained to compare the results by both methodologies.

In the control cultures (without Pb), and using these techniques, it was found that the metal was not detected either internally or externally in any of the three microorganisms.

On the other hand, the results observed after Pb pollution for each of the three cyanobacteria are as follows.

Oscillatoria sp. PCC 7515 is seen to accumulate Pb in the EPS, which can be observed in the EDX spectrum (Figs. 6.3a and 6.3b). Furthermore, the ultrathin sections of this microorganism show abundant high electron-dense inclusions of different sizes in its cytoplasm (Fig. 6.3c). EDX analysis of these, demonstrate that are PP inclusions (P and Ca peaks) and that they retain lead (Pb main peak at 10.5 KeV indicated by arrow) (Fig. 6.3d). These results are in accordance with those obtained by Jensen and Sicko (1974).

In the samples of *Chroococcus* sp. PCC 9106 the results obtained by SEM-EDX and TEM-EDX are similar to those shown in *Oscillatoria* sp. PCC 7515 and they demonstrate this microorganism's ability to accumulate Pb extra- and intracelullarly (Fig. 6.4).

Finally, *Spirulina* sp. PCC 6313 shows a similar ability to retain Pb in the EPS (Figs. 6.5a and 6.5b). Ultrathin sections show expanded thylakoids, high electron-dense PP inclusions and polyhydroxyalkanoates (PHA) granules, identified previously in an optical microscope by Sudan Black stain (Fig. 6.5c). This cyanobacterium also accumulates Pb in PP inclusions (Fig. 6.5d). Furthermore, the presence of PHA granules in *Spirulina* sp. PCC 6313, probably demonstrate a nitrogen limitation in the lead-polluted cultures, that has also been shown in *Synechocystis* sp. strain PCC 6803 (Schlebusch and Forchhammer, 2010).

Taken together, the results described in this section show that the three cyanobacteria are able to accumulate the metal externally (in the EPS) and internally in the intracytoplasmatic PP inclusions.

Previous studies also showed that *Microcoleus* sp. one of the dominant cyanobacteria in the Ebro Delta microbial mats accumulates lead in the PP inclusions (Burnat et al., 2010).

Lead detection in cyanobacteria from polluted microcosms by SEM-EDX and TEM-EDX

In order to ascertain whether cyanobacteria can be bioindicators of lead pollution in microbial mats, a microcosm was polluted with a concentration of 10 mM and the results were compared with a control microcosm (unpolluted). In this case, since the images of natural environments do not allow us a clear view of the variety of microorganism, due to the high microbial density and the presence of EPS, which masks the results, a CLSM analysis of the *in vivo* diversity was done previously. In the unpolluted microcosm *Oscillatoria*-like was the dominant cyanobacterium while *Phormidium*-like and *Lyngbya*-like were the most abundant in the polluted microcosm. In the latter, very small and thin unidentified filaments were also present (Figs. 6.6a and 6b).

SEM image of the control microcosm shows a community of microorganisms covered by polysaccharides (Fig. 6.7a). No Pb is detected by EDX in these samples. The detected elements are: iron (Fe) from metal sulfides, silicon (Si) from diatoms and calcium (Ca) from photosynthetic bacteria. These elements are always present in microcosms from Ebro Delta microbial mats (Fig. 6.7b).

In the polluted microcosm, a great cellular lysis of *Oscillatoria*-like is evident, probably due to the high lead concentrations. On the other hand, in these conditions the cyanobacteria *Lyngbya*-like and *Phormidium*-like are selected (Fig. 6.7c), in accordance to the images obtained by CLSM. EDX analysis of these microorganisms gives similar results (Fe, Si and Ca peaks), to those obtained in the control microcosm, but in this case Pb is also detected (Fig. 6.7d).

The ultrathin sections obtained by TEM of *Phormidium*-like and *Lyngbya*-like from the polluted microcosm show, as in the cyanobacteria cultures studied in this work, an increase in PP inclusions. An ultrathin section of *Phormidium*-like is showed in Fig. 6.7e TEM-EDX microanalysis also detects Pb as well as the elements P and Ca that can already be seen in the control microcosm. No other toxic metals that could be polluting the original mat are detected in the PP inclusions (Fig. 6.7f).

Conclusions

Considering all the results presented in this paper, it can be concluded that the three cyanobacteria studied could be good bioindicators of pollution by Pb. Furthermore, if these results are compared to those obtained in the polluted microcosm, at a higher concentration than the tested in the cultures, we observe a positive selection of *Lyngbya*-like and *Phormidium*-like with respect to *Oscillatoria*-like, in the control experiment.

All cyanobacteria studied in this work - both cultures and microcosms - accumulate PP inclusions that increase with stress, due to lead pollution and the fact that all these inclusions retain this metal.

On the other hand, according to the results obtained, it is possible to link bioaccumulation processes to the toxicological effects of the lead, since this metal is toxic at any concentration. Lead also, as has been demonstrated in microcosm experiments, causes changes in the diversity, thus the ecological importance of metal pollution.

We believe that the application of these techniques open up broad prospects for future studies of metal ecotoxicity, as they will allow us to determine whether pollution by other heavy metals could have occurred in natural environments.

Acknowledgments

Fig. 6.1. CLSM images and *λscans* plots of unpolluted and polluted cyanobacteria cultures. *Oscillatoria* sp. PCC 7515 **(a)**; *Chroococcus* sp. PCC 9106 **(c)** and *Spirulina* sp. PCC 6313 **(e)** from unpolluted cultures are represented in CLSM images. Scale bars represent 50, 25 and 10 *μ*m, respectively. In these confocal images the pseudo-colour palette 4 (Leica Aplication Suite, Leica Microsystems CMS GmbH) was used, where warm colours represent the maximum intensities and cold colours respresent the low intensities of fluorescence. *λscans* plots of *Oscillatoria* sp. PCC 7515 **(b)**; *Chroococcus* sp. PCC 9106 **(d)** and *Spirulina* sp. PCC 6313 **(f)** polluted with different Pb concentracions. 2D plots represent the MFI spectra: emission wavelenght, *x* axis; MFI, *y* axis.

Fig. 6.2. Rapid test to detect lead in cyanobacteria by SEM-EDX. Analysis points indicated by arrows from *Oscillatoria* sp. PCC 7515 **(a)**, *Chroococcus* sp. PCC 9106 **(c)** and *Spirulina* sp. PCC 6313 **(e)** obtained by SEM. Scale bars represents 10, 10 and 20 μm, respectively. Pb peak showed in the spectrum (obtained by SEM-EDX) is indicated in each case by arrow **(b)**, **(d)** and **(f)**.
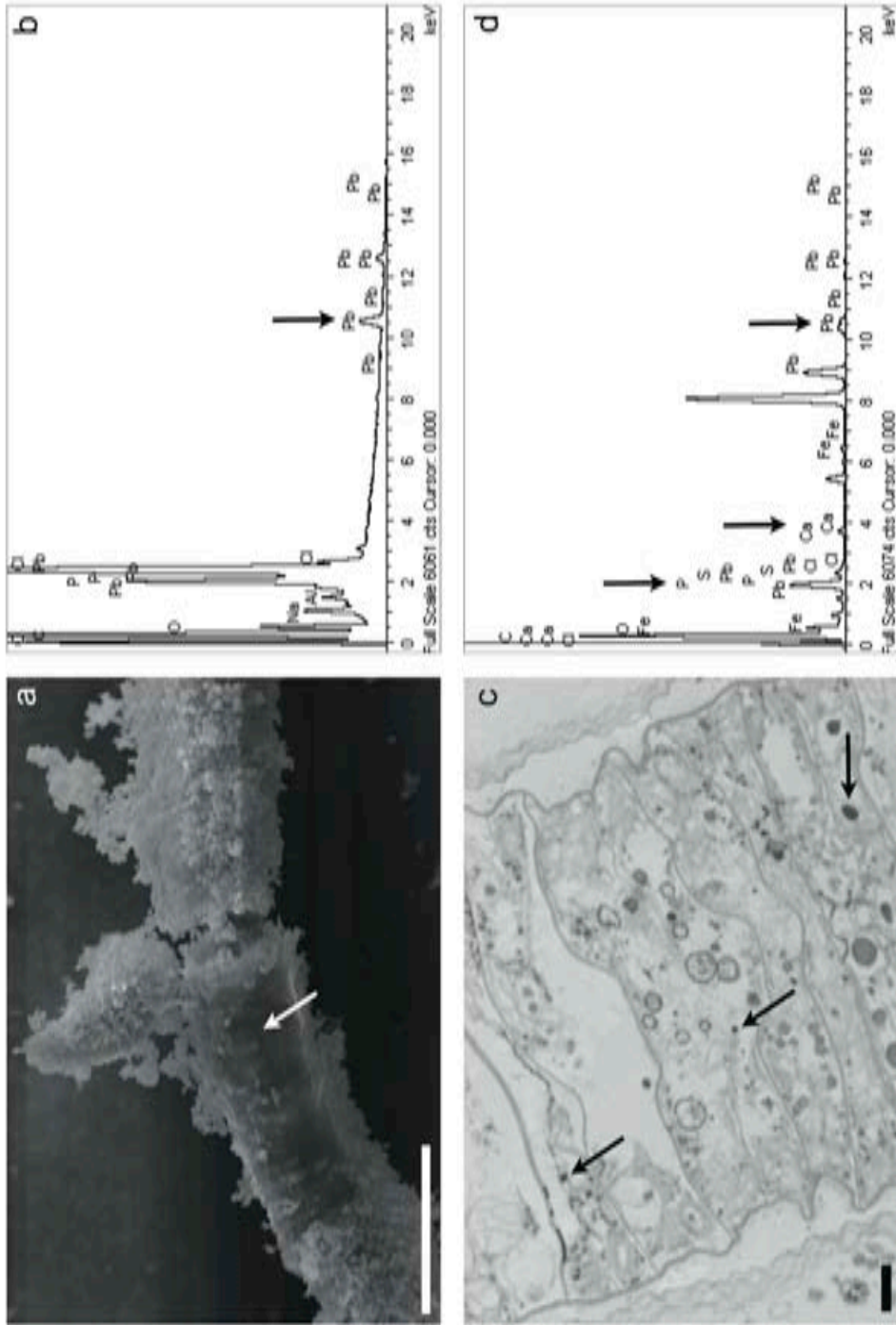
Fig 6.3. Lead sequestration by *Oscillatoria* sp. PCC 7515. Analysis point indicated by arrow from *Oscillatoria* sp. PCC 7515. Scale bar represents 20 *μm* **(a)**. Pb peak showed in the spectrum obtained by SEM-EDX (see arrow) **(b)**. High electron-dense inclusions from an ultrathin section of *Oscillatoria* sp. PCC 7515 are indicated by arrows. Scale bar represents 2 *μm* **(c)**. Pb, Ca and P peaks showed in the spectrum obtained by TEM-EDX from high electron-dense (see arrows) **(d)**.
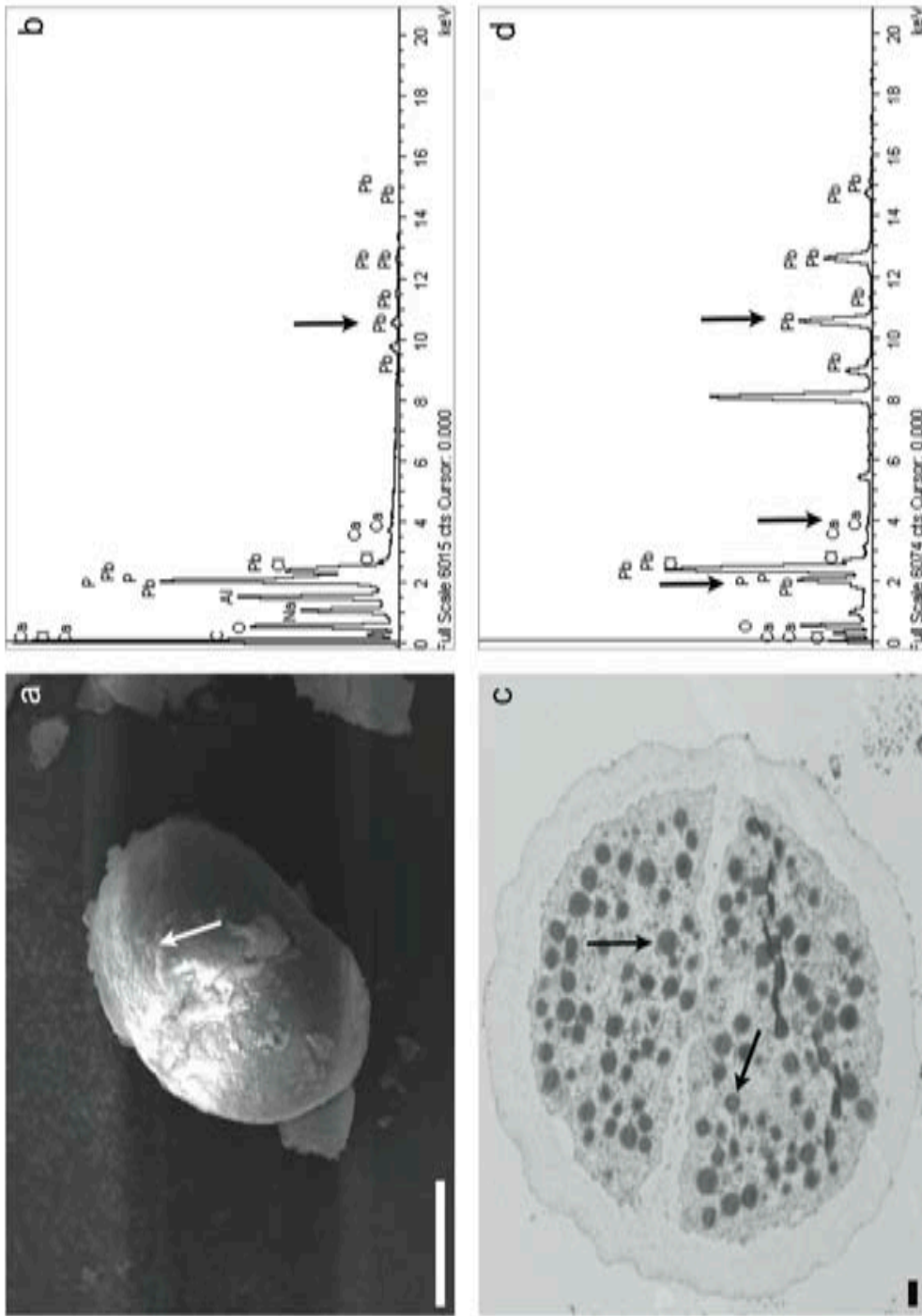
Fig 6.4. Lead sequestration by *Chroococcus* sp. PCC 9106. Analysis point indicated by arrow from *Chroococcus* sp. PCC 9106 obtained by SEM. Scale bar represents 10 μm **(a)**. Pb peak showed in the spectrum obtained by SEM-EDX (see arrow) **(b)**. High electron-dense inclusions from an ultrathin section of *Chroococcus* sp. PCC 9106 are indicated by arrows. Scale bar represents 2 μm **(c)**. Pb, Ca and P peaks showed in the spectrum obtained by TEM-EDX from high electron-dense inclusions (see arrows) **(d)**.

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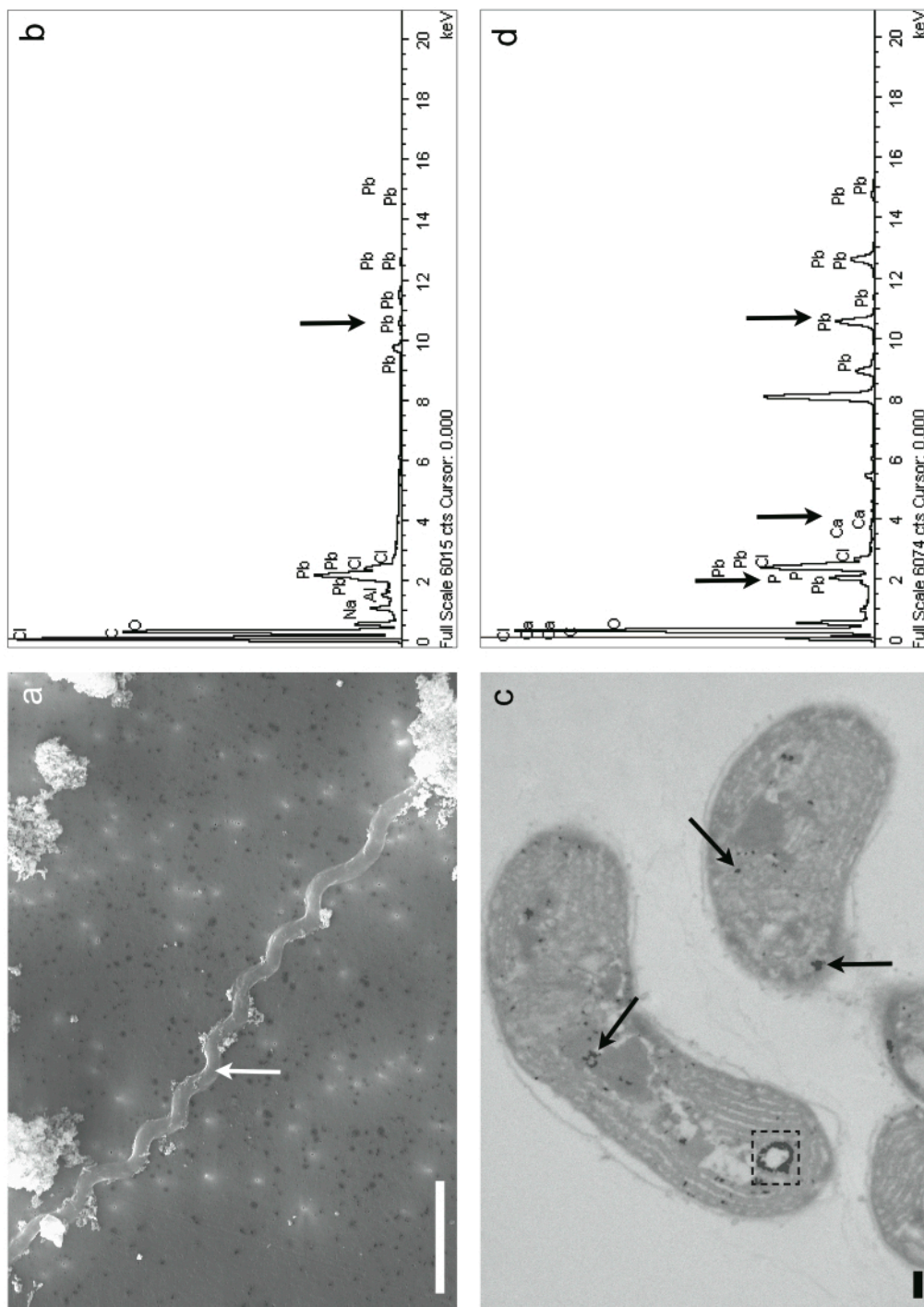
Fig. 6.5. Lead sequestration by *Spirulina* sp. PCC 6313. Analysis point indicated by arrow from *Spirulina* sp. PCC 6313 obtained by SEM. Scale bar represents 10 *μm* **(a)**. Pb peak showed in the spectrum obtained by SEM-EDX (see arrow) **(b)**. High electron-dense inclusions from an ultrathin section of *Spirulina* sp. PCC 6313 are indicated by arrows. PHA granule is marked with a dashed rectangle. Scale bar represents 0.2 *μm* **(c)**. Pb, Ca and P peaks showed in the spectrum (obtained by TEM-EDX) from high electron-dense inclusions (see arrows) **(d)**.
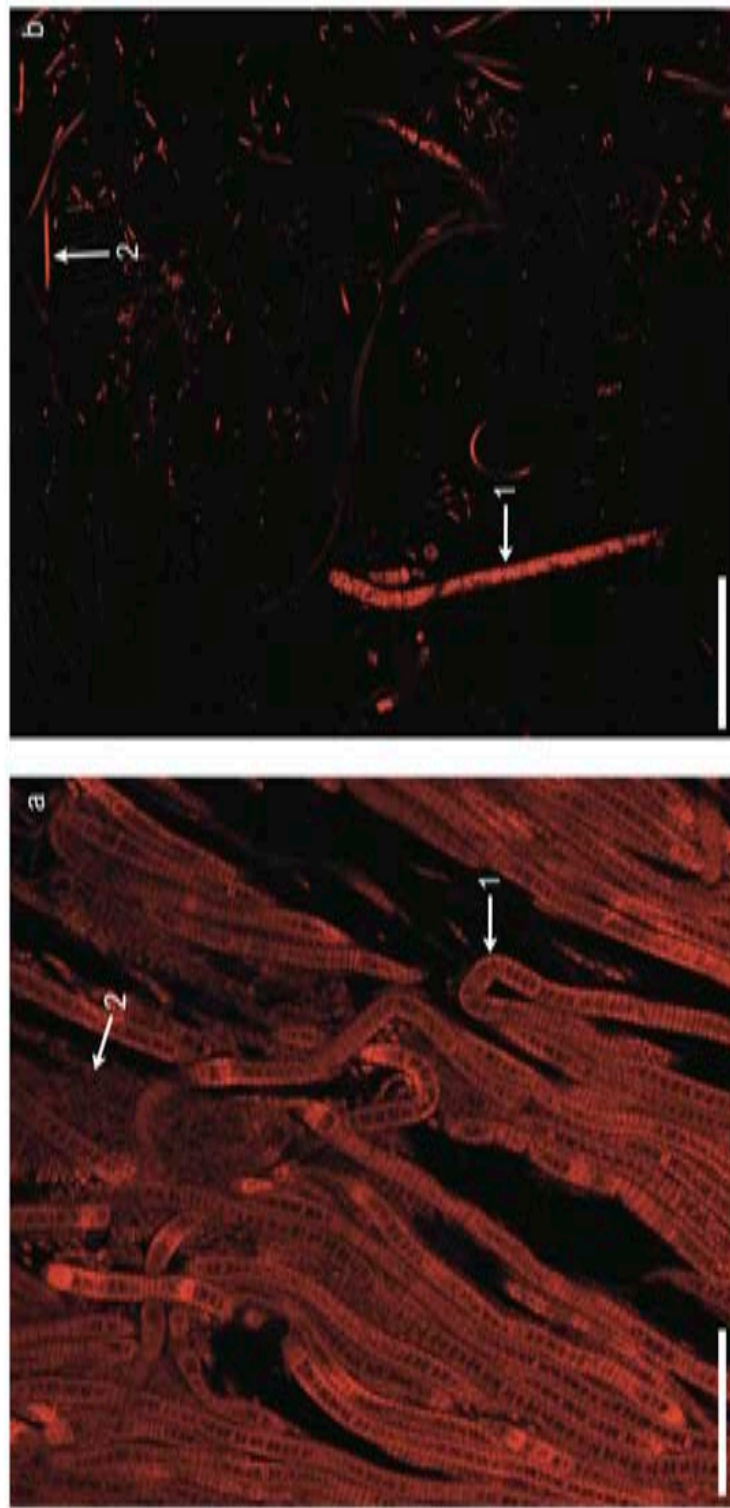
Fig. 6.6. CLSM images from unpolluted and polluted microcosms. CLSM image from the unpolluted microcosm shows *Oscillatoria*-like (**1**) and *Microcoleus*-like (**2**). Scale bar represents 50 μm (**a**). CLSM image from polluted microcosm shows *Lyngbya*-like (**1**) and *Phormidium*-like (**2**). Scale bar represents 50 μm (**b**).
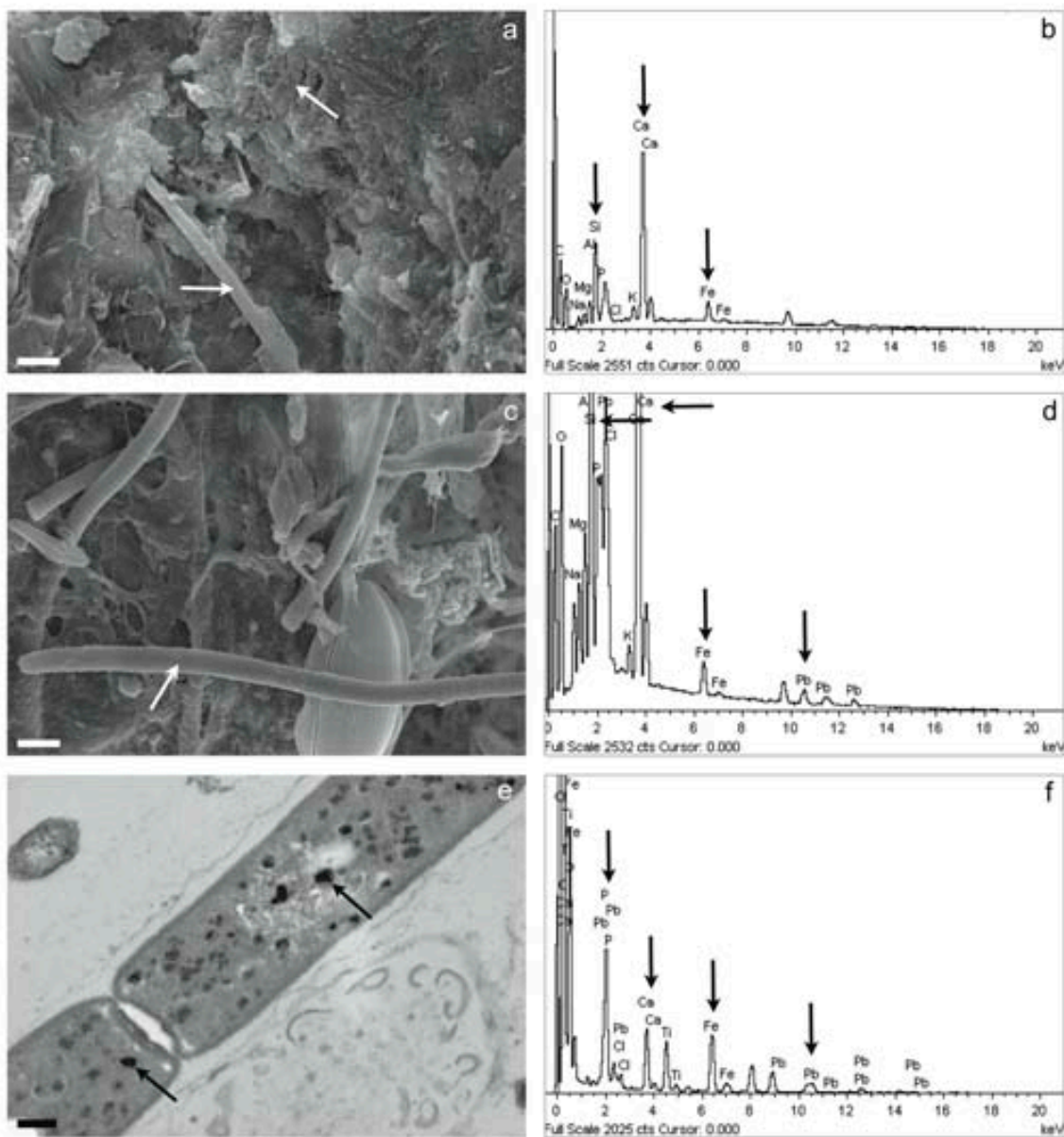
Fig 6.7. Lead sequestration by cyanobacteria in microcosm experiments. Analysis points indicated by arrows from cyanobacteria in the uncontaminated microcosm obtained by SEM. Scale bar represents 10 μm **(a)**. Fe, Si and Ca peaks showed in the spectrum obtained by SEM-EDX (see arrows) **(b)**. Analysis point indicated by arrow from *Lyngbya*-like in the contaminated microcosm obtained by SEM. Scale bar represents 10 μm **(c)**. Fe, Si, Ca and Pb peaks showed in the spectrum obtained by SEM-EDX (see arrows) **(d)**. High electron-dense inclusions from an ultrathin section of *Phormidium*-like in the polluted microcosm are indicated by arrows. Scale bar represents 0.2 μm **(e)**. Fe, P, Ca and Pb showed in the spectrum obtained by TEM-EDX from high electron-dense inclusions are (see arrows) **(f)**.

