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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-*λscan*) 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.

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.

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The CLSM coupled to a spectrofluorometric detector (λ*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-λ*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 °C and 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.

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. λ*scan function*

Pigment analysis of DE2009 microalga cultures was determined by λ*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*λ, 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.

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 μm^{-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 °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 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.

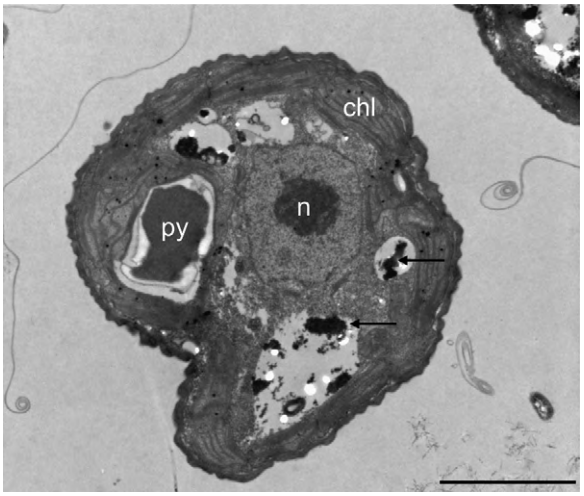

Fig. 1. Ultrathin section of the DE2009 microalga. Chloroplast showing thylakoids (chl), nucleus (n), pyrenoid (py) and HE inclusions (indicated by arrows). Scale bar represents 2 μm.

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 ($\mathrm{mgC\,cm^{-3}}$) in the control experiment to 3.82 ± 0.80 ($\mathrm{mgC\,cm^{-3}}$) 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

