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GIOVANNI COLICA

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Preamble

The aims of this thesis were to test the adsorption efficiency of cyanobacterial biomasses of polysaccharide producing strains and to realize a pilot plant for galvanic waste treatment based on biosorption.

First of all the different strains were tested at laboratory scale with solutions containing one or more metals, in order to gradually approach the reality of galvanic industries, where several metals at different concentrations are present at the same time in the wastes. To realize the pilot plant we tried to combine the protocols established during laboratory experiments with the necessities of the final user, the industry, by designing and realizing three prototypes.

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Chapter 1
Introduction

1. Why biosorption

1.1 Pollution

The form of habitat degradation more subtle and still more diffuse is pollution, mainly caused by synthetic pesticides and fertilisers used in agriculture, by chemical products, wastewaters and sewage released by industries, urban settlements and intensive farming, by toxic gases emitted by factories and cars, by sediments originating from hillsides subject to erosion. These forms of pollution are not always immediately visible and perceivable, though they are by now part of the world where we live. The effects of pollution on climate and on the quality of water and air represent a major concern, as they are not only a threat for biodiversity, but also a great problem for humans and their health (Primack, 2003). Environmental contamination operated by one or more pollutants can be defined as:

1. The consequence of a human action able to modify the properness of conditions or the availability and quality of resources in a defined space and time interval (Vighi, 1998)
2. When a measurable damage occurs to a biological system, then the environmental contamination becomes pollution (Vighi, 1998)

Pollution is never qualitative, *i.e.* linked to the presence/absence of one or more substances, but quantitative. A resource is contaminated when it contains an excess of impurities, either natural or anthropic. It follows that to declare a resource polluted it is, first of all, necessary to establish how many natural "pollutants" were present in that resource, and in which doses, before the supposed impact. The tolerance range due to the capability of the resource to perform non-lethal absorption and the tolerance limits allowed for the intended use should also have been taken into consideration to determine whether the resource is utilizable or not. The water originating from a foundry, being polluted with the heat accumulated within the plant, cannot be released in streams or other water bodies, but it could be useful for domestic central heating. The water that cannot be used for domestic purposes, could find an application in agriculture, while that unsuitable for agriculture application could be used in cooling systems. It is then possible to define water as polluted when it becomes unsuitable for the use to which it was addressed, including its reintroduction in the environment.

Art. 2 of the Convention for Protection Of The Mediterranean Sea Against Pollution (Convention of Barcelona) states that: «Pollution» means the introduction by man, directly or indirectly, of substances or energy into the marine environment resulting

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in such deleterious effects as harm to living resources, hazards to human health, hindrance to marine activities including fishing, impairment of quality for use of sea water and reduction of amenities.

In the EEC Council Directive No. 76/464 of 1976, May 4, concerning pollution derived from dangerous substances released in water bodies within the Community territory, Art. 1, paragraph 2e, states: «Pollution» means the discharge by man, directly or indirectly, of substances or energy into the aquatic environment, the results of which are such as to cause hazards to human health, harm to living resources and to aquatic ecosystems, damage to amenities or interference with other legitimate uses of water.

It appears evident that many definitions of "pollutant" exist, albeit they are more often linked to the interest of the writer party than actually aimed at producing a universally accepted definition. Then, we will find definitions that differ in their targets and tolerance limits according to whether the writer is a naturalist, a hygienist, an environmental technician or a jurist. It is therefore possible to conclude that an unequivocal definition of pollution does not exist and maybe will never be reached. Therefore, it will be necessary to analyse definitions and laws specific to each single case that has to be treated.

1.2 Who pollutes?

There is a widespread opinion that the bigger an industrial site the higher the pollution it produces. This idea is, however, as diffuse as incorrect.

It is true that on the surface of an industrial site it is possible to find amounts (kg/m^2) of reagents and intermediate and final products that highly exceed the tolerance range of an area even more extended than that occupied by the industrial site, and then are, potentially, highly polluting in case of accidental release. For example, in the case of Chernobyl, the failure of the cooling system led to an explosion followed by radioactive fall-out, which has interested the whole Europe (NEA Committee on Radiation Protection and Public Health, 1995).

It is also true that a big industrial site, if built according to the safety regulations, has plant engineering and structural design suited to contain the majority of the predictable accidents, besides being regularly controlled to verify the efficiency of the plant. Unfortunately, this does not happen with common people, who pollute, consciously or not, starting from the assumption that their impact is certainly negligible compared to the immensity of nature, and are out of any kind of control if not that operated by their own conscience. The simplest example is the paper thrown in the street floor. It is small, insignificant and out of law's control but if we multiply this paper by the number of inhabitants of a town, all the papers together constitute a significant volume of waste illegally disposed of, because simply abandoned in the street and not taken to the structure in charge of the disposal. On the other hand, everybody would be outraged if the same amount of wastes were to be discharged all together in the centre of the town. So, pollution is due to everybody who lacks a social conscience and is allowed to do it with impunity.

Other major sources of pollution are (O.E.C.D., 2003a; O.E.C.D., 2003b; O.E.C.D., 1992; I.E.A., 2002; Baird Colin, 2001):

- Fertilizers used in agriculture, often without a correct canalisation system for irrigation and meteoric waters, with the result that nutrients are washed away from soils to rivers, lakes and seas where they can cause eutrophication.
- Pesticides used in agriculture that, washed away by rain or irrigation systems, enter surface and deep aquifers, determining their pollution.

- The huge number of transportation vehicles in urban areas producing high concentrations of noxious gases, *e.g.* NOx and SOx, which cause several respiratory diseases.
- Urban sewerage positioned in wrong places or poorly treated wastewaters.
- Widespread bad-manners.

It is therefore worth noting that, besides great polluters or significant but occasional accidental release of high amounts of pollutants, the crowd of small polluters has an environmental impact similar or even higher than the above mentioned.

1.3 Why do we pollute?

The polluter pollutes because he needs to remove materials –wastes, that cause immediate damage from an economic, hygienic, aesthetic, of encumbrance, etc. point of view, in the more economically convenient way for himself, which usually means to remove the wastes from his own field of action. Unfortunately, due to the high degree of urbanisation, his own field of action ends where that of his neighbours starts, so that the cost of disposal, in terms of environmental quality, that he has saved is finally paid by his neighbours. Air and water streams are the removal vehicles most diffuse and economic to use. It is possible to see how all this becomes real by looking at the market laws that govern world economy. To make a product more convenient all the expense items not directly referable to the realisation cost, including disposal and possible environmental damages that can be produced, are excluded from the total cost. The total cost is given by the sum of the commercial cost and the cost of the ecological footprint related to realisation, production, use, non-use, and end of use of the product under examination. The discrepancy between the market cost and the actual total cost is only apparent, as the consumer will eventually pay the entire cost, if not from an economical point of view, in terms of health or renouncement to some resources. On the other hand, the producer has an advantage in maintaining the two cost components separate, so the costs appear lower and the expenses due to the environmental impact are transferred to the community or to the people who are subjected to the pollution, among which, owing to the large diffusion of this *modus operandi*, the polluter himself and his family and friends. The producer is directly or indirectly contaminated by his actions, if he consciously or not acts out of the laws, and/or by the actions of other polluters-neighbours who adopt his same illegal conduct. Therefore, the behaviour of the polluter damaging himself and his family appears senseless. Only the lack of knowledge of the entity and consequences of the phenomenon justifies the apparent contempt for the good of the people the polluter loves. If, on the contrary, he will be aware of this danger he will not hesitate to pay the cost of the cleaning up or of a different strategy of design and production or of a different lifestyle.

To quote examples of what has been written above:

- Everybody wants to have sewerage and waste collection, but nobody wants a wastewater treatment plant, a garbage dump or an incinerator close to home.
- Everybody wants to have electric energy or natural gas in their houses, but nobody wants a power plant, a high-voltage line or a gas meter.
- Everybody knows that some jobs are useful though not healthy, but nobody wishes for himself or his child to end up doing one of them.
- Everybody wants to watch television or listen to the radio or use mobile phones, but nobody wants an antenna on the roof of their houses.

Concluding, we pollute because:

- There is a lack of knowledge on the effects of pollution on our own and public health.
- To save money on collection and disposal of wastes.
- For the low value attributed to environmental quality compared to the enjoyment of other goods such as cars, motor-boats, stay in crowded tourist resorts, etc., even if these have a negative impact on the environment.

1.4 To eliminate pollution

«The only way not to have pollution is not produce it!» (Primak, 2003)

Once pollution has been produced, it can only be removed from the environment by concentrating and disposing pollutants in an adequate site in the ecologically most proper way or, better, by reuse.

It is important to elucidate that total removal of a pollutant from air as well as from water or soil is impossible for chemico-physical reasons but it is possible to remove it in an amount close to the totality. This is acceptable if we think that completely pure water and air do not exist in nature. We can then state that cleaning up consists in the process of reducing the concentration of the pollutant to values included in the tolerance range estimated for that specific environment. To determine these limits, that must consider the effects of the pollutant on the different aspects of the complex trophic nets of the ecosystem, it is increasingly common to use bio-indicators and bio-markers as reference terms to monitor and verify the state of health of the environment (Morpurgo, 1996; Cotta Ramusino, 1998; Bargagli, 1998; Fossi, 1998).

Many research groups are working to optimize this methodology to read nature, by means of sampling systems for bio-indicators and bio-markers and of mathematical and statistical systems more and more efficient. This investigation approach appears more effective in describing complex natural ecosystems than only physical parameters as temperature, pH, and relative and total concentrations.

In this operation of environmental restoration the economic aspect has to be considered: the cost of the removal operation increases as the concentration of the pollutant gets closer to zero. Unfortunately cost and depuration efficiency are not directly proportional according to a linear function but according to an exponential curve. Then, if an expense of 1 is necessary to arrive at a certain pollutant concentration, for example in water, to half this concentration the expense will be 100. All this, obviously, poses a big economical-moral dilemma.

It is therefore more convenient to try to reduce pollutants during the design of the product, according to the philosophy known as "a new way of producing", based both on waste recycling and on plant re-design. This aims to reduce the production of pollutants and produce those unavoidable in forms easily recyclable, by integrating the recycling systems within the production lines.

2. Biosorption

At the end of the '80s a group of researchers started to develop new technologies for depuration and recycling of waters polluted by heavy metals derived from working cycles of mines, galvanic and leather tanning industry: in 1986 at the "16th Biotechnology and Bioengineering Symposium" the first studies on the use of biomaterial for treat-

ment of polluted water and industrial wastewater were presented. Since the beginning these biomaterials has entered the market of the systems for polluted waters and industrial wastewater treatment, consisting of an average annual expense in the order of billion dollars, as a concrete alternative to conventional systems for water treatment both for their low production and operating costs and for the possibility to recover metals. These benefits are even more appreciable when referring to the programmatic points of the Agenda 21, result of the Conference of Rio de Janeiro on the environment.

The ability of cells to absorb and metabolise heavy metals, called bioaccumulation, was known for a long time. In toxicology, the bioaccumulation or biological accumulation is the process by which toxic substances (*e.g.* DDT) are accumulated within an organism at increasing concentrations moving towards higher trophic levels. This accumulation occurs through respiration, ingestion or contact. The term «bioaccumulation» was introduced between the '50s and the '60s by a group of American naturalists, among which Rachel Louise Carson in *Silent Spring* (1962), who found high DDT concentrations in the organisms of some bird species. Following this finding, DDT was banned from the USA and many other countries in 1973. The idea of using cell metabolic pathways to treat wastewaters was, however, discarded because of the need to use viable cells, often with high operating costs, and of the slowness of the assimilation processes, which made this technique unfeasible for industrial purposes, as industrial application implies the treatment of large volumes of wastewater in a short time. This technique nowadays is still studied for soil reclamation and phytodepuration that will be described later on.