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7. Results and General discussion

The aim of this PhD thesis study has been to use electron microscopy techniques to determine how different photrophic and heterotrophic microorganisms are affected by metals, as well as their capacity to capture them. To do this, and as indicated in Chapter 2 (Material and methods) different microorganisms isolated from the natural environment (Ebro Delta microbial mats) have been used, together with strains of cyanobacteria from the Pasteur culture collection (PCC). The metals tested were Pb and Cu, as both have been detected in the Ebro River, and also because the former is very toxic, showing no biological function, while the latter, by contrast, is an essential element at low doses, while at high doses it is toxic. The techniques used, involving CLSM, SEM, TEM and EDX coupled with the latter two microscopes, have been optimized and used with different purposes. CLSM-*λscan* has been used to determine sensitivity to metal (tolerance-resistance) while SEM-EDX and TEM-EDX respectively have been used to determine external and intracellular metal biocapture.

This section discusses the importance of adapting the sample preparation protocols to the metal to be tested and the features of each microscope, and EDX in particular, to determine the effect and capture of metals in the heterotrophic and phototrophic microorganisms indicated above, which have been selected because they are the most abundant in Ebro Delta microbial mats. Once the effectiveness of these methodologies has been proven, the same tests have been carried out in microcosms in order to obtain good bioindicators of metal pollution, with the ultimate aim of applying them in the natural environment.

The protocols used are then optimized and the possible sources of metal pollution from outside the samples are determined. Secondly, the results obtained are summarized and presented in the form of articles (Chapters 3, 4, 5 and 6) for the selection of bioindicators. These results are also presented in tables so that they can be discussed more easily.

7.1 Methodological optimization

In general, the standard protocols described in Chapter 2 on Material and Methods, have been followed, so this section describes only the improvements applied to these protocols to clear up the interaction of the metals tested with external contamination (presence of metals in sample supports and electron microscopes).

In the experiments carried out with CLSM-$\lambda scan$ to determine Pb sensitivity in photosynthetic microorganisms, the protocol described by Burnat et al., (2010) has been followed. However, in this study a greater number of MFI analyses (70 ROIs) have been carried out in order to obtain more reliable results.

The methodological optimization has basically focused on analysis by EDX coupled with SEM and TEM. As mentioned-above, the EDX is a technique used for the elemental analysis of a sample. The technique is based on the analysis of X-rays emitted by matter in response to collisions with charged particles coming from an energy beam of the electron microscope. The number and energy of emitted X-rays in a sample can be measured semi-quantitatively by an energy dispersive spectrometer that can be coupled to both SEM and TEM.

Concerning EDX, one of the main disadvantages of its use is the possible interaction between metals that may occur at protocol and at sample observation level.

Protocol optimization

In this case, the concentration of the metal tested, the staining used and the metallization of the samples has been considered.

In the experiments carried out using different metal concentrations it has been observed that, in the case of Cu, the concentration of metal used must be taken into account, as at high concentrations (greater than 5 mM Cu), the Cu is precipitated, contaminating even the resin in which the samples are placed to be analysed by TEM-EDX. The same happens with analysis by SEM-EDX, in which Cu can be detected in the filter retaining the sample. On the other hand in the experiments in which lower doses have been used (1 µM in the case of DE2009 microalga, Alvaro Burgos personal communication) no metal precipitation has occurred that would mask the results. For this reason, it

is recommended using low concentrations of metal in all cases, both Pb and for Cu, to prevent precipitation.

In addition, the protocols used to prepare the ultrathin section for TEM include solutions that dye the samples, providing contrast. The most commonly used are uranyl, phosphotungstic acid and lead citrate. The use of lead citrate has therefore been ruled out for evaluating Pb capture both in intracytoplasmic inclusions and in the cytoplasm of the microorganisms, to prevent interference with this metal.

Concerning the preparation of samples for SEM, in order to obtain good contrast with them, metallizing is carried out with a noble metal. Whether the metal used for metallizing the samples, has an energy peak in the element spectrum overlapping the metal to be evaluated has been taken into account. This is why Au has been used, as its energy peak does not overlap either with Pb or with Cu.

Sample observation

In this section, the support grids and the presence of metals in electron microscopes have been considered.

Given the importance placed on the selection of support grids, we also wanted to evaluate their composition, considering those most frequently used: Cu, Au, Ni and Ti (Fig. 7.1 and 7.2).

From the results obtained in the EDX spectra it is deduced that, in the case of Pb contamination, any of the grids mentioned above could be used. By contrast, in the case of Cu-polluted samples, Ni or Ti ones should be used if the data obtained by SEM-EDX is considered (Fig. 7.1). However, if the SEM-EDX and TEM-EDX results are compared, it is concluded that the TEM specime holder, shows traces of Cu and Cr (Fig. 7.2). It is for this reason that, for experiments with Cu, it is necessary to subtract the value corresponding to the Cu peak determined as control from the maximum Cu peak of the sample analysed.
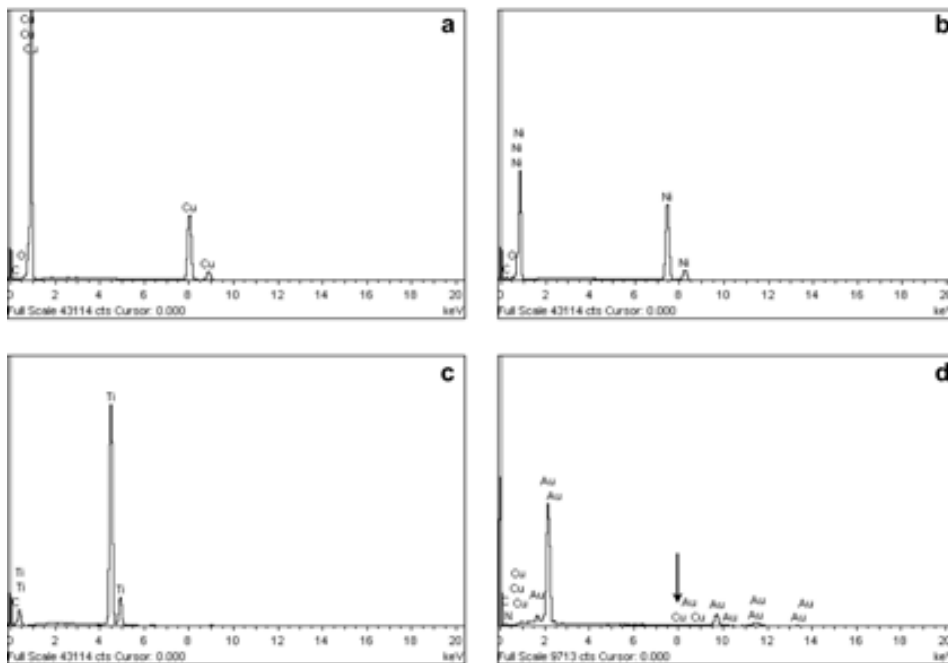
Fig. 7.1. EDX spectra obtained by SEM from Cu **(a)**, Au **(b)**, Ti **(c)** and Ni **(d)** grids. Cu peak is indicated by arrow.
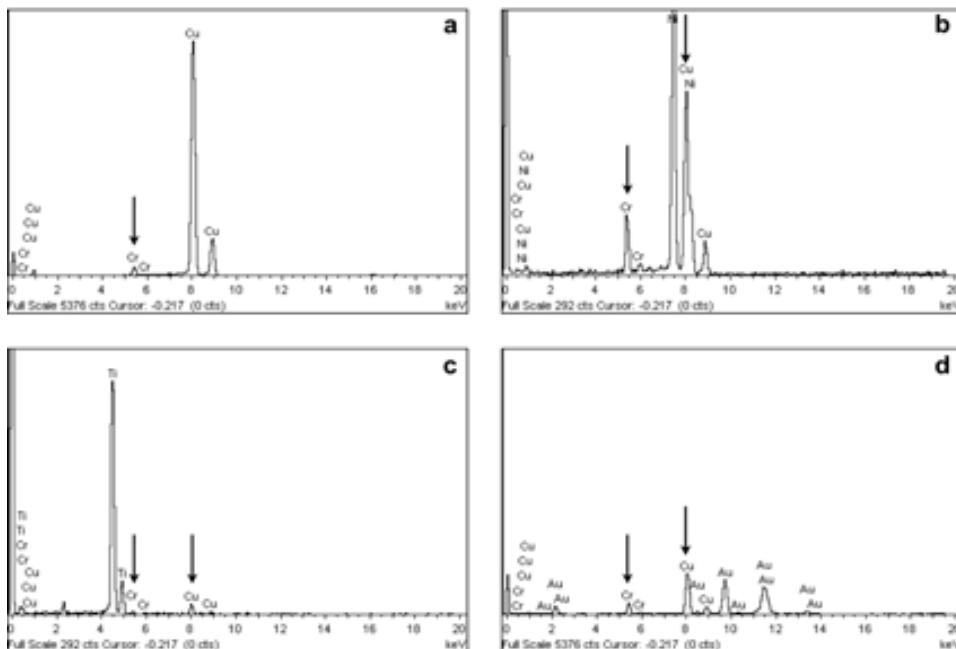
Fig. 7.2. EDX spectra obtained by TEM from Cu **(a)**, Au **(b)**, Ti **(c)** and Ni **(d)** grids. Cu and Cr peaks are indicated by arrows.

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7.2. Comparative study of the results obtained and general discussion.

For this goal it has been determined the *in vivo* effect of Pb on different phototrophic microorganisms (tolerance-resistance) (CLSM-*λscan*), and the ability of these microorganisms to capture metals extra- and/or intracellularly (SEM-EDX and TEM-EDX) as described in Chapters 3, 4, 5 and 6.

7.2.1 Determination of the effect of metals (tolerance-resistance) on phototrophic microorganisms, using CLSM-*λscan*.

The first objective of this study has been to determine the sensitivity to Pb of the different phototrophic microorganisms (directly isolated from the natural environment and also some of the culture collection) using CLSM coupled to a spectrofluorometric detector (CLSM-*λscan*).

The minimum inhibitory concentration (MIC) technique is commonly used to determine the sensitivity of microorganisms to metals or antibiotics, requiring the microorganism tested to have growth capacity in liquid and solid mediums. This means the technique is valid for the majority of heterotrophic microorganisms and for some phototrophic microorganisms. However, this method cannot be applied in filamentous cyanobacteria that show slow and or irregular growth. On the other hand, high-performance liquid chromatography (HPLC) (Chakrabortys et al., 2010), which gives indirect biomass values for a photosynthetic population based on the pigments extracted from it, would also be rejected because this method does not allow the determination of the effect of a metal directly at cell level, and requires long protocols.

This is the reason for the interest in seeking other methods allowing us to determine the effect of metals on these microorganisms in a more precise way, as well as being able to determine this *in vivo* effect at individual cell level.

Confocal laser scanning microscopy (CLSM) is an outstanding optical microscopy technique because allows accurate and non-destructive optical sectioning giving high-resolution images (optical sections) where out-of-focus views are eliminated. It is also a very interesting technique for observing phototrophic microorganisms (algae and cyanobacteria) because they emit natural fluorescence and staining protocols are not

required for this kind of sample, avoiding the need to use of long and exhaustive protocols.

At the same time this methodology means it is easy to differentiate morphotypes of phototrophic microorganisms living in mixed populations. CLSM has made it possible to characterize cyanobacteria in microbial mats and determine the biomass of different types of cyanobacteria in these habitats at micrometer scale (Solé et al., 2003; 2007; 2009).

CLSM-$\lambda scan$ provides an *in vivo* analysis considering the state of the pigments performed in individual cells without manipulating the sample. In this work, we have optimized the $\lambda scan$ function of the CLSM to determine the sensitivity of different phototrophic microorganisms to lead. An spectrofluorometic detector ($\lambda scan$ function) coupled to the microscope allows us to determine the effect of a metal at photosynthetic pigment level.

Table 7.1 shows the minimum metal concentration (Pb and Cu) with a toxic effect on photosynthetic microorganisms. The data referring to Cu are pending publication or have not yet been determined.

Table 7.1. Minimum metal concentration (Pb and Cu) with toxic effect on different phototrophic microorganisms by means of CLSM-$\lambda scan$.

Microorganism	Pb	Cu	References
DE2009 microalga	0.5 mM	5 μM	Maldonado et al, 2010b (Pb) / Unpublished results (Cu)
Oscillatoria sp. PCC 7515	0.1mM	ND	Maldonado et al, 2010b (Pb) / ND (Cu)
Chroococcus sp. PCC 9106	0.5 mM	100 nM	Maldonado et al, 2010b (Pb)/ Unpublished results (Cu)
Spirulina sp. PCC 6313	0.1 mM	100 nM	Maldonado et al, 2010b / Unpublished results (Cu)
Microcoleus sp.	0.25 mM	ND	Burnat et al., 2010 / ND (Cu)

ND: not determined

These results demonstrate that *Chroococcus* sp. PCC 9106 and DE2009 microalga show greater tolerance to Pb than *Oscillatoria* sp. PCC 7515 and *Spirulina* sp. PCC 6313.

Previous results, in *Microcoleus* sp. (Burnat et al., 2010), show that this cyanobacteria tolerates a minimum concentration of 0.25 mM Pb which is lower than that presented by *Chroococcus* sp. PCC 9106 and DE2009 microalga and greater than that of *Oscillatoria* sp. PCC 7515 and *Spirulina* sp. PCC 6313.

In the case of Cu, the minimum concentration with a toxic effect is 100 nM in the collection cultures tested. This value is much lower than that obtained for Pb (0.1 mM). The same thing happens in DE2009 microalga, with 5 μM for Cu and 0.5 mM for Pb. These results allow us to conclude that Cu, despite being a metal considered essential for phototrophic microorganisms, is much more toxic than Pb.

Surosz and Palinska, 2004 have also demonstrated in *Anabaena flos-aquae* that Cu at high concentrations causes the expansion of thylakoids in this cyanobacteria and lower production of photosynthetic pigments, largely chl *a*.

The CLSM-*λscan* technique is very sensitive because allows us to detect changes in fluorescence intensity at 100 nM.

7.2.2. Sequestration of lead and copper by phototrophic and heterotrophic microorganisms, using SEM-EDX and TEM-EDX.

The second aim of this study has been to determine the capacity of different microorganisms to accumulate lead or copper, both externally in the EPS and internally in PP inclusions. Alongside this and with the same aim, experiments have been carried out in microcosms contaminated with Pb to evaluate the capture capacity of photosynthetic microorganisms *in situ*.

7.2.2.1 Extracellular capture of lead and copper by photrophic and heterotrophic microorganisms, using SEM-EDX.

Biosorption (extracellular metal capture) is used in biorepair processes to describe the passive accumulation of metals and radioactive elements by samples of biological origin (Schiewer and Volesky 2000).

As has already been said, the most abundant microorganisms in microbial mats are cyanobacteria and the majority of these microorganisms show EPS envelopes that can be present in the form of sheaths or slimes. In tables 7.2 and 7.3 it can be seen that all the microorganisms analysed, including heterotrophic microorganisms, show both Pb and Cu in the EPS in the elemental analysis spectra.

Also, the cyanobacteria in the microcosms contaminated with Pb, which have been studied in this work, capture the metal in the EPS.

With respect to the functions the EPS may have, various authors have suggested protection against dehydration and UV radiation, biomineralization, phagocytosis, adhesion capacity to the surrounding substrate and capacity to sequester metals (Pereira et al., 2011). The latter is also demonstrated in this experimental work carried out.

Meanwhile, Decho (1999) observed an increase in EPS production in different microorganisms when they grow in the presence of toxic compounds. Also, in this PhD thesis study, a tendency to increase the thickness of the EPS in a directly proportional way to the concentration of the metal tested has been observed through the images obtained by SEM and TEM.

Table 7.2: Sequestration of Pb by different phototrophic and heterotrophic microorganisms using electron microscopic techniques coupled to EDX.

Microorganism	SEM	TEM	Reference
DE2009 microalga	+	+	Maldonado et al., 2010b
Oscillatoria sp. PCC 7515	+	+	Maldonado et al., 2011
Chroococcus sp. PCC 9106	+	+	Maldonado et al., 2011
Spirulina sp. PCC 6313	+	+	Maldonado et al., 2011
Microcoleus sp.	+	+	Burnat et al., 2010
Lyngbya–like*	+	+	Maldonado et al., 2011
Phormidium-like*	+	+	Maldonado et al., 2011
Paracoccus sp. DE2007	+	-	Baratelli et al., 2011
Micrococcus sp. DE2008	+	-	Maldonado et al., 2010a
Unidentified heterotrophic bacterium DE2010	+	ND	————

ND: not determined
* from microcosms experiments

Table 7.3. Sequestration of Cu by different phototrophic and heterotrophic microorganisms using electron microscopy techniques coupled to EDX.

Microorganism	SEM	TEM	Reference
DE2009 microalga	+	NC	Unpublished results
Oscillatoria sp. PCC 7515	+	NC	Unpublished results
Chroococcus sp. PCC 9106	+	NC	Unpublished results
Spirulina sp. PCC 6313	+	NC	Unpublished results
Microcoleus sp.	-	ND	Burnat et al., 2010
Lyngbya-like*	ND	ND	————
Phormidium-like*	ND	ND	————
Paracoccus sp. DE2007	+	ND	Unpublished results
Micrococcus sp. DE2008	+	-	Maldonado et al., 2010a
Unidentified heterotrophic bacterium DE2010	ND	ND	————

ND: not determined
NC: not conclusive
* from microcosms experiments

7.2.2.2 Intracellular capture of lead and copper by photrophic and heterotrophic microorganisms, using TEM-EDX.

Bioaccumulation is defined as the intracellular accumulation of metals by microorganisms. It consists in the first place of biosorption in the cell surface followed by transport and accumulation either in intracellular compartments or directly in the cell cytoplasm.

In this study it has been possible to show that all the cyanobacteria studied and the DE2009 microalga accumulate Pb in the PP inclusions (Table 7.2). It has been shown using the EDX spectra, that these inclusions are PP because they contain P and also divalent cations of Ca and/or K (Figs. 3.4d, 3.5d, 5.4d, 6.3d, 6.4d, 6.5d and 6.7f). The accumulation of metals in these inclusions have also been demonstrated in other microorganisms: algae (Perverly et al., 1978), yeasts (Raguzzi et al., 1988) and some cyanobacteria (Petterson et al., 1985; Surosz and Palinska, 2004; Pereira et al., 2011; Burnat et al., 2010; Jensen et al., 1982).

It has been demonstrated that these inclusions are formed due to some kind of stress, for example restricted access to nutrients like nitrogen (Stevens et al., 1985) or a high concentration of metals in the environment, as has been observed in the results presented in the figures previously mentioned in this study.

Meanwhile, Surosz and Palinska, (2004) suggest that cyanobacteria play an important role in the compartmentalization and mobilization of heavy metals in the aquatic medium, as has also been corroborated for Pb-polluted microcosms experiments.

By contrast, despite showing PP inclusions, the heterotrophic bacteria tested (*Paracoccus* sp. DE2007 and *Micrococcus luteus* sp. DE2008) have all given a negative result for metal capture (Tables 7.2 and 7.3).

In the case of Cu, the results have not been considered conclusive due to problems of external contamination with this metal, as has already been mentioned in the methodological optimization section. Given that TEM-EDX is a semi-quantitative technique it should be stated that an increase in the Cu peak has been observed in the PP inclusions, greater than that observed in other parts of the *Chroococcus* sp. PCC 9106 cell (Marina Seder-Colomina personal communication). In any case, it would be neces-

sary to carry out greater experimentation to obtain more conclusive results. Concerning the sensitivity of the analysis by SEM-EDX and TEM-EDX, new experiments are carried out using a wide range of concentrations, both for Pb and for Cu.