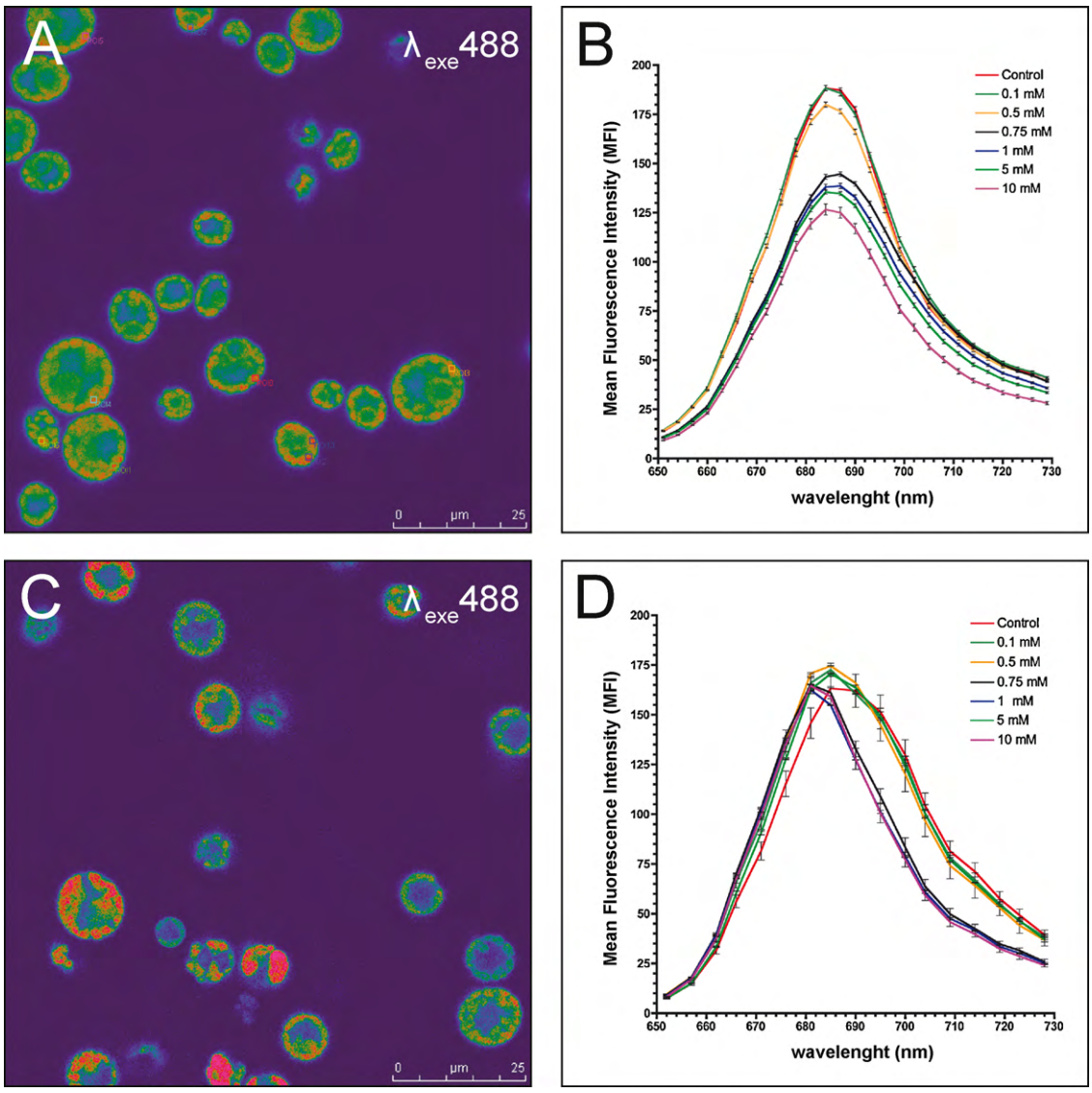

Fig. 2. CSLM images and λ*scans* plots of DE2009 microalga growing at pH 7 and pH 4. (A) and (C) represent CLSM images from a non-Pb treated cultures of DE2009 growing at pH 7 and 4 respectively. In these confocal images the pseudo-colour palette 4 (Leica Aplication Suite, Leica Microsystems CMS GmbH) 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.)

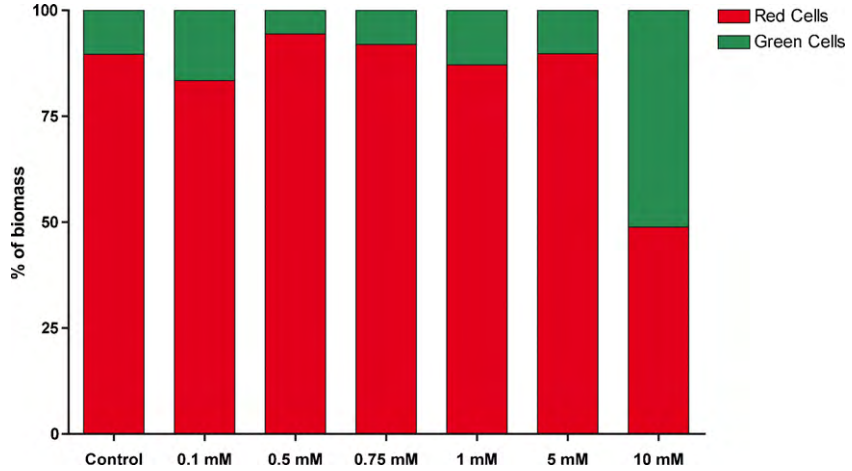

Fig. 3. Relative abundance of red and green DE2009 microalga cells at different Pb concentrations (expressed as a percentage of biomass). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

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 *Microcoleus* 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),

