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Confocal Laser Scanning Microscopy Coupled to a Spectrofluorometric Detector as a Rapid Tool for Determining the In Vivo Effect of Metals on Phototrophic Bacteria

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Abstract In this paper, we determine for the first time the in vivo effect of heavy metals in a phototrophic bacterium. We used Confocal Laser Scanning Microscopy coupled to a spectrofluorometric detector as a rapid technique to measure pigment response to heavy-metal exposure. To this end, we selected lead and copper (toxic and essential metals) and *Microcoleus* sp. as the phototrophic bacterium because it would be feasible to see this cyanobacterium as a good biomarker, since it covers large extensions of coastal sediments. The results obtained demonstrate that, while cells are still viable, pigment peak decreases whereas metal concentration increases (from 0.1 to 1 mM Pb). Pigments are totally degraded when cultures were polluted with lead and copper at the maximum doses used (25 mM $Pb(NO_3)_2$ and 10 mM $CuSO_4$). The aim of this study was also to identify the place of metal accumulation in *Microcoleus* cells. Element analysis of this cyanobacterium in the above mentioned conditions determined by Energy Dispersive X-ray microanalysis (EDX) coupled to Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM), shows that Pb (but not Cu) accumulates externally and internally in cells.

Keywords *Microcoleus* sp. · Heavy metals · Confocal laser scanning microscopy · Spectrofluorometric techniques · Electron microscopy techniques · Energy dispersive X-ray microanalysis

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In recent years, cyanobacteria have been the object of research into metal biosorption and toxicity studies (Li et al. 2004; Gong et al. 2005; Solisio et al. 2006). Nevertheless, few is known about the effect of different heavy metals in individual living cells which is needed to predict the impact of heavy metals on the natural ecosystems.

Confocal Laser Scanning Microscopy (CLSM), based on natural pigment fluorescence emitted by cyanobacteria, is being shown to be an excellent methodology for different types of studies related with phototrophic microorganisms. This optical microscopy technique avoids the need of either manipulating or staining the samples. Moreover, this microscopy allows accurate and non-destructive optical sectioning that generates high-resolution images (optical sections grouped into stacks) where out-of-focus is eliminated. Using CLSM, our work group has made it possible to characterize and identify these photoautotrophic microorganisms in Ebro delta microbial mats (Spain) and also, with the aid of image-analysis computer systems (CLSM-IA), to determine their individual genera biomass accurately and reliably for a large number of samples and stacks of CLSM images (Solé et al. 2007). The efficacy of this method has been demonstrated in highly diverse microbial mat (benthic stratified coastal sediments) samples, oil polluted and unpolluted, in which cyanobacteria are the most abundant (Sole et al. 2009). Few years ago Roldan et al. (2004) proposed a new application of the CLSM coupled to a spectrofluorometric detector, which provides simultaneous 3D information on photosynthetic microorganisms and their fluorescence spectra profiles within thick assemblages. The relevant application of their work was that this setup allowed the discrimination of cells with particular fluorescence spectra profiles within a colony, and the correlation of morphology and individual cell states.

Our paper details the first ever application of *lambda scan* function of CLSM to determine the effect of two heavy metals (lead and copper) on pigments of *Microcoleus* sp., the most abundant cyanobacterium in Ebro delta microbial mats.

Complementary studies using scanning electron microscopy (SEM), transmission electron microscopy (TEM) and energy dispersive X-ray microanalysis (EDX) coupled to SEM and TEM are also made to test the ability of *Microcoleus* sp. for intra- and extracellular biocaption of heavy metals.

Materials and Methods

Microcoleus consortium was isolated from microcosms using the method described by Diestra et al. (2005). Cultures of this consortium were grown into mineral Pfenning medium (Pfennig and Trüper 1992) in anoxic conditions and incubated in light at 15 μE $m^{-2}s^{-1}$ at 27°C.