Today many papers have been published on treatment of wastewater and polluted surface or aquifer water by means of biomaterials of different origin applied in various ways. It is then necessary to shed some light on the terminology to be used:

1. Active Biosorption: it is a mechanism that exploits viable cells and their ability to absorb heavy metals and other inorganic substances. These mechanisms are not induced, but they are part of the normal physiology of the cell, where they are responsible for nutrient uptake (Fig. 1). Through the same metabolic pathways substances that are useless for cell life can be also absorbed and these can possibly kill the cell and/or be accumulated and/or be released. When these substances are accumulated within the cells, as cells do not posses metabolic pathways or other systems to release them, the phenomenon is called Bioaccumulation. Active biosorption is largely used for Phytodepuration and Phytoremediation, processes that exploit the ability of plant cells to absorb several elements or compounds from soil and to accumulate the catabolism by-products in the vacuoles. This phenomenon is widely studied for soil reclamation because when the right plants are grown, it is sufficient to harvest them to eliminate the pollutants (Phytoremediation). In the case of phytodepuration, plants and their root web are used to create a bio-filter which actively absorbs, from water flowing within the soil, organic and inorganic substances, *e.g.* ammonium for their growth, leaving a nutrient load low enough to allow water discharge in streams.
2. Passive Biosorption: in this case it is not necessary to have viable cells. This process is based on the ability of some cell constituents, such as cell wall, membrane, capsule or released polysaccharide, to bond the compounds to be eliminated. As metabolic pathways are not involved, cell viability is useful only for growth and doubling, which however is usually carried out in separate sites with respect to the application. Passive biosorption, being based on chemical rather than biological reactions, is faster than active biosorption and therefore can represent an

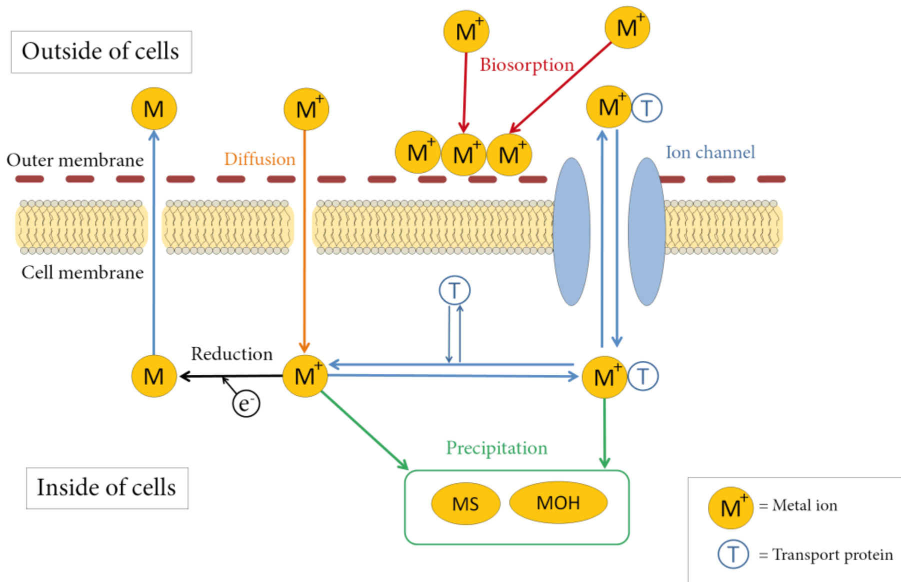

Figure 1 : Biosorption mechanisms and active and passive cation transport through the cell membrane.

interesting alternative to conventional chemico-physical systems usually utilised in industrial processes. In the passive biosorption two main categories of interaction between the substance to be removed and the biosorbent material can be distinguished: the Absorption, in which atoms or molecules of the substance to be removed penetrate, in a homogenous way, inside the biosorbent material, giving origin to a new system in which the two substances are indistinguishably mixed, and the Adsorption, in which the substance to be removed accumulates at the contact surface with the biosorbent material without becoming mixed with it. In this case, the biosorbent material and the substance to be removed behave as immiscible substances and the process is an inter-phase accumulation, in a way similar to other reactions studied by the inter-phase chemistry. This field of research is strictly connected to the functionalisation of a surface, a technique aimed at modifying the composition of a surface by incorporating elements or functional groups that produce the desired effects or ameliorate the properties of the surface or interface itself, like in this study, where the characteristics of the biosorbent material are ameliorated by specific pre-treatments. In detail, the study and the analysis of the structure and properties of an inert-phase surface involves both chemical and physical analytical techniques. This kind of studies, started among the others by Irving Langmuir, had a huge acceleration around 1920 thanks to the availability of the scanning and tunnel effect electron microscopes. This increased interest is part of the more general interest in nanotechnology and biosorption studies, in which the inter-phase accumulation of the solute to be removed is at the base of the process. Langmuir for-

mulated one of the equations that allows to better understand the biosorption reactions, used to model adsorption of a monatomic layer where all the adsorption sites have the same affinity for the adsorbed surface (Langmuir Equation). The first ones to observe adsorbing phenomena were C.W.Scheele in 1773 for gases and Lowitz in 1785 for solutions (Volesky, 2003). Today, many adsorbing phenomena have been identified in physical, chemical and biological processes, such as in galvanic bath electrodes or in cell membranes or in the use of activated carbon for water depuration. This thesis work is based on adsorbing principles and can be considered an extensive example of it.

3. Sorption: it is a more general term that can be used instead of adsorbing to avoid details concerning chemico-physical reaction occurring at the contact surface between the solute (sorbate) and the sorbent material.
4. Biosorption: this is the term used to identify a type of adsorbing based on the use of a solid phase (the sorbent material) that can be made of different materials of biological origin such as lignin, chitin, cellulose, polysaccharide, or biomasses of plant, animal, bacterial origin, selected on the basis of their chemico-physical features and of their sorbing power in relation to the wastewater to be treated and to the systems available for the treatment.

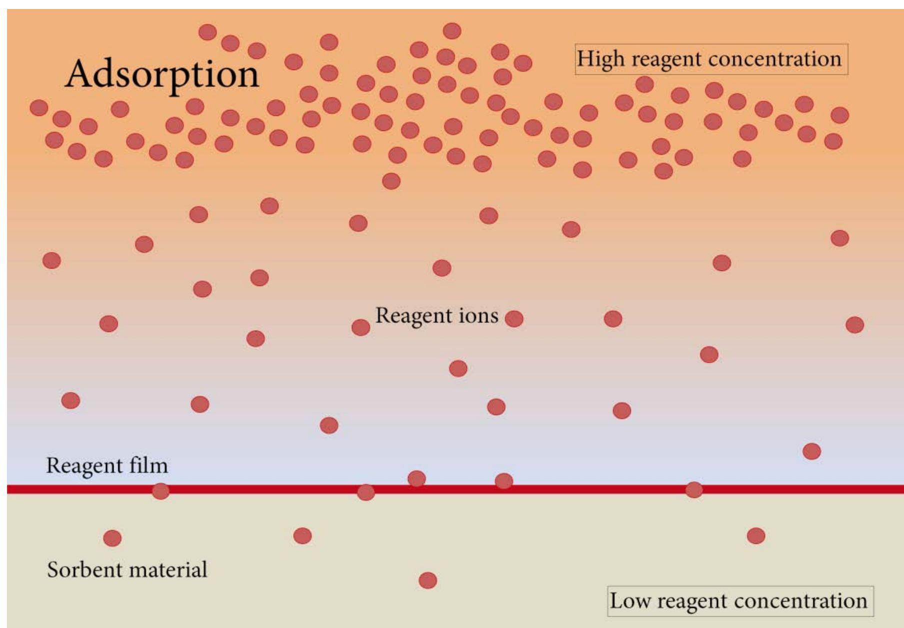

Figure 2 : Concentration gradient is the driving force that moves the solute towards the biosorbing material and determines the formation of a solute film surrounding the biosorbent itself.

Biosorption is the most common term used in the literature to indicate the adsorbing phenomena without describing the type of adsorbing material and the specific reactions occurring between the two phases.

In general, all the biosorption reactions are based on the same functioning scheme, in which a difference in concentration between the solution to be treated, rich in cer-

tain molecules or atoms, and the biosorbent material, poor or even free of these molecules/atoms, is established. When the solution and the biosorbent material are in contact, the latter starts to bond at its surface the molecules/atoms to be removed, creating a film surrounding the material itself. In this way, at the inter-phase between the solution and the biosorbent material a low solute concentration is present, that creates an osmotic force able to attract molecules/atoms of the solute towards the biosorbent material. At the beginning, the biosorbent will be enveloped by a film of molecules/atoms of solute that, in a successive step, will start to penetrate from this surface film towards the interior of the sorbent material until this process is allowed by the structure of the material itself (Fig. 2) (Volesky, 2003).

2.1 Potentiality of biosorption

The increasing diffusion of modern technologies has also increased the need for raw materials, leading to such an increase in their cost that, compared to few years ago, optimisation of production lines, recycling of working by-products and of products at the end of their life-cycle is becoming convenient. In this frame, biosorption appears, especially in the treatment of industrial wastewaters rich in heavy metals, as a good alternative to conventional chemico-physical treatment systems for both manufacturing and waste-waters, due to the possibility of metal recovery at low running costs.

2.2 Heavy metals and their effects on human health

Data reported, unless differently specified, are from: Guidelines for Drinking-water Quality. Third Edition. WHO-OMS, 2006.

Heavy metals are among the main problems for human health because they contaminate the main food resources, the environment and the water we drink. This contamination is mainly ascribable to production cycles involving heavy metals, by now essential in industrial sectors like mechanical, electric, electronic, tanning, galvanic, and oil industry. In nature biomagnification concentrates heavy metals in the animals at the top of the Elton pyramid, often reaching lethal doses.

Heavy metals are defined as: cations with specific weight higher than 5 g cm^{-3} and a normal concentration in natural waters lower than 1 mg l^{-1}. They have variable solubility in water, function of solubility of the anionic species present in the solution, with which they can precipitate as insoluble compounds (hydroxides, carbonates, chlorides, sulphides, etc.). They generally show high mobility in acidic and reducing conditions and precipitate in alkaline and oxidant conditions. Another important factor controlling heavy metal diffusion is their ability to form complexes (organic or inorganic). Toxicity towards organisms and these latter assimilation capability depend on the chemical form in which metals are present in the solution (salt, oxide, complex, etc.) and from their oxidation state. Toxic metals can be divided in two great groups: the first one is composed by metals which, in trace, are necessary for metabolism, but noxious at high doses and includes As, Cr, Co, Cu, Ni, Se, Va and Zn. The second group, including Pb, Hg, Cd, Ur, Ag and Be is composed of toxic metals without recognized nutritional value (Inthorn, 2001). They are mainly originated from industrial wastes, garbage dump percolation, urban meteoric wastewater and from specific sources described in detail hereafter:

- Iron: in a reducing environment and in anaerobic conditions iron is present in the soluble divalent form, as soon as the environment becomes oxidant it turns into

the trivalent state and precipitates (as hydroxide) due to its low solubility. It is the cause of the yellow-pink colour of water due to ferric precipitate and of malodour and turbidity; it favours the formation of iron bacteria in mucilaginous masses that can obstruct pipes.