As a summary of what has been explained, it is observed that all the cyanobacteria and DE2009 microalga analysed in this study could be considered good bioindicators of pollution. However, DE2009 microalga have been discounted for this purpose because they are not abundant in Ebro Delta microbial mats, although they could offer future prospects for studies of metal bioremediation.

Finally, considering the results obtained, it is proposed to select *Oscillatoria* sp. and *Microcoleus* sp. as the best bioindicators because they are dominant in the natural environment, larger in size, tolerant to high concentrations of metals and have capacity to accumulate them extra- and intracellularly.

We will now list the conclusions and indicate future prospects that could emerge from the experimental study presented.

8. Conclusions and Future prospects

Conclusions obtained based on methodological optimization

For the objectives set, firstly the techniques involving the use of CLSM, SEM, TEM and EDX coupled to these last two microscopes have been optimized, both for preparing the samples and for observation.

1. The samples preparation protocols have been the standard ones described in Material and Methods. However, considering the metals used in this study Pb and Cu, it was advisable not to use lead citrate as a stain for the ultrathin sections (TEM-EDX) in lead contamination experiments to prevent overlap with this metal and it is also recommended for metallisation (SEM-EDX) of samples to use Au, as this does not cause interference either with Pb or with Cu.

2. The metal compositions of 4 types of grids, the most commonly used: Au, Cu, Ti and Ni were analysed by TEM-EDX. With respect to these, for the ultrathin sections, Ti and Ni are recommended, as these metals show no interference either with Pb or with Cu. However, it has been shown that the TEM holder specimen has traces of Cu and Cr, so this external contamination should be considered when the analysis of the contaminated samples with these metals is required.

3. CLSM-*λscan* is mainly suitable in cases where it is necessary to analyse the tolerance-resistance of phototrophic microorganisms against a metal, because the observation is *in vivo*, with no handling of the sample and with immediate observation. SEM-EDX and TEM-EDX are also the best methods of checking the capacity of a microorganism to accumulate a metal extracellularly in the EPS and to determine metal accumulation within the cytoplasm, respectively.

Conclusions from the results obtained

The overall aim has been to seek good indicators of lead and copper pollution. The conclusions obtained using each of the techniques tested are detailed below.

4. CLSM-*λscan* has made it possible to detect the tolerance-resistance of phototrophic microorganisms. *Chroococcus* sp. PCC 9106 and DE2009 microalga show greater tolerance to Pb (0.5 mM) than *Oscillatoria* sp. PCC 7515 and *Spirulina* sp. 6313 (0.1 mM). In the case of Cu, the minimum concentration tolerated by *Chroococcus* sp. PCC 9106 and *Spirulina* sp. PCC 6313 is 100 nM, allowing us to conclude that Cu, although it is considered an essential metal, is more toxic than Pb. The technique explained is very precise as it detects the toxic effect in cells at nM level.

5. SEM coupled to EDX allows us to determine that all the microorganisms studied (phototrophic and heterotrophic) have capacity to accumulate Pb and Cu externally in the EPS.

6. TEM coupled to EDX has allowed us to determine that all the phototrophic microorganisms studied accumulate Pb in PP inclusions. On the other hand, heterotrophic bacteria, although they show such inclusions, do not accumulate this metal. The results obtained in the samples contaminated by Cu have not been considered conclusive, as external contamination from the microscope has occurred, masking the results obtained.

7. With respect to the contamination experiments for metals in microcosms, only Pb has been tested, and it has been shown that, as with cultures, all phototrophic microorganisms growing in these polluted artificial ecosystems are capable of accumulating this metal both externally and internally, always in PP inclusions.

8. Considering all the results obtained it can be concluded that all cyanobacteria and DE2009 microalga analysed could be considered good metal pollution indicators. However, *Oscillatoria* sp. and *Microcoleus* sp. have been selected as the best indicators of metal pollution in the natural environment because: they are the most abundant, largest in size, tolerant to high concentrations of metals and, finally, capable of accumulating metal extra- and intracellularly.

Future prospects

The study presented opens up new short- and long-term prospects for research. In the short-term and in accordance with the results obtained both in cultures and in microcosms, cyanobacteria, and in particular *Microcoleus* sp. and *Oscillatoria* sp., will be used as microorganisms indicating metal pollution directly in the natural environment. In forthcoming experiments, ultrathin sections will be obtained directly from different samples of the green layer of Ebro Delta microbial mats and the TEM-EDX spectra of the PP inclusions of the cyanobacteria present will be analysed. The future aim is to determine whether the zone is pristine or if it is exposed to any type of metal pollution. Before this study, a rapid overview of research into metal capture in the EPS will be carried out using SEM-EDX.

In the longer term, the determination by CLSM-$\lambda scan$ of the tolerance-resistance to several metals of different photosynthetic microorganisms grown in mixed cultures will be considered and the effect of more than one metal at a time on a single microorganism will be tested. The aim in this case will be the study of the possible interactions between microorganisms and metals. As was mentioned before, the samples are observed *in vivo* and without manipulation and the sensitivity of this technique is of the order of 100 nM. The results that can be obtained will provide greater knowledge about the direct interactions occurring between microorganisms and metals in the natural habitat. This technique will also make it possible to carry out new experiments to analyse the physiological state of cells under different culture conditions.

Finally, in phototrophic microorganisms such as: DE2009 microalga or *Microcoleus* sp., in which greater metal accumulation has been seen using SEM-EDX and TEM-EDX, future studies of capture efficiency could be carried out by chemical analysis. Based on the results obtained, it might be considered that these microorganisms would be useful in industrial bioremediation processes.

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Annex I

LB medium

Ingredient	Composition g L^{-1}
Triptone	10
Yeast extract	5
NaCl	10
Destilled water	1 L

Pfennig Mineral Medium

For 1 L

Deionized water	**850 mL**
A Solution	**10 mL**
B Solution	**10 mL**
C Solution	**10 mL**

Autoclave 15', 121ºC, after cooling add:

D Solution	**1 mL**
E Solution	**100 mL**
F Solution	**10 mL**
H Solution	**25 mL**

· A Solution

Ingredient	Composition g L^{-1}
NH_4Cl	30
K_2HPO_4	30
Deionized water	to 1 L

Adjust pH to 3 adding HCl

· B Solution

Ingredient	Composition g L^{-1}
$CaCl_2 \cdot 2H_2O$	20
$MgCl_2 \cdot 6H_2O$ / $MgSO_4 \cdot 7H_2O$	20/25
KCl	20
Deionized water	to 1 L

· C Solution

Ingredient	Composition g L^{-1}
[1]	
Na$_2$-EDTA	500
FeSO$_4$·7H$_2$O	200
ZnSO$_4$·7H$_2$O	10
MnCl$_2$ 4H$_2$O	3
[2]	
H$_3$BO$_3$	30
CoCl$_2$·6H$_2$O	20
CuCl$_2$·2H$_2$O	1
[3]	
NiCl$_2$·6H$_2$O g	2
[4]	
Na$_2$MoO$_4$·2H$_2$O	3

Add 1 mL of [1] [2] [3] and [4]
in deionized water to 1 L

Adjust pH to 3 adding HCl

· D Solution

Ingredient	Composition g L^{-1}
Cyancobalamin	0.02
Deionized water	to 1 L

Sterilise by filtration

· E Solution

Ingredient	Composition g L^{-1}
Na$_2$CO$_3$ anh	20
Deionized water	to 1 L

· F Solution

Ingredient	Composition g L^{-1}
Na$_2$S 7-9H$_2$O	24
Na$_2$CO$_3$ anh	21
Deionized water	to 1 L

· H Solution

HCl 35%	88.3 mL
Deionized water	to 1 L

BG-11 Medium

Ingredient	Composition g L^{-1}
$NaNO_3$	1.5
$K_2HPO_4 \cdot 3H_2O$	0.04
$MgSO_4 \cdot 7H_2O$	0.075
$CaCl_2 \cdot 2H_2O$	0.036
Citric acid	0.006
Ferric ammonium citrate	0.006
EDTA	0.001
Na_2CO_3	0.04
Trace metal Mix A5+Co*	1 mL
Deionized water	to 1L

pH after autoclaving and cooling: 7.4

ASN-III medium

Ingredient	Composition g L^{-1}
NaCl	25
$MgCl_2 \cdot 6H_2O$	2
KCl	0.5
$NaNO_3$	0.75
$K_2HPO_4 \cdot 3H_2O$	0.02
$MgSO_4 \cdot 7H_2O$	0.075
$CaCl_2 \cdot 2H_2O$	0.5
Citric acid	0.03
Ferric ammonium citrate	0.003
EDTA	0.0005
Na_2CO_3	0.04
Trace metal Mix A5+Co*	1 mL
Deionized water	to 1 L

pH after autoclaving and cooling: 7.5

*Trace Metal Mix A5+Co

Ingredient	Composition g L^{-1}
H_3BO_3	2.86
$MnCl_2 \cdot 4H_2O$	1.81
$ZnSO_4 \cdot 7H_2O$	0.222
$Na_2MoO_4 \cdot 2 H_2O$	0.390
$CuSO_4 \cdot 5H_2O$	0.079
$Co(NO_3)_2 \cdot 6H_2O$	0.0494
Deionized water	to 1 L

MO medium

Medium MO is prepared as MN but with nitrate ($NaNO_3$) ommited.

MN medium

Medium MN is medium BG-11 prepared at half strength (except for trace metal mix A5+Co, which is added as in BG-11) in 750 mL of seawater.

pH after autoclaving and cooling: 8.3

Annex II

Isolation and identification of a bacterium with high tolerance to lead and copper from a marine microbial mat in Spain

**Juan Maldonado · Elia Diestra · Lionel Huang · Ana M. Domènech ·
Eduard Villagrasa · Zully M. Puyen · Robert Duran · Isabel Esteve · Antonio Solé**

Abstract A bacterial strain (DE2008) was isolated from a consortium of microorganisms living in the microbial mats of the Ebro Delta with the ability to grow under conditions of high concentrations of lead and copper. Strain DE2008 has been characterized by microscopic and metabolic techniques and identified by sequencing of PCR-amplified 16S rRNA gene fragments as *Micrococcus luteus*. The strain is highly resistant to lead and copper. *Micrococcus luteus* DE2008 grew optimally until levels of 1 mM of $Pb(NO_3)_2$ and $CuSO_4$, respectively, and was completely inhibited at 3 mM $Pb(NO_3)_2$ and at 1.5 mM $CuSO_4$. Elemental analysis determined by energy-dispersive X-ray spectroscopy (EDX) coupled with scanning electron microscopy and transmission electron microscopy from polluted cultures of DE2008 show that this microorganism can biosorpt Pb and Cu in exopolysaccharide envelopes, without accumulating them inside the cells.

Keywords *Micrococcus luteus* DE2008 · Lead · Copper · Electron microscopy · EDX

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Introduction

Contaminated environments, such as soils and sediments polluted by crude oil, heavy metals, or pesticides can be considered as extreme conditions for microbial growth. The effects of these contaminants in different populations have been reported (Radwan and Sorkhoh 1993; Al-Hasan et al. 1998). The bioremediation capacity of phototrophic micro-organisms (cyanobacteria and algae), heterotrophic bacteria, yeast, and fungi have been investigated (Gong et al. 2005; Binupriya et al. 2006).

Gram-positive bacteria are known to possess high metal sorption capacities. The ability of the cell wall of *Bacillus subtilis* to interact with different heavy metals has been much studied (Tunali et al. 2006). Although several papers have shown that the cell walls of Gram-positive cocci such as those of *Staphylococcus xylosus* and *Micrococcus luteus* have an affinity for metal ions (Nakajima and Tsuruta 2004; Ziagova et al. 2007), the metal sorption by the cell walls has still to be characterized. Indigenous microbes in nature tolerate high concentrations of heavy metals and may play an important role in restoring contaminated soils. For example, Wei et al. (2009) recently identified *Agrobacterium* (CCNWRS33-2) from the Taibai gold mining region in China as the bacterial strain most resistant to heavy metals. This bacterium was able to grow in up to 2 mM of copper and lead.

Stratified benthic marine ecosystems (microbial mats) are often exposed to heavy metals (Sánchez-Chardi et al. 2007). The microorganisms that compose these ecosystems are mainly cyanobacteria, oxygenic phototrophic micro-organisms, which are primary producers on these habitats (Stal 2000). Our group of work has studied the diversity and biomass of cyanobacteria in oil-polluted and unpolluted microbial mats around Europe (Diestra et al. 2004; Solé et

al. 2009). Over the last few years, we have also isolated a consortium, formed mainly by a single cyanobacterium, *Microcoleus chthonoplastes*, and by different heterotrophic bacteria (Diestra et al. 2005; Sánchez et al. 2005). In addition, this consortium was able to degrade crude oil (García de Oteyza et al. 2004) and tolerate heavy metals (Burnat et al. 2009).

Recently, we have isolated a Gram-positive heterotrophic bacterium (DE2008) from the above-mentioned consortium, with the ability to grow in high concentrations of heavy metals. Two metals were selected for this work, Pb and Cu. The first one is a toxic metal, whilst the second one is an essential metal with known biological functions in all organisms (Roane and Pepper 2000).

The goals of this work are to identify this bacterium and to determine the maximum concentration levels of both metals in which the bacterium can grow. At the same time, we are trying to determine whether the strain's ability to tolerate such high concentrations of heavy metals is from an ability to accumulate Pb and/or Cu externally in sheaths or internally in intracytoplasmic inclusions.

Materials and methods

Isolation, biochemical and physiological characterization of the strain DE2008 Inoculums from a *Microcoleus* consortium (Diestra et al. 2005) were transferred to Luria-Bertani (LB) agar medium containing tryptone (10.0 g L^{-1}), yeast extract (5.0 g L^{-1}), and NaCl (10.0 g L^{-1}), pH 7.0. The cultures were incubated in darkness at 27°C. Different colonies were obtained, one of which was streaked on LB agar and isolated in pure culture. The morphological characteristics of strain DE2008 were examined with an Olympus BH2 conventional light microscope. Different stain methods were used to characterize the DE2008 bacterium (Gram, Toluidine Blue, Wirtz-Conklin, and Negative stains). Motility was determined with an optical microscope using the hanging-drop technique. The biochemical assays were made using the API 20 NE strip-identification system (BioMerieux, Marcy l'Étoile, France). Oxidase activity was analysed by oxidation of 1% *p*-aminodimethylaniline oxalate (Cowan and Steel 1965). Catalase activity was determined by the presence of bubbles with a 3% H$_2$O$_2$ solution (Takeuchi et al. 1996). Starch hydrolysis was analyzed as described by Cowan and Steel (1965).

The effect of different physical–chemical parameters were also tested on bacterial strain DE2008. The pH range for growth was determined by incubating cells in LB medium at 27°C for 4 days at the following pH: 2, 3, 4 (LB broth medium), 5, 6, 7, 8, 9, 10, and 11 (LB agar medium). NaCl tolerance was measured in LB agar medium at

concentrations of 0, 1, 2, 3, 4, 5, 6, 7, 8, and 10% (w/v). To determine the temperature range, the growth of the strain at different temperatures (8–43°C) was tested.

Phylogenetic characterization Genomic DNA was extracted from 2 ml of an overnight DE2008 culture in liquid LB using UltraCleanTM Soil DNA Isolation Kit (Mobio Laboratories, Carlsbad, USA) according to manufacturer's instructions. The 16S rRNA gene fragment was obtained by PCR amplification using primers 8F (5′-AGAGTTT GATCCTGGCTCAG-3′) and 1489R (5′-TACCTTGTTAC GACTTCA-3′), according to the procedure previously described (Bordenave et al. 2004). The following PCR conditions were applied: 35 cycles of 10 min at 95°C, 45 s at 95°C, 45 s at 51°C, and 1.5 min at 72°C, with a final step of 10 min at 72°C. PCR product was then purified using the GFX™ PCR DNA and Gel Band Purification Kit as directed by the supplier (GE Heathcare, UK). The sequence of the purified DNA fragment was obtained using the BigDye Terminator Cycle Sequence Kit (versions 1.1; Applied Biosystems, Foster City, USA) on an ABI PRISM310 Genetic Analyzer (Applied Biosystems).

The sequence was compared to those present in the NCBI database (http://www.ncbi.nlm.nih.gov) using the BLAST program. For phylogenetic analysis, a dataset containing GenBank 16S rRNA gene sequences was used. Pairwise evolutionary distances based on 1,455 unambiguous nucleotides were computed by the method of Jukes and Cantor (1969) with MEGA version 3.0 software (Kumar et al. 2004). The phylogenetic tree was constructed using neighbor-joining (Saitou and Nei 1987). Confidence in the tree topology was determined by bootstrap analysis using 1,000 resampling of the sequences.

Effects of Pb and Cu on the growth of the strain DE2008 Overnight cultures of DE2008 strain grown on LB medium were inoculated into LB medium supplemented with different Pb(NO$_3$)$_2$ or CuSO$_4$ concentrations: 0.1, 0.5, 1, 1.5, and 2 mM. The cultures were incubated in an orbital shaker (180 rpm) at 27°C for 12 h. Growth rate was determined by measuring the increase in turbidity (absorbance at 550 nm; Greenblatt et al. 2004) every 30 min with a Beckman Coulter DU 730 Life/Science UV/Vis spectrophotometer.

The specific growth rate (μ) was calculated for each Pb and Cu concentration using the following formula (Wang 2005).

$$\mu = \frac{1}{OD_0} \times \frac{(OD_t - OD_0)}{(T_t - T_0)}$$

OD$_t$ and OD$_0$ represent the optical density (550 nm) of the cultures at the time *t* (final time) and 0 (initial time), and T$_t$ and T$_0$ represent homologous times (h).

In order to determine the minimum inhibitory concentration (MIC) for lead and copper, two techniques were used: the agar diffusion method and an assay by tube dilution.

For the agar diffusion method, Petri plates containing LB agar medium evenly inoculated with the test microorganism were prepared. Known amounts (20 μL) at different concentrations of metals were placed on the surface of the agar. Milli-Q water solutions without metals were used as a control experiment. Plates were incubated at 27°C for 48 h to determine zones of inhibition of bacterial growth.

For the assay by tube dilution, series of culture were prepared, each one containing medium with a different concentration of the metal. All tubes of the series were inoculated with the test microorganism. Cultures without metals were used as a control experiment. The tubes were incubated at the same conditions mentioned above.

Heavy metal accumulation capacity of DE2008 To test the ability of the strain to capture heavy metals (intra- or/and extra-cellularly), polluted cultures with the highest concentrations of $Pb(NO_3)_2$ and $CuSO_4$ (5 and 25 mM) were assayed to obtain a rapid response to both metals (Burnat et al. 2009). To determine structural characteristics and for the heavy metals localization in DE2008 cells, scanning electron microscopy (SEM), transmission electron microscopy (TEM) and energy-dispersive X-ray spectroscopy (EDX) were used.

For SEM analysis, samples of DE2008 cultures were fixed in 2.5% glutaraldehyde Millonig buffer phosphate for 2 h and washed four times in the same buffer. They were then dehydrated in successively increasing gradient concentrations of acetone (30, 50, 70, 90, and 100%) and dried by critical-point drying. Finally, all samples were mounted on metal stubs and coated with gold. A Jeol JSM-6300 scanning electron microscope (Jeol, Tokyo, Japan) was used to view the images.

For X-ray analysis, cultures were homogenously distributed and filtered on polycarbonate membrane filters. These filters were then dehydrated and dried by the same procedure as culture samples. An EDX Link Isis-200 (Oxford Instruments, Bucks, England) operated at 20 kV coupled to SEM was used.

For TEM analysis, samples of DE2008 cultures were fixed in 2.5% glutaraldehyde Millonig buffer phosphate for 2 h and washed four times in the same buffer. Samples were post-fixed in 1% OsO_4 at 4°C for 2 h, and washed four times in the same buffer. They were then dehydrated in a graded series (50, 70, 90, 95, and 100%) of acetone and embedded in Spurr resin. Ultrathin sections (70 nm) were mounted on carbon-coated copper grids and stained with uranyl acetate and lead citrate. Samples were viewed in a Hitachi H-7000 transmission electron microscope (Hitachi, Tokyo, Japan). Sections of 200–300 nm thickness were not stained and then mounted on titanium grids for EDX. Samples were analyzed with a Jeol Jem-2011 transmission electron microscope (Jeol, Tokyo, Japan).

Data analysis Data obtained from growth cultures (control and different metal concentrations) were compared with a Student's *t* test with 95% significance ($p < 0.05$). Statistical analyses were performed with SPSS software (version 15.0 for Windows).

Results and discussion

Identification of the strain DE2008

Cells of strain DE2008 are facultative aerobic, Gram-positive cocci, (1.5 μm in size), non-motile, encapsulated, and non-spore-forming. Colonies grow in LB agar are cream-colored, low convex, smooth and circular. Growth occurs between 8 and 43°C (optimum 27°C) and at pH between 3 and 11. No growth occurs in the presence of more than 8% of NaCl.

The biochemical assays indicated that strain DE2008 hydrolyzes glucose, arabinose, mannose, maltose, adipic acid, malic acid, tributirine, and trisodium citrate, even after 8 days of growth. Starch cannot be hydrolyzed. The strain can reduce the nitrates to nitrites, but denitrification does not occur.

In addition, electron microscopy techniques (SEM and TEM) were used to determine morphological parameters. The images obtained by SEM indicate that the bacterium occurs in tetrads, irregular clusters or regular packets, and without flagella (Fig. 1a). Ultrathin sections show the characteristic cell wall of Gram-positive bacteria, and inside the cytoplasm few high electron-dense (HE) inclusions were detected (Fig. 1b).

16S rRNA gene sequence analysis (Fig. 2) revealed that the isolate belonged to the Actinobacteria class and was closely related to representatives of the genus *Micrococcus*. According to 16S rRNA gene sequence comparison, the isolate showed 99% similarity with the type strain *M. luteus* DSM 20030[T] (AJ536198) and *M. luteus* strain Ballarat (AJ409096). This value was over the 97% similarity level indicating that the isolate belongs to the same species. We thus indentified the isolate DE2008 as *M. luteus*.

Effects of Pb and Cu at different concentrations on *Micrococcus luteus* DE2008 growth

The Pb effect on the strain varied significantly depending on the concentrations. The differences were not statistically
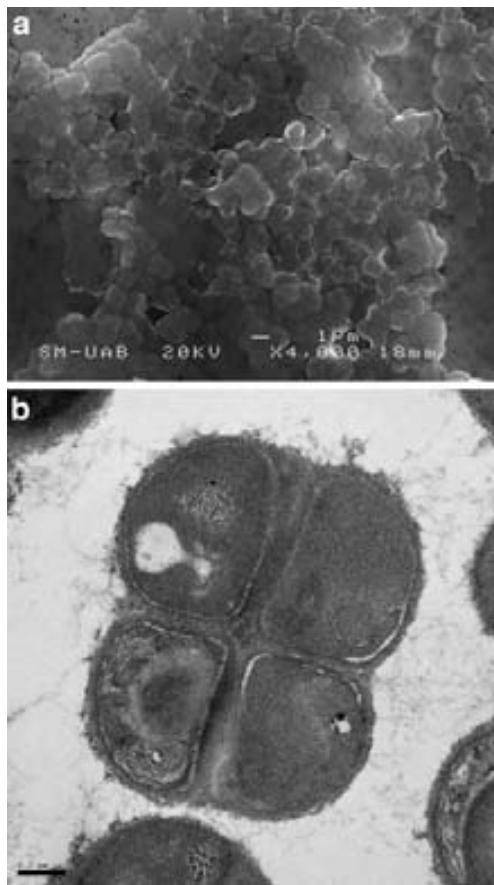
Fig. 1 Electron microscopy images of *Micrococcus luteus* DE2008. **a** SEM image showing clusters. *Scale bar* 1 μm. **b** TEM image showing a single tetrad surrounded by EPS. *Scale bar* 0.2 μm

significant ($p<0.05$) between the control experiment and 0.1, 0.5, and 1 mM Pb(NO$_3$)$_2$. Thus, these concentrations had only a small effect on the growth of the strain. The differences were statistically significant ($p<0.05$) between the control experiment and 1.5 and 2 mM Pb(NO$_3$)$_2$ (Fig. 3a). The Pb MIC was 3 mM.