Lead (Pb) and cooper (Cu) stock solutions were prepared as $Pb(NO_3)_2$ and $CuSO_4$ (Merck KGaA, Darmstadt, Germany) with deionized water and were then sterilized by filtration with polycarbonate membrane filters. Heavy metal stock solutions were prepared 24 h before use and kept in darkness at 4°C.

Stock solutions of Pb and Cu were added to *Microcoleus* consortium cultures with Pfenning mineral medium to reach a final concentration of 0.25, 0.50, 0.75, 1, 25 mM $Pb(NO_3)_2$ and 10 mM $CuSO_4$, and pH was adjusted to 4 with HCl 1N in order to minimize precipitations of metals. Cultures were incubated in the light at 15 μE $m^{-2}s^{-1}$ at 27°C during 9 days.

Pigment analysis of *Microcoleus* consortium cultures polluted with heavy metals was determined by *lambda scan* (*λscan*) function of CLSM (Leica TCS SP2 AOBS; Leica, Heidelberg, Germany), using the method described by Roldan et al. (2004). This technique made it possible to obtain information about the photosynthetic pigments of the microorganisms on the basis of the emission wavelength region and the fluorescence intensity emitted. Each image sequence was obtained by scanning the same *xy* optical section throughout the visible spectrum.

In a first experiment, cultures where polluted with the maximum concentrations of $Pb(NO_3)_2$ (25 mM) and $CuSO_4$ (10 mM), with the aim to induce a rapid response from *Microcoleus* sp. to the high concentrations of metals. The excitation of the sample was carried out: (1) at 488 nm (λ_{exe} 488), with a λ step size of 40 nm for emission wavelength between 500 and 800 nm, and (2) at 561 nm (λ_{exe} 561), with a λ step size of 25 nm for emission wavelength between 575 and 800 nm.

Images were adquired at the z position at which the fluorescence was maximal, and acquisitions setting were not modified thorough the experiment.

In order to measure the mean fluorescence intensity (MFI) of the $xy\lambda$ data sets obtained with CLSM, the Leica Confocal Software was used. The regions-of-interest (ROIs) function of the software were used to measure the spectral signature. For each sample, 10 ROI's of 1 μm^2 taken from filaments of cyanobacterium were analysed. The mean and standard error for each sample and λ_{exe} used, was calculated.

A second experiment with *λscan* function was used with the same procedure mentioned above. In this case, different concentrations of $Pb(NO_3)_2$, and two control cultures (pH 4 and pH 7) were used in order to determine the dose–response emission profile. Excitation of the sample was carried out at 488 nm (λ_{exe} 488), with a λ step size of 40 nm for emission wavelength between 500 and 800 nm. For each sample, 40 ROI's of 1 μm^2 taken from filaments of cyanobacterium were analysed. The mean and standard error for each sample was calculated.

For TEM analysis, samples of *Microcoleus* consortium cultures were fixed in 2.5% glutaraldehyde Milloning buffer phosphate (Milloning 1961) (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 (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 mounted on titanium grids for energy dispersive X-ray microanalysis in order to avoid elemental substitution during the analysis. Samples were analyzed with a Jeol Jem-2011 transmission electron microscope (Jeol LTD, Tokyo, Japan).

For SEM analysis, samples of *Microcoleus* consortium cultures were fixed in 2.5% glutaraldehyde Milloning buffer phosphate (0.1 M, pH 4) 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 LTD, Tokyo, Japan) was used to view the images.

For energy dispersive X-ray microanalysis coupled to SEM, consortium cultures were homogenously distributed and filtered on polycarbonate membrane filters. These filters were then dehydrated and dried by the same procedure as culture samples, but no metal fixing and gold coating were

carried out in order to avoid elemental substitution during the analysis. An energy dispersive X-ray spectrophotometer (EDX) Link Isis-200 (Oxford Instruments, Bucks, England) operated at 20 kV coupled to SEM was used.