- Manganese : its behaviour is similar to that of iron.
- Zinc: mainly produced by farmed animal manure (it is present in the feed). At more than 3 mg l^{-1} it confers an astringent flavour to water and causes opalescence and deposit formation in the pipes. US Food and Drug Administration (U.S.F.D.A.) recommends a daily intake of zinc of 12.5 mg for adults, 5 mg for 1-10 years old children, 15 mg for 11 (or more) years old males and 12 mg for females. Women should assume daily 30 mg of zinc during pregnancy and 15 mg during lactation. A good average diet provides 10-15 mg daily. Zinc has a low toxicity, however prolonged intake of high doses brings side effects. Daily doses as high as 660 mg of zinc sulphate were given to aged patients leading to minimal side effect, like diarrhoea. Other symptoms of zinc excess are nausea, vertigo, loss of muscular coordination, drowsiness, gastrointestinal problems, apathy, kidney failure, anaemia, vomiting. These are symptoms of acute toxicity and in case of appearance the intake dose should be reduced.
- Copper: similar to zinc, it shows the same effects at concentrations higher than 1 mg l^{-1}. Many industrial activities as production of paint, paper, oil-derived products and conductors release high amounts of this metal in the environment, causing toxic effects and damages in living organisms (S g et al., 1995). Man can assume it with water (copper pipes), food (kitchen utensils), intrauterine coils, dental amalgams. When present in excess in the human body it substitutes zinc in the enzymatic chains, leading to a weakening of the immune system and to dermatological diseases such as ungual diseases and alopecia.
- Nickel: it is present in food, cigarettes, dental amalgams, beverages. At high concentrations it reduces RNA-polymerase and ATPase enzymatic activity (Palermo and Gallo 2000). It is toxic to man if intake levels are too high. It is possible to find excessively high nickel values in people who suffered from heart attack, stroke, uterine cancer, burn and toxaemia of pregnancy. Allergy to earrings containing nickel alloys and used to pierce ears often occurs. Dentists often use surgical tools made of nickel alloys. Nickel toxicity increases when it combines with carbon to form nickel carbonyl. This compound is obtained during several industrial processes and it is also a component of cigarette smoke. Researches carried out on rats have shown that the amount of nickel responsible for lung cancer can be obtained by smoking 15 cigarettes per day for one year. In the animals nickel toxicity appears as limb swelling and lipid and oxygen depletion in the liver. Nickel accumulates in the liver, in bones and in the aorta. Among the symptoms of nickel poisoning there are migraine, vertigo, nausea, vomit, respiratory problems, interference with Krebs cycle enzymes, skin rash, chest pain and cough. Hair analysis allows identification of nickel intoxication.

The following metals are extremely toxic:

- Arsenic: it forms inorganic (oxides) and organic, of anionic type, complexes highly soluble and mobile in porous medium. It is present in wastewaters from pigment and metal alloys manufacturing processes; it is present also in insecticides and herbicides.
- Cadmium: it is present in wastes from galvanic, tanning and pottery industry but the main source of environmental discharge is represented by chemical fertilizers;

moreover, food refining brings to the removal of zinc, that has a protective function against cadmium, which, on the contrary, is not removed; another important source is cigarette smoke, with carcinogenic effects.
- Mercury, lead, selenium: they are present in industrial wastes. Mercury can derive from washing waters of mine wastes from cinnabar extraction.
- Radioactive elements: they are alpha and beta radiation emitters. Caesium originates from nuclear power plant accidents and is easily adsorbed by clayey soils.
- Chromium: This element will be treated in detail, here and in other parts of the thesis, as it was selected as the main metal on which to carry out the experimentation of this thesis and to show how many effects heavy metals can have on both human health and the environment. Owing to its chemico-physical characteristics, chromium (Cr) (atomic number 24, atomic weight 51.99, density 7.19 g/cm^3, melting point 1850-1900 °C, boiling point 2672 ± 20 °C, vapour pressure 1 Torr at 1616 °C) is soluble in HNO_3, insoluble in water, can be diluted in HCl and H_2SO_4 and easily bonded to other metals. It exist in several stable oxidation states (0, +3, +6) and in others less stable (+2, +4, +5). In the natural environment, in the presence of water, there is an equilibrium between the tri- and hexavalent species. All the compounds of trivalent chromium are easily oxidizable to hexavalent chromium compounds in alkaline environment, while in acidic environment the opposite process occurs. These features are fundamental for absorption. The hexavalent chromium exists in solution as hydrogen chromate ($HCrO_4^-$), chromate (CrO_4^{2-}) and dichromate ($Cr_2O_7^{2-}$), which solubility is pH dependent and highly variable, from insoluble to highly soluble (Shupack, 1991). Trivalent and hexavalent chromium in galvanic baths are mostly present in ionic form, while in solids they are mainly present as compounds. In the galvanic technique, hexavalent chromium is typically obtained from chromium trioxide, commonly called chromic acid (or chromic anhydride).

Surface treatments using chromium include the following finish:
- Passivation with hexavalent chromium, applied on zinc covering and on aluminium, to improve the behaviour towards corrosion and the adhesion of successive organic coats (paint), respectively. The passivation layer contains hexavalent chromium.
- Passivation with trivalent chromium, applied on zinc covering as an alternative to passivation with hexavalent chromium. The passivation layer contains mainly chromium in the trivalent form, but the presence of the hexavalent in equilibrium with the trivalent form cannot be excluded.
- Metallic chromium with valence zero, applied as decorative finish (thickness lower than 1 μm) or as a technical-functional finish (hard chromium, thickness of tens to hundreds μm). Decorative chromium can be obtained from both hexavalent and trivalent chromium baths, hard chromium only from hexavalent chromium. Chromium electroplating, independently from the type of bath used, are made of solid metallic chromium.

Chromium (VI) can be absorbed by man in several ways:
- The digestive tract represents the physiological way of absorption of chromium as an essential element. The extent of the absorption depends on several factors, as valence, water-solubility, gastric acidity, gastro-duodenal transition time (De Flora, 1987). Absorption varies from 0.1% to 4.5% and is higher for hexavalent than for trivalent chromium compounds. It should be pointed out that, at the concen-

trations present in normal diets, the stomach reduces hexavalent to trivalent chromium. Abstinence from food and achylia increase the amount of metal absorbed in the digestive tract.

- Skin absorption is influenced by the type of compound (higher for hexavalent chromium), the concentration in the solution and the contact time. Conditions favouring hexavalent chromium penetration through the skin are: increase of skin pH, inflammation, breaks, use of surfactants.
- Inhalation represents the most important absorption way in professional exposure for both chromium (III) and chromium (VI) (Bozena Baranowska-Dutkiewicz, 1981). While inhalation of water-soluble compounds is characterised by a rapid absorption at all levels, including the deep respiratory tract, the dynamic of less soluble particles is little known. Up to 97% of the particles containing trivalent chromium smaller than 5 µm are kept within the lung. The very soluble hexavalent chromium compounds can, instead, be absorbed in the first part of the respiratory tract (Foa, 1988).

Once inside the human organism the trivalent chromium compounds mainly bond to blood plasmatic proteins. The hexavalent compounds can be transferred inside the erythrocytes and then be reduced to the trivalent state (Langard, 1982). The distribution of chromium inside the organism depends on its valence and on the membrane permeability to different compounds. Once inside the cell, hexavalent chromium is in part reduced to the trivalent state and in few percents remains at the hexavalent state. The anion chromate easily passes through cell membranes and is reduced within the cells, by means of complex mechanisms involving reducing systems (ascorbate, glutathione, cysteine) and enzymatic activities (cytochrome P-450, aldehayde oxidase and NADP(H)-quinone oxidoreductase or DT-diaforase) (Aiyar, 1992). The reduction of hexavalent chromium to trivalent chromium facilitates the formation of bonds to DNA. The metabolic reduction of chromium represents, then, a mechanism of limiting threshold for the biological effects in vivo (Petrilli, 1998). In non-professionally exposed subjects the concentration of chromium in the different tissues is extremely low; phenomena of accumulation of the assumed species occur in lung, spleen, kidney and liver, without an evident relation with age. In the professionally exposed subjects, the lung represents the most important site of chromium accumulation, as it was evidenced in welders. Chromium in the blood (CrB) of subjects belonging to general population has a mean value of 0.23 µg l^{-1} (Apostoli, 1997). The mean value of chromium concentration in the serum of non-professionally exposed subjects is 0.1 - 1 µg l^{-1}. An Italian national investigation carried out in several centres, showed that the geometric mean of chromium in the urine of non-professionally exposed subjects was 0.08 µg l^{-1}, with a range of concentration from “no detectable” to 0.34 µg l^{-1} (Apostoli, 1997). In spite of all this, trivalent chromium is an essential element necessary for many functions; in particular, it plays an active role in the synthesis of the factor that induces glucose tolerance, necessary for the peripheral action of insulin. Recommended daily intake with food is of about 60 - 70 µg, amount guaranteed by normal diets.

Metallic chromium, as all the metals, has a neutral charge and low or nil biological reactivity. There is no evidence of negative effects on humans, animals and environment.

Trivalent chromium, stable and scarcely reactive, cannot pass through membranes. This is the reason for which trivalent chromium has been classified as not hazardous for tissues and DNA. The current state of knowledge indicates that it does not cause allergy.

Hexavalent chromium is highly reactive and has a high oxidative potential. It passes easily though membranes and has strongly irritant - corrosive, sensitizing, mutagenic and carcinogenic properties. Pathology caused by hexavalent chromium is essentially chronic and from professional exposure. Emergencies are usually localised and mainly concern skin and respiratory system (Donald, 1999).

Cutaneous alterations include irritative contact dermatitis and chronic ulcer due to the oxidant action of hexavalent chromium, and allergic dermatitis following a sensitization action (Brock, 2003).

The respiratory system represents the target of the oxidant and corrosive action of the metal, with a chronic bronchitis as a result for professional exposure to hexavalent chromium compounds. Chromium nephrotoxicity is expressed as a weak tubule affection, is mainly attributable to high absorption and is transitory (Franchini, 1988).

Exposition to hexavalent chromium is able to induce a series of DNA alterations, such as dot-like mutations and chromosome aberrations (DNA *strand breaks*, DNA *interstrand cross-links*, DNA-*protein cross-links* with adduct formation) and macromolecular modifications due to the production of reactive oxygen species, as well as lipid peroxidation phenomena with subsequent structural and functional membrane damage (Dayan, 2001).

The experimental data available indicate the importance of the reduction of hexavalent chromium to trivalent chromium within the organism. The reaction of reduction that hexavalent chromium compound can experience, as well as the interactions with other molecular targets, mainly nucleic acids, could represent the basis of its genotoxic effects. The final product of this reaction is represented by trivalent chromium. During reduction to trivalent chromium stable bonds with DNA and proteins, such as actin, and the aminoacids cysteine, histidine, serine, threonine and tyrosine could be formed. Chromate at intermediate valence can behave as a co-carcinogenic agent with compounds that cause oxidative damage through generation of 8-oxo- guanine (Sugden, 2002).

