Ultrastructural Changes In *Microcoleus chthonoplastes* Growing In The Presence Of Crude Oil. Applications for Ecological Studies

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A consortium of microorganisms with the capacity to degrade crude oil was isolated from artificial microcosms which simulate polluted Ebro delta microbial mats (Tarragona, Spain). The consortium was formed mainly by a cyanobacterium identified by CLSM, as *Microcoleus chthonoplastes* and by different heterotrophic bacteria. When analyzing *Microcoleus* sp. by TEM, ultrastructural changes within cells growing with and without oil were observed. A quantitative ultrastructural study was performed with samples taken from cells growing in pristine and polluted cultures. No inclusions were detected in the pristine cultures, while the majority of cells growing in polluted cultures had from $1.1x10^{-4}$ to 68.18 % of their volume occupied by highly electrodense inclusions. Elemental analysis of these inclusions using spot setting of the scanning transmission mode in conjunction with an energy dispersive X-ray spectrometer showed that they contain mainly P and Ca, as cytoplasmic polyphosphate granules also do. In this paper we try to discuss if the presence of these granules can be used as indicator of ecotoxicity caused by crude oil in natural ecosystems.

Keywords: *Microcoleus*, crude oil, CLSM, SEM, TEM and X-ray spectometer.

1.Introduction

Microbial mats are benthonic stratified ecosystems only a few millimeters thick, supporting extreme conditions for life. Cyanobacteria are the most abundant microorganisms in these ecosystems and are recognized by the greenish layer that they form on the mat surface. Cyanobacteria are oxygenic phototrophic microorganisms that use light as a source of energy and water as an electron donor. They represent a highly diverse group that colonize all types of extreme habitats and mainly microbial mats [6,7,17,18]

These natural environments have been repeatedly polluted with petroleum due to accidental spills. Interest in studying polluted microbial mats began after the Gulf War in 1991. Studies of these ecosystems demonstrate the existence of cyanobacteria in the areas contaminated with petroleum. Al-Hasan et al. [2], demonstrated that the non axenic cultures of *Microcoleus chthonoplastes* and *Phormidium corium*, isolated from contaminated microbial mats from the Gulf of Arabia, were able to degrade *n*-alkanes. Furthermore, other authors have demonstrated that different strains of oxic phototrophic bacteria were also able to degrade petroleum compounds [12,13]. It is important to point out that many of these studies could not be carried out with axenic cultures and therefore the role of cyanobacteria in directly degrading crude oil has been questioned. Cyanobacteria often form associations with heterotrophic bacteria and it has consequently been proposed that the latter bacteria are responsible in some cases for degrading crude oil [1]. Moreover very little is known about the role of *Microcoleus*

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chthonoplastes, the cyanobacterium which dominates in microbial mats ecosystems, in crude oil utilization in laboratory cultures.

Our research team has used CLSM to study the diversity of cyanobacteria, in particular *Microcoleus chthonoplastes*, and their biogeographic distribution in different microbial mats from all over Europe with different degrees of petroleum contamination [4,7,21]. This technique has been also used to establish a method for determining the overall and individual biomass of each cyanobacterium at a microscale level [16]. To determine the role of *Microcoleus chthonoplastes* in the degradation of petroleum, we prepared experimental systems in the laboratory (microcosms) that simulate the microbial mats from the Ebro delta, and contaminated these with crude oil [10]. Diestra et al. [5] isolated a consortium of microorganisms from these microcosms in laboratory cultures. The consortium was formed mainly by a cyanobacterium identified as *Microcoleus chthonoplastes* and by different heterotrophic bacteria that were identified with molecular techniques [14]. The consortium was involved mainly in the degradation of aliphatic heterocyclic organo-sulphur compounds [8].

The *Microcoleus chthonoplastes* cells of the consortium growing with and without petroleum showed important ultrastructural differences. A large number of highly electrodense (HE) inclusions were observed in all cells of the cultures contaminated with Casablanca and Maya oil. The aim of this work is to analyze the nature of these intracytoplasmatic inclusions and to carry out a quantitative and comparative study of the inclusions of *Microcoleus chthonoplastes* grown both in the presence and absence of crude oil.