Results and Discussion

We have studied the effect of two heavy metals ($Pb(NO_3)_2$ and $CuSO_4$) on *Microcoleus* sp. using the *lambda scan* (*λscan*) function of CLSM, which allowed us to evaluate the physiological state of *Microcoleus* sp., from photosynthetic pigments, in laboratory cultures.

The consortium of *Microcoleus* is formed by *Microcoleus* sp., a filamentous phototrophic oxygenic cyanobacterium, and different heterotrophic bacteria encased in a dense exopolysaccharide (EPS) envelope (Diestra et al. 2005; Sánchez et al. 2005). This bacterial aggregate grows by forming a dense mucilaginous mass in liquid medium and green filamentous colonies in solid medium. Given that

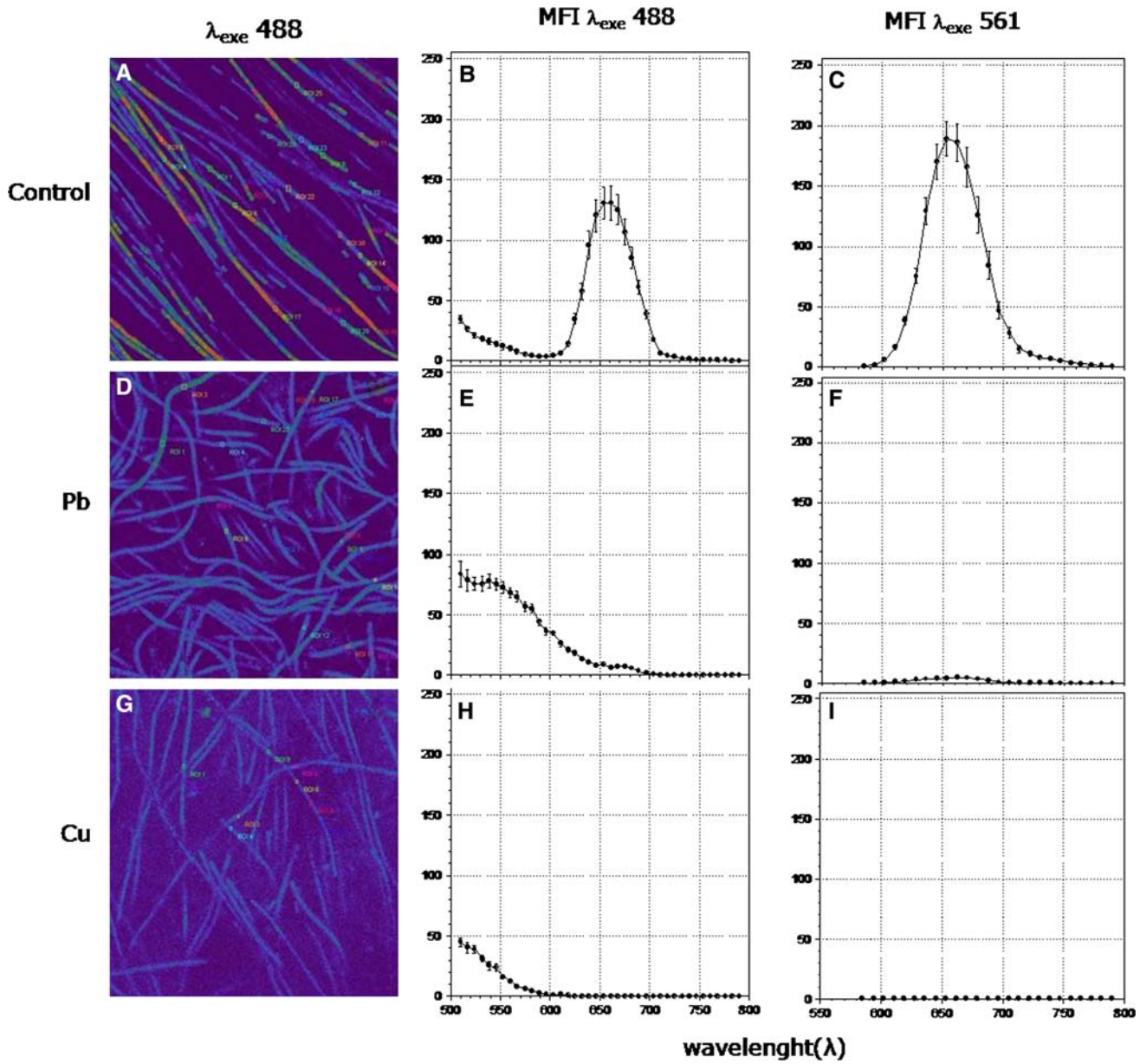

Fig. 1 CLSM images and *lambda scans* of *Microcoleus* consortium control cultures and polluted cultures with lead and copper. (*A*), (*D*) and (*G*) represent pseudocolor confocal *xyz* optical section corresponding to the autofluorescence of Chl *a* and phycobilines of control culture, Pb-polluted culture and Cu-polluted culture, respectively. *Warm colours* are representative of maximum intensities and *cold colours* represent low intensities of fluorescence. Spectral profiles were derived at λ_{exe} 488 nm (*B*, *E*, *H*) and at λ_{exe} 561 nm (*C*, *F*, *I*). Two-dimensional plots represent the mean fluorescence intensity (MFI) spectra: *emission wavelength*, *x* axis; *MFI*, *y* axis

the consortium only contains one type of cyanobacteria identified as *Microcoleus* sp., the spectra can be attributed to this cyanobacterium.

Two different experiments were prepared: (A) The first one using high concentrations of both metals to promote stress response in cells. (B). The second one, to determine the effect of $Pb(NO_3)_2$ in cultures of *Microcoleus* sp. at different concentrations of this metal.