De Flora (2000) has reconsidered the results of about 650 studies reported in the literature, by using 32 different chromium compounds in genotoxicity assays at short time with different genetic targets. The great majority of the studies with positive results were carried out with hexavalent chromium compounds. The trivalent chromium compounds, though more reactive with the purified nucleic acid, did not induce genotoxic effects in most of the studies carried out on intact cells, though they could produce a variety of effects on sub-cellular targets and acellular systems. Many epidemiological studies indicate an association between the work in industrial sectors using chromium and neoplasia of the respiratory system. Methodology and results of several mortality studies have been collected in a literature review by Langard (1990). There is a strong evidence of association between lung, nasal fossae and paranasal sinus cancer risk and production of hexavalent chromate and dichromate, pigment production and galvanic chromium-plating. The fact that promotion of cancer to the respiratory system by hexavalent chromium is documented for only three occupational situations, in spite of the great number of individuals exposed during different working activities, could depend from the necessity of being exposed to high doses of hexavalent chromium to have tumour induction (De Flora, 2000). There are no evidences about the risk of cancer from exposition to aerosol containing metallic chromium or trivalent chromium compounds; at present in the literature there are no reported cases or epidemiological studies confirming the hypothesis that trivalent chromium compounds represent a cancer risk for humans,

except for the insoluble compounds. The International Agency for Research on Cancer (IARC) has concluded that:
- There is a sufficient evidence of carcinogenicity for hexavalent chromium compounds, used in chromate and pigment production, and galvanic chromium-plating
- There is insufficient evidence of carcinogenicity for metallic chromium and trivalent chromium compounds
- In animals, there is a sufficient evidence of carcinogenicity for calcium, zinc, strontium and lead chromate and a limited evidence of carcinogenicity for chromic acid and sodium dichromate. Insufficient evidence of carcinogenicity is found for metallic chromium and trivalent chromium compounds.
- Therefore, metallic chromium and trivalent chromium compounds are nor classifiable as carcinogenic for men.

In the light of the information reported above it is possible to draw the following general conclusions:
1. Chromium is a ubiquitous essential element, which intestinal absorption with water and food is regulated by homeostatic mechanisms.
2. Chromium was demonstrated to be toxic mainly in its stable hexavalent species and in its labile (penta and tetra) forms, which are produced during the reduction from hexavalent to trivalent chromium.
3. Chromium is toxic through skin contact and inhalation. This toxicity was demonstrated mainly (for skin diseases) or exclusively (for cancer) in some working activities.
4. The electro-coated objects release low quantities of chromium in its metallic species, that is inactive from the toxicological point of view.
5. In case of presence of hexavalent chromium in food, *e.g.* for an accidental contamination, once absorbed through the gastrointestinal tract by a homeostatic regulation mechanism, it would be rapidly and completely reduced to trivalent chromium.
6. The low release of chromium, if demonstrated, from chromium-plated object to be used in food preparation is not a risk for human health.

2.3 Conventional systems for wastewater treatment

The increasing industrialization in the last two decades has intensified the flux of metals in the ecosphere, driving industries towards the development of several systems for reclamation of the environment from metals and systems more and more efficient and safe to treat industrial wastewaters, with reduction of heavy metals and their possible recovery and reuse. At present these systems include techniques like chemical oxidation and reduction, separation through membranes, liquid extraction, active charcoal extraction, ion exchange, electrolytic treatments, electric precipitation, coagulation, flocculation, evaporation, precipitation with hydroxides and sulphates and crystallization (Montgomery, 1985). These methods differ in terms of efficiency and cost, but all of them concentrate metal in sewage, usually excluding the possibility of reuse it, or, at least, of reuse it without having recycling costs exceeding those of extraction and treatment.
- Filtration: it is one of the simplest and less expensive systems to treat waters. It is based on grilled barriers or membranes with "pores" varying from Ø>100 mm to

Ø<1µm, through which the water flows due to gravity or under pressure. Often more filtering units are assembled in a way to operate an increasingly finer filtration. Besides grills and membranes, sands with different granulometry are used to filter water because of their low cost and easiness of finding, especially for plants treating large volumes of water. Unfortunately, this system is inefficient for depuration of chemical substances in solution.

- Sedimentation: except for the cost of realisation of the plant, this is a low cost system. It uses the gravitational attraction acting on the substances in suspension to precipitate them and create the water column fractionation, finally allowing to separate the pollutants from "clear" water. Unfortunately, this is as well a poorly efficient system, mainly for those pollutants in solution that have very long sedimentation times. Often to obviate this problem, flocculants agents are associated to this system as they accelerate precipitation.
- Evaporation: it is a system far more efficient than the two above, but it is much more expensive as it requires an energy input sufficient to evaporate the volume of water to treat. Another problem of this system is the incapability to separate water from those substances with an evaporation point near or lower to that of water.
- Ionic capture: this term indicates a whole family of systems for the reduction of the ion load in the water. The most diffuse systems are:
  1. Ion exchange resins
  2. Galvanic cells
  3. Reagents favoring ion precipitation
  4. pH variations to start precipitation reactions

Unfortunately, the cost of water treatment with these systems is directly, if not exponentially, proportional to the efficiency. The closer to water purity the higher the cost of depuration. As a matter of fact none of the water treatment plants adopts a unique system, but always a combined array of systems, that allows to increase their efficiency and reduce costs.

2.4 Biosorption

Bioabsorption is estimated to have a potential, only for North America, of US $ 27 million / year if compared to the present market of environmental investments (Volesky, 2001b).

Biosorption operated by microbial cells represents at present a valid alternative to the conventional systems described above (Volesky, 1994). The chemico-physical characteristics of cell walls and external polysaccharides, both capsular and released in the culture medium (RPS), seem to allow, besides the isolation of metal ions from the treated solutions, a certain degree of selectivity in the bioabsorption process.

Metal ions are involved in all the aspects of microbial life. At low concentrations ions play a fundamental role in a number of vital function of microorganisms, from stabilisation of protein structure to the role of co-factors in many enzymes. However, when metal ion concentrations increase too much, their beneficial effect changes to toxic (Hughes, 1989). As a response to this toxicity some microorganisms have developed defence mechanisms, among which the ability to convert metal ions to insoluble metallic complexes, that, precipitating outside the cytoplasm of the microorganism, return to the external environment in crystal form. This normal biological process

could already represent an effective solution to the majority of the problems associated with metals released in the environment by industries.

In the literature the potentiality of many organisms in biosorption is reported. Lead and cadmium are effectively removed from solutions by brown algae such as *Ascophyllum* and *Sargassum*. Fungi like *Rhizopus* and *Absidia* have shown an excellent biosorption capability for lead, cadmium, copper, zinc and uranium. Species of *Saccharomyces* can remove silver, cadmium, cobalt and copper. Gold, cadmium, chromium, copper, iron and manganese can be removed by *Bacillus* sp. (Volesky, 1995).

The biomass behaves, in all these cases, as a chemical compound exchanging ions with the only difference that it has a biological origin. In particular, the chemical characteristics of the constituents of algal, fungal and bacterial cell walls and, only for bacteria, also of the released polysaccharides, rich in amino, carboxylic, phosphate and sulphate groups, are determining the efficiency of biomass as a biosorbent material. At present some types of biomass are produced at large scale thanks to industrial fermentors, as in the case of *Rhizopus* and *Bacillus subtilis*, or to collection from abundant marine reservoirs, as for the brown algae *Sargassum* and *Ecklonia*. These biomasses are the base of the present bioabsorption processes, because they can accumulate metals (Pb, Cd, U, Cu, Zn, Cr and others) up to more than 25% of their dry body weight like (Volesky, 2003)(Tab. 1).

Bioabsorption is influenced by environmental factors like pH, temperature, ion competition and specific metabolic features that can influence cell microenvironment. Other factors influencing the biosorption capability can be the variation in the chemical composition of the cell wall, of the capsule or, in general, of the released polysaccharides (when produced). In any case metals can deposit around the cells as phosphate, sulphates and oxides.

The advantages of bioabsorption compared to traditional systems of water treatment can be summarised as follows:

- High efficiency at low concentrations of metals/radionuclides
- Efficient operativeness in a wide range of pH and temperature
- No interference by calcium and magnesium with the ion exchange process, as it occurs with synthetic resins
- Easiness of absorbed metal recovery
- Possibility to obtain microbial biomass at low costs in fermentation plants
- Selectivity of the used organism, possibly allowing an easy selective recovery of the metal ions.

To these advantages another should be added, that is the use of non-vital biomass, which demonstrated to be even more efficient than the vital one, as it does not suffer from the interferences due to the specific physiology of each organism used:

- Non-vital biomass does not need nutrients neither for culture media nor for maintenance.
- There are no problems of contamination.
- Non-vital biomass is not susceptible to the toxic effects, leading to better results than those obtained with vital biomass, as the defence mechanisms of the cells are lacking.
- The metabolic regulation problems that occur when using vital biomasses are absent.
- There is not the problem of the recovery of the vital biomass after use.

Table 1 : Some of the results obtained by several research groups in the field of biosorption.

Strain used	Removed metal	System of use	Efficiency and application
Pseudomonas fluorescence	Pb, Zn	Immobilised on PVC and packed in columns	Used in the Hungarian chemical company
Citrobacter spp.	Cd,Cu, Pb, U	Immobilised in polyacrylamide gel columns	80-90% removed. It is possible to use it for long time
Bacillus subtilis	Cd,Cu, Ni, Pb, Zn, Hg, Ag	Free biomass or grains packed in reactors	Agent for metal recovery of the Advanced mineral technology Inc. AMT-Bioclaim
Psedomonas, Micrococcus, Saccharomyces	Au, Pd		98% removed from industrial and mining sewage
Streptomycesviri-do - chromogenes	U	Immobilised in polyacrylamide gel columns	80 - 100 % removed
Rhizopus arrhizus (obtained as reject item of industrial fermentation)	U, Th	Packed dry in reactors	170 mg of uranium accumulated per gram of biomass dry weight - 18% of dry weight
Rhizopus arrhizus	Cd		30- 130 mg per gram of biomass dry weight
Aspergillus niger Penicillium chrysogenum	Ag, Zn		Used in Austria
Fungal biomass killed with high temperature	Au, Cu, Pt, Zn		17 kg of fungi capture 1g of Pt, patented in the USA
Trichoderma viride	Cu	Immobilised in China clay	5.94- 127.5 mg of metal per gram of dry biomass
Aspergillus oryzae	Cd	Immobilised on reticulated sheeting	99% removed
Chlorella vulgaris	Au, Ag, Hg, Zn, Cu, U	Immobilised in polyacrylamide gel columns	From marine water
Dead algae		Stucked in a silicate matrix	90% removed, AlgaSORB

(Source : Biotechnology for Removal and recovery of Metals from Industrial Wastes Chemical Engineering Journal)

2.5 Possible industrial use of biosorption

Italian industry, and mainly the small and medium-sized companies, with few exceptions, invests only little resources for research and especially for research in fields not directly linked to the main production lines. A part from the final users, even those who are in charge of plant realisation make few investments in research and development of innovative technologies. They base their offers on plants conceptually obsolete, even if integrated with automatic controls aimed at reducing the number of operators. The lack of attitude to invest in research and innovation, causes the companies to buy well-known technologies, of which they can quantify the cost of purchase, operation, duration and yield, rather than invest on new technologies of which they are not able to quantify a priori costs and profit margin. This leads researchers work-

ing in strict contact with companies to be guided by applicative tasks already in the base research phase, distorting and preventing its normal development (Volesky, 2003; Volesky, 2001b). Starting from the choice of the possible biomaterial to study, biomass of either animal, plant, algal or microbial origin, it is necessary to consider that the final user will need continuous supplies of big amounts, as he is presently doing with ion exchange resins. Therefore, the biomaterial selected should be very abundant or easy to find in nature or easy to produce in huge amounts, at low costs and with small overall dimensions of the production and storage system, to favour the location within the company structure. In brief, to transfer the biosorption from a laboratory to an industrial scale it would be necessary to:

- Simplify all the processes to allow non-specialised staff to operate them.
- Reduce costs, usually by reducing precision and renouncing to sterility.
- Integrate the new treatment system within the already existing plants.