2.Methods

2.1 Sample Treatment and Growth Conditions

Artificial microcosms that simulate marine microbial mats were prepared according to the method described by Llirós et al. [10]. Some of these microcosms were polluted with crude oil after inoculation with samples from Ebro delta microbial mats. After two months, samples were taken from these microcosms in order to obtain cyanobacteria cultures with the capacity to degrade crude oil.

Cyanobacteria enrichment cultures were prepared by introducing a portion of the microbial mat into mineral Pfenning medium [20] containing 3% (v/v) Casablanca or Maya petroleum. Casablanca has a high content of aliphatic hydrocarbons and Maya is rich in sulphur and aromatic compounds. *Microcoleus* consortium cultures were isolated in liquid medium from the enrichment cultures, and incubated in anoxic conditions in the light at 15 $\mu Em^{-2}s^{-1}$ and 27ºC [5].

2.2 Microscopy Techniques

Samples from polluted and unpolluted cultures (control experiments) were analyzed using CLSM in accordance with the method described by Solé et al. [15]. Samples were viewed in a Leica True Confocal Scanner TCS 4d (Leica Laser-technik GmbH, Heidelberg, Germany).

For Scanning electron microscopy (SEM), samples were fixed in 2.5% glutaraldehyde and washed in buffer phosphate. They were then centrifuged and dehydrated in successively increasing concentrations of acetone (30%, 50%, 70% and 100%). Finally, all samples were mounted on metal stubs and coated with a layer of gold. The images were viewed with a Hitachi S-570 Scanning Electron Microscope.

For thin sectioning, samples from liquid and solid media were suspended in 2.5% glutaraldehyde sodium cacodylate buffer (0.1M; pH 7.4) for two hours. The samples were washed in the same buffer, post-fixed with 1% OsO_4 (at 4ºC for two hours) and washed three times in the same buffer. Samples were centrifuged at 5000xg for 10 minutes and the pellet obtained was mixed with an equal volume of 2% pure agar. The agar was cut into small cubes, dehydrated in a graded series (30%, 50%, 70%, 100%) of acetone, and then rinsed twice in 100% propylene oxide. Finally, samples were embedded in Spurr resin. An LKB ultramicrotome was used for sectioning. Ultrathin sections were mounted on carbon-

coated grids, stained with saturated uranyl acetate and Reynolds lead citrate and viewed in a Hitachi H-7000 Transmission Electron Microscope (TEM) (Hitachi Ltd. Tokyo, Japan).

2.3 Measurements, Data Processing and Statistical Analysis

Ultrathin sections, defined as cell profiles, were processed for each sample: 34 for the control culture (unpolluted), 37 for the culture polluted with Casablanca oil, and 38 for the culture polluted with Maya oil. Images from the TEM were enlarged 30,000 times in order to obtain accurate measurements. Image-analysis software *Metamorph* 0.5v was used to calculate the volume of each cell profile. The same procedure was used to calculate the volume of all inclusions. Stereological corrections were made considering cell profiles and inclusions as ellipsoids with an equal area. All structures appearing in each cell profile were counted. A total of 210 (cultures growing with Casablanca oil) and 434 (cultures growing with Maya oil) inclusions were measured for each sample.

Numerical density (number of HE inclusions per cell profile) and volume density, which is the volume occupied by the inclusions in reference to cell profile volume, were also calculated.

Data processing and statistical analyses were performed with the Statistical Package for Social Sciences (SPSS) [11] on a digital Vax-11/780 Computer at the Computing Centre of the Autonomous University of Barcelona. Separate one-sample Kolmogoroff-Smirnoff tests for goodness of fit were performed for all parameters tested in order to determine normality of distribution. The results indicated that data were distributed normally within each sample, which indicates that the thin sections were selected randomly from within the samples. Parametric tests could therefore be applied to our data.

2.4 X-Ray Microanalysis

Elemental analysis of cell components was carried out in the TEM (Jeol JEM-2011 (Jeol LTD, Tokio, Japan)) operating at 200 Kv, by placing the probe spot mode on the HE inclusions and on cytoplasmic sectors for a period of 200s each, with a dead time of about 3%. The probe diameter was 20 nm.