Similar results were obtained with cultures containing Cu. However, differences in toxicity were observed from 1 mM indicating that copper had a stronger effect than lead (Fig. 3b). The Cu MIC was 1.5 mM. Although Wei et al. (2009) verified that Cu increased the growth rate of the strain CCNWRS33-2 at this concentration, our results are more in accordance with those obtained by Chaudri et al. (2000), which demonstrated a negative effect of Cu on rhizobial species in soils.

Micrococcus luteus growing at 0, 0.1, 0.5, and 1 mM of Pb(NO$_3$)$_2$ had a specific growth rate of 0.5, 0.49, 0.44, and 0.32 respectively. The same microorganism growing at the same concentrations of CuSO$_4$ had a specific growth rate of 0.49, 0.25, 0.27, and 0.18 respectively. These results demonstrated the high tolerance of *M. luteus* DE2008 to heavy metals. The genus *Micrococcus* seems to be well suited to grow under extreme conditions such as in amber (Greenblatt et al. 2004), in the upper stratosphere at very low atmospheric pressures (Bamji and Krinsky 1966), and in unusual metabolic sources for nutrition (Sims et al. 1986; Doddamani and Ninnekar 2001; Zhuang et al. 2003; Tallur et al. 2008).

Micrococcus luteus DE2008 grows at higher metal concentrations than those described for other strains. For example, Roy et al. (2008) proved that the *Synechocystis* sp.
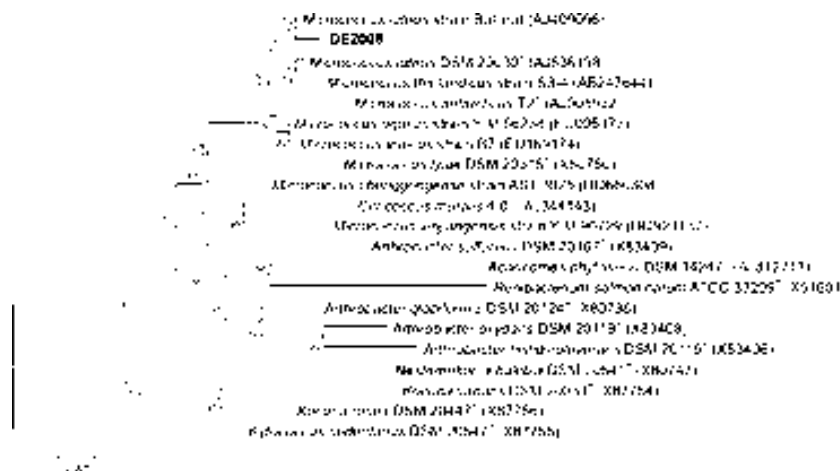
Fig. 2 Phylogenetic position of the isolate DE2008 based on the 16S rRNA gene sequence analysis. Phylogenetic distances and tree construction were carried by the neighbor-joining method with MEGA (Phylogenetic Tree-Mega Software). The tree was rooted with the sequence of *Kytococcus sedentarius* DSM20547^T. Values at nodes represent percentage occurrence based on 1,000 bootstrap replications. *Scale bar* corresponds to 0.5 substitutions per 100 nucleotide positions. Accession numbers are given in parentheses
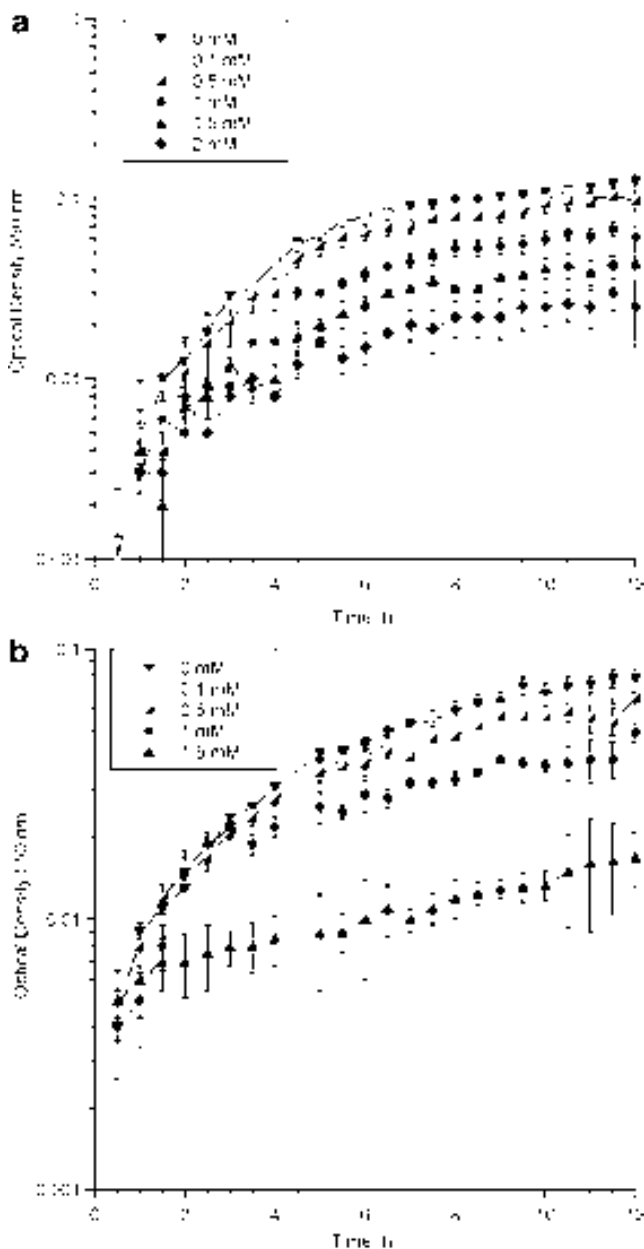
Fig. 3 Growth rates of *Micrococcus luteus* DE2008 growing between 0 and 2 mM Pb(NO$_3$)$_2$ (**a**) and between 0 and 1.5 mM CuSO$_4$ (**b**)

growth was completely inhibited at 1.9 mM Pb^{2+}. Although *M. luteus* DE2008 is unable to grow at the highest concentrations assayed, it maintains its capacity to bioaccumulate Pb and Cu.

Heavy metal accumulation in *Micrococcus luteus* DE2008

With the aim of proving whether *M. luteus* DE2008 could capture metals, cells from cultures with and without metals were analyzed by EDX coupled to SEM and TEM.

Cells from cultures without metals show thick envelopes of EPS surrounding the cells when they are analyzed by SEM and TEM. Few HE inclusions inside the cells were observed. The EDX images obtained coupled to SEM and TEM and used as a control experiment corroborated that neither of the two contaminants metals were detected either outside or inside the cells.

*Micrococcus luteus* DE2008 from cultures containing Pb was analyzed by the same above-mentioned procedures. No important differences in the structure of *M. luteus* DE2008 were observed in the cells from Pb (Fig. 4c). The analysis of the energy-dispersion spectra coupled to SEM demonstrated that Pb was found in EPS (Fig. 4b). The ultrathin sections of the cells also exhibited discernible changes (distortion of the cells) after exposure to Pb. A high number of HE inclusions, which can be observed in the ultrathin sections of cells grown in the presence of Pb, were evident (Fig. 4c). These inclusions were identified as polyphosphate granules (PPG) (see peak of P; Fig. 4d). In many cases, similar inclusions have been found when cells are grown in adverse culture conditions (Sicko 1972; Jensen and Sicko 1974; Stevens et al. 1985). However, their role in other functions such as a detoxification by sequestering heavy metals has been suggested (Goldberg et al. 2001). The analysis of the energy-dispersion spectra of the inclusions confirmed that Pb was not accumulated in PPG inside the cells. No significant peaks of Pb were visible.

Equivalent experiments were made in *M. luteus* DE2008 cultures containing Cu. When the structure of the cell from Cu cultures was compared with that of cells from Pb cultures, we could not observe any effect of Cu on the cell structure. The analysis of the energy-dispersion spectra coupled to SEM proved that this microorganism was able to accumulate Cu outside but not inside the cells (Fig. 5). Cu peaks in Fig. 5d are not relevant because they belong to the TEM holder support, which is made of Cu.

These results indicated that the EPS envelopes have a great affinity for both metals. It is well known that the EPS matrix can protect cells against toxic compounds such as metals that can be toxic to cells, and further, that the presence of these metals can overproduce exopolymer secretion (Decho 1994). Specifically, uronic acids and sulphate groups present in EPS may interact with various metals thereby immobilizing them (Stal 2000).

From these results, it can be concluded that *M. luteus* DE2008 grew optimally until levels of 1 mM of Pb(NO$_3$)$_2$ and CuSO$_4$, respectively, and was completely inhibited at 3 mM Pb(NO$_3$)$_2$ and at 1.5 mM CuSO$_4$. Moreover, the

Fig. 4 **a** SEM image of *Micrococcus luteus* DE2008 culture treated with 25 mM Pb (NO₃)₂ forming aggregates (*arrow*). *Scale bar* 1 μm. **b** X-ray analysis spectra coupled to SEM, see the main Pb peak at 10.5 keV (*arrow*). **c** Ultrathin sections of the same bacteria showing high electron-dense (HE) inclusions (*arrow*). *Scale bar* 500 μm. **d** X-ray analysis spectra coupled to TEM from HE inclusions. No peaks of Pb were detected
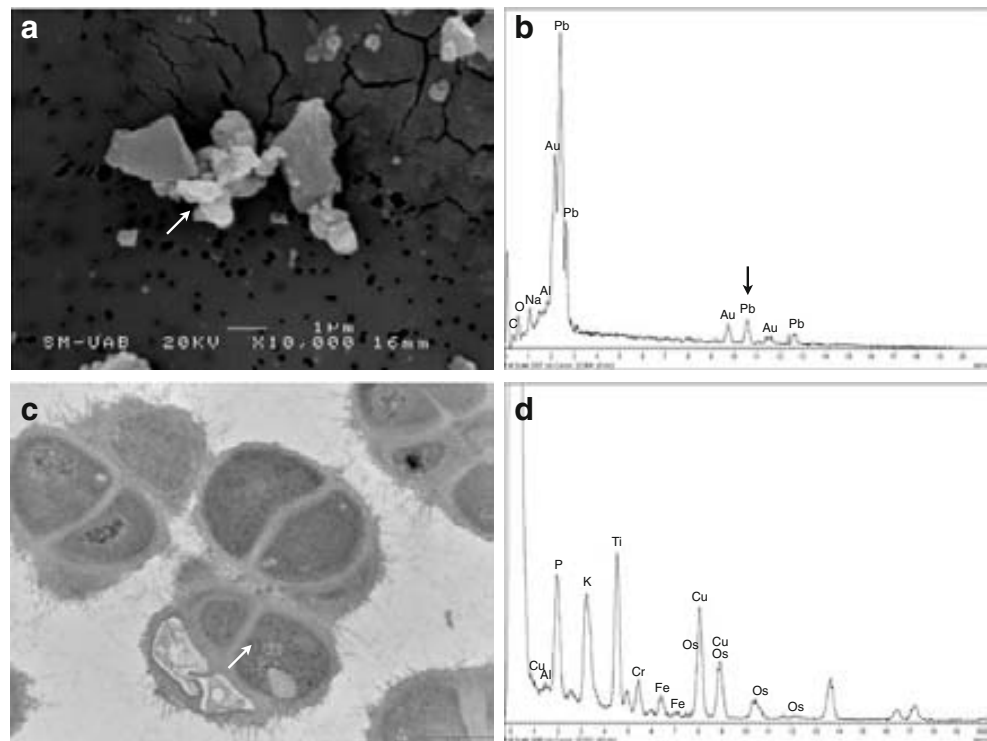
strain can tolerate higher concentrations of both metals by extracellular biosorption.

On the other hand, *M. luteus* DE2008 can be considered an indigenous microorganism, which probably plays a very important role in removing Pb and Cu from polluted natural marine ecosystems. In future studies, this microorganism could be considered a promising candidate for the removal of heavy metals from wastewaters.

Fig. 5 **a** SEM image of *Micrococcus luteus* DE2008 culture treated with 25 mM CuSO₄. **b** X-ray analysis spectra coupled to SEM. Note the main Cu peak at 8 keV (*arrow*). **c** Ultrathin sections of the same bacteria showing high electron-dense (HE) inclusions (*arrow*). *Scale bar* 200 μm. **d** X-ray analysis spectra coupled to TEM from HE inclusions. Cu peaks are irrelevant because they belong to the TEM holder
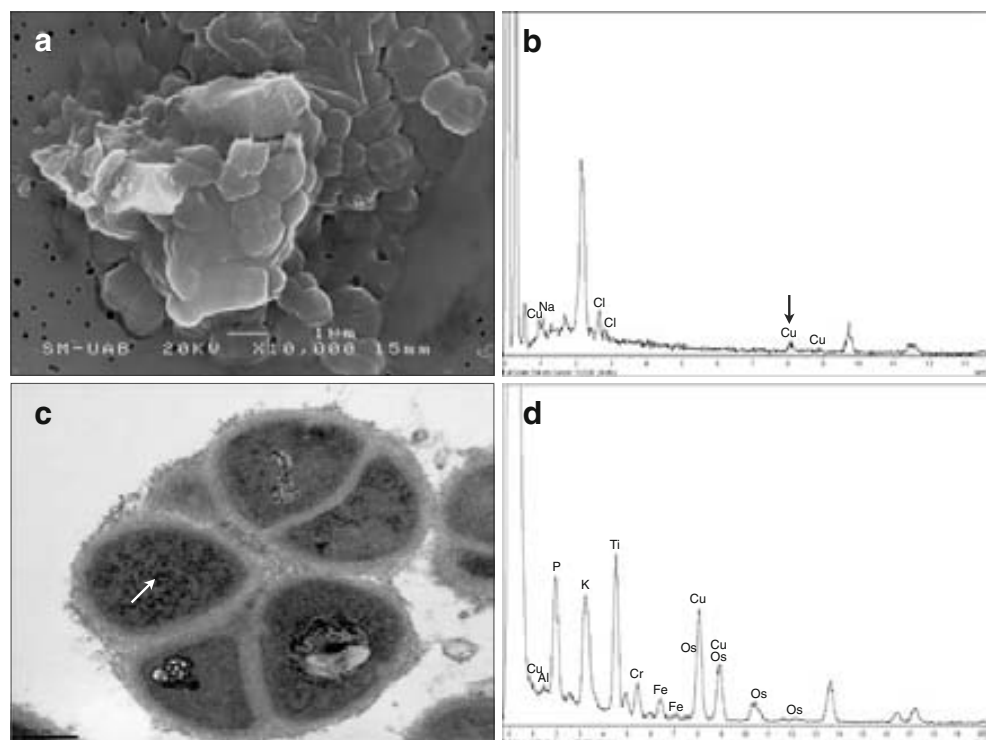
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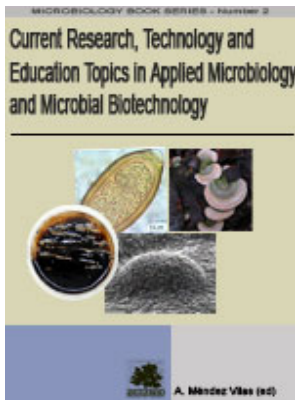
Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology

Microbiology Book Series – 2010 Edition

Badajoz (Spain), 28 June 2010

Dear Dr. Diestra,

I am pleased to inform you that your paper titled "*Electron microscopy techniques and energy dispersive X-ray applied to determine the sorption of lead in Paracoccus sp. DE2007*" has been accepted for inclusion in the forthcoming number of the Microscopy Book Series titled "*Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology*", a divulgation/educationally-oriented publication on Applied Microbiology and Microbial Biotechnology. As an active member of the research community in this area (*Microbial Ecology, 2005;50(4):580-8; Applied Microbiology and Biotechnology, 2004;2:226–232; Microbial Ecology, 2005;50:580-588; Annals of Microbiology, 2010;60:113–120*) we appreciate the time you spent preparing this article based on your research and/or knowledge with a more divulgative approach.

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Electron microscopy techniques and energy dispersive X-ray applied to determine the sorption of lead in *Paracoccus* sp. DE2007

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Paracoccus sp. DE2007 was isolated from a consortium of microorganisms in Ebro delta microbial mats. The isolated strain was identified according to its biochemical and molecular characteristics as *Paracoccus* sp. DE2007 [1]. The strain was able to grow in solid medium in the presence of heavy metals and crude oil.

In the present work electron microscopy techniques coupled to Energy Dispersive X-ray were used to determine if this strain is able to capture lead extra- and/or intra-cellularly because lead has been detected in Ebro delta as a toxic pollutant.

The results indicate that *Paraccoccus* sp. DE2007 has a great affinity for lead, which is absorbed into the external envelope but not into the cytoplasmic space.

Keywords: *Paracoccus* sp. DE2007, lead, SEM, TEM, EDX

1. Introduction

Among the possible applications of the capacity of microorganisms to interact with metals, biosorption and bioaccumulation are a potential option for replacing abiotic techniques in the removal of toxic metals [2].

The biosorption process takes place mainly at the cell surface, where there are a large variety of cellular compounds that can bind metals, such as the peptidoglycan, teichoic acids and lipoteichoic acids and several polysaccharides and proteins [3]. Functional groups containing O-, N-, S- or P- are directly involved in the binding of metals. The most important of these groups are: carbonyl (ketone), carboxyl, sulfhydryl (thiol), sulfonate, thioether, amine, secondary amine, amide, imine, imidazole, phosphonate and phosphodiester [3]. The mechanisms involved in metals binding to the cell surface are metabolically passive and include electrostatic interactions, Van der Waals forces, covalent bonds, redox interactions, extracellular precipitation, mineral nucleation and combinations of these [4]. The extracellular polymeric substance (EPS) is currently receiving much attention as it is able to flocculate and absorb metal ions and enhance colonization and microbial growth through the formation of biofilms [5]. Many microorganisms produce EPS, which is a mixture of biopolymers composed mainly of polysaccharides, as well as proteins, nucleic acids, lipids and humic substances. The EPS is also located in the intercellular space of microbial aggregates and forms the structure and architecture of the biofilm matrix. Its secretion and features depend on the species and growing conditions. It has been observed that some bacteria can increase their EPS production in the presence of toxic compounds (e.g. metals) as a defence mechanism [6].

On the other hand, bioaccumulation is defined as the intracellular accumulation of metals. It consists first in biosorption to the cell surface, followed by transport and concentration in intracytoplasm compartments. The extracellular heavy metals can move in the cytoplasm through two processes: the first is rapid, nonspecific, and usually based on the chemiosmotic gradient (passive transport), and since it is used by a wide variety of substrates it is expressed constitutively; the second transport system has a high specificity for the substrate, is slower and often uses adeninatriphosphate hydrolysis (ATP) as the energy source (active transport), but can also function by osmotic gradient. The latter system is inducible and is produced by the cell during stress conditions or cell starvation [7]. The metals released into the cell bind or chelate different cellular components before being incorporated into cellular compartments, if they are suitable. The captured metal can form complexes with compounds such as proteins rich in thiol groups physiologically present in cells (enzymes of metabolic pathways) or synthesized in the presence of metals (metallothioneins and phytochelatins) and glutathione (a compound that determines oxidative stress in cells) [3].

The need for more new products to bioremediate polluted ecosystems makes it necessary to find microorganisms with unique capabilities to capture metals. These microorganisms are often found and isolated in polluted environments where there is high selective pressure.

In recent years a *Microcoleus* consortium that is capable of degrading crude oil [9] was isolated in our laboratory by Diestra et al. [8] from Ebro delta microbial mats (Tarragona-Spain). The *Microcoleus* consortium was formed by a phototrophic cyanobacterium and different heterotrophic microorganisms. *Microcoleus* sp. DE2005 [8] and *Micrococcus* sp. DE2008 [10] were isolated and their capacities to tolerate or biocapture heavy metals were determined. Although *Paracoccus* sp. DE2007 isolated from the same consortium was also identified by Diestra et al. [1], its capacity to biosorption and/or bioaccumulate heavy metals was not determined.

Despite the extensive information available on the genus *Paracoccus*, very little is known about its ability to biocapture heavy metals. Since both the *Microcoleus* consortium and some of the isolated microorganisms mentioned above are able to biosorption or bioaccumulate heavy metals, the objective of this study was to determine whether *Paracoccus* DE2007 has this ability. For this purpose we used electron microscopy techniques in combination with an electron dispersive X-ray detector. We select lead among heavy metal because it is very toxic and has no biological functions and because it has been detected in Ebro delta.

2. Material and Methods

2.1. Bacterial strain and culture conditions:

Paracoccus sp. DE2007 was isolated from a *Microcoleus* consortium [1]. Cells were grown in Luria Bertani (LB) liquid medium containing tryptone (10.0g/l), yeast extract (5.0 g/L) and NaCl (10.0 g/L) at pH 7 and 27ºC for 24h. Lead stock solution was prepared with Pb $(NO_3)_2$ (Merck KGaA, Darmstadt, Germany) in deionized water and was sterilized by filtration with polycarbonate membrane filters. To test whether *Paracoccus* sp. DE2007 could grow in the presence of lead. Pb stock solution was added to LB liquid medium to reach a final concentration of 5mM. Cultures were incubated at 27ºC for 24h.

2.2. Scanning electron microscopy (SEM) and energy dispersive X-ray (EDX) microanalysis:

For SEM analysis, samples of *Paracoccus* sp. cultures were fixed in 2.5% glutaraldehyde (Millonig's buffer) [11] (0.1M and pH7) for 2 h and washed four times in the same buffer. They were then dehydrated in successively increasing gradient concentrations of ethanol (30, 50, 70, 90 and 100%) and dried by critical point. Finally, all samples were mounted on metal stubs and coated with gold. A JEOL JSM-6300 scanning electron microscope (JEOL LTD, Tokyo, Japan) was used to view the images.
For energy dispersive X-ray microanalysis, cells were homogenously distributed and filtered with polycarbonate membrane filters. These filters were dehydrated and dried by critical point and coated with gold. An energy dispersive X-ray spectrophotometer (EDX) Link Isis-200 (Oxford Instruments, Bucks, England) operated at 20 kV coupled to SEM was used.

2.3. Transmission electron microscopy (TEM) and energy dispersive X-ray (EDX) microanalysis:

For TEM analysis, samples of *Paracoccus* sp. DE2007 cultures were fixed in 2.5% glutaraldehyde (Millonig's buffer) 0.1 M, pH 7 for 2 h and washed four times in the same buffer. Samples were post-fixed in 1% OsO_4 at 4ºC for 2 h, and washed four times in the same buffer. They were then dehydrated in a graded series (50, 70, 90, 95 and 100%) of acetone and embedded in Spurr's resin. Ultrathin sections (70 nm) were mounted on carbon coated copper grids and stained with uranyl acetate and lead citrate. Samples were viewed with a Hitachi H-7000 transmission electron microscope (Hitachi LTD, Tokyo, Japan). Sections with a thickness of 200 nm were not stained with lead citrate in order to avoid elemental substitution during the analysis. They were mounted on gold and copper grids for energy dispersive X-ray microanalysis. The samples were analyzed with a JEOL JEM-2011 transmission electron microscope (JEOL LTD, Tokyo, Japan).