Companies want that a bioremediation plant can treat large quantities of wastes/waters in a short time and with a high efficiency, besides occupying little space and having a cost of design, realisation, installation, operation and management close to zero. Taken note of these considerations about the industrial application, the two ways now followed by researchers in the field of biosorption are (Volesky, 2003):

1. To prepare the biomaterial, of whatever origin and whatever treatment it would need before its final use, in a form or inside an apparatus easy to integrate in the pre-existing systems of waste treatment. For example, the biomaterial could be prepared in grains, similar to the ion-exchange resins, so that it could be possible to substitute the resins in the columns already in use. These grains should satisfy several requirements, like being compact, do not disintegrate during use, do not release substances in the solution to be treated, and allow an easy desorbing and recovery of the substance (metal or other) of interest. Moreover, the cost of installation, operation and management at short and medium term should allow a rapid amortization.
2. To create plants expressly dedicated to biosorption, not aiming at the integration with the present wastewater treatment systems, but instead at their complete replacement. This choice has the advantage to be focused on the biosorbent material features, trying to exploit them at their best both in the biosorption phase and in the following phase of desorbing, regeneration of the biosorbent and recovery of the substance of interest, whether they are metals or not. Companies, however, are often reluctant to adopt this strategy as they would be obliged to change their whole wastewater treatment line with time and costs of realisation much higher than with the strategy described at point 1. Moreover, this strategy implies the impossibility to rapidly convert the plants in case of problems and a difficulty in quantifying the costs.

2.6 Polysaccharide producing cyanobacteria and biosorption

Cyanobacteria, in the past also named blue-green algae, are photoautotrophic prokaryotes including a wide variety of species, differing for morphology, physiology and biochemical properties (De Philippis, 2003; De Philippis, 2001a; Madigan, 2003). The three main forms of cyanobacteria are referable to the genera *Synechococcus*, *Oscillatoria*, *Nostoc*; this means that they include both unicellular and filamentous forms, each showing different morphological types. They are widely distributed

in nature in terrestrial, marine and freshwater habitats. In general, they are more tolerant than algae to extreme conditions where often they represent the dominant or the only photosynthetic organisms, like in geothermal springs, saline lakes and other extreme environments. Many members of this microbial group can be found on the surface of soil and rocks and, occasionally, even inside rocks. In desert soil experiencing high solar radiation, cyanobacteria often form extended biological crusts, formed by cells remaining dormant for most of the year and growing during the short rain periods. In shallow marine bays, where water is relatively hot, cyanobacteria can form thick stratifications. In freshwater lakes, especially the eutrophic or hypertrophic ones, they can originate blooms. Some cyanobacteria are symbionts of ferns, liverworts, cycads and they often represent the phototrophic component of lichens. In the case of the fern *Azolla* it was demonstrated that the endophitic cyanobacterium (a species of the genus *Anabaena*) fixes molecular nitrogen that is then, in part, utilized by the plant.

For many cyanobacteria the base composition of DNA was determined. That of the unicellular forms varies from 35 to 71% in G+C, suggesting a scarce relation among organisms of this group, while in the heterocystous forms the G+C percent is less variable, from 39 to 46%. From a phylogenetic point of view, cyanobacteria tend to group mainly according to morphological lines.

Cyanobacteria nutritional requirements are simple as they do not usually need vitamins and can utilize several nitrogen forms, as nitrate, ammonium and, in many species, molecular nitrogen, that can be fixed directly in vegetative cells or in specialized cells named heterocysts. Most cyanobacteria are obligate photoautotrophs, though some species are able to assimilate simple organic compounds, such as glucose and acetate, in the presence of light (photo assimilation), while few other species, mainly filamentous, are able to grow in the dark using glucose or other sugars as carbon and energy sources.

Many products of cyanobacterial metabolism are of great commercial interest (*e.g.* spirulina biomass), while others are potentially dangerous for man and environment, like some potent toxins, especially when produced during blooms, because in case of ingestion of contaminated waters they can be lethal for both man and animals (Madigan, 2003).

Cell wall structure in cyanobacteria is similar to that of gram-negative bacteria and contains a peptidoglucan layer inside and a lipoprotein layer outside. Many cyanobacteria are able to synthesize a coating, of still unclear evolutionary significance, that can be a polysaccharidic mucilage or a sheath, besides releasing in the culture medium polysaccharidic material (RPS) of high commercial and industrial potential for its peculiar characteristics (Kratochvil, 1998)(Fig. 3).

Chemical reactivity properties of these polymers show that they are complex anionic heteropolymers containing, in the 80% of cases, from six to ten different monosaccharides and, in 90% of cases, one or more uronic acids. All of them has non-saccharidic components, like peptidic, acetylic, pyruvate or sulphate groups (De Philippis, 2000a). These considerations have driven the two main lines of research on the possible use of cyanobacterial biomasses in metal biosorption from solutions: one addressed to the optimization of the complete removal and the other one to a selective removal. In both cases there are two sublevels of research, one aiming at understanding the chemico-physical mechanisms underlying the functioning of such biosorbent materials, and the other one at investigating the possible application as an integrated part of the industrial treatment systems presently in use.

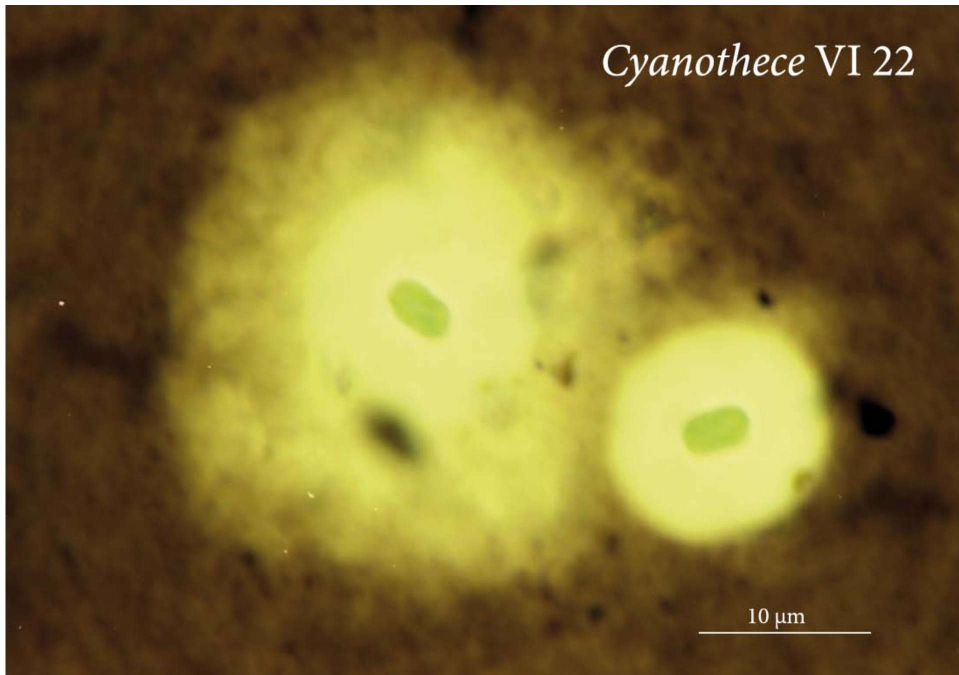

Figure 3 : *Cyanothece* VI 22 with contrast coloration obtained with India ink. Magnification 40x with range extender 0,8 x. It is worth noting the capsule, well defined, around green cells and the polysaccharide glow diffusing in the culture medium (RPS).

In the case of total removal, the research aims at exploiting all the characteristics proper of biosorption, like its good efficiency down to very low metal concentrations and the low cost of production and maintenance of biomasses, by searching for biomasses with the most convenient cost/efficiency ratio. In the case of selective removal, the search is focused on strains that produce proteins, carbohydrates and bio-polymers able to operate a selective removal of metal cations. To understand this aspect of selectivity too few studies have been carried out, but trials on selection efficiency performed by using different biomasses with several multimetal solutions, have shown that, probably, the factors involved are ionic valence of cations together with the dimension of the respective ionic radium and, mainly, with the chemico-physical characteristics of the biomolecules. These features determine biomolecule ability of adsorption and conformational change during the reaction with cations (Micheletti, 2007; Colica, 2004). The utilization of cyanobacterial biomasses has many potential advantages compared to those tested so far, like:

- Low production cost due to the low nutritional requirements of cyanobacteria. The fact that these organisms are present in every natural environment, from the poles to deserts, is an indication of their low nutritional requirements and of their high adaptation capability. The type of metabolism, that needs only water, light and carbon dioxide, favours these organisms in hostile habitats.
- High diversity of the produced polysaccharides, many of which are species-specific. Therefore a great number of bio-complexes are available to be tested for their efficiency in the biosorption sector (research in other sectors, such as medical, pharmaceutical, cosmetic and food has started several years ago).

2.7 Cyanobacterial polysaccharides

The cyanobacterial strains tested has shown the ability to produce polysaccharide capsule and to release polysaccharide in the culture medium in amounts, determined through the carbohydrate content in the culture, similar to proteins (component used as reference for microbial biomass) or even double the protein concentration, as it is shown in table 2 for some of the tested strains. The analysis of the monosaccharidic composition of these polysaccharides, carried out by ion exchange chromatography, shows a great variability among different strains. It is noteworthy that only glucose, fucose and mannose are present in every polysaccharide analyzed, while nearly all of them lack ribose and the other monosaccharides are present in variable percentage in the different polymers (Mannelli, 2007; De Philippis, 1998; De Philippis, 2000; De Philippis, 2000a; De Philippis, 2001; Vincenzini, 1990) (Tab 3).

The high amount of uronic acids found in most of the strains is of special interest for biosorption: from about 32% of EPS dry weight in strain PCC7936 to about 5% in strain VI13, besides the presence in all the analysed strains of sulphate, pyruvate and acetate groups. This latter group do not form ester bonds with the sugar to which is bonded, and then is ionically active (Mannelli, 2007) (Tab. 4).

These composition differences were correlated with the total and selective removal power shown by the different strains, but the results were not conclusive (Colica, 2004). It is, therefore, possible that this variable polysaccharide composition plays a key role in the biosorbent power, but it is also possible that this is due to other factors yet not known as, for example, the three-dimensional structure of the polysaccharide or the structural changes induced when the polysaccharide is in contact with the metal. It has been noted, in experimentations still in progress, that cultures with polysaccharide or polysaccharide alone behave in different ways from polymer to polymer when in contact with the metal, precipitating or remaining fluid in suspension, gelling or remaining in solution.

Table 2 : Characterization of biomass through dry weight, protein and carbohydrate concentrations (*Cyanothece* CE 4, PE 14, TI 4, VI 22 and *Nostoc* PCC7936).

Strain	Protein	Carbohydrate	Dry weight
	mg/L	mg/L	mg/L
CE 4	124,7	109,4	290
PE 14	151,4	93,6	280
TI 4	30,9	30,3	320
VI 22	101,1	231,3	600
PCC 7936	232,4	173,8	430

Table 3 : Monosaccharidic composition of polysaccharides obtained from the cyanobacterial strains, determined by ion exchange chromatography (Mannelli, 2007).