3.Results and discussion

In the last years, we have isolated and characterized a consortium of microorganism able to degrade crude oil [5]. As described in this paper, and to illustrate the role of the consortium in oil degradation, different experiments were carried out. Culture aliquots from the previously described consortium were growing both in presence and absence of light, carbonates, and crude oil. Aerobic and anaerobic conditions were also tested. The consortium grew in the presence of light, which it means that the consortium's heterotrophic depended on the growth of *Microcoleus chthonoplastes* for its own growth. Furthermore, no growth whatsoever was detected in the absence of light.

Concerning the carbon source, the consortium grew in the presence of carbonates as well as of crude oil; nevertheless, the oil provided the optimum growth for the consortium.

These studies also showed that this consortium was able to grow in the presence of crude oil, degrading aliphatic heterocyclic organo-sulfur compounds as well as alkylated monocyclic and polycyclic aromatic hydrocarbons [8].

Recently, we have characterized this oil-degrading consortium through the analysis of the 16S rRNA gene sequences. The results indicated that most of the clones found in the polluted culture correspond to well-known oil-degrading and nitrogen-fixing microorganisms, and belong to different phylogenetic groups, such as the Alpha, Beta, and Gamma subclasses of Proteobacteria, and the Cytophaga/Flavobacteria/Bacteroides group. The presence of organisms closely related to well-known nitrogen fixers such as *Rhizobium* and *Agrobacterium* suggested that at least some of the cyanobacteria-associated heterotrophic bacteria were responsible for nitrogen fixation and degradation of hydrocarbon compounds inside the polysaccharidic sheath, whereas *Microcoleus* provided a habitat and a source of oxygen and organic matter [14].

In the present work, we analyze *Microcoleus* sp., ultrastructural changes within cells growing with and without oil. First, a set of images showing the *Microcoleus* consortium grown in pristine and polluted conditions are shown in figures 1A, 1B, 1C and 1D.

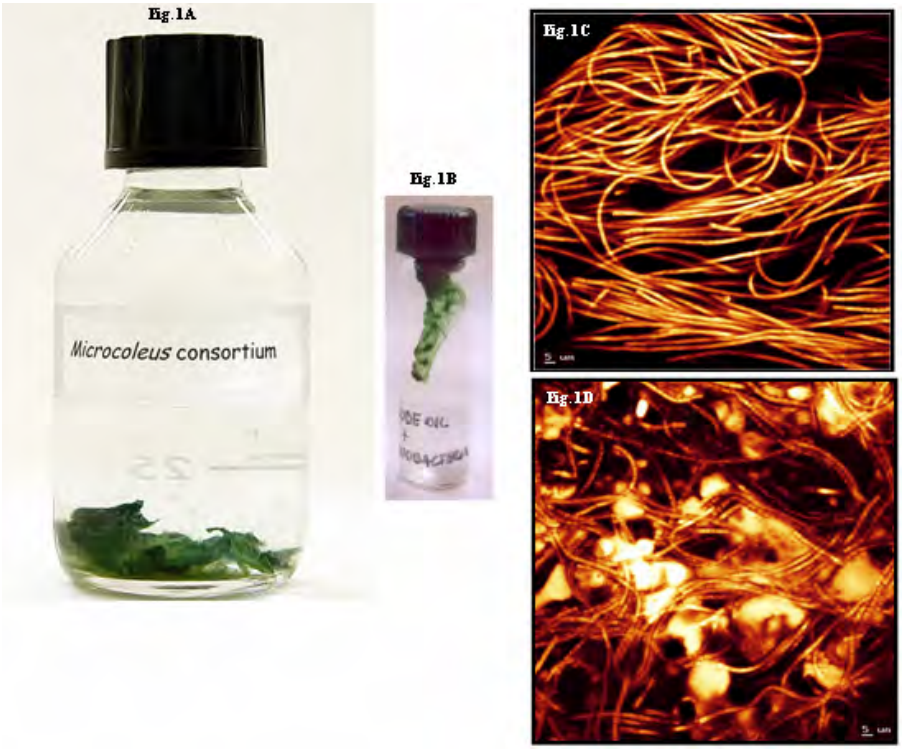

Fig. 1: *Microcoleus chthonoplastes*, from consortium, growing in pristine cultures **(A)**, and in Maya oil **(B)**. CLSM images showing filaments of *Microcoleus chthonoplastes* in control experiments **(C)** and in polluted cultures **(D)**.