(A) In the first experiment, the results obtained using the *λscan* function of CLSM by measuring the spectrum of absorption of chlorophyll *a* pigment are represented in Fig. 1. Unpolluted cultures showed a peak of maximum absorption at 660 nm corresponding mainly to Chl *a* and phycobilins, both to λ_{exe} 488 nm (Fig. 1b), as to λ_{exe} 561 nm (Fig. 1c). In contrast, pigments were degraded when cultures were polluted with lead and copper at the maximum doses used. In both cases, no fluorescence emission was detected neither when cultures were excited with λ_{exe} 488 nm (Fig. 1e, h) nor with λ_{exe} 561 nm (Fig. 1f, i). These results agree with those obtained by other authors (Massieux et al. 2004) that showed that metals can affect the physiology of the phototrophic community.

(B) In the second experiment, the fluorescence emission of *Microcoleus* sp. pigments was detected when cultures were excited with λ_{exe} 488 nm. The results showed that the pigment peak decreases while the concentration of the metal increases from 0 mM $Pb(NO_3)_2$ (control culture) to 1 mM, while cells are still viable (Fig. 2).

On the other hand, with the aim of proving whether *Microcoleus* sp. could capture metals, unpolluted and polluted cultures were prepared and analysed by EDX coupled to SEM and TEM. The concentrations of $Pb(NO_3)_2$ and $CuSO_4$ assayed were higher than those used by other authors in similar studies, to obtain a rapid response from *Microcoleus* sp. to the high concentrations of metals. Unpolluted cultures were analysed by SEM (Fig. 3a) and by energy dispersive X-ray microanalysis coupled to SEM (Fig. 3b). In the ultrathin sections of the same cells, thylakoids can clearly be differentiated, in which the photosynthetic pigments are contained (Fig. 3c). The thylakoids were expanded, which could be attributed to the acidic conditions used in all experiments, to favour the dissolution of heavy metals. Neither of the two metals used as contaminants were detected by energy dispersive X-ray microanalysis coupled to TEM (Fig. 3d).