3. Results

We used SEM and TEM coupled to EDX to study the effect of lead on *Paracoccus* sp. DE2007 in order to determine the capacity of this microorganism to capture heavy metals.
Paracoccus sp. DE2007, as mentioned above, was isolated from a *Microcoleus* consortium formed by this cyanobacterium and different heterotrophic bacteria encased in a dense exopolysaccharide (EPS) envelope [8, 12]. In liquid medium this bacterial aggregate formed a dense mucilaginous mass, and in solid medium it formed green filamentous colonies.
In this work *Paracoccus* sp. DE2007 cultures were prepared with a 5mM lead concentration with the aim of determining whether *Paracoccus* sp. DE2007 can capture metals. Unpolluted and polluted cultures were prepared and analyzed by EDX coupled to SEM and TEM (Fig. 1 and 2).
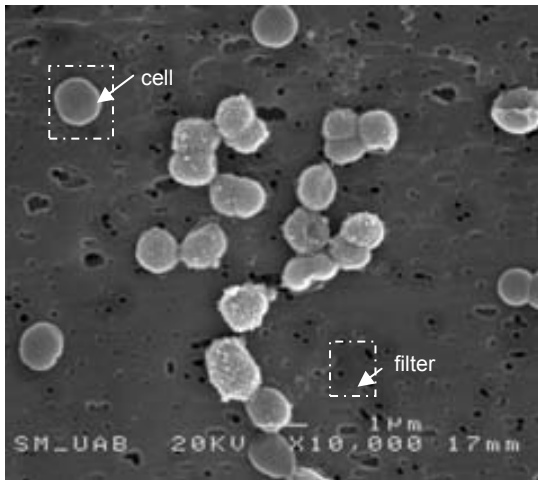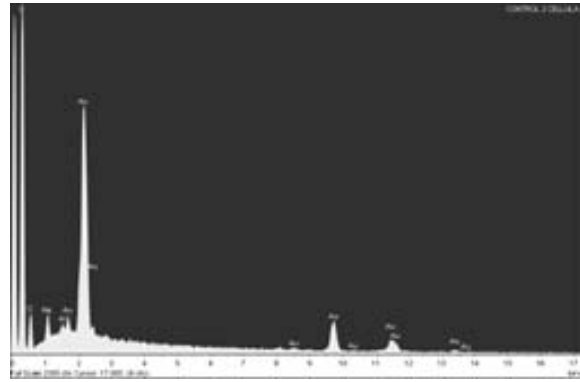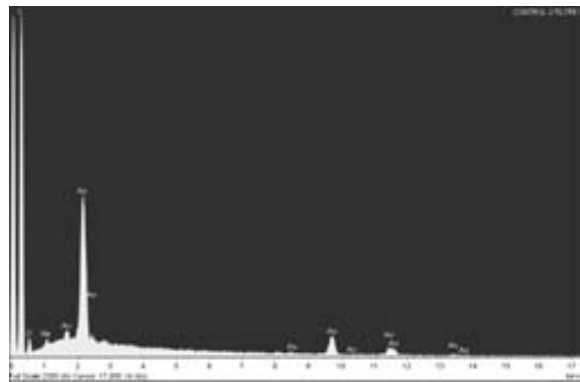
Figure 1: Unpolluted cultures of *Paracoccus* sp. DE2007 cells are analyzed by SEM-EDX (1a). Lead is not detected in either the cells (1b) or the filter (1c). Scale bar = 1μm in 1a.
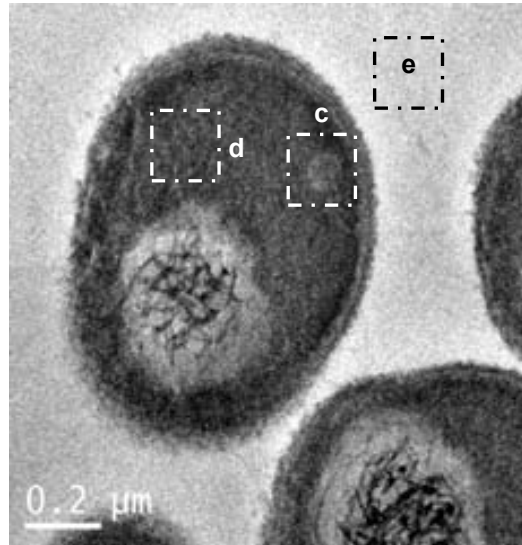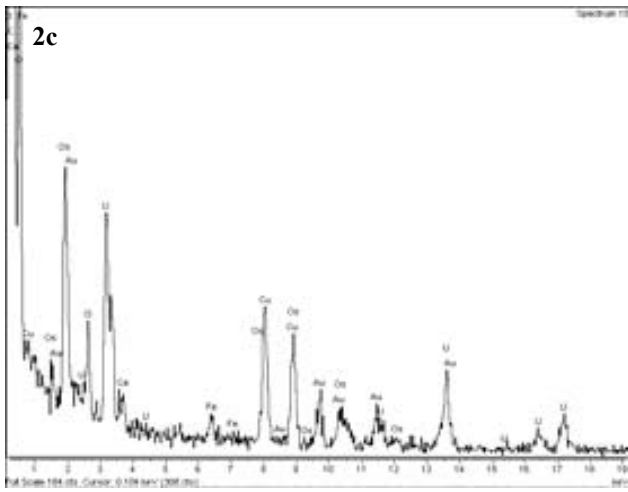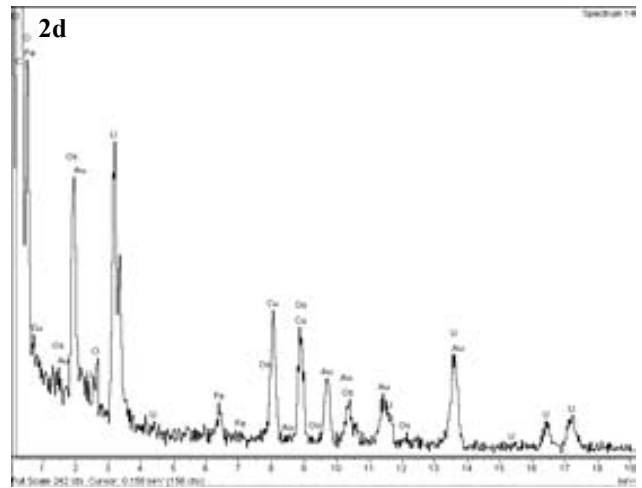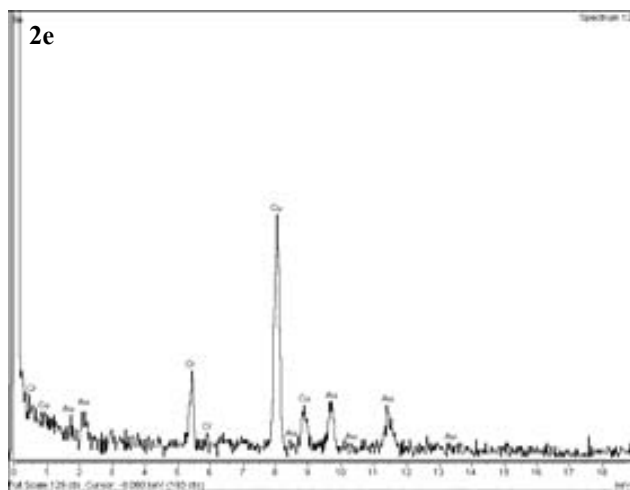
Figure 2: Unpolluted culture of *Paracoccus* sp. DE2007 cells showing vesicles in the cytoplasm (arrows) (2a). Cells were analyzed by TEM-EDX (2b). EDX microanalysis spectra show the absence of lead in: the vesicles (2c), the cytoplasm (2d) and the Spurr's resin. (2e). Au and Cu peaks are due to the gold grids. Scale bars = 200nm in 2a and 2b.

Individual cells and groups of 2 to 4 cells are view in SEM image (Fig. 1a). The EDX analysis of the cells shows that lead is not detected in the cells (fig. 1b) or in the filter (Fig. 1c). The ultrathin sections show cells with a wall and membrane structure characteristic of Gram-negative cells. In the cytoplasm some vesicles that are only very slightly

electrodense can be observed (Fig. 2a, indicated by arrows), similar to those found by other authors [13]. We assumed that these vesicles have lipid content as they were positively stained with Sudan Black (data not shown). The results obtained in the TEM-EDX analysis (Fig. 2b) evidence the absence of lead in: the cytoplasm, the vesicles and the resin (Figs. 2c-2e).

Cells from Pb-polluted cultures exhibit morphological and ultrastructural changes (Figs. 3 and 4). The SEM image shows cells that are more deformed and which have a higher tendency to aggregate (Fig. 3a). The EDX analysis evidences the presence of lead in the cells (Fig. 3b, indicated by an arrow); however, no lead was found in the filters in which the cells are retained (Fig. 3c).

3a

3b

3c
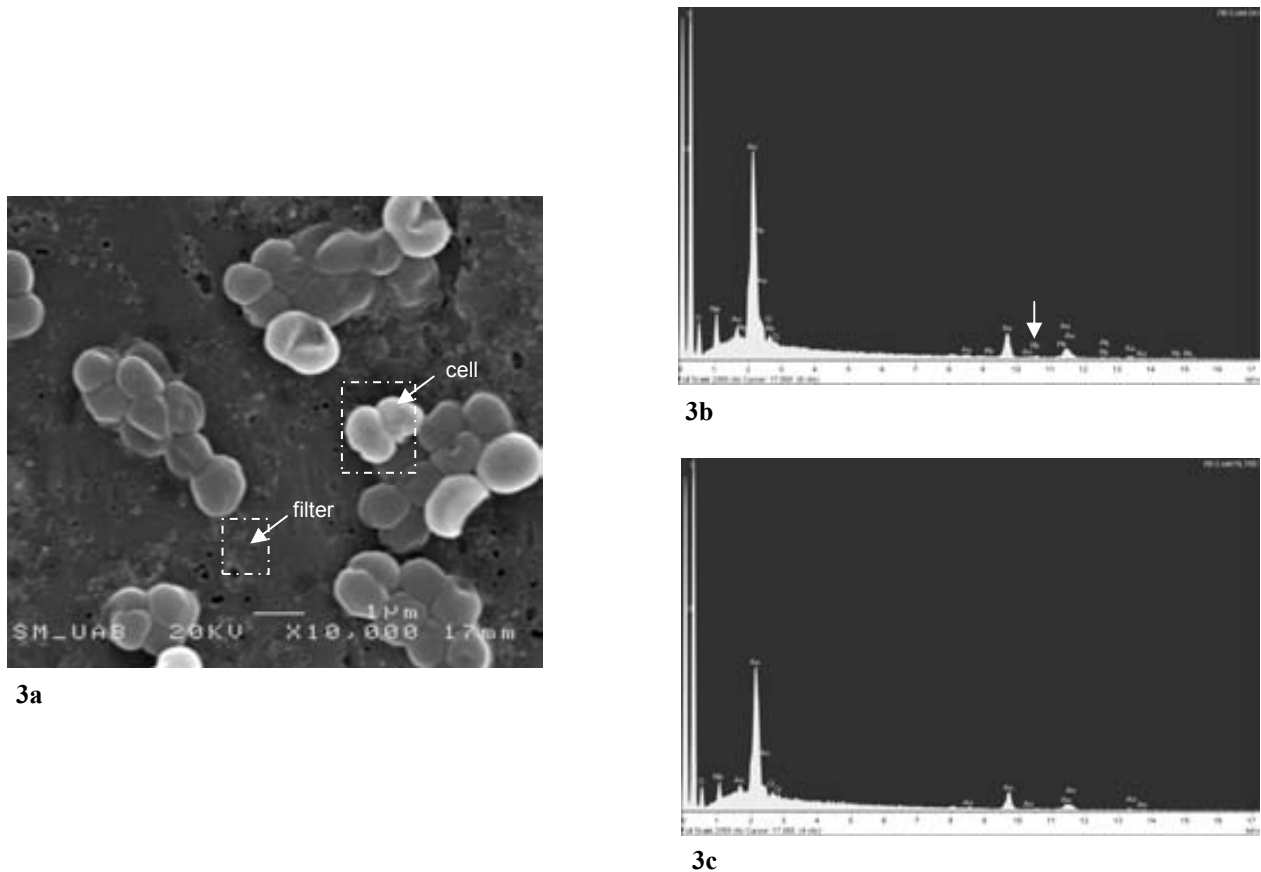
Figure 3: Cells of *Paracoccus* sp. DE2007 grown in the presence of 5mM of lead (3a) Scale bar = 1µm. X-ray analysis spectra coupled to SEM show a Pb peak (arrow) (3b) and the absence of lead in the filter (3c).
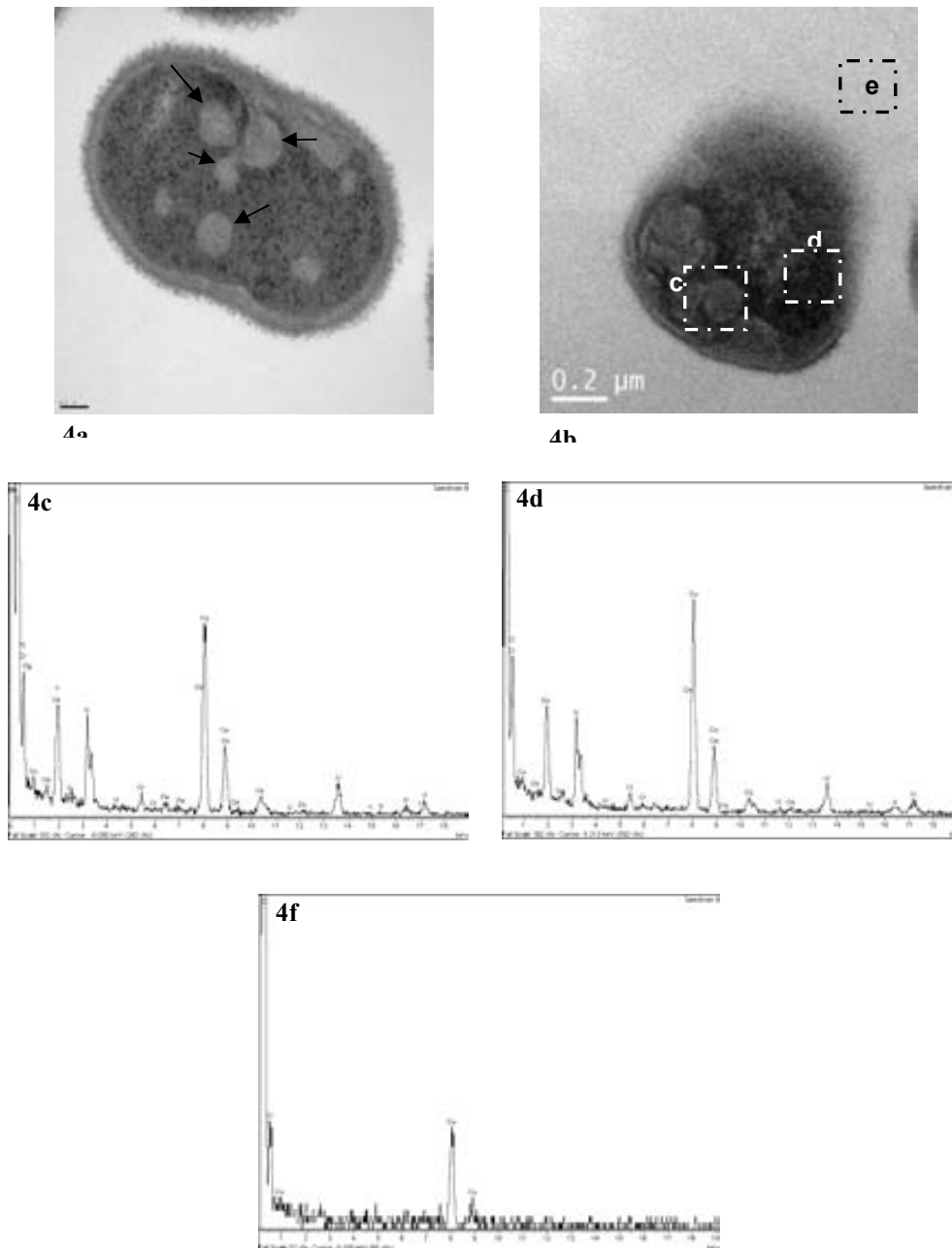
Figure 4: *Paracoccus* sp. DE2007 cells grown in 5mM lead salts show an increase in the number of intracitoplasmic vesicles (arrows) (4a). Cells were analyzed by TEM-EDX (4b). EDX microanalysis spectra show the absence of lead in: the vesicles (4c), the cytoplasm (4d) and the Spurr's resin. (4e). Scale bars = 100nm in 2a and 200 nm in 2b.

The TEM analysis of the same cells shows an increase in the intracytoplasmic vesicles at the ultrastructural level (Fig. 4a, also indicated by arrows). The TEM-EDX analysis (Fig. 4b) gives evidence that lead is not retained in either the vesicles (Fig. 4c) or the cytoplasm (Fig. 4d). The resin in which the samples were embedded was also analyzed as a control, and as expected it gave a negative result (Fig. 4e).

Copper and gold were detected in the spectra of the unpolluted cells because the gold grids are made of Cu covered with Au.

4. Discussion

The phylum *Proteobacteria* is composed of an extensive and diverse group of bacteria, some of which, especially some *Pseudomonas* species, have been demonstrated to have the ability to absorb lead [4, 14].

The members of the genus *Paracoccus* are metabolically versatile and are widely distributed, both in land and marine environments. The isolation of some strains in polluted environments evidences the capacity of these organisms to tolerate the presence of toxic compounds. Moreover, their ability to grow heterotrophically on a large number of organic compounds allows them to degrade some organic pollutants [15, 16, 17].

In the present work we studied the ability of *Paracoccus* sp. DE2007 to biocapture lead. As mentioned above, lead was chosen as it is a very toxic metal, even at low concentrations, has no biological function in the cell and because it has been found to be a pollutant in the Ebro delta microbial mats [18]. The *Paracoccus* sp. DE2007 strain that we studied was isolated from a microorganism consortium from a sample from the Ebro delta [8]. This strain also grew in the presence of crude oil and heavy metals (lead and copper) [1], which indicates its capacity to tolerate these toxic compounds.

To determine whether the *Paracoccus* sp. DE2007 strain could capture or bioaccumulate lead, two high resolution microscopy techniques, SEM and TEM, were employed coupled with an energy dispersive X-ray technique. Using these techniques together allowed us to analyze the microorganisms morphostructurally and evaluate the presence and also the location of the lead.

The energy dispersive X-ray spectra obtained with SEM detected the presence of lead extracellularly (Fig. 3b), which shows that the *Paracoccus* sp.DE2007 strain captures the metal in the EPS envelope.
Paracoccus sp. DE2007 produces a large amount of EPS in response to the presence of lead in the polluted cultures, which was observed with optical microscopy by means negative staining (data not shown). The increase in the EPS production is in accordance with the results obtained by other authors for other strains of the same genus, which can even eventually produce biofilms in particular culture conditions [19].

The presence of the extracellular polymeric substance in the cell is considered promising for bioremediation applications to eliminate heavy metals [20].

However, lead was not detected in either the cytoplasm or in any of the intracellular structures using TEM-EDX analysis of the *Paracoccus* sp. DE2007 cultures grown in the presence of lead (Fig. 4a). Therefore, *Paracoccus* sp. DE2007 does not bioaccumulate lead.

5. Conclusions

1. SEM and TEM coupled with EDX constitute a set of methodologies that allow a quick diagnosis of whether a microorganism can capture a metal extra- or intra-cellularly. Although in the present work we only assayed one metal (lead), it is possible to obtain information about any metal and in different types of microorganisms.
2. The *Paracoccus* sp. DE2007 strain is able to capture lead extracellularly in its EPS envelopes, but it does not bioaccumulate lead intracellularly. However, the number of intracellular vesicles increases considerably in presence of lead. This could be related to an increase in the synthesis of lipid compounds that usually do not have metal affinity but which play an important role as a carbon reserve in microorganisms under stress conditions, such as the exposure to heavy metals.

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Sequestration and *in vivo* effect of lead on DE2009 microalga, using high-resolution microscopic techniques

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ARTICLE INFO

ABSTRACT

Algae are primary producers in a wide variety of natural ecosystems, and these microorganisms have been used in bioremediation studies. Nevertheless, very little is known about the *in vivo* effect of heavy metals on individual living cells.

In this paper, we have applied a method based on confocal laser scanning microscopy and *lambda scan* function (CLSM-λ*scan*) to determine the effect of lead (Pb), at different concentrations, on the DE2009 microalga. At the same time, we have optimized a method based on CLSM and image-analysis software (CLSM-IA) to determine *in vivo* biomass of this microorganism. The results obtained by *lambda scan* function indicated that the pigment peak decreases while the concentration of metal increases at pH 7. On the other hand at pH 4 there is no good correlation between the concentration of metal and the intensity of the emission of fluorescence of the pigment. Also, in some cases a displacement of the Chl *a* peak towards 680 nm is produced. Total and individual biomass determined by CLSM-IA shows statistically significant differences between unpolluted and 10 mM polluted cultures.

Complementary studies using electron microscopy techniques coupled to energy dispersive X-ray microanalysis (EDX) demonstrate that the microalga can sequestrate Pb extra- and intracellularly.

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1. Introduction

Microalgae and cyanobacteria are the most important primary producers in stratified laminated ecosystems, such as microbial mats, which cover large extensions of marine coastal environments [1–5].

In the last few years, we have isolated a consortium of microorganisms, from Ebro delta microbial mats, dominated by a single cyanobacterium, *Microcoleus* sp., and different heterotrophic bacteria [6,7]. Recently we have isolated a new phototrophic microorganism, a microalga (DE2009) from the same habitat. Given that *Microcoleus* sp. was able to tolerate lead and copper [8] in this study we propose an analysis of whether DE2009 microalga is able to sequestrate heavy metals.

Phototrophic microorganisms have been frequently used in biosorption research [9–12]. Metals are one group of contaminants frequently involved in marine environmental pollution. It is known that some metals at low concentrations, participate in different metabolic routes (essentials), but at high concentrations they are toxic for many living organisms; while others metals always

have a toxic effect [13]. Different methods have been proposed to study the toxic effect of heavy metals on microalgae, but most authors conclude that the metal concentration that affects growth in microalgae is variable and depends of many different factors, including the ability to accumulate heavy metals [14,15]. Algal surfaces have been found that containing different chemical function groups that differ in affinity and specificity towards these metals [16–18].

Although the capacity of some microalgae to capture heavy metals has been described, little is known about the effect of these metals in individual living cells, which is needed to predict the impact of heavy metals on natural ecosystems. In this study we selected Pb as a toxic metal and because the microbial mats studied are located in a lead-polluted area of the Ebro delta [19].

Confocal laser scanning microscopy (CLSM) based on natural pigment fluorescence emitted by phototrophic microorganisms is proving to be an excellent methodology for different types of studies related to these microorganisms. This optical microscopy technique avoids the need for either manipulating or staining the samples and allows accurate and non-destructive optical sectioning that generates high-resolution images, where out-of-focus is eliminated. Due to its high resolution, it is easy to differentiate morphotypes of phototrophic microorganisms living in mixed populations, because they emit natural fluorescence.

The CLSM coupled to a spectrofluorometric detector ($\lambda scan$ function), provides simultaneous three-dimensional information on photosynthetic microorganisms and their fluorescence spectra profiles in stratified ecosystems, such as microbial mats and biofilms. The most significant application is the discrimination of cells with specific fluorescence spectra profiles within a colony, and the correlation of morphology and individual cell states [20].

In this paper, we have applied CLSM-$\lambda scan$, to determine the *in vivo* effect of Pb (at different concentrations) on DE2009 microalga and CLSM-IA to determine their total and individual biomass.

Complementary studies using scanning electron microscopy (SEM), transmission electron microscopy (TEM) and energy dispersive X-ray microanalysis (EDX) coupled to SEM and TEM were also performed to test the capacity of DE2009 microalga for extra- and intracellular uptake of Pb.