The images of the same samples obtained by Scanning Electron Microscopy show individuals filaments of *Microcoleus* (Fig. 2A) and the presence of different heterotrophic bacteria inside or attached to exopolysaccharide sheath which surround the cyanobacteria (Fig. 2B).

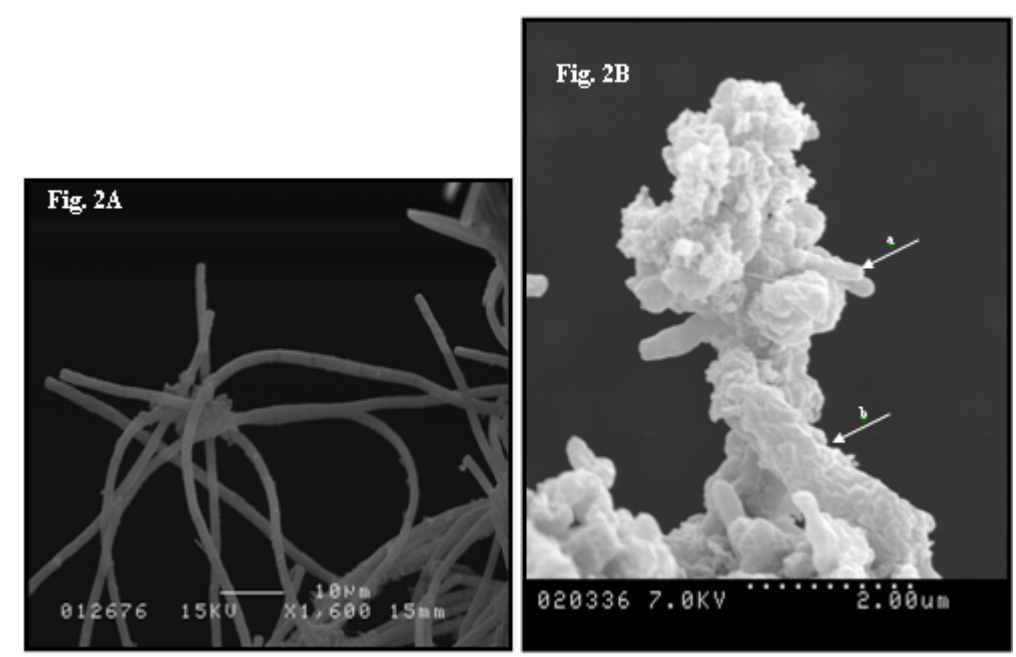

Fig. 2: Scanning electron micrograph of *Microcoleus chthonoplastes* filaments **(A).** SEM image shows bundles of *Microcoleus chthonoplastes* and heterotrophic bacteria **(a)** surrounded by EPS **(b)**, **(B)**.

When *Microcoleus chthonoplastes* cells were growing in the presence of Casablanca or Maya oil, there were HE inclusions in most of the cell sections analyzed by means of TEM (Fig. 3B and 3C), while no inclusions were detected in cells growing without oil (Fig. 3A).

Ultrathin sections of cyanobacteria reveal granules that were usually ellipsoidal, with a wide range of electron densities. The volume of high electrodense inclusions varies greatly (from $1.3x10^{-4}$ to 2.93 μm^3 in Casablanca oil and from $1.3x10^{-4}$ to 38.35 μm^3 in Maya oil) as well as their number and location in the cells. HE inclusions can be sublimate several times in the beam. This process, which leaves characteristic "holes" in the granules, was described by Baxter and Jensen [3] for polyphosphate (PPB) granules inside cyanobacterial cells. In the ultrastructure of *Microcoleus chthonoplastes,* clear differences were seen between cells growing with and without crude oil. In both cases, ultrathin sections revealed the presence of tylakoids (Fig. 3A, 3Bb and 3Cb). However, *Microcoleus chthonoplastes* growing in the presence of Casablanca and Maya oil have numerous HE inclusions of different sizes distributed uniformly throughout the cytoplasm of the cyanobacteria (Fig. 3A and 3B).