	Monosaccharidic mmol %										
EPS	Fucose	Rhamnose	Arabinose	Galactose	Glucose	Mannose	Xylose	Fructose	Ribose	Galacturon-ic Acid	Glucuronic Acid
Cyanospira capsulata	4,1	0,0	29,5	0,0	27,3	27,5	0,0	0,2	0,1	11,0	0,3
Cyanothece CE4	11,6	0,0	0,0	0,0	46,6	41,8	0,0	0,0	0,0	0,0	0,0
Cyanothece PE14	2,1	9,2	10,2	0,0	17,0	49,7	11,8	0,0	0,0	0,0	0,0
Cyanothece TI4	34,9	0,0	4,8	14,4	25,3	5,7	4,9	0,0	0,0	0,0	10,0
Cyanothece VI22	32,9	0,1	18,3	1,2	21,9	13,2	7,4	0,0	0,0	0,0	5,0
Cyanothece VI13	39,7	0,0	0,0	1,6	31,8	14,8	7,3	0,0	0,0	0,0	4,7
Cyanothece ET5	19,7	17,8	0,0	10,9	20,2	11,2	14,5	0,0	0,0	5,6	0,0
Cyanothece TP10	1,9	0,0	1,4	0,0	52,3	20,2	1,3	0,0	0,0	4,5	18,5
Cyanothece 16Som2	26,9	0,0	4,1	0,0	41,7	12,6	0,0	0,0	0,0	3,0	11,7
Cyanothece PE13	15,1	2,9	1,1	18,5	34,2	10,4	12,4	0,0	3,8	0,0	1,7
Nostoc PCC7936	5,7	2,1	0,6	16,7	22,7	16,1	4,4	0,0	0,0	6,9	24,8

Table 4 : Composition in sulphate groups, total sugars and uronic acids of exopolysaccharides obtained from the cyanobacterial strains (Mannelli, 2007).

EPS produced by strain:	Sulphate groups % of raw EPS	Total sugars % of dry EPS	Uronic acids % of raw EPS
Cyanospira capsulata	0.59	34.09	11,4
Cyanothece CE4	18.66	23.99	0,0
Cyanothece PE14	15.50	45.11	0,0
Cyanothece TI4	26.94	42.19	10,0
Cyanothece VI22	28.24	67.12	5,0
Cyanothece VI13	17.07	47.93	4,7
Cyanothece ET5	3.21	6.73	5,6
Cyanothece TP10	3.58	4.73	23,0
Cyanothece 16Som2	14.64	47.11	14,7
Cyanothece PE13	25.08	55.02	1,7
Nostoc PCC 7936	0.36	24.02	31,8

The high amount of uronic acids found in most of the strains is of special interest for biosorption: from about 32% of EPS dry weight in strain PCC7936 to about 5% in strain VI13, besides the presence in all the analysed strains of sulphate, pyruvate and acetate groups. This latter group do not form ester bonds with the sugar to which is bonded, and then is ionically active (Mannelli, 2007) (Tab. 4).

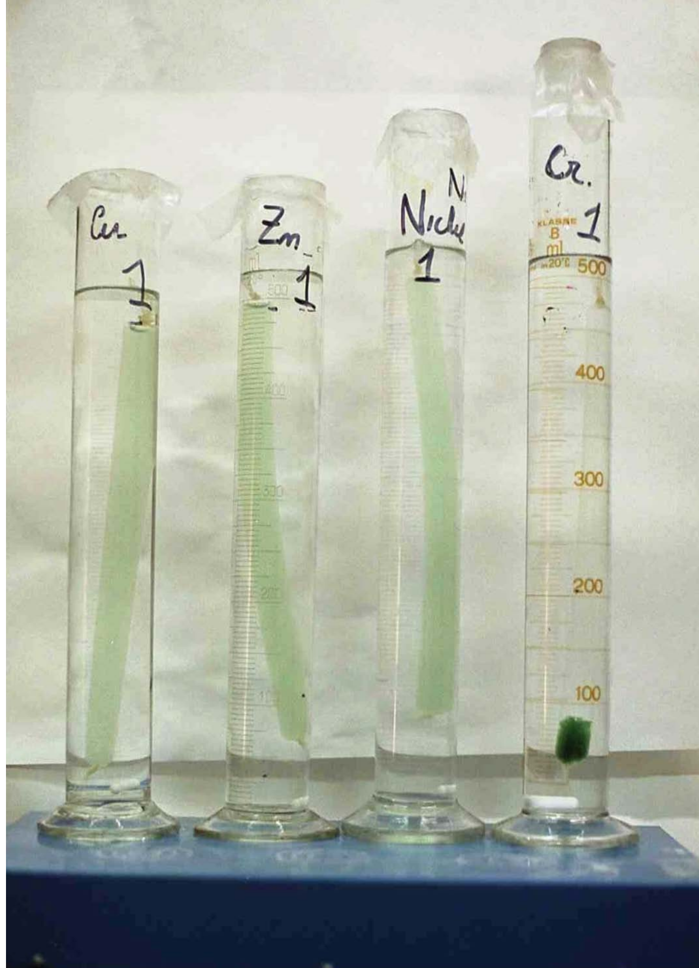

Figure 4 : Different behaviours of the same biomass (*Cyanothece TI4*) during biosorption. The metal in the solution is written on the cylinder wall. It is worth noting that biomass in the right cylinder, containing a solution of Cr(III), is precipitated inside the confinement system, a dialysis tube containing the cell suspension.

These composition differences were correlated with the total and selective removal power shown by the different strains, but the results were not conclusive (Colica, 2004). It is, therefore, possible that this variable polysaccharide composition plays a key role in the biosorbent power, but it is also possible that this is due to other factors yet not known as, for example, the three-dimensional structure of the polysaccharide or the structural changes induced when the polysaccharide is in contact with the metal. It has

been noted, in experimentations still in progress, that cultures with polysaccharide or polysaccharide alone behave in different ways from polymer to polymer when in contact with the metal, precipitating or remaining fluid in suspension (Fig. 4), gelling (Fig. 5) or remaining in solution.

Cr(III). In the right cylinder there are two dialysis tubes containing 500 mg of polysaccharide each. At the beginning of the experiment the polysaccharide was a dense but fluid solution, while during the trial it has gelled entrapping air bubbles at its interior. Once ended the test, polysaccharide extracted from the dialysis membranes remained compact, as it is shown in the detail at the bottom left of the picture.

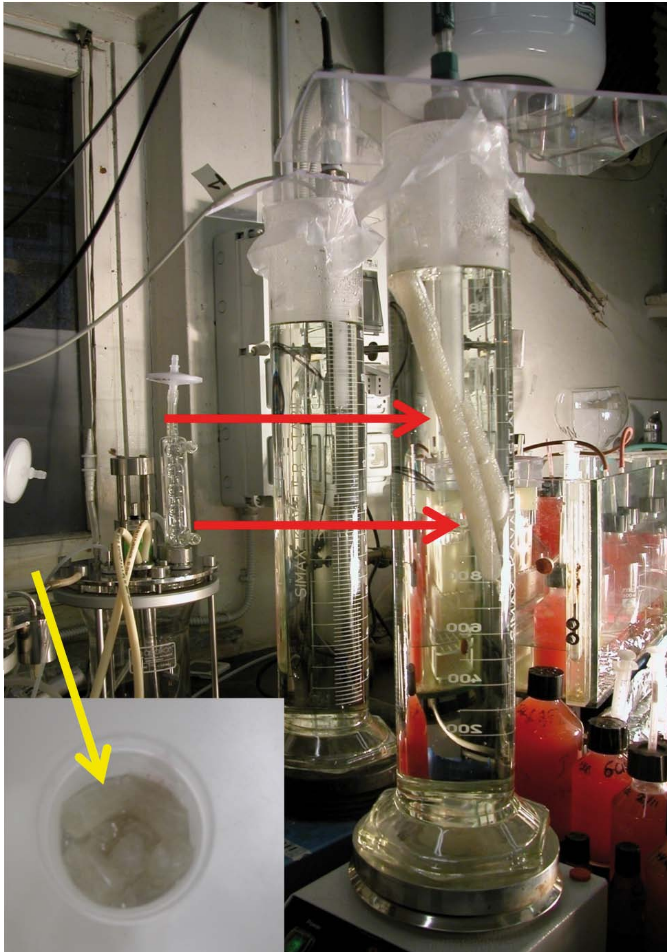

Figure 5 : Biosorption trials carried out with the polysaccharide produced by *Cyanothece TI4* confined in dialysis membranes. Solution contained 50 ppm of Cr(III). In the right cylinder there are two dialysis tubes containing 500 mg of polysaccharide each. At the beginning of the experiment the polysaccharide was a dense but fluid solution, while during the trial it has gelled entrapping air bubbles at its interior (indicated by red arrows). Once ended the test, polysaccharide extracted from the dialysis membranes remained compact, as it is shown in the detail at the bottom left of the picture (indicated by yellow arrow).

2.8 Chromate (CrO_4^{2-}) biosorption

(Due to the summary character of this paragraph the references on which it is based are reported here after: Nourbakhsh, 1994; Rocio, 2004; Maja, 2004; Helena, 2006; Rapoport, 1995; Muter, 2001; Bai, 2002; Melo, 2004; Cervantes, 2001; Pillichshammer, 1995; Ishibashi, 1990; Arslan, 1987; Yi-Tin, 1995)

The toxic effects for human and environmental health of chromate (CrO_4^{2-}), previously discussed, have led many researchers to study biosorption applied to wastewaters and bioremediation of soils contaminated by this dangerous substance. Typing "biosorption chromium (VI)" on Google Scholar, the search engine finds more than 750 links to publications treating Cr(VI) biosorption by using biomasses of different origin. Even if the biomasses tested are of different origin and the tests are carried out in different conditions, most of the publications are based on few common principles:

- The ability of Cr(VI) to penetrate cell membranes.
- The reduction of Cr(VI) to Cr(III) during oxidation of cell constituents.
- The impermeability of cell membrane to Cr(III).
- The capability of certain cells to survive Cr(VI) aggression and bioaccumulate or expel Cr(III).
- The capability of some biomasses to adsorb Cr(VI) on cell walls.
- The capability of some biomasses to adsorb the Cr(III) produced by the reduction of Cr (VI).

On the basis of these studies the possibility to use polysaccharide producing cyanobacteria in the treatment of wastewaters rich in chromate $(CrO_4)^{2-}$ was hypothesized. The ability of chromate to oxidize organic matter is known for a long time and is the base of the widely use reaction to determine COD, in which chromate has the function of oxidizing agent: it is reduced to Cr(III), while it oxidizes the organic matter, making possible the quantitative determination of the oxidized material.

3. Aim of the thesis

From the studies carried out in the laboratory, biosorption seems to be a very promising technology for the future but, being still in the development phase, it is almost completely absent from the industrial sector.

This analysis has been the starting point for this thesis work, that aimed at investigating the possible solutions to make biosorption feasible and cost-effective for companies.

For this purpose, we intended to realize a pilot plant for Cr(VI) treatment, trying to face the requests and the issues of the companies interested to this type of plant:

- To contain the production, storage and operating cost of biomasses.
- To have a system that can be integrated within the already existing treatment lines, reducing to the minimum the necessary modifications.
- To have an easy and economic system to dispose the biomasses at the end of their life cycle as biosorbent material.
- To be able to recover the metals, possibly in a selective way, at the end of the biosorption cycle.

In the first phase of the thesis work, carried out in the laboratory, the goal was to test the biosorbent power of a number of cyanobacterial strains with mono- and mul-

ti-metal solutions to select those with the highest potential for industrial wastewater treatment.

To evaluate the possibility of producing biomass at low cost, studies on the possible culture systems and, considering their abundance and diffusion, on the use of freshwater cyanobacterial blooms for biosorption have been carried out. In fact, the use of cyanobacterial blooms would zeroing the production cost, adding to this the advantage of reducing the negative effects due to the blooms in freshwaters. These negative effects range from eutrophication to health threats due to the release of toxins by the huge cyanobacterial biomass growing in drinking waters (Fig.6).

Another goal of the research was to study systems for biosorption treatment of waters with reduced plant operating costs, exploiting already existing technologies readapted for the specific use with cyanobacteria.

It was moreover evaluated the possibility to recover the adsorbed metal in a form easy and convenient for recycling.

Figure 6 : Cyanobacterial bloom in a canal connected to Lake Dian Chi , near Kun Ming (China).