Lead polluted cultures were analysed by the same procedures mentioned above. No important differences in the structure of *Microcoleus* sp. were detected in contaminated cultures with lead (Fig. 4a) but the analysis of the energy-dispersion spectra coupled to SEM demonstrated that Pb

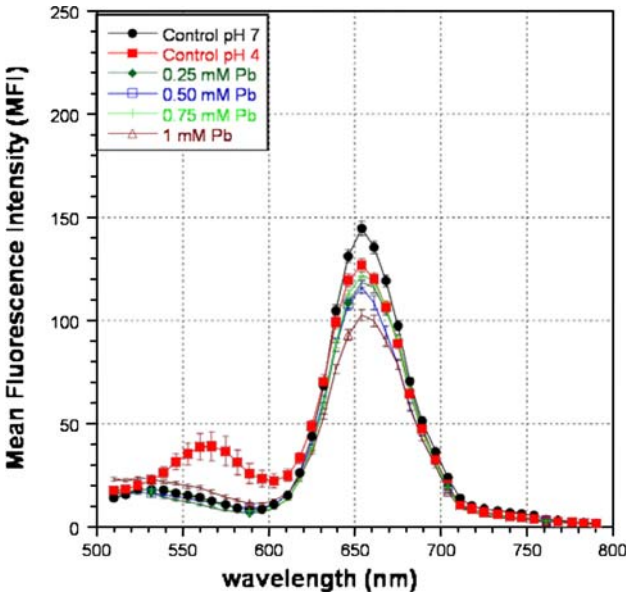

Fig. 2 *Lambda scans* of *Microcoleus* consortium control cultures and polluted cultures with lead at different concentrations. The spectral profiles were derived at λ_{exe} 488 nm. Two-dimensional plot represent the mean fluorescence intensity (MFI) spectra: *emission wavelength*, *x* axis; *MFI*, *y* axis

was found in EPS (Fig. 4b). The ultrathin sections of the *Microcoleus* sp. also exhibit discernible changes after exposure to $Pb(NO_3)_2$. The distortion of cells, as well as the appearance of large intrathylakoidal spaces is shown in Fig. 4c. It is also important to highlight the increase in the number of electrodense inclusions which can be observed in the ultrathin sections of cells grown in the presence of $Pb(NO_3)_2$. These inclusions, peripherally distributed in the periplasmic space of the cells, were identified as polyphosphate granules (see peaks of P and Ca in Fig. 4d). Similar inclusions had been in many cases indicative of the fact that the cells are in adverse culture conditions (Sicko 1972; Stevens et al. 1985), but may also perform other functions such as that of a detoxification mechanism by sequestering heavy metals (Goldberg et al. 2001). The analysis of the energy-dispersion spectra of the inclusions demonstrated that $Pb(NO_3)_2$ was also accumulated inside the cells in polyphosphate inclusions. High peaks of Pb were clearly visible in the same figure.

Figure 5 illustrates a scanning electron micrograph of *Microcoleus* sp. polluted with $Pb(NO_3)_2$ (Fig. 5a), back-scattered electron intensity (Fig. 5b), and the elemental analysis for Pb (Fig. 5c) and for carbon (C) (Fig. 5d) of the same view, showing the localizations of Pb and C along the exopolysaccharide envelopes and filaments, respectively (dots). Localizations of Pb, which are congruent with EPS envelopes, suggest a surface binding mechanism.

Similar experiments were made in *Microcoleus* sp. cultures contaminated with copper. No important