Data obtained for inclusions indicate that numerical density was about two times higher for cells growing in Maya oil than in Casablanca oil; probably due to Maya oil is rich in sulphur and aromatic compounds, and in general more toxic for bacteria. The number of HE inclusions formed per cell section was markedly different, depending on the crude oil tested (an average of 6 inclusions per cell in Casablanca oil, and 11 inclusions per cell in Maya oil). Volume density of the inclusions was $1.1x10^{-4}$ to 5.07 % in Casablanca oil and $1.1x10^{-4}$ to 68.18 % in Maya oil. Differences in the medians are observed in the statistical analysis (after carrying out a parametric Mann-Whitney U test with a significance level of 10% (α: 0.1) ($p< 0.064$)).

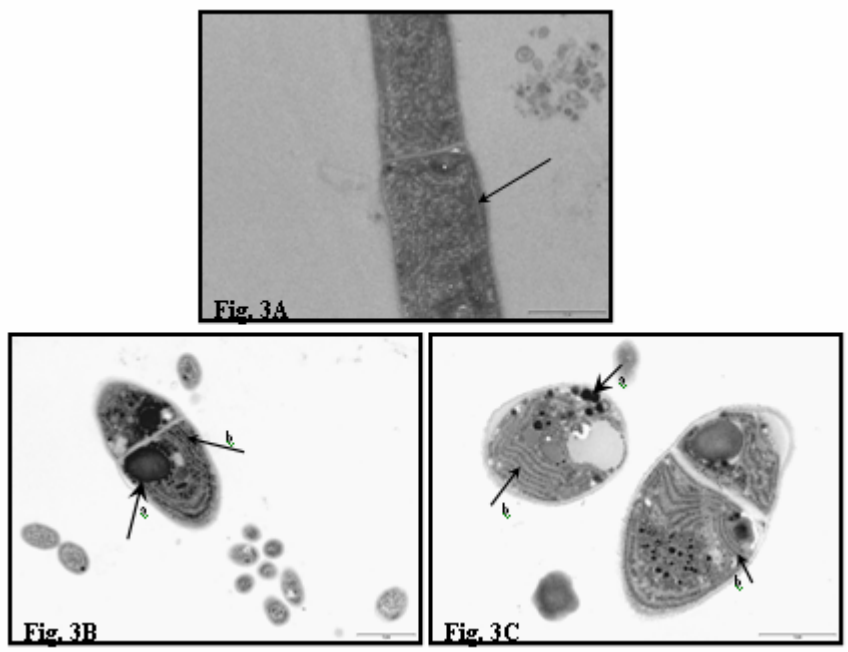

Fig. 3: Ultrathin section of a *Microcoleus* filament growing in pristine conditions **(A)** and in polluted conditions **(B and C)**. Thylakoids **(a)** and HE inclusion **(b)** are indicated by arrows. Bars represent 1μm.

Elemental analysis of the HE inclusions was carried out using the transmission mode of the TEM in conjunction with a PGT energy dispersive X-ray spectrometer.

Peaks obtained from the HE inclusions and the cytoplasmic spectra are shown in figures 4A and 4B. The HE inclusions mainly showed P and Ca (Fig. 4A). This pattern was very similar to that described by Jensen [9] for cyanobacteria in polyphosphate (PPB) granules. The authors demonstrated that, under normal growth conditions, the PPB granules were small and cells only had a few of these. They concluded that PPB granules were accumulated under stress conditions.

As well as the spectrum shown in Figure 4A, the HE inclusions also contained Fe peaks. Torres M. et al. [19] show that the PPB inclusions can play an important role in taking up metals, and therefore we suppose that the presence of Fe in the inclusions could be due to this metal being taken up from the culture medium used for growing the cyanobacteria (the mineral medium Pfenning contains 200 mg of $FeSO_4.7H_2O$). The Cu peaks observed in Figs. 4A and 4B were a spectral contaminant from copper grids, and the Os peaks in Fig. 4B were a contaminant from the sample fixation process.

In conclusion, although the presence of crude oil increases consortium's growth, the constant contact that bacteria in the culture have with the petroleum could induce accumulation of HE inclusions inside *Microcoleus* cells. The presence of these inclusions might be considered to indicate ecotoxicity in coasts along which the presence of crude oil is suspected, and in particular in the microbial mats within which these cyanobacteria are usually dominant.