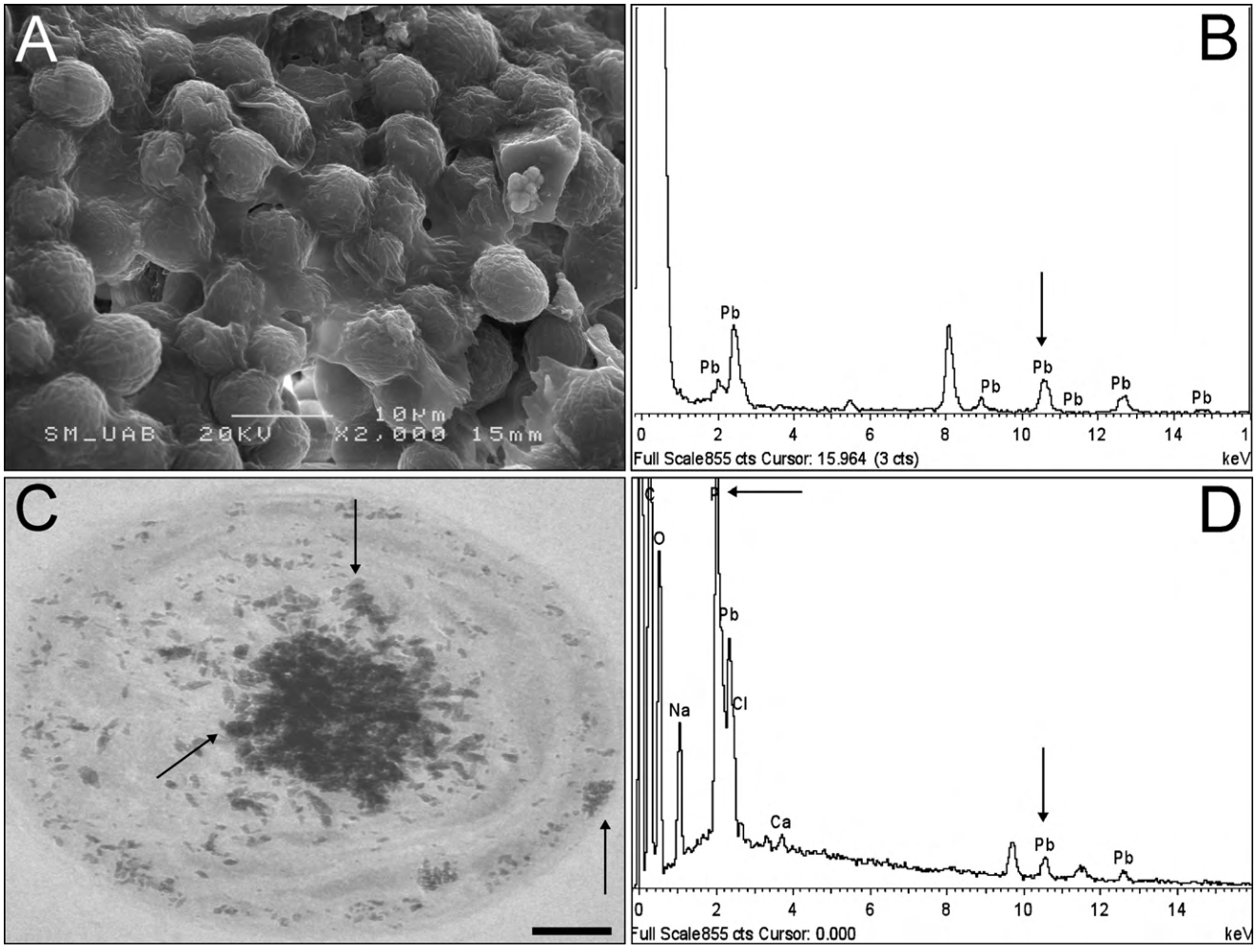

Fig. 4. DE2009 microalga culture treated with 10 mM Pb. SEM image. Scale bar represents 10 μm (A). EDX spectrum coupled to SEM. Arrow indicates the main Pb peak at 10.5 keV (B). Ultrathin section of DE2009 microalga. Arrows indicate the distribution of HE inclusions. Scale bar represents 0.2 μm (C).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