Chapter 2
Materials and methods

1. Cyanobacteria used during the experiments

The experimentation started with a preliminary investigation to select the strains suitable for industrial application, by verifying the adsorbent power of 12 biomasses of different origin and characteristics. For strain selection, besides the biosorption characteristics, the costs of cultivation for the production of high amounts of biomass has been taken into account in view of a possible industrial application.

For each cyanobacterial strain tested a summary table including taxonomic collocation is reported together with photographs obtained with different techniques of light microscopy (LEV & SPICER 1964a; Reddy et al. 1996b; Scott & Dorling 1965b):

- Microscopic photographs of the crude sample without dye or contrast media to show the morphology of the cells ad their dimensions; this information is very useful to determine the method of use and the design of the plant.
- Photograph with India ink to emphasize the capsule and the released polysaccharide. The technique allows to evidence these structures because the granules of which the ink is composed can not penetrate the capsule and the layers of water-soluble RPS which are present immediately close to the cells. In this way a well defined clear area around the cells evidencing the capsule and a glow fading towards the external, due to the water-soluble RPS enveloping the cell, appear. For *Cyanothece* it was necessary a dilution with distilled water of about 10 times the culture volume to reduce the effect of the salts of the culture medium which cause the precipitation of the ink and then make the sample useless.
- Photographs with the Alcian blue dye to evaluate the presence/absence and, possibly, distribution of the acidic polysaccharides, as this dye bonds to the acidic polysaccharide colouring them of cyano blue.

The biomasses composed of mixed strains originate from cyanobacterial blooms from the same lake (Lake Dian Chi, People's Republic of China) collected at different times, to evaluate the possibility to use these blooms as source.

The biomasses used have been:

1. *Nostoc* PCC7936
2. *Cyanothece* TI 4
3. *Cyanothece* ET 5
4. *Cyanothece* CE 4
5. *Cyanothece* PE 14
6. *Cyanothece* VI 13
7. *Cyanothece* VI 22
8. *Cyanothece* 16SOM2
9. *Cyanospira capsulata*
10. *Bloom in Lake Dian Chi, August 2007*
11. *Bloom in Lake Dian Chi, July 2007*
12. *Bloom in Lake Dian Chi, August 2003*

To give a complete picture of the strains used in the experiments several observations using different microscopy dyeing techniques were performed to optimise the visualisation of the sample and photographs were taken to document these observations (Fig. 7).

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For cell measurement their dimensions in the pictures were compared to the 35.0-mm-photo frame by using the microscope magnification factor.

In some cases coloured filters or phase interference were used to optimise contrast or to increase the quality of the image.

1.1 *Nostoc* PCC7936

- Phylum : Cyanobacteria
- Subsection : IV, heterocystous
- Order : Nostocales
- Family : *Nostocaceae*
- Genus : *Nostoc*
- Strain : PCC 7936
- Coidentità : ATCC 43530 , UTEX 2094
- Cluster : Unknown
- G+C (mol %) : not determined
- a. k. a (also known as) : *Anabaena doliolum*
- Culture medium : $BG11_0$
- Properties : nitrogen fixer, aerobic, heterocystous
- Isolated by : Wolk < Singh in 1979/80
- References : Pasteur Institute website (De Philippis, Sili & Vincenzini 1996;De Philippis & Vincenzini 1998;K.D.Pandey & K.Kashyap 1986;Singh & Singh 1964)
- Notes: it has been isolated as *Anabena doliolum*, then re-classified as *Nostoc* but without classifying the species and the former name was maintained.
- Sampling site: Varanasi, India
- Habitat : It was isolated from paddy fields in the farm of the Banaras Hindu University, near Varranasi, India
- Photographs : figg. 8, 9, 10.

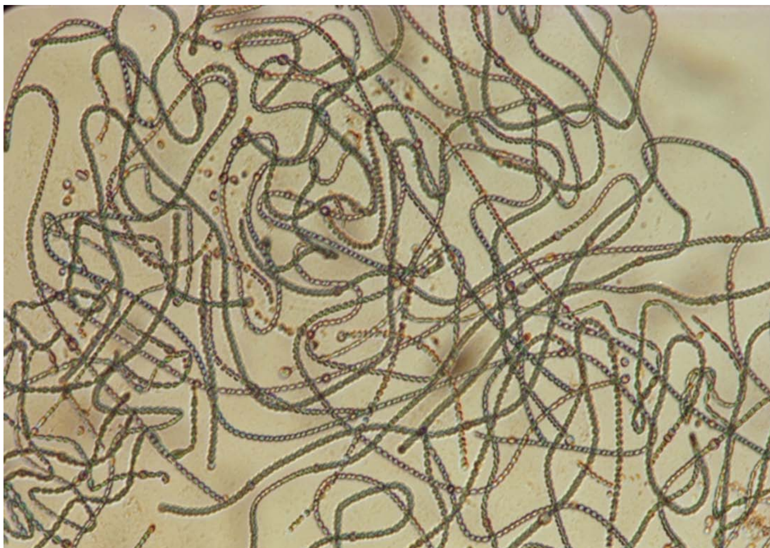

Figure 8: Nostoc PCC7936 in a picture taken at 20x magnification to appreciate the filamentous nature of the strain and the length of the trichomes.

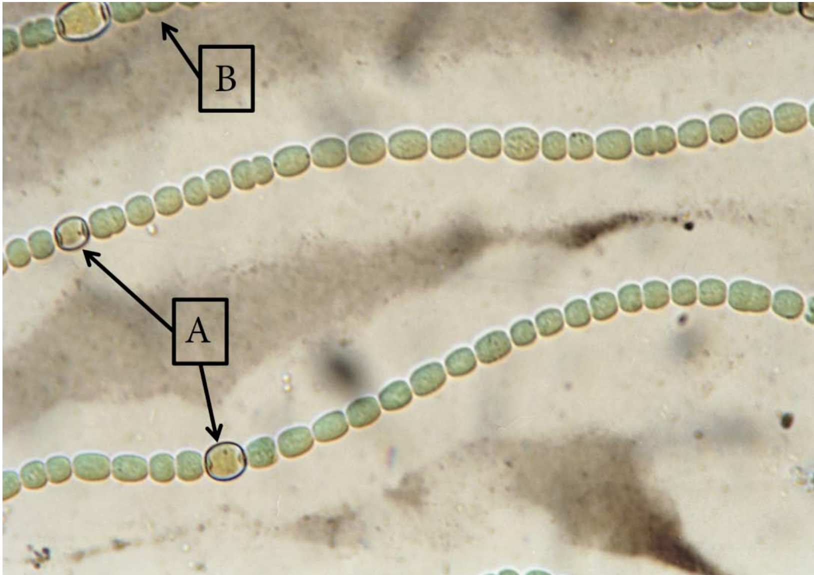

Figure 9: Nostoc PCC7936. Detail of the filaments, photographed at a magnification 100x with India ink to highlight the polysaccharidic layer enveloping the filament. It is possible to note that in correspondence of the heterocysts (A), the section of the EPS is reduced, as these cells do not produce polysaccharide. In some cases filaments are deprived of the polysaccharidic sheathing (B).

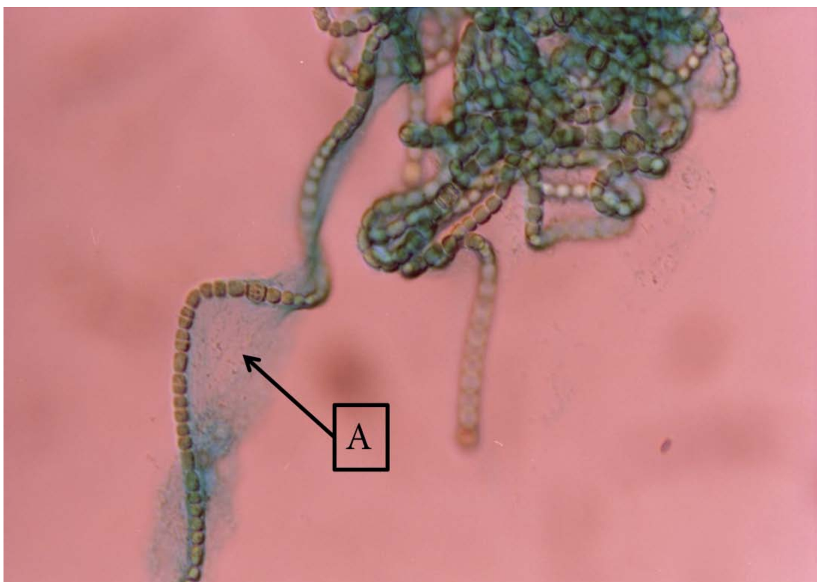

Figure 10: Nostoc PCC 7936 photographed after dyeing with Alcian blue, which bonded to the polysaccharide (A) and illuminated through red filters to optimise contrast. Magnification 25x.

1.2 *Cyanothece* TI 4

- Phylum : Cyanobacteria
- Subsection : I
- Order : Chroococcales
- Group : *Cyanothece*
- Strain : TI 4
- G+C (mol %) : 41 - 49
- Culture medium : A M A
- Isolated by : N. Feminò < M.C.Margheri in 1995/96
- References: (De Philippis et al. 1998;M.Sarrica 1996).
- Sampling site : Tindari, Sicily, Italy.
- Habitat : coastal pond of high salinity water.
- Photographs: figg. 11, 12, 13.

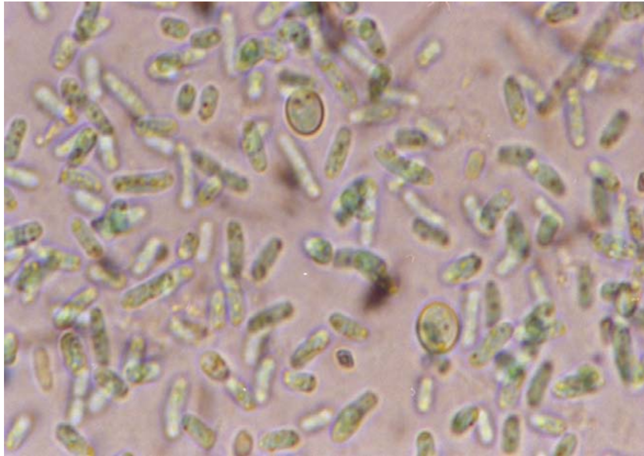

Figure 11: *Cyanothece* TI 4 : magnification 100x.

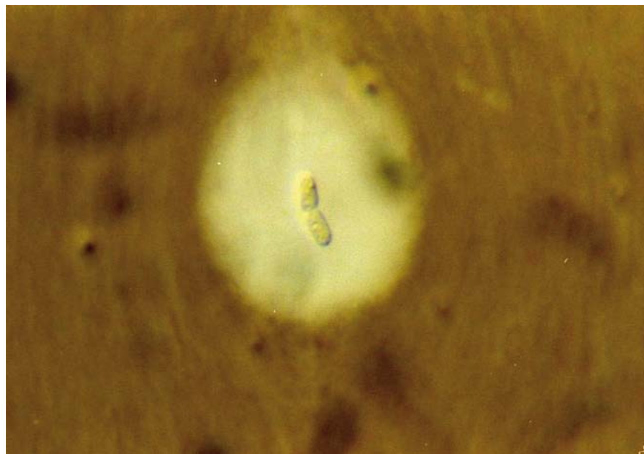

Figure 12: *Cyanothece* TI 4 : with India ink to evidence the capsule. Magnification 40x with range extender 0.8x.