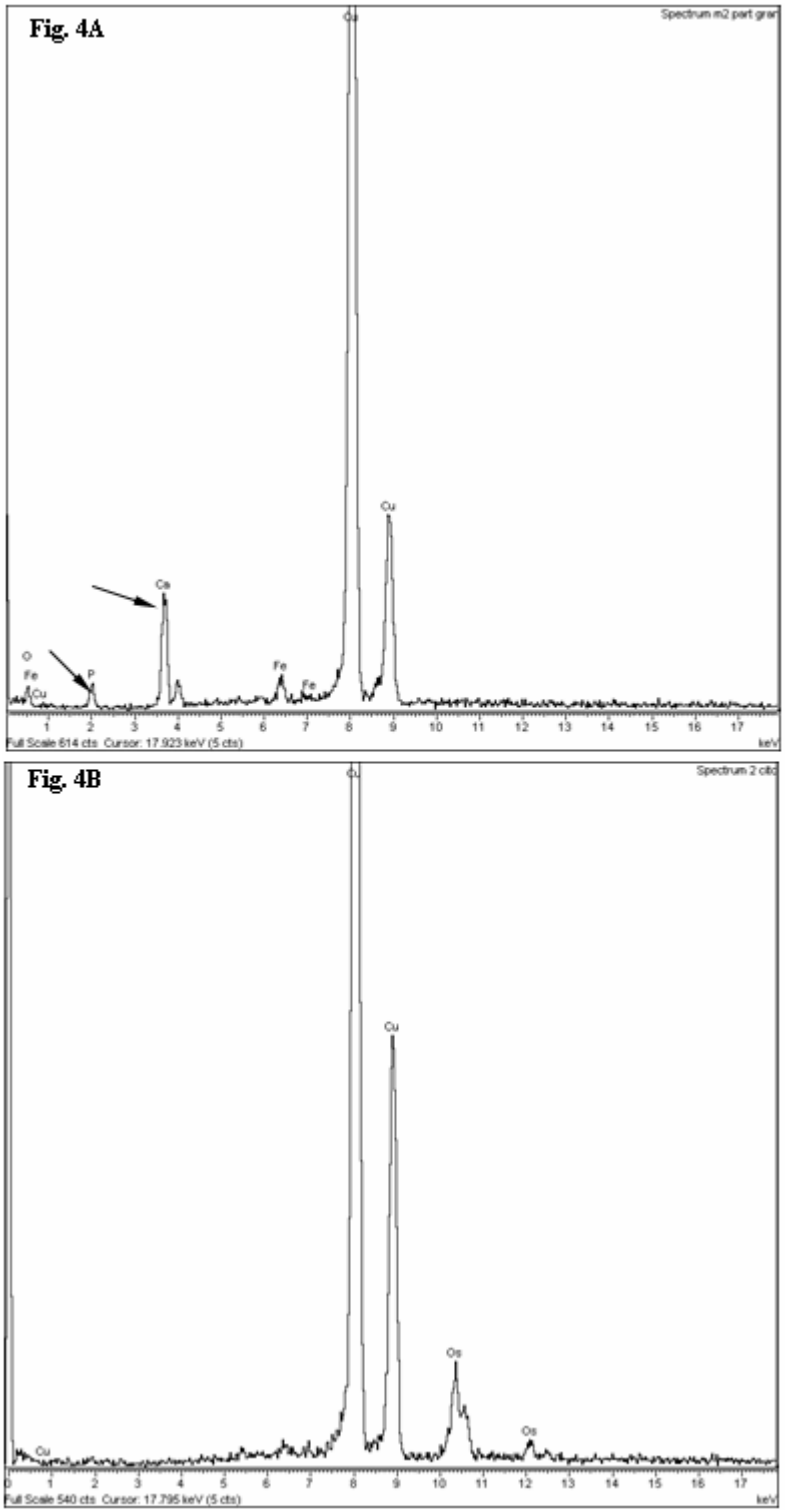

Fig. 4: X-ray spectrum showing the main elements in HE inclusions **(A)** and in cytoplasm **(B)**. The units along the X axis are electron volts (KeV); and Y axis is the number of X-rays detected. Peaks of Ca and P are indicated by arrows.

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