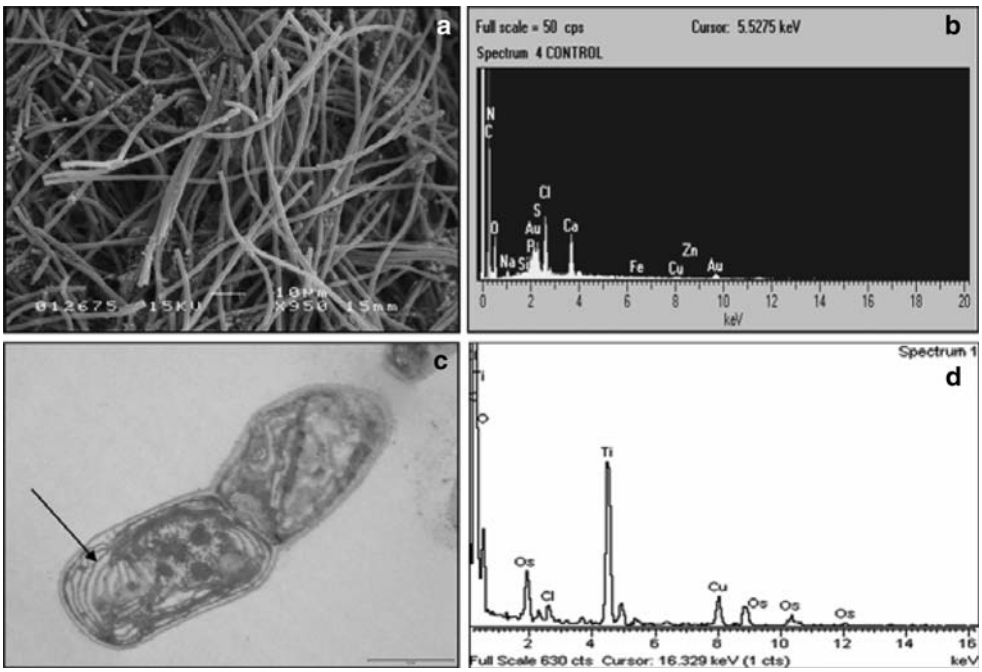

Fig. 3 Non-treated *Microcoleus* consortium cultures. **a** SEM image. *Scale bar* represents 10 μm. **b** Energy dispersive X-ray spectra coupled to SEM. **c** Ultrathin section. Cells showing expanded thylakoids are indicated by *arrow*. *Scale bar* respresent 1 μm. **d** Energy dispersive X-ray microanalysis spectra coupled to TEM from cytoplasm of the cyanobacterium

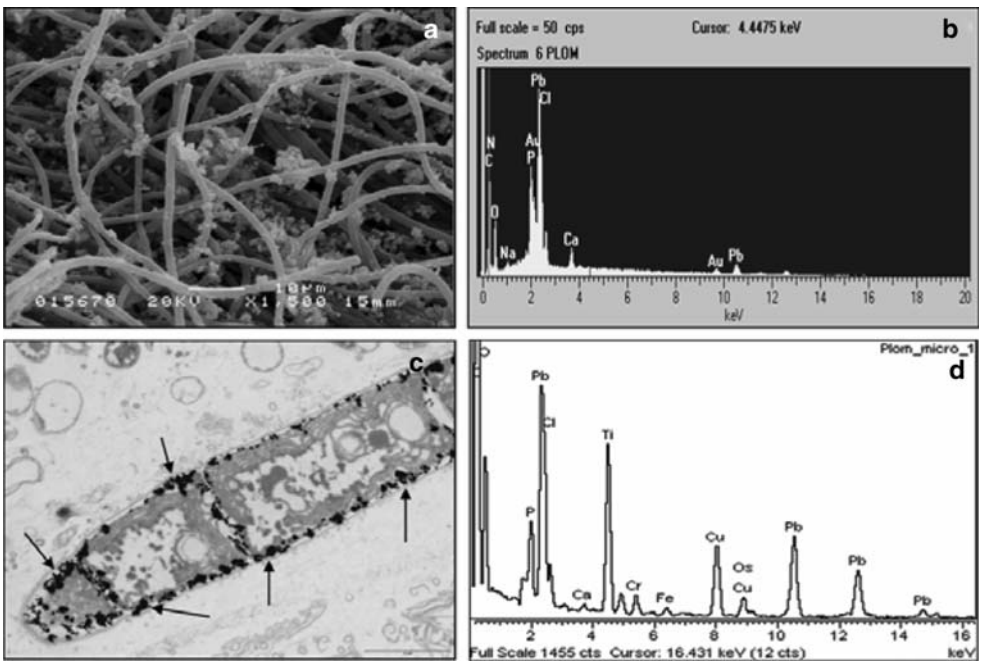

Fig. 4 *Microcoleus* consortium culture treated with 25 mM Pb. **a** SEM image. *Scale bar* represent 10 μm. **b** Energy dispersive X-ray microanalysis spectra coupled to SEM. **c** Ultrathin section. *Arrows* indicate the distribution of electrodense inclusions. *Scale bar* respresent 1 μm. **d** Energy dispersive X-ray microanalysis spectra coupled to TEM from electrodense inclusions