2. Experimental

2.1. Culture conditions

Cultures of DE2009 microalga were grown at $27\,^{\circ}C$ and $15\,\mu E\,m^{-2}\,s^{-1}$ in liquid mineral Pfennig medium at two pHs (7 and 4) and at different concentrations (0, 0.1, 0.5, 0.75, 1, 5 and 10 mM) of lead $(Pb(NO_3)_2)$ for 9 days.

2.2. Confocal laser scanning microscopy

The confocal experiments were performed using a confocal laser scanning microscope (Leica TCS SP5; Leica Heidelberg, Germany).

2.2.1. $\lambda scan$ function

Pigment analysis of DE2009 microalga cultures was determined by $\lambda scan$ function of CLSM. This technique provides information on the state of the photosynthetic pigments of phototrophic microorganisms on the basis of the emission wavelength region and the fluorescence intensity emitted (autofluorescence). Each image sequence was obtained by scanning the same *xy* optical section throughout the visible spectrum. Images were acquired at the *z* position at which the fluorescence was maximal, and acquisition settings were constant throughout the experiment. The sample excitation was carried out with an Argon Laser at 488 nm (λ_{exe} 488) with a λ step size of 3 nm for an emission wavelength between 510 and 752 nm.

In order to measure the mean fluorescence intensity (MFI) of the $xy\lambda$, CLSM data sets obtained by means of the Leica Confocal Software (Leica Microsystems CMS GmbH) were used. The regions-of-interest (ROIs) function of the software was used to measure the spectral signature. For each sample, 70 ROIs of $1\,\mu m^2$ taken from DE2009 microalga cells were analysed.

2.2.2. Biomass estimations

Confocal images were obtained using the CLSM mentioned above. Two types of fluorescence at cell level from the same DE2009 microalga were observed in images obtained in all cultures analysed. Red (red cells) and green (green cells) were distinguished on screen as pseudo-colours. For that reason, a *sequential scan* in two channels was carried out from each same *xy* optical section. On the first channel, samples were excited with a diode 561 nm (λ_{exe} 561) and the emission of fluorescence was captured between 670 and 794 nm (red pseudo-colour). On the second channel, samples were excited with an Argon Laser at 488 nm (λ_{exe} 488) and the emission of fluorescence was captured between 550 and 575 nm (green pseudo-colour). Finally, 10 red and 10 green confocal images were obtained from all cultures studied.

Total biomass estimations from the red and green algal cells were obtained separately. Moreover, individual biomass for both

types of cells was studied. Finally, total and individual biomass was estimated for each metal concentration.

In this paper, we have used a modification of the method described by Solé et al. [21] using CLSM and a free image-processing analysis software, *ImageJ v1.41.* (CLSM-IA). This method was used to determine the percentage between red and green pixels of DE2009 microalga and their biomass from the different cultures studied in this work.

The method used in this paper is as follows: for total biomass each pair of images (red and green) from an identical *xy* optical section were opened in their original format (8-bit, 1024×1024 pixels) as tiff images and the corresponding overlay image was obtained. These three images were transformed to binary images (black/white) using different thresholds. Values of 70 and 25 were applied respectively to red and green images from 0.1, 0.5, 0.75, 1 and 5 mM metal concentrations. Conversely, threshold values of 50 and 60 were applied respectively to red and green images from the 10 mM metal concentration.

In order to determine the percentage between the red and green fluorescences at pixel level the image calculator function of the *ImageJ* was used. To obtain images with cells showing up only as red fluorescence, all green fluorescence was subtracted from the image. In the same way, red fluorescence is subtracted from the image when greens only are obtained. In both cases to clean the images it was necessary to filter out the red and green pixels. A smoothing filter (median filter with a radius of 2.0 pixels) was then applied to the images.

To obtain biovolume values, the Voxel Counter plug-in was applied to these filtered images [22]. This specific application calculates the ratio of the thresholded voxels (the red or green microalga volume), to all voxels from the binary image determined. The biovolume value obtained (Volume Fraction) was finally multiplied by a conversion factor of $310\,fgC\,\mu m^{-3}$ to convert it to biomass [23,24].

To calculate the individual biomass, 30 red and 30 green cells were selected using *ImageJ* software and then the cells were analysed following the same protocol described above.

2.3. Scanning electron microscopy

To determine whether DE2009 microalga was able to capture Pb extracellularly, cultures polluted with 10 mM Pb were incubated under the same conditions as previous experiments. The following procedure was used: cultures were fixed in 2.5% glutaraldehyde Millonig buffer phosphate (0.1 M pH 4) for 2 h and washed four times in the same buffer. They were then dehydrated in a graded series (30%, 50%, 70%, 90%, and 100%) of ethanol and dried by critical-point. The samples were mounted on metal stubs and coated with gold and then viewed in a Jeol JSM-6300 scanning electron microscope (Jeol Ltd., Tokyo, Japan). For X-ray analysis, cultures were filtered on polycarbonate membrane filters. These filters were then dehydrated and dried by the same procedure used for culture samples. An EDX Link Isis-200 (Oxford Instruments, Bucks, England) operated at 20 kV coupled to SEM was used.

2.4. Transmission electron microscopy

In order to assess whether DE2009 microalga was able to capture the metal intracellularly, cultures polluted with 10 mM Pb were incubated under the same conditions as previous experiments. The following procedure was used: cultures were fixed in 2.5% glutaraldehyde Millonig buffer phosphate (0.1 M pH 4) for 2 h and washed four times in the same buffer. Samples were post-fixed in 1% OsO_4 at $4\,^{\circ}C$ for 2 h, and washed four times in the same buffer. They were then dehydrated in a graded series (30%, 50%, 70%, 90%, and 100%) of acetone and embedded in Spurr resin. To show a better

quality image, ultrathin sections of 70 nm were mounted on carbon coated copper grids and stained with acetate and lead citrate. Samples were viewed in a Hitachi H-7000 electron microscope (Hitachi Ltd., Tokyo, Japan). To determine the capacity of polyphosphate inclusions for accumulating Pb, sections of 200 nm thick mounted on titanium grids were used for energy dispersive X-ray microanalysis. Samples were analysed with a Jeol Jem-2011 (Jeol Ltd., Tokyo, Japan).

2.5. Statistical analysis

Means and standard errors for each sample parameter determined in this study were calculated using SPSS software (version 15.0 for Windows). Data obtained for λ scan experiments were compared using a Student's t test with a 95% significance ($p < 0.05$). Data obtained from percentages, and biomass was compared separately in the same way. All the statistical analyses were performed with the same software.

2.6. Molecular characterisation

Genomic DNA was extracted from an DE2009 overnight culture in Pfennig medium using UltraClean™ Microbial DNA Isolation Kit (Mobio Laboratories, Carlsbad, USA) according to manufacturer's instructions. The 18S rRNA gene fragment was obtained by PCR amplification using SR1 (5′-TACCTGGTTGATCCTGCCAG-3′) and Euk516 (5′-ACCAGACTTGCCCTCC-3′) primers [25], using PureTq™ Ready-To-Go™ PCR (GE Healthcare). The PCR conditions were those described in [25]. The PCR product was then purified using the QIAquick PCR purification Kit (Quiagen) as directed by the supplier. Both complementary strands were sequenced separately at the SECUGEN sequencing company (S.L. Madrid, Spain).

3. Results and discussion

3.1. Characterisation of the DE2009 microalga

DE2009 microalga was isolated from the Ebro delta microbial mats. Cells are spherical, with a diameter of 7–9 μm. Ultrathin sections of cells show the thylakoids grouped into bands (inside the chloroplast); the nucleus and the pyrenoid. High electron-dense inclusions (HE) inside the cytoplasm, were identified as polyphosphate granules (PPG). In pristine cultures (without Pb) no exopolysaccharides (EPS) were detected surrounding the cell wall (Fig. 1).

According to 18S rRNA gene sequence comparison, the closest cultured relatives were representatives of the *Scenedesmus* genus: *Scenedesmus pectinatus* (AB037092), *Scenedesmus acutiformis* (AB037089) and *Scenedesmus vacuolatus* (X56104) with 99% similarity.

However, the lack of ultrastructural similarity and the low variability of this marker among different closely related green algae genera makes it difficult to assign this isolate to the genus *Scenedesmus* until more informative markers are sequenced.

3.2. Effect of Pb on DE2009 microalga

Different concentrations of Pb were used to study its effect on DE2009 microalga by CLSM. Two different experiments were prepared:

(A) The first experiment was performed to determine the *in vivo* effect of Pb on microalga pigments by means of the λ scan function of CLSM.

This method, allowed us to evaluate the physiological state of the microalga at single-cell level, considering changes in Chloro-
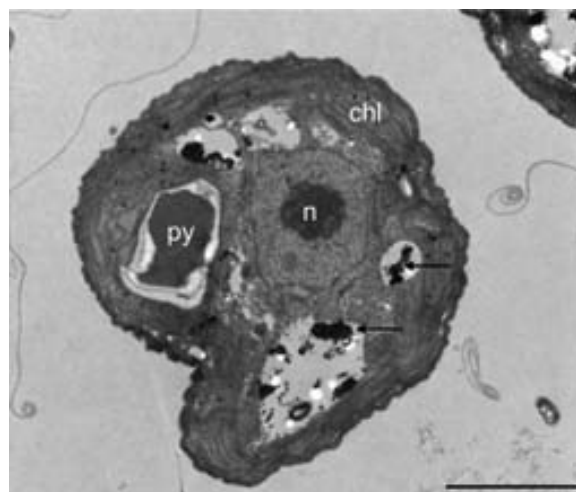

Fig. 1. Ultrathin section of the DE2009 microalga. Chloroplast showing thylakoids (chl), nucleus (n), pyrenoid (py) and HE inclusions (indicated by arrows). Scale bar represents 2 μm.

phyll *a* (Chl *a*) (maximum absorption at 685 nm). Cultures of DE2009 microalga were grown at pHs 7 and 4 and at different Pb concentrations.

An *xyz* optical section corresponding to the autofluorescence detected in control cultures growing at pH 7 is shown in Fig. 2A. The results demonstrate that the pigment peak decreases while the concentration of metal increases from 0 mM Pb (control culture) to 10 mM Pb. The Chl *a* peak at the different Pb concentrations followed the same pattern as to that the obtained for the control culture (Fig. 2B).

An *xyz* optical section corresponding to the autofluorescence detected in control cultures growing at pH 4 is shown in Fig. 2C. At pH 4 there is no good correlation between the concentration of metal and the pigment's intensity of the fluorescence emission. In some cases, a displacement of the Chl *a* peak towards 680 nm is produced (Fig. 2D). The differences in the effect of the metal on the cultures grown at both pHs could be attributed to the greater toxicity of the metal at pH 4.

Nevertheless, in both cases the Pb effect varied significantly according to the metal concentration used. The differences were not statistically significant ($p < 0.05$) between the control experiments and 0.1 mM Pb. However, statistically significant differences were observed between control and 0.5, 0.75, 1, 5 and 10 mM Pb cultures as pH 7 as pH 4.

(B) The second experiment, was performed to determine changes in total and individual biomass.

Changes in DE2009 microalga biomass depending on different Pb concentrations were studied in cultures growing at pH 4 and in the same light and temperature conditions.

To determine total biomass, previously, the red and green fluorescence pixel counts were measured, as mentioned in Section 2. The former ranged from $91,365.80 \pm 15,695.33$ (control experiment) to $13,972.90 \pm 3083.46$ (at 10 mM Pb) and the latter varied from $10,593.70 \pm 1687.01$ (control experiment) to $30,529.40 \pm 17,706.84$ (at 10 mM). The conversion of this data into biomass makes it possible to observe that the red cell biomass was drastically decreased from 27.01 ± 4.64 (mgC cm^{-3}) in the control experiment to 3.82 ± 0.80 (mgC cm^{-3}) at 10 mM Pb. In Fig. 3 these results are expressed as percentages for each Pb concentration. On comparing the growth of DE2009 microalga in unpolluted and 10 mM polluted cultures it is observed in the former case, red cells represent 89.61% and green cells
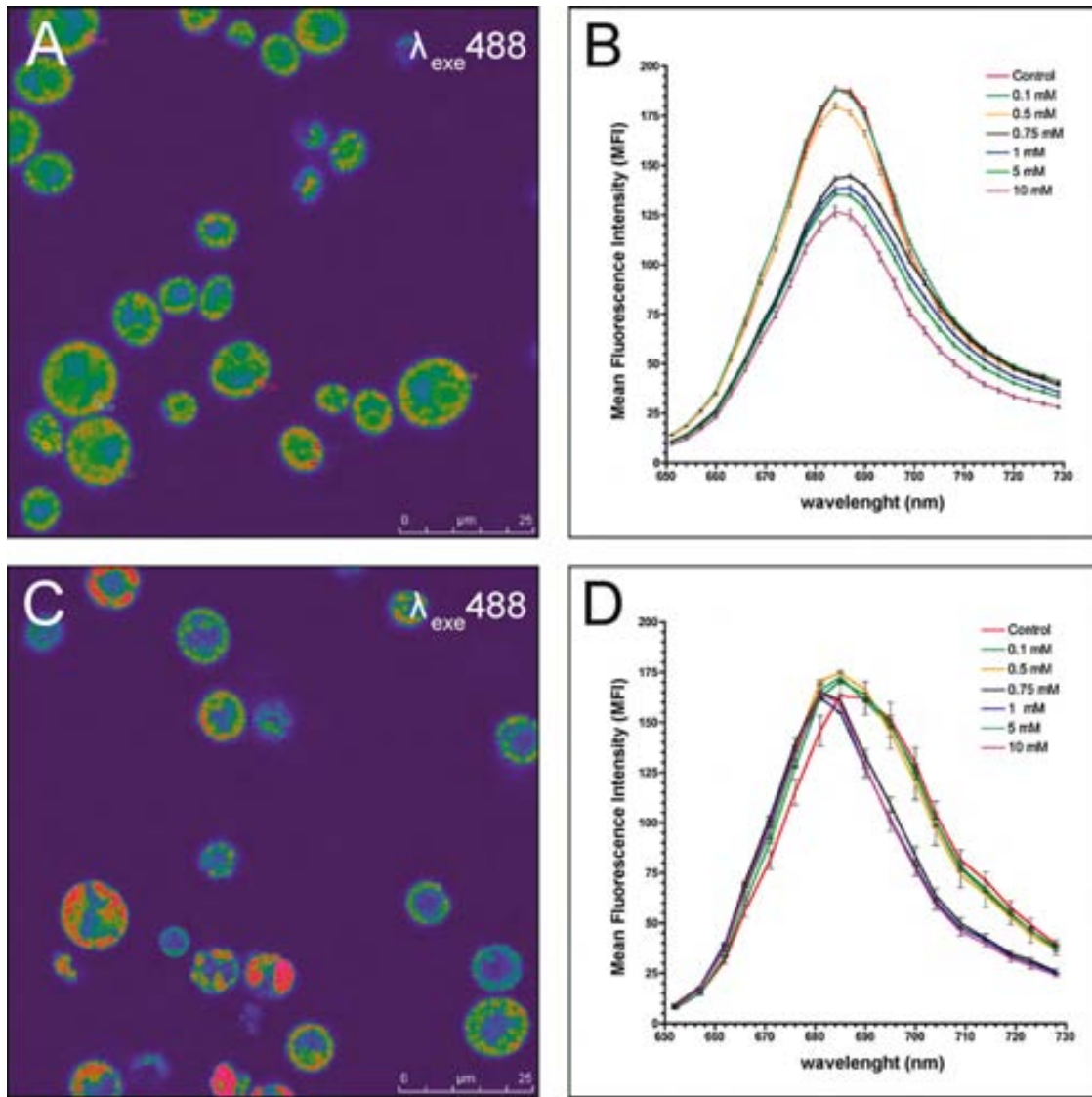
Fig. 2. CSLM images and λ*scans* plots of DE2009 microalga growing at pH 7 and pH 4. (A) and (C) represent CLSM images from a non-Pb treated cultures of DE2009 growing at pH 7 and 4 respectively. In these confocal images the pseudo-colour palette 4 (Leica Aplication Suite, Leica Microsystems CMS GmbH) was used, where warm colours represent the maximum intensities and cold colours represent the low intensities of fluorescence. λ*scans* of DE2009 microalga cultures treated with different Pb concentrations at pH 7 (B) and pH 4 (D). 2D plots represent the MFI spectra: emission wavelength (650–730 nm), *x* axis; MFI, *y* axis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)
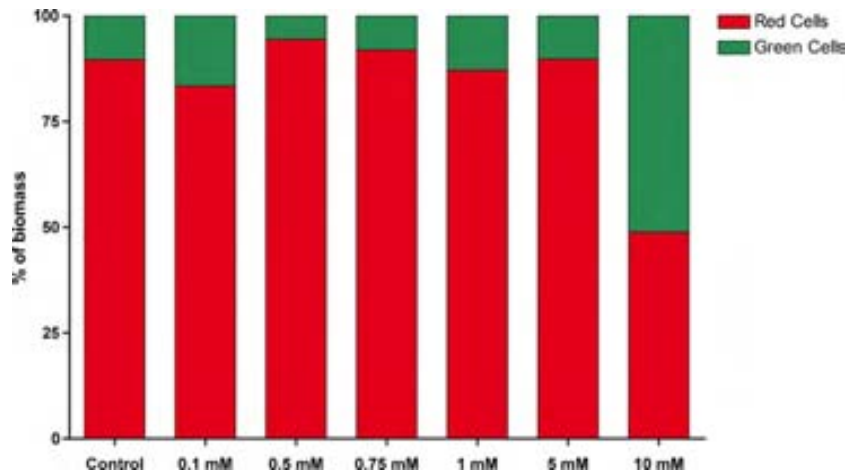

Fig. 3. Relative abundance of red and green DE2009 microalga cells at different Pb concentrations (expressed as a percentage of biomass). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

10.38%. In the latter case however, red cells represent 48.83% and green cells 51.16%. This data probably indicates that red cells could be considered physiologically active and green cells inactive.

To determine changes in individual biomass, only the red cells were considered for applying the CLSM-IA. In this case the pixel counts ranged from 595.87 ± 30.08 (control experiment) to 432.87 ± 25.21 at 10 mM Pb.

The cellular biomass, obtained from this data, decreased from 0.173 ± 0.09 (mgC cm^{-3}) to 0.128 ± 0.007 (mgC cm^{-3}). Statistically significant differences between the control culture (without lead) and 10 mM Pb cultures were found.

The results obtained both for the total and in individual biomass indicate the toxic effect at the highest concentration tested (10 mM Pb).

These experiments demonstrated the high *in vivo* tolerance of DE2009 to Pb. This microalga grows in higher metal concentrations than those described for other cyanobacteria and microalgae. For example, Roy et al. [26] demonstrated that the *Synechocystis* sp. growth was completely inhibited at 1.9 mM Pb^{2+}, while [27] found that the maximum concentration of Pb tolerated by different microalgae was 0.03 mM Pb^{2+}. In the first case, the cyanobacterium was unable to grow at the concentrations used in this work, and in the second case, the time used for growth was 72 h, a shorter period of time when compared to the time used in our experiments (9 days).

The results obtained with DE2009 microalga also show a higher tolerance to Pb than that observed for the heterotrophic bacterium *Micrococcus luteus* DE2008 [28] and the cyanobacterium *Micro-coleus* sp. [29], both microorganisms forming part of the same indigenous consortium.

3.3. Heavy metal accumulation in DE2009 microalga

With the aim of proving whether the DE2009 microalga could capture metals, cells from cultures with and without Pb were analysed by EDX coupled to SEM and TEM. In control cultures Pb was not detected either externally or internally.

Cultures containing Pb were also analysed using the same above-mentioned procedure. In this case, major differences in the structure of DE2009 microalga were observed. A higher excretion of EPS was found surrounding the cells (Fig. 4A) and the EDX coupled to SEM demonstrated that Pb was found in EPS (Fig. 4B). It has been proved that different microorganisms have an EPS matrix which can protect cells against toxic compounds such as metals and that its presence can overproduce exopolymer secretion [30]. Also, more specifically, uronic acids and sulphate groups present in EPS may interact with various metals thereby immobilizing them [31].

Moreover, the ultrathin sections of DE2009 microalga also exhibited discernible changes (distortion of the cells) after exposure to Pb. An increase in the HE inclusions was evident (Fig. 4C),

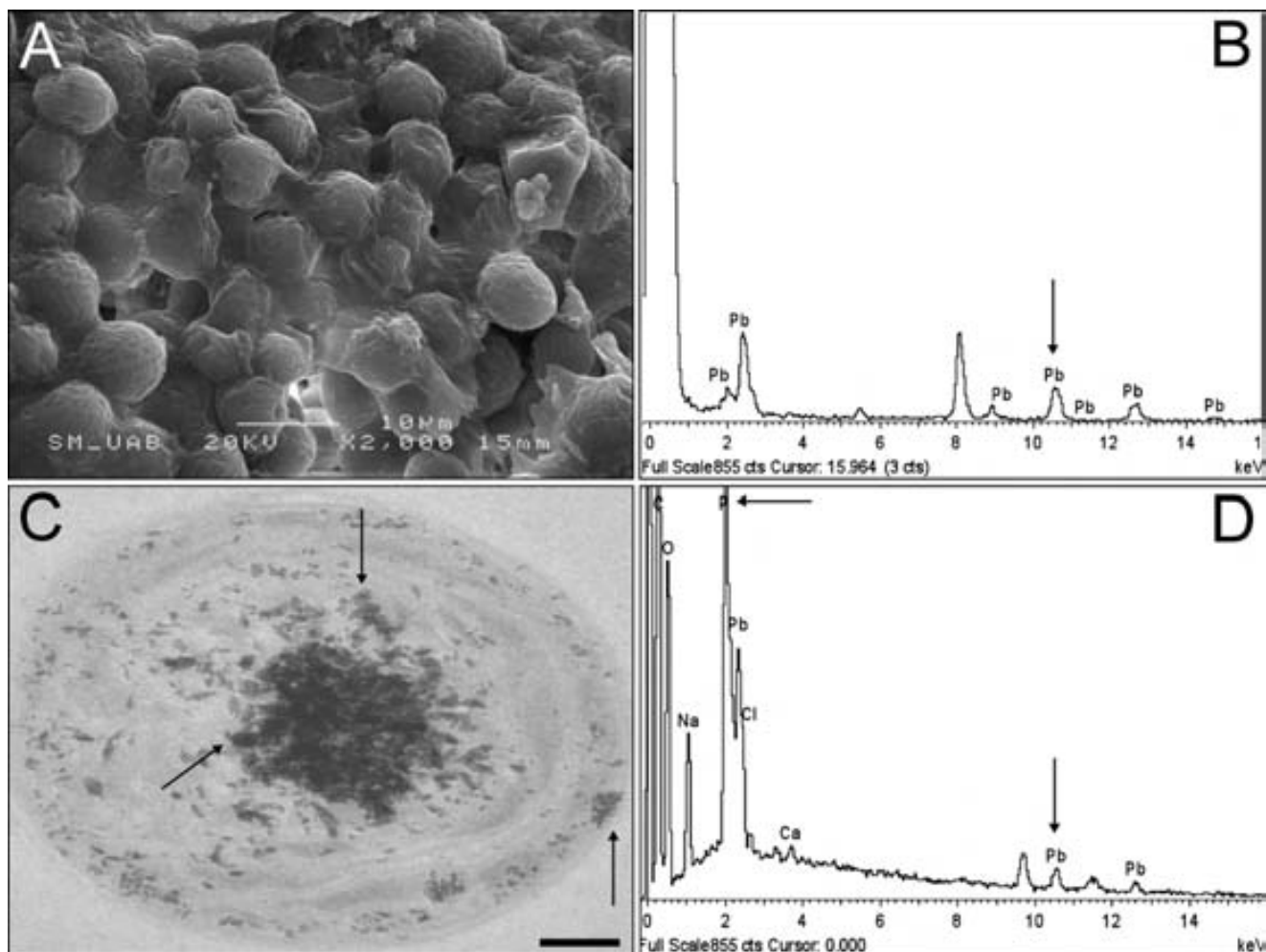**Fig. 4.** DE2009 microalga culture treated with 10 mM Pb. SEM image. Scale bar represents 10 μm (A). EDX spectrum coupled to SEM. Arrow indicates the main Pb peak at 10.5 keV (B). Ultrathin section of DE2009 microalga. Arrows indicate the distribution of HE inclusions. Scale bar represents 0.2 μm (C). EDX spectrum coupled to TEM from HE inclusions. Arrow indicates the main Pb peak at 10.5 keV (D).

when comparing the cellular ultrastructure of the microalga grown in unpolluted and polluted cultures. These inclusions were identified as polyphosphate granules (PPG) (see peak P indicated by an arrow, Fig. 4D). In many cases, similar inclusions have been found when cells are grown in adverse culture conditions [32–34]. The results obtained through the energy dispersive X-ray analysis of the inclusions, confirmed that Pb was also accumulated in PPG inside the cytoplasmic space. A significant Pb peak was detected (Fig. 4D). These results agree with studies of Goldberg et al. [35], which suggested that these kind of inclusions had a detoxifying effect by sequestering heavy metals.