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References

[1] R. Guerrero, J. Urmeneta, G. Rampone, Distribution of types of microbial mats at the Ebro Delta, Spain, BioSystems 31 (1993) 135–144.
[2] I. Esteve, D. Ceballos, M. Martínez-Alonso, N. Gaju, R. Guerrero, Microbial mats: structure, development and environmental significance, in: L.J. Stal, P. Caumette (Eds.), NATO ASI Series G: Ecological Sciences, Springer, Heidelberg, 1994, pp. 4165–4420.
[3] T. Nakagawa, M. Fukui, Phylogenetic characterization of microbial mats and streamers from a Japanese alkaline hot spring with a thermal gradient, J. Gen. Appl. Microbiol. 48 (2002) 211–222.
[4] A. Wieland, M. Kuhl, L. McGowan, A. Fourcans, R. Duran, P. Caumette, T. Garcia de Oteyza, J.O. Grimalt, A. Solé, E. Diestra, I. Esteve, R.A. Herbert, Microbial mats on the Orkney Islands revisited: microenvironment and microbial community composition, Microb. Ecol. 46 (2003) 371–390.
[5] A. Fourçans, T. Garcia de Oteyza, A. Wieland, A. Solé, E. Diestra, J. van Bleijswijk, J.O. Grimalt, M. Kuhl, I. Esteve, G. Muyzer, P. Caumette, R. Duran, Characterization of functional bacterial groups in a hypersaline microbial mat community (Salins-de-Giraud, Camargue, France), FEMS Microbiol. Ecol. 51 (2004) 55–70.
[6] E. Diestra, A. Solé, M. Martí, T. Garcia de Oteyza, J.O. Grimalt, I. Esteve, Characterization of an oil-degrading *Microcoleus* consortium by means of confocal scanning microscopy, scanning electron microscopy and transmission electron microscopy, Scanning 27 (2005) 176–180.
[7] O. Sánchez, E. Diestra, I. Esteve, J. Mas, Molecular characterization of an oil-degrading cyanobacterial consortium, Microb. Ecol. 50 (2005) 580–588.
[8] M. Burnat, E. Diestra, I. Esteve, A. Solé, *In situ* determination of the effects of lead and copper on cyanobacterial populations in microcosms, PLoS One 4 (7) (2009) e6204.
[9] R. De Philippis, R. Paperi, C. Sili, M. Vincenzini, Assessment of the metal removal capability of two capsulated cyanobacteria, *Cyanobacteria capsulata* and *Nostoc* PCC7936, J. Appl. Phycol. 15 (2003) 155–161.
[10] L.Y. Heng, K. Jusoh, C.H. Ling, M. Idris, Toxicity of single and combinations of lead and cadmium to the cyanobacteria *Anabaena flos-aquae*, Bull. Environ. Contam. Toxicol. 72 (2004) 373–379.
[11] R. Gong, Y. Ding, H. Liu, Q. Chen, Z. Liu, Lead biosorption and desorption by intact and pre-treated *Spirulina maxima* biomass, Chemosphere 58 (2005) 125–130.
[12] C. Solisio, A. Lodi, P. Torre, A. Converti, M. Del Borghi, Copper removal by dry and re-hydrated biomass of *Spirulina platensis*, Bioresour. Technol. 97 (2006) 1756–1760.
[13] M. Valls, V. de Lorenzo, Exploiting the genetic and biochemical capacities of bacteria for the remediation of heavy metal pollution, FEMS Microbiol Rev. 26 (2002) 327–338.
[14] I. Moreno-Garrido, L.M. Lubian, A.M. Soares, Influence of cellular density on determination of EC(50) in microalgal growth inhibition tests, Ecotoxicol. Environ. Saf. 47 (2000) 112–116.
[15] S.N. Luoma, P.S. Rainbow, Why is metal bioaccumulation so variable? Biodynamics as a unifying concept, Environ. Sci. Technol. 39 (2005) 1921–1931.
[16] D. Kaplan, D. Christiansen, S.M. Arad, Chelating properties of extracellular polysaccharides from *Chlorella* spp., Appl. Environ. Microbiol. 53 (1987) 2953–2956.
[17] C.J. Tien, Biosorption of metal ions by freshwater algae with different surface characteristics, Process. Biochem. (2002) 605–613.
[18] C.J. Tien, Copper adsorption kinetics of cultured algal cells and freshwater phytoplankton with emphasis on cell surface characteristics, J. Appl. Phycol. 17 (2005) 379–389.