differences in the structure of this cyanobacterium were detected in Cu-contaminated cultures. The spectra of the energy dispersive X-ray microanalysis coupled to SEM demonstrated that this microorganism was not able to accumulate Cu outside the cell. The distortion of cells, as well as the appearance of large intrathylakoidal spaces, were similar to those obtained in cells polluted by $Pb(NO_3)_2$, but no polyphosphate granules and no Cu was detected inside the cells (data not shown).

On the other hand, SEM and TEM coupled to EDX, allows us into determine the capacity of *Microcoleus* sp. to bioaccumulate Pb externally and inside the cells (in intracytoplasmic inclusions). This cyanobacterium could be considered a promising bacterium for removing lead from wastewater in a future work.

In conclusion, this work constitutes the first attempt to study in vivo the cellular response to heavy metal exposure. We thought that CLSM-*lambda scan* could be a

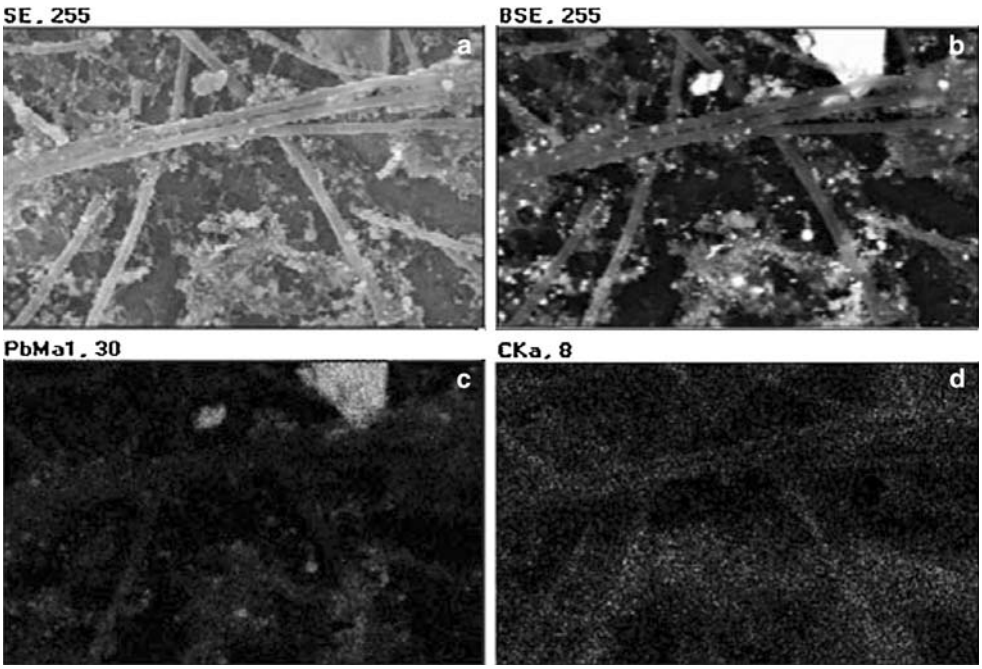

Fig. 5 SEM image of *Microcoleus* sp. filaments surface (**a**), back-scattered electron intensity (**b**), and elemental analysis for lead (**c**) and carbon (**d**) of same view

feasible and easily performed method to make predictive studies regarding the effect of metal on *Microcoleus* sp. The proposed method can also be applied to determining the effect of different metals on distinct types of phototrophic microorganisms.

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