Our results also suggested that the DE2009 microalga has a great affinity for Pb both extra- and intracellularly.

4. Conclusions

In conclusion, we consider that the CLSM-λscan could be a rapid technique for studying in vivo the cellular responses to heavy metal pollution. At pH 7 there is and inverse correlation between the intensity of pigment's fluorescence emission and the concentration of essayed metal. At pH 4 there is no good correlation between the concentration of metal and the pigment's intensity of the fluorescence emission.

Moreover, this method combined with the values obtained by means of CLSM-IA enables evaluation of the changes in total and individual biomass depending on the Pb concentration used. Total and individual biomass is also drastically reduced at 10 mM Pb in the experiments performed at pH 4.

On the other hand, the DE2009 microalga has the ability to remove Pb extra- and intracellularly. As DE2009 microalga is an indigenous microorganism in marine coastal stratified ecosystems, this microalga is probably involved in removing Pb from these habitats.

Acknowledgments

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Selection of bioindicators to detect lead pollution in Ebro delta microbial mats, using high-resolution microscopic techniques

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ABSTRACT

Lead (Pb) is a metal that is non-essential to any metabolic process and, moreover, highly deleterious to life. In microbial mats – benthic stratified ecosystems – located in coastal areas, phototrophic microorganisms (algae and oxygenic phototrophic bacteria) are the primary producers and they are exposed to pollution by metals. In this paper we describe the search for bioindicators among phototrophic populations of Ebro delta microbial mats, using high-resolution microscopic techniques that we have optimized in previous studies. Confocal laser scanning microscopy coupled to a spectrofluorometric detector (CLSM-λscan) to determine *in vivo* sensitivity of different cyanobacteria to lead, and scanning electron microscopy (SEM) and transmission electron microscopy (TEM), both coupled to energy dispersive X-ray microanalysis (EDX), to determine the extra- and intracellular sequestration of this metal in cells, were the techniques used for this purpose.

Oscillatoria sp. PCC 7515, *Chroococcus* sp. PCC 9106 and *Spirulina* sp. PCC 6313 tested in this paper could be considered bioindicators for lead pollution, because all of these microorganisms are indigenous, have high tolerance to high concentrations of lead and are able to accumulate this metal externally in extracellular polymeric substances (EPS) and intracellularly in polyphosphate (PP) inclusions.

Experiments made with microcosms demonstrated that *Phormidium*-like and *Lyngbya*-like organisms selected themselves at the highest concentrations of lead assayed.

In the present study it is shown that all cyanobacteria studied (both in culture and in microcosms) present PP inclusions in their cytoplasm and that these increase in number in lead polluted cultures and microcosms. We believe that the application of these microscopic techniques open up broad prospects for future studies of metal ecotoxicity.

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1. Introduction

Many metals are released from natural and anthropogenic sources into aquatic environments and can be bioaccumulated in sediments and in aquatic biota, agriculture and industry being the most important sources of this kind of pollution. The toxicity of metals in biological systems depends on their bioavailable form and on different biotic and abiotic factors (Roane and Pepper, 2000).

Faced with this problem, research has been undertaken on how metals affect the diversity, biomass, structure and activity of microbial communities in soil and sediments, including microbial mats (Boivin et al., 2005; Massieux et al., 2004; Ranjard et al., 2006).

In the past years our research group has studied the Ebro delta microbial mats. These mats are coastal benthic stratified ecosystems formed by vertically laminated microbial communities, which develop coloured layers (Esteve et al., 1992; Guerrero et al., 1999). They are located in the NE of Spain (40°40′N, 0°40′E) and although the Ebro delta comprises an area of 320 km², the region occupied by these ecosystems covers 3 km². This area has been polluted by many metals, mainly from the Ebro river (Mañosa et al., 2001).

Cyanobacteria are the most abundant prokaryotic microorganisms living in the upper layers of microbial mats. They are photoautotrophic bacteria, having chlorophyll *a* (chl *a*) as their major photosynthetic pigment. Among cyanobacteria, *Microcoleus chthonoplastes* is the most abundant cyanobacterium in Ebro delta microbial mats (Esteve et al., 1994; Mir et al., 1991; Solé et al., 2009). This cyanobacterium is also dominant in hypersaline environments (García-Pichel et al., 1996) and in the hot deserts (Campbell, 1979).

In previous works, we have studied the cyanobacterial diversity in microbial mats with *Microcoleus* sp., *Oscillatoria* sp., *Pseudanabaena* sp., *Phormidium* sp. and *Spirulina* sp. as the most abundant among the filamentous type and *Chroococcus* sp., *Gloeopcasa* sp., *Synechocystis* sp. and *Mixosarcina* sp. among the unicellular type (Diestra et al., 2004; Fourçans et al., 2004; Solé et al., 2003; Wieland et al., 2003).

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Fig. 1. CLSM images and λ*scans* plots of unpolluted and polluted cyanobacteria cultures. *Oscillatoria* sp. PCC 7515 (a); *Chroococcus* sp. PCC 9106 (c) and *Spirulina* sp. PCC 6313 (e) from unpolluted cultures are represented in CLSM images. Scale bars represent 50, 25 and 10 μm, respectively. In these confocal images the pseudo-colour palette 4 (Leica Aplication Suite, Leica Microsystems CMS GmbH) was used, where warm colours represent the maximum intensities and cold colours respresent the low intensities of fluorescence. λ*scans* plots of *Oscillatoria* sp. PCC 7515 (b); *Chroococcus* sp. PCC 9106 (d) and *Spirulina* sp. PCC 6313 (f) polluted with different Pb concentracions. 2D plots represent the MFI spectra: emission wavelenght, *x* axis; MFI, *y* axis.

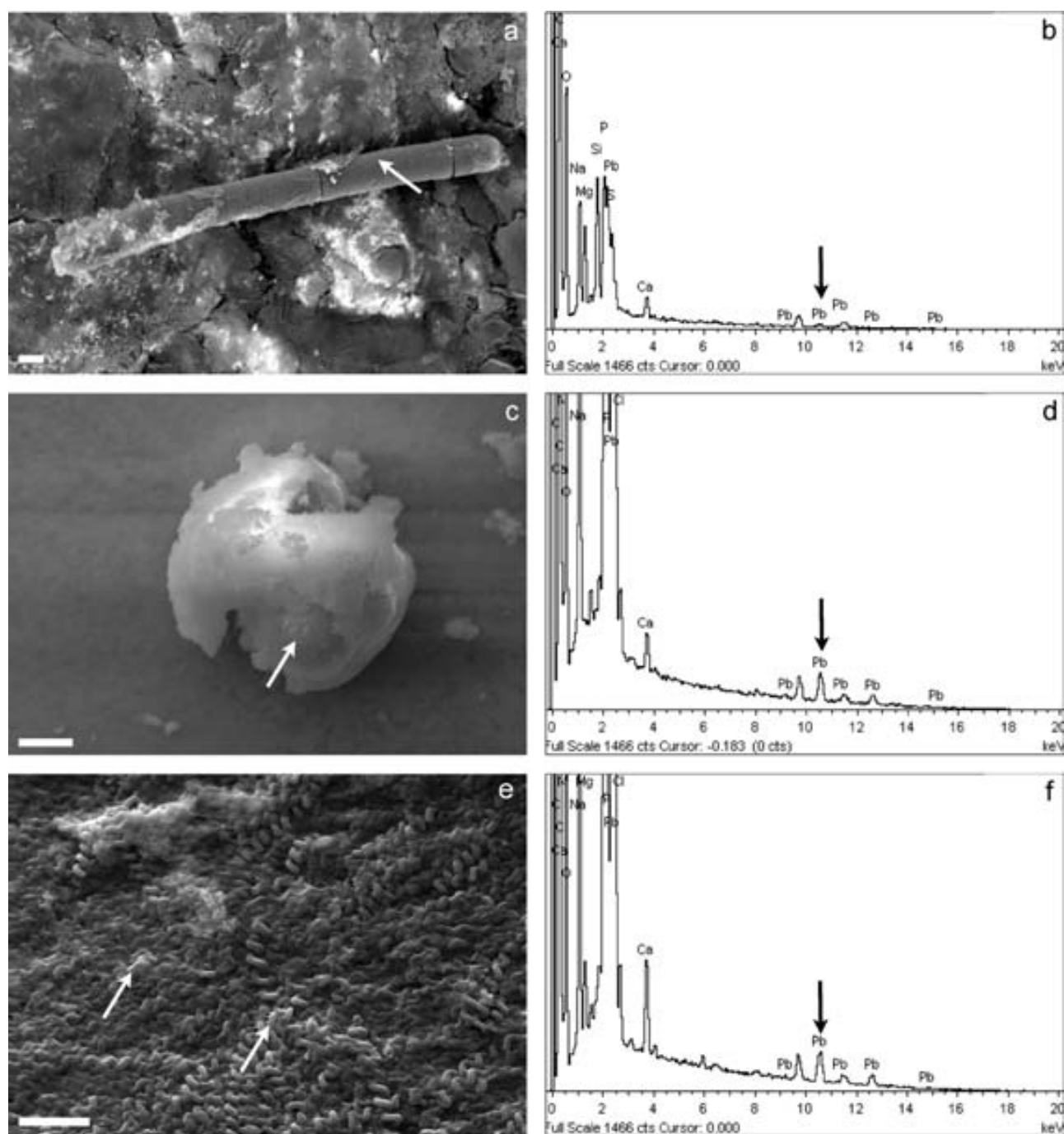**Fig. 2.** Rapid test to detect lead in cyanobacteria by SEM-EDX. Analysis points indicated by arrows from *Oscillatoria* sp. PCC 7515 (a), *Chroococcus* sp. PCC 9106 (c) and *Spirulina* sp. PCC 6313 (e) obtained by SEM. Scale bars represents 10, 10 and 20 μm, respectively. Pb peak showed in the spectrum (obtained by SEM-EDX) is indicated in each case by arrow (b), (d) and (f).

Communities living in microbial mats are often subjected to strong environmental changes, including the presence of pollutants, among the metals. Many studies have been carried out to demonstrate the capacity of microorganisms to sequestrate metals: on bacteria (Borrego and Figueras, 1997), fungi (Lo et al., 1999), algae (Mehta and Gaur, 2001) and cyanobacteria (De Philippis et al., 2003; Incharoensakdi and Kitjaharn, 2002).

Although some species of plants, invertebrates, fish, birds and mammals have been used as bioindicators of contamination and water quality (Azevedo et al., 2009; Faria et al., 2010) and soil quality (Garitano-Zavala et al., 2010; Gjorgieva et al., 2011; Sánchez-Chardi et al., 2007), nothing is known about the use of algae and cyanobacteria as metal bioindicators in polluted microbial mats.

As was mentioned above, given the ecological importance of metal pollution and considering that no bioindicators have been described for this type of pollution in microbial mats; we propose, in this work, to select bioindicators for lead.

We consider the following characteristics to select the bioindicators: ubiquity and abundance of the selected microorganisms; ease of growth under laboratory conditions; sensitivity to the chosen metal and the ability to accumulate it.

In the recent years, our working group has optimized high-resolution microscopy techniques (CLSM, SEM-EDX and TEM-EDX)
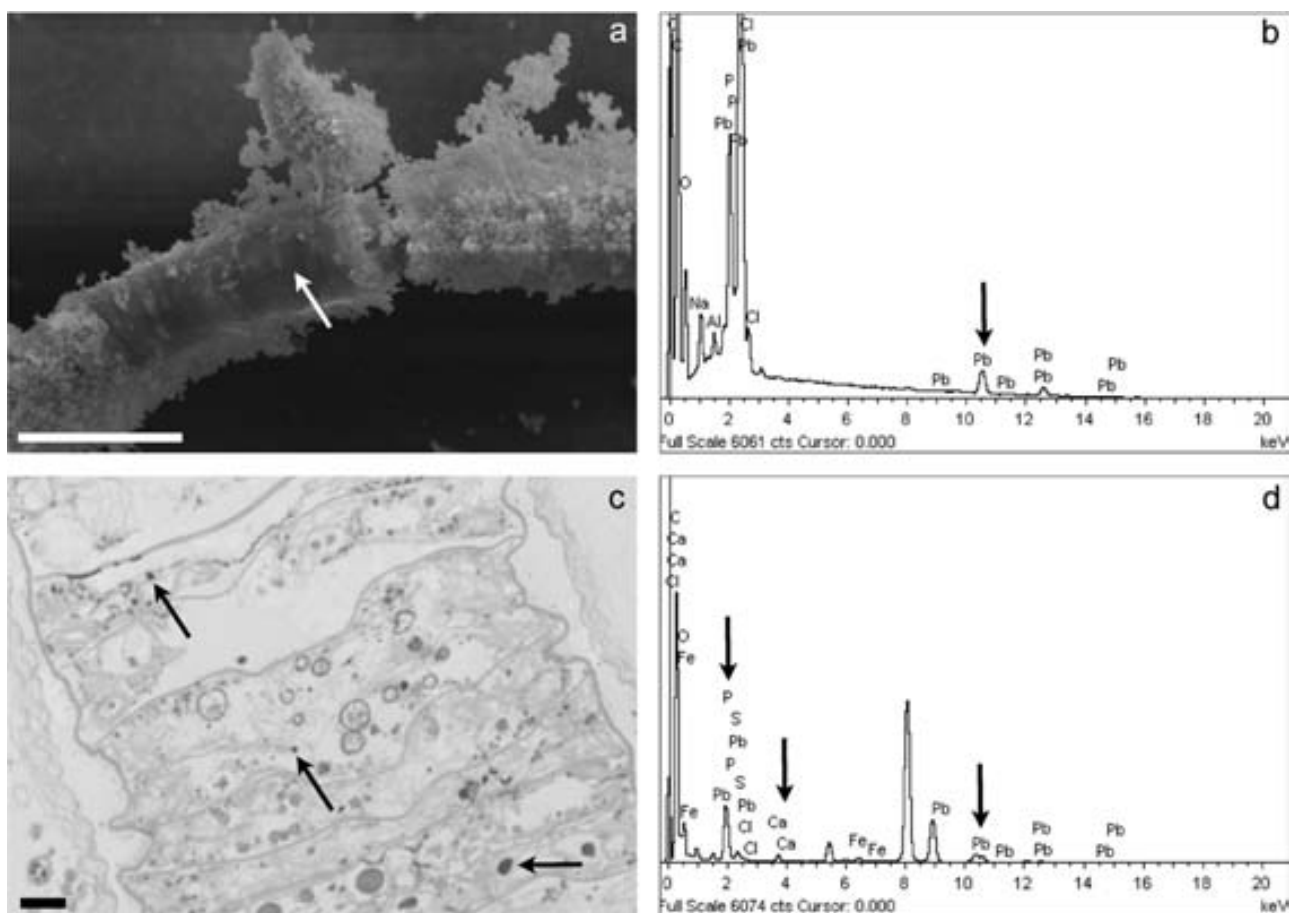
Fig. 3. Lead sequestration by *Oscillatoria* sp. PCC 7515. Analysis point indicated by arrow from *Oscillatoria* sp. PCC 7515 obtained by SEM. Scale bar represents 20 μm (a). Pb peak showed in the spectrum obtained by SEM-EDX (see arrow) (b). High electron-dense inclusions from an ultrathin section of *Oscillatoria* PCC 7515 are indicated by arrows. Scale bar represents 2 μm (c). Pb, Ca and P peaks showed in the spectrum obtained by TEM-EDX from high electron-dense (see arrows) (d).

to determine *in vivo* the effect of different metals on phototrophic microorganisms (sensitivity) and to detect the ability of these microorganisms to capture metals extra- and/or intracellularly (Burnat et al., 2009, 2010; Maldonado et al., 2010).

For this research, we chose three cyanobacteria from the Pasteur culture collection of cyanobacteria (PCC) that are abundant in the Ebro delta microbial mats. Lead was chosen because it is a non-essential toxic metal and in the past, lead pellets were reported to have been found in high densities in the Ebro river sediment (Mateo et al., 1997).

The aim of this work is therefore to assess the potential of different cyanobacteria to be considered bioindicators of lead pollution in coastal microbial mats, using high-resolution microscopy techniques.

2. Material and methods

2.1. Cyanobacterium strains and culture conditions

Oscillatoria sp. PCC 7515, *Chroococcus* sp. PCC 9106 and *Spirulina* sp. PCC 6313, were chosen from the PCC (Herdman et al., 2005) because they are always present in Ebro delta microbial mats. The first strain was cultivated in Marine medium (MN) with nitrate omitted (Rippka et al., 1979) and the other two strains were grown in a 1:1 (v/v) BG11 and ASN III mixture medium (Rippka et al., 1979). All culture strains were grown at 27 °C and 15 μE m^{-2} s^{-1}.

2.2. Sampling site and microcosms setup

Microbial mat samples were collected from the Ebro delta on October 2009. Samples were taken in 55 mm × 43 mm × 88 mm poly(methyl methacrylate) boxes and transferred to the laboratory. Two microcosm experiments were prepared, one microcosm was used as a control experiment (unpolluted) and the other microcosm was polluted with 10 mM [Pb(NO$_3$)$_2$] solution. The microcosms were maintained in laboratory conditions for 9 days, after which the metal solution was removed from the polluted microcosm. Samples for electron microscopy and CLSM were taken with glass cores Ø (6 mm).

2.3. Confocal laser scanning microscopy

Cultures of the cyanobacterium strains were polluted at different Pb concentrations: 0 (unpolluted culture), 0.1, 0.5, 0.75, 1 and 2 mM (polluted cultures). All experiments were performed for a period of 9 days under the same culture conditions mentioned in Section 2.1.

Pigment analysis was carried out by the λscan function of CLSM (Leica TCS SP5; Leica Heidelberg, Germany). This technique made it possible to obtain information about the state of pigments on the basis of the emission wavelength region and the fluorescence intensity emitted. Each image sequence was acquired by scanning the same *xy* optical section throughout the visible spectrum. Images were acquired at the *z* position at which the fluorescence was maximal, and acquisition settings were constant throughout each
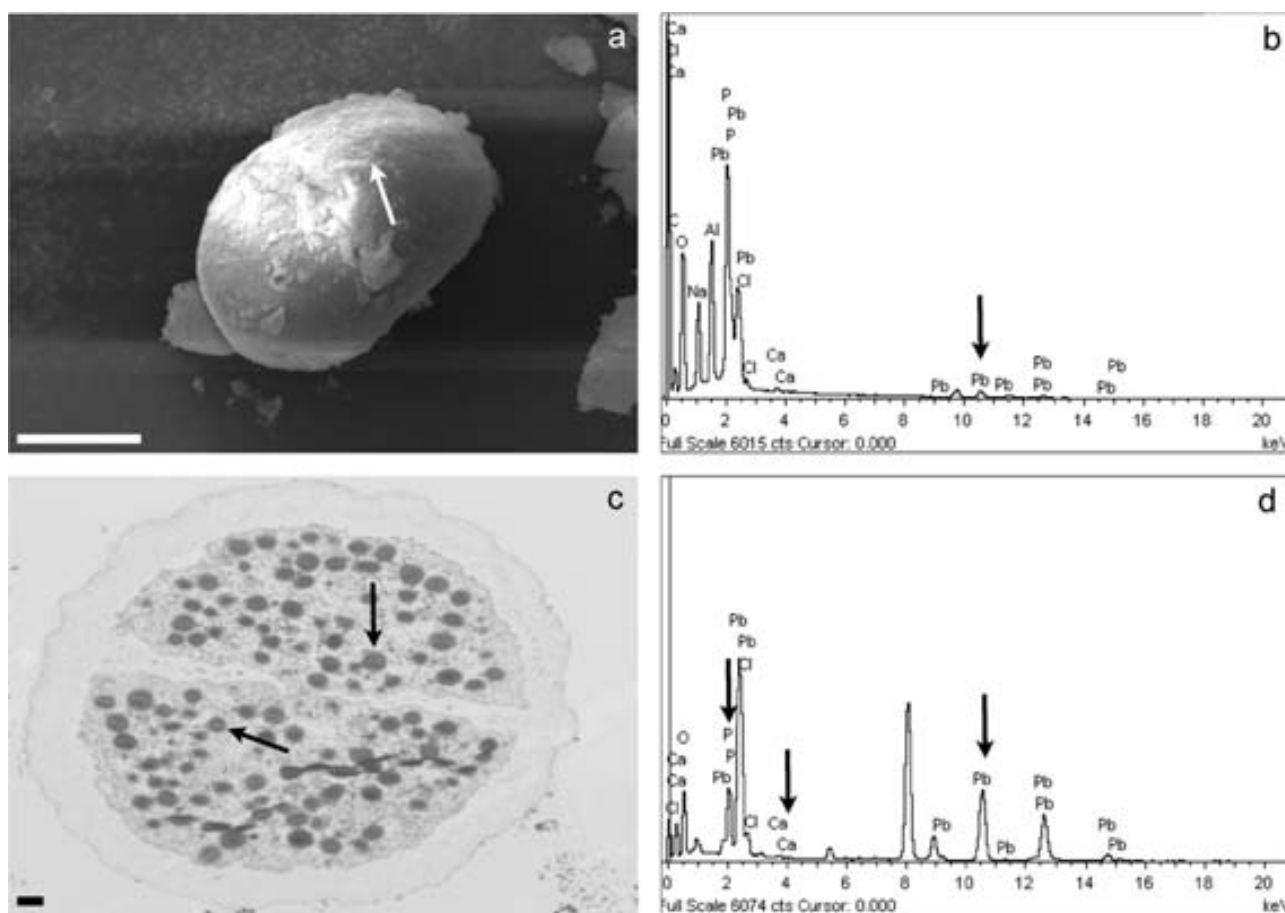
Fig. 4. Lead sequestration by *Chroococcus* sp. PCC 9106. Analysis point indicated by arrow from *Chroococcus* sp. PCC 9106 obtained by SEM. Scale bar represents 10 μm (a). Pb peak showed in the spectrum obtained by SEM-EDX (see arrow) (b). High electron-dense inclusions from an ultrathin section of *Chroococcus* PCC 9106 are indicated by arrows. Scale bar represents 2 μm (c). Pb, Ca and P peaks showed in the spectrum obtained by TEM-EDX from high electron-dense inclusions (see arrows) (d).

experiment. The sample excitation was carried out with an Argon Laser at 488 nm (λ_{exe} 488) with a λ step size between 3 and 4.74 nm for an emission wavelength between 510 and 752 nm.

In order to measure the mean fluorescence intensity (MFI) of the $xy\lambda$ data sets, the Leica Confocal Software (Leica Microsystems CMS GmbH) was used. The regions-of-interest (ROIs) function of the software was used to measure the spectral signature of the samples. 70 ROIs of 1 μm^2 taken from cells from each sample were analyzed.