[19] A. Sánchez-Chardi, M.J. Lopez-Fuster, J. Nadal, Bioaccumulation of lead, mercury, and cadmium in the greater white-toothed shrew, *Crocidura russula*, from the Ebro Delta (NE Spain): sex- and age-dependent variation, Environ, Pollution 145 (2007) 7–14.
[20] M. Roldán, F. Thomas, S. Castel, A. Quesada, M. Hernández-Marine, Non-invasive pigment identification in single cells from living phototrophic biofilms by confocal imaging spectrofluorometry, Appl. Environ. Microbiol. 70 (2004) 3745–3750.
[21] A. Solé, J. Mas, I. Esteve, A new method based on image analysis for determining cyanobacterial biomass by CLSM in stratified benthic sediments, Ultramicroscopy 107 (2007) 669–673.
[22] W.S. Rasband, ImageJ. US National Institutes of Health, Bethesda, MD, USA. http://rsb.info.nih.gov/ij (1997–2010).
[23] J.C. Fry, Direct methods and biomass estimation, Meth. Microbiol. 22 (1990) 441–485.
[24] J. Bloem, M. Veninga, J. Shepherd, Fully automatic determination of soil bacterium numbers, cell volumes, and frequencies of dividing cells by confocal laser scanning microscopy and image analysis, Appl. Environ. Microbiol. 61 (1995) 926–936.
[25] B. Diez, C. Pedros-Alio, T.L. Marsh, R. Massana, Application of denaturing gradient gel electrophoresis (DGGE) to study the diversity of marine picoeukaryotic assemblages and comparison of DGGE with other molecular techniques, Appl. Environ. Microbiol. 67 (2001) 2942–2951.
[26] S. Roy, A.N. Ghosh, A.R. Thakur, Uptake of Pb(2+) by a cyanobacterium belonging to the genus *Synechocystis*, isolated from East Kolkata Wetlands, Biometals 21 (2008) 515–524.
[27] B. Debelius, J.M. Forja, A. DelValls, L.M. Lubian, Toxicity and bioaccumulation of copper and lead in five marine microalgae, Ecotoxicol. Environ. Saf. 72 (2009) 1503–1513.
[28] J. Maldonado, E. Diestra, L. Huang, A.M. Domènech, E. Villagrasa, Z.M. Puyen, R. Duran, I. Esteve, A. Solé, Isolation and identification of a bacterium with high tolerance to lead and copper from a marine microbial mat in Spain, Ann. Microbiol. 60 (2010) 113–120.
[29] M. Burnat, E. Diestra, I. Esteve, A. Solé, Confocal laser scanning microscopy coupled to a spectrofluorometric detector as a rapid tool for determining the in vivo effect of metals on phototrophic bacteria, Bull. Environ. Contam. Toxicol. 84 (2010) 55–60.
[30] A.W. Decho, Exopolymers in microbial mats: assessing their adaptative roles, in: L.J. Stal, P. Caumette (Eds.), Microbial Mats. Structure, Development and Environmental Significance, Springer-Verlag, Berlin, Heidelberg, 1994, pp. 215–219.
[31] L.J. Stal, Cyanobacterial mats and stromatolites, in: B.A. Whitton, M. Potts (Eds.), The Ecology of Cyanobacteria, Kluwer Academic Publishers, Dordrecht, The Netherlands, 2000, pp. 61–120.
[32] L.M. Sicko, Structural variation of polyphosphate bodies in blue-green algae, in: J. Arceneaux (Ed.), Ann. Proc. Electron., Micros. Soc. Amer., Los Angeles, CA, 1972, pp. 218–219.
[33] T.E. Jensen, L.M. Sicko, Phosphate metabolism in blue-green algae. I. Fine structure of the "polyphosphate overplus" phenomenon in *Plectonema boryanum*, Can. J. Microbiol. 20 (1974) 1235–1239.

[34] S.E. Stevens Jr., S.A. Nierzwicki-Bauer, D.L. Balkwill, Effect of nitrogen starvation on the morphology and ultrastructure of the cyanobacterium *Mastigocladus laminosus*, J. Bacteriol. 161 (1985) 1215–1218.

[35] J. Goldberg, H. González, T.E. Jensen, W.A. Corpe, Quantitative analysis of the elemental composition and the mass of bacterial polyphosphate bodies using STEM EDX, Microbios 106 (2001) 177–188.