2.4. Scanning electron microscopy

Scanning electron microscopy coupled to EDX (SEM-EDX) was used in order to assess whether the selected cyanobacteria strains and cyanobacteria from the polluted microcosm were able to capture Pb extracellularly. Cultures of the three strains and microcosms experiments were both polluted with 10 mM Pb for a period of 9 days. This concentration was used to obtain a rapid response to Pb from cyanobacteria.

The following procedure was used: samples were fixed in 2.5% glutaraldehyde Millonig buffer phosphate (0.1 M pH 4) for 2 h and washed four times in the same buffer. They were then dehydrated in graded series (30%, 50%, 70%, 90%, and 100%) of ethanol and dried by critical point. The samples were mounted on metal stubs, coated with gold and viewed in a Zeiss EVO® MA 10 scanning electron microscope (Carl Zeiss NTS GmbH, Oberkochen, Germany). An EDX Link Isis-200 (Oxford Instruments, Bucks, England) operated at 20 kV coupled to the microscope was used.

2.5. Transmission electron microscopy

TEM was used to determine the ultrastructure of cyanobacteria and TEM coupled to EDX (TEM-EDX) to assess whether the cyanobacteria strains and cyanobacteria from the polluted microcosm were able to bioaccumulate the studied metal intracellularly. Culture conditions were the same as described for scanning electron microscopy.

The following procedure was used: samples were fixed in 2.5% glutaraldehyde Millonig buffer phosphate (0.1 M pH 4) for 2 h and washed four times in the same buffer. Samples were post-fixed in 1% OsO$_4$ at 4 °C for 2 h, and washed four times in the same buffer. They were then dehydrated in graded series (30%, 50%, 70%, 90%, 100%) of acetone and embedded in Spurr resin. To show a better quality image, ultrathin sections of 70 nm were mounted on carbon-coated copper grids and stained with uranil acetate and lead citrate. Samples were viewed in a Hitachi H-7000 electron microscope (Hitachi Ltd., Tokyo, Japan). To analyze the samples with EDX, sections of 200 nm thick mounted on carbon-coated titanium grids were used. These sections were not stained with lead citrate in order to avoid elemental substitution during the analysis. Samples were analyzed with a Jeol Jem-2011 (Jeol Ltd., Tokyo, Japan). An EDX Link Isis-200 (Oxford Instruments, Bucks, England) operated at 20 kV coupled to this microscope was used.

2.6. Statistical analysis

The MFI analysis of $\lambda scan$ experiments, were carried out by one-way analysis of variance (ANOVA) and Tukey and Bonferroni's
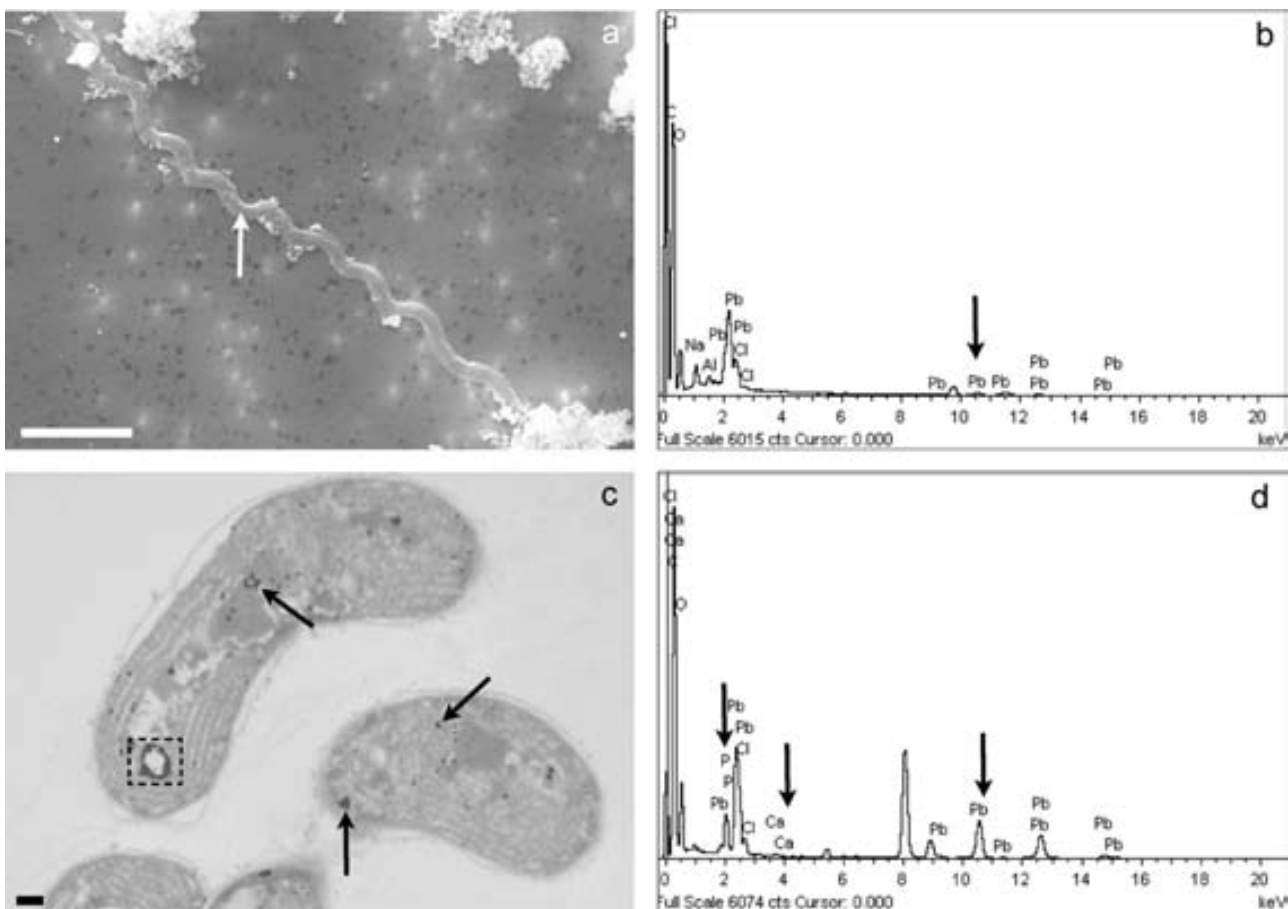
Fig. 5. Lead sequestration by *Spirulina* sp. PCC 6313. Analysis point indicated by arrow from *Spirulina* sp. PCC 6313 obtained by SEM. Scale bar represents 10 μm (a). Pb peak showed in the spectrum obtained by SEM-EDX (see arrow) (b). High electron-dense inclusions from an ultrathin section of *Spirulina* PCC 6313 are indicated by arrows. PHA granule is marked with a dashed rectangle. Scale bar represents 0.2 μm (c). Pb, Ca and P peaks showed in the spectrum (obtained by TEM-EDX) from high electron-dense inclusions (see arrows) (d).
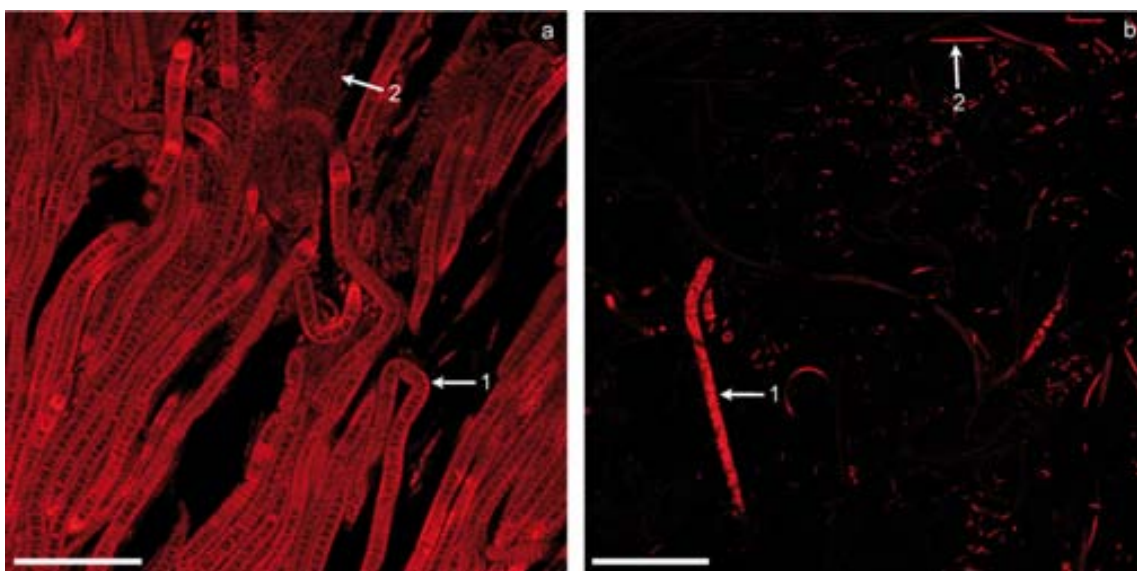

Fig. 6. CLSM images from unpolluted and polluted microcosms. CLSM image from the unpolluted microcosm shows *Oscillatoria*-like (1) and *Microcoleus*-like (2). Scale bar represents 50 μm (a). CLSM image from polluted microcosm shows *Lyngbya*-like (1) and *Phormidium*-like (2). Scale bar represents 50 μm (b).
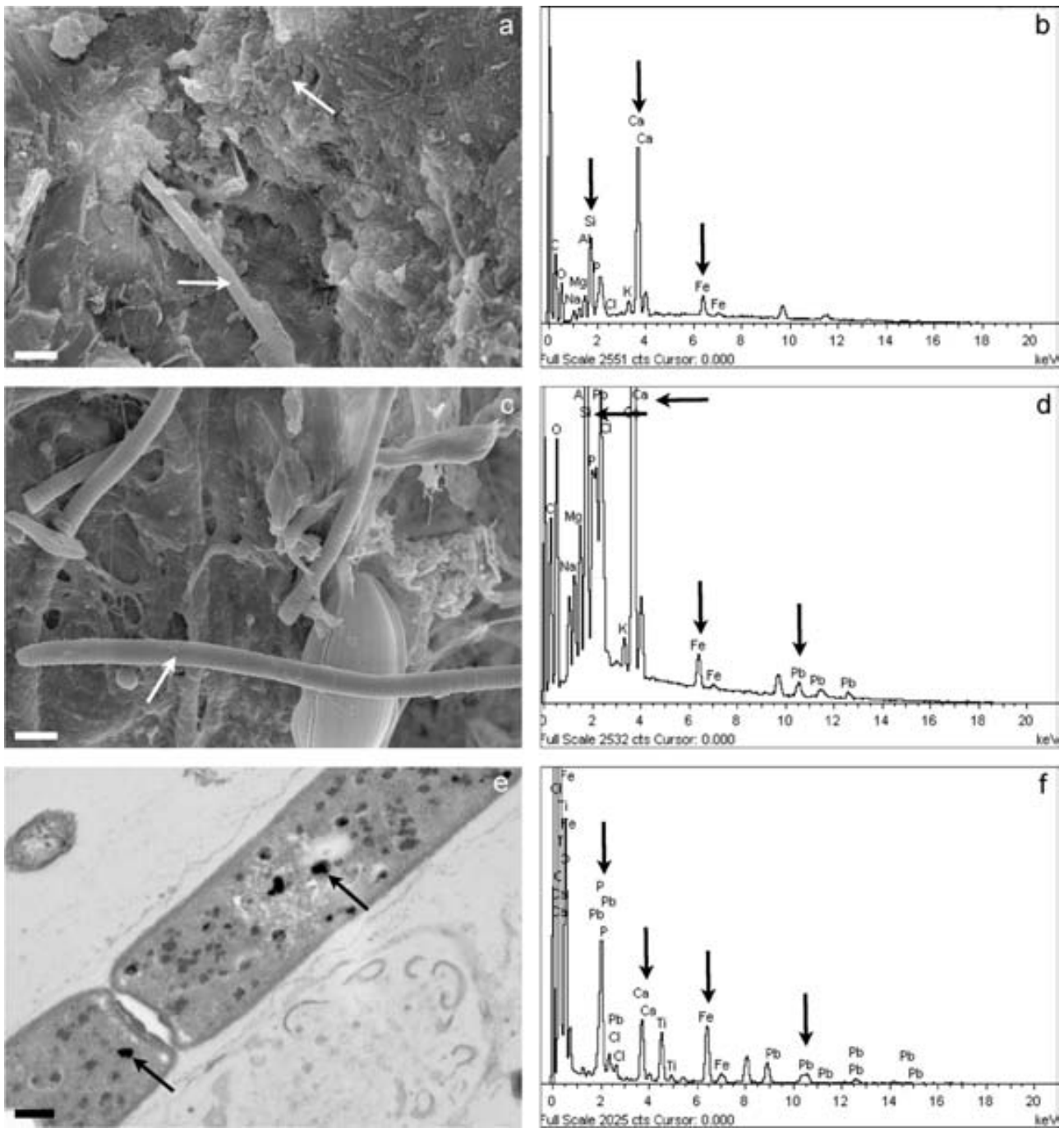
Fig. 7. Lead sequestration by cyanobacteria in microcosm experiments. Analyses points indicated by arrows from cyanobacteria in the uncontaminated microcosm obtained by SEM (a). Fe, Si and Ca peaks showed in the spectrum obtained by SEM-EDX (see arrows) (b). Analysis point indicated by arrow from *Lyngbya*-like in the contaminated microcosm obtained by SEM (c). Fe, Si, Ca and Pb peaks showed in the spectrum obtained by SEM-EDX (see arrows). (d). High electron-dense inclusions from an ultrathin section of *Phormidium*-like in the polluted microcosm are indicated by arrows (e). Fe, P, Ca and Pb showed in the spectrum obtained by TEM-EDX from high electron-dense inclusions are (see arrows) (f).

comparison *post hoc* tests. Significant differences were accepted at $p < 0.05$. The analyses were performed using SPSS software (version 19.0 for Mac OS X).

3. Results and discussion

The bioindicators were chosen on the basis of whether the microorganisms being studied fulfilled the aforementioned conditions: (a) ubiquity and abundance of the chosen microorganisms; (b) ease of growth under laboratory conditions; (c) sensitivity to the chosen metal; (d) the ability to accumulate it.

3.1. Lead-sensitivity of cyanobacteria strains determined by means of the CLSM-λscan function

The three cyanobacteria studied in this work grew both in liquid medium and solid medium. These media and the culture conditions have been mentioned above in Section 2.1. With the liquid medium cultures (unpolluted and polluted) as a starting-point, the corresponding experiments were performed using CLSM.

The CLSM-λscan experiments corresponding to each of the cyanobacteria tested were performed to determine sensitivity to Pb and its effect *in vivo*. To this end, the state of pigments was considered by means of the maximum fluorescence signal

detected at: 575 nm [phycoerythrin (PE)] for *Oscillatoria* sp. PCC 7515 (Bryant, 1982); 680 nm [chlorophyll *a* (chl *a*)] for *Chroococcus* sp. PCC 9106 and 652 nm [chlorophyll *a* (chl *a*)] for *Spirulina* sp. PCC 6313.

(A) The autofluorescence detected in control cultures of *Oscillatoria* sp. PCC 7515 corresponding to a *xyz* optical section is shown in Fig. 1a. In the plot the maximum fluorescence peak is shown, which corresponds to 575 nm (Fig. 1b). The results demonstrate that the fluorescence peak decreases while the Pb concentration increases from 0 mM (control experiment) to 2 mM. The 0.1 mM fluorescence spectrum followed the same pattern as the control culture, although at the other concentrations (0.5, 0.75, 1 and 2 mM) there is a displacement of the fluorescence peak from 575 nm towards to 570 nm. In this study a blue-shift of the MFI from 0.5 mM has also been observed, which corresponds with the changes in the PE pigment. An analog effect has been demonstrated by Murthy and Mohanty (1991), in *Spirulina platensis* growing in the presence of mercury and at high temperatures (Murthy et al., 2004). Statistically significant differences were found between all the conditions tested ($F = 236.980$) ($p < 0.05$). Using the Tukey and Bonferroni comparison tests, the only concentrations that were not significantly different were those of 0.5 mM and 0.75 mM, as these concentrations have the same effect on the MFI. The minimum metal concentration (when compared with the control) that affected the intensity of the pigment was 0.1 mM Pb.

(B) *Chroococcus* sp. PCC 9106 *xyz* optical section corresponding to the autoflurorescence observed in control cultures is shown in Fig. 1c. The control spectrum shows the maximum fluorescence peak at 682 nm, which decreases while the Pb concentration increases (from 0 to 2 mM). The 0.1 and 0.5 mM fluorescence spectra follow the same pattern as control cultures, but at 0.5 mM the fluorescence intensity is lower. From 0.75 mM there is a decrease in the MFI reaching the lowest levels and no fluorescence was detected at the highest concentration tested (2 mM). Statistically significant differences were found between all the conditions tested ($F = 1709.574$) ($p < 0.05$). The minimum metal concentration (when compared with the control) that affected the intensity of the pigment was 0.5 mM Pb.

(C) As in the two experiments mentioned above, a *Spirulina* sp. PCC 6313 *xyz* optical section corresponding to the autofluorescence in control cultures is shown in Fig. 1e. A maximum fluorescence peak is detected at 652 nm. Moreover, at the highest concentrations (1 and 2 mM) the MFI is drastically reduced (Fig. 1f). The 2 mM concentration was not included in this figure because no fluorescence signal was detected. Statistically significant differences were found between all the conditions tested ($F = 1109.582$) ($p < 0.05$). The minimum metal concentration (when compared with the control) that affected the intensity of the pigment was 0.1 mM Pb. In Fig. 1f, the fluorescence spectra obtained at 0.5 mM and 0.75 mM do not correspond to the expected trend, making this particular case difficult to interpret.

From these results, it can be concluded that *Oscillatoria* sp. PCC 7515 and *Spirulina* sp. PCC 6313 are sensitive to lead at the concentration of 0.1 mM, while *Chroococcus* sp. PCC 9106 is sensitive at 0.5 mM. In all three cases there is an inverse correlation between the MFI and the concentration of the metal (Fig. 1b, d and f). These results are consistent with those obtained by Massieux et al. (2004), who showed that metals could affect the physiology and photosynthetic activity of the phototrophic community.

3.2. Lead sequestration in cyanobacteria strains by SEM-EDX and TEM-EDX

A rapid test by SEM-EDX was performed on each one of the cyanobacteria studied to determine the minimum concentration at which metal uptake occurs (considering the results obtained by CLSM-λscan). Moreover, in polluted cultures analyzed we also tested different parts of the filters to be sure that Pb was retained only in cells.

The result was positive in all of them for the minimum metal concentration used, and negative for the control (Fig. 2).

With the purpose or ascertaining whether the three strains of cyanobacteria could accumulate metal internally, cultures of each strain were polluted with 10 mM, to increase the signal, and analyzed by TEM-EDX. In all cases, images by SEM-EDX were also obtained to compare the results by both methodologies.

In the control cultures (without Pb), and using these techniques, it was found that the metal was not detected either internally or externally in any of the three microorganisms.

On the other hand, the results observed after Pb pollution for each of the three cyanobacteria are as follows.

Oscillatoria sp. PCC 7515 is seen to accumulate Pb in the EPS, which can be observed in the EDX spectrum (Fig. 3a and b). Furthermore, the ultrathin sections of this microorganism show abundant high electron-dense inclusions of different sizes in its cytoplasm (Fig. 3c). EDX analysis of these demonstrates the presence of PP inclusions (P and Ca peaks) which retain lead (Pb main peak at 10.5 KeV indicated by arrow) (Fig. 3d). These results are in accordance with those obtained by Jensen and Sicko (1974).

In the samples of *Chroococcus* sp. PCC 9106 the results obtained by SEM-EDX and TEM-EDX are similar to those shown in *Oscillatoria* sp. PCC 7515 and they demonstrate this microorganism's ability to accumulate Pb extra- and intracelullarly (Fig. 4).

Finally, *Spirulina* sp. PCC 6313 shows a similar ability to other cyanobacteria to retain Pb in the EPS (Fig. 5a and b). Ultrathin sections show expanded thylakoids, high electron-dense PP inclusions and polyhydroxyalkanoate (PHA) granules, identified previously in an optical microscope by Sudan Black stain (Fig. 5c). This cyanobacterium also accumulates Pb in PP inclusions (Fig. 5d). Furthermore, the presence of PHA granules in *Spirulina* sp. PCC 6313, probably demonstrate a nitrogen limitation in the lead-polluted cultures, that has also been shown in *Synechocystis* sp. strain PCC 6803 (Schlebusch and Forchhammer, 2010).

Taken together, the results described in this section show that the three cyanobacteria are able to accumulate the metal externally (in the EPS) and internally in the intracytoplasmatic PP inclusions.

Previous studies also showed that *Microcoleus* sp. one of the dominant cyanobacteria in the Ebro delta microbial mats, accumulates lead in the PP inclusions (Burnat et al., 2010).

3.3. Lead detection in cyanobacteria from polluted microcosms by SEM-EDX and TEM-EDX

In order to ascertain that cyanobacteria can be bioindicators of lead pollution in microbial mats, a microcosm was polluted with a lead concentration of 10 mM and the results were compared with a control microcosm (unpolluted). In this case, since the images of natural environments do not allow us a clear view of the variety of microorganism, due to the high microbial density and the presence of EPS, which masks the results, a CLSM analysis of the *in vivo* diversity was done previously. In the unpolluted microcosm *Oscillatoria*-like was the dominant cyanobacterium while *Phormidium*-like and *Lyngbya*-like were the most abundant in the polluted microcosm. In the latter, very small and thin unidentified filaments were also present (Fig. 6a and b).

SEM image of the control microcosm shows a community of microorganisms covered by polysaccharides (Fig. 7a). No Pb is detected by EDX in these samples. The detected elements are: iron (Fe) from metal sulfides, silicon (Si) from diatoms and calcium (Ca) from photosynthetic bacteria. These elements are always present in microcosms from Ebro delta microbial mats (Fig. 7b).

In the polluted microcosm, a great cellular lysis of *Oscillatoria*-like organisms was evident, probably due to the high lead concentrations. On the other hand, in these conditions the *Lyngbya*-like and *Phormidium*-like cyanobacteria are selected (Fig. 7c), in accordance to the images obtained by CLSM. EDX analysis of these microorganisms gives similar results (Fe, Si and Ca peaks), to those obtained in the control microcosm, but in this case Pb is also detected (Fig. 7d).

The ultrathin sections obtained by TEM of *Phormidium*-like and *Lyngbya*-like cyanobacteria from the polluted microcosm show, as in the cyanobacteria cultures studied in this work, an increase in PP inclusions. An ultrathin section of *Phormidium*-like cyanobacteria is shown in Fig. 7e. TEM-EDX microanalysis also detects Pb as well as the elements P and Ca that can already be seen in the control microcosm. No other toxic metals that could be polluting the original mat are detected in the PP inclusions (Fig. 7f).

4. Conclusions

Considering all the results presented in this paper, it can be concluded that the three cyanobacteria studied could be good bioindicators of pollution by Pb. Furthermore, if these results are compared to those obtained in the polluted microcosm, at a higher concentration than the tested in the cultures, we observe a positive selection of *Lyngbya*-like and *Phormidium*-like cyanobacteria with respect to *Oscillatoria*-like organisms, in the control experiment.

All cyanobacteria studied in this work – both cultures and microcosms – accumulate PP inclusions that increase with stress, due to lead pollution and the fact that all these inclusions retain this metal.

On the other hand, according to the results obtained, it is possible to link bioaccumulation processes to the toxicological effects of the lead, since this metal is toxic at any concentration. Lead also, as has been demonstrated in microcosm experiments, causes changes in the diversity, thus the ecological importance of metal pollution.

We believe that the application of these techniques open up broad prospects for future studies of metal ecotoxicity, as they will allow us to determine whether pollution by other metals could have occurred in natural environments.

Acknowledgments

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