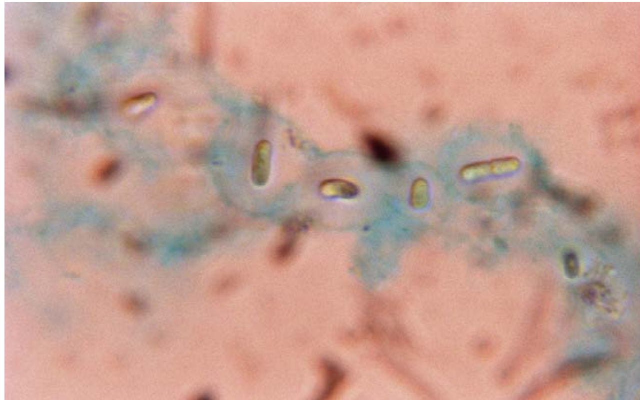

Figure 13: *Cyanothece* TI 4 : with Alcian blue to evidence the ionically active sites of the capsule. Magnification 100x.

1.3 *Cyanothece* ET5

- Phylum : Cyanobacteria
- Subsection: I
- Order : Chroococcales
- Group : *Cyanothece*
- Strain : ET5
- Mol % G+C : 41 - 49
- Culture medium : A M A
- Isolated by : M.C.Margheri < M. Sarrica in1995/96
- References: (De Philippis et al. 1998; M.Sarrica 1996).
- Sampling site : coast of Villasimius, Sardinia, Italy
- Habitat : hypersaline
- Photopgraphs : figg. 14, 15, 16, 17, 18.

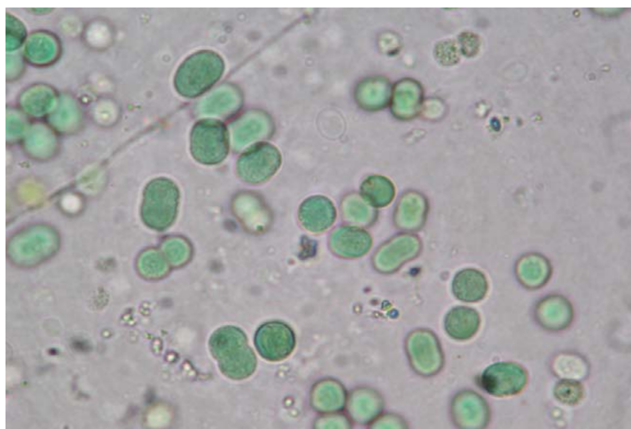

Figure 14: *Cyanothece* ET 5 : magnification 100x

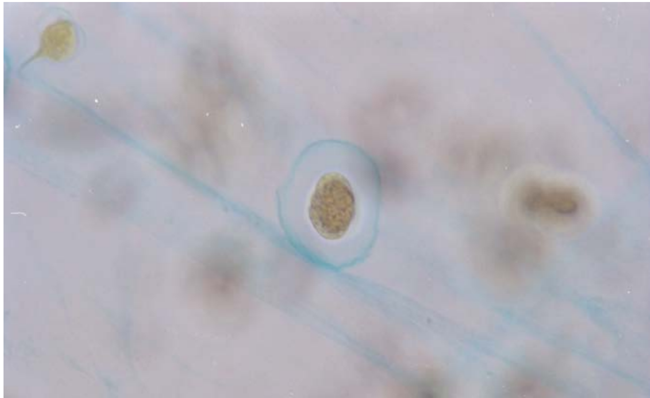

Figure 15: *Cyanothece* ET5 : with Alcian blue to evidence the ionically active sites of the capsule. Magnification 100x.

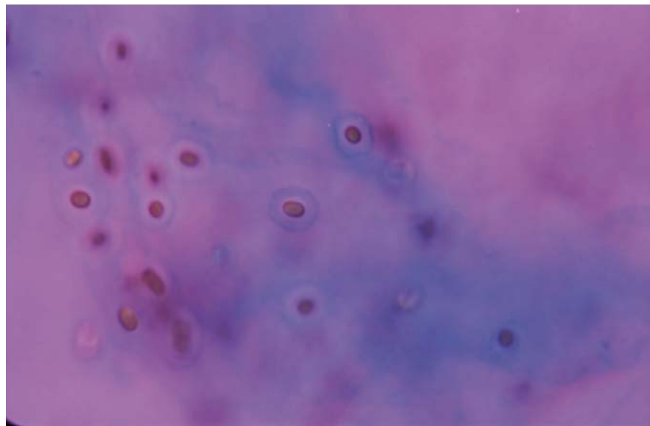

Figure 16 : *Cyanothece* ET5 : with Alcian blue to evidence the ionically active sites of the capsule. Magnification 60x.

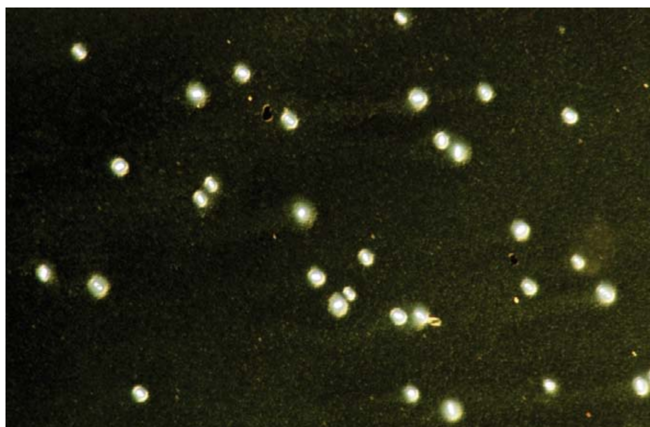

Figure 17: *Cyanothece* ET5 : with India ink to evidence the capsule. Magnification 40x

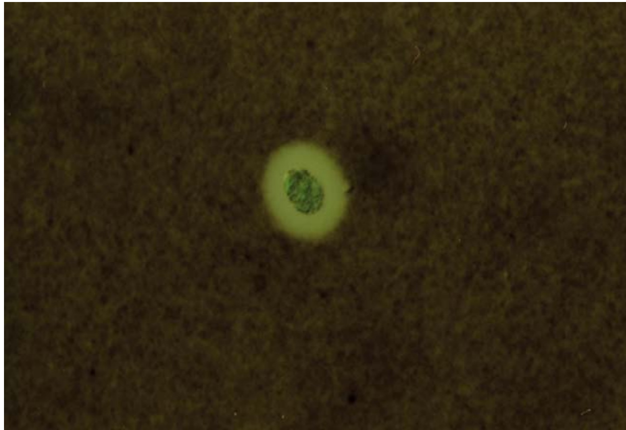

Figure 18: Cyanothece ET5 : with India ink to evidence the capsule. Magnification 100x.

1.4 Cyanothece CE4

- Phylum : Cyanobacteria
- Subsection : I
- Order : Chroococcales
- Group : *Cyanothece*
- Strain : CE4
- Mol % G+C : 41 - 49
- Culture medium : A M A
- Isolated by : M.C.Margheri < M. Sarrica in 1995/96
- References: (De Philippis et al. 1998;M.Sarrica 1996).
- Sampling site : Salt marshes, Cervia, Italy
- Habitat : hypersaline
- Photographs : figg. 19, 20, 21, 22, 23.

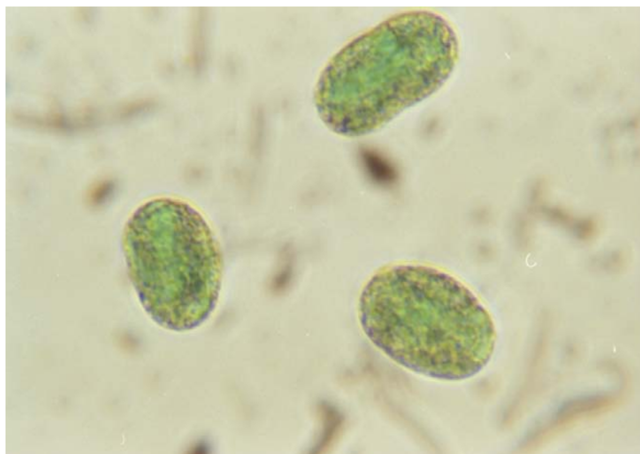

Figure 19: *Cyanothece* CE 4 : Magnification 100x with range extender 0.8x

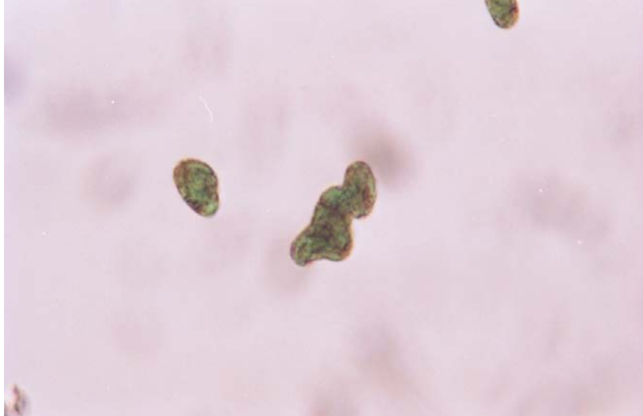

Figure 20: Different forms of *Cyanothece* strain CE 4. Magnification 40x.

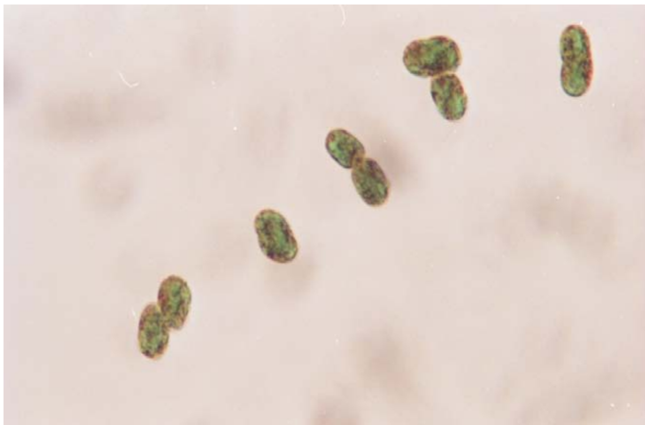

Figure 21: *Cyanothece* CE 4 undergoing cell division. Magnification 40x.

Figure 22: *Cyanothece* CE 4 : with India ink to evidence the capsule. Magnification 100x with range extender 0.8x.

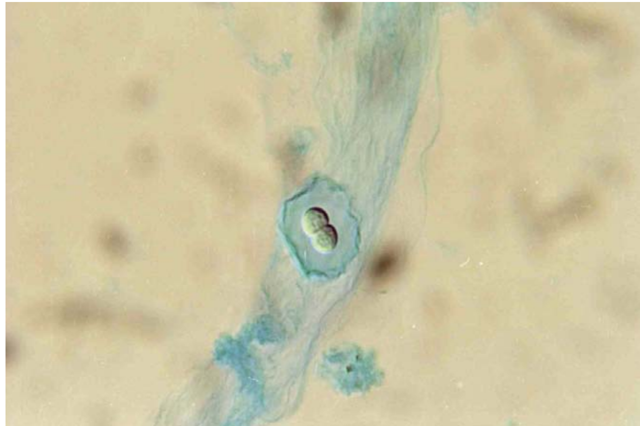

Figure 23: *Cyanothece* CE 4 : with Alcian blue to evidence the sites active in ion exchanges. Magnification 40x.

1.5 *Cyanothece* PE 14

- Phylum : Cyanobacteria
- Subsection : I
- Order : Chroococcales
- Group : *Cyanothece*
- Strain : PE 14
- Mol % G+C : 41 - 49
- Culture medium : A M A
- Isolated by : M.C.Margheri < M. Sarrica in 1995/96
- References: (De Philippis et al. 1998;M.Sarrica 1996).
- Sampling site : South coast of Peloponnese, Greece
- Habitat : coast
- Photographs : figg. 24, 25, 26.

Figure 24: *Cyanothece* PE 14 : Magnification 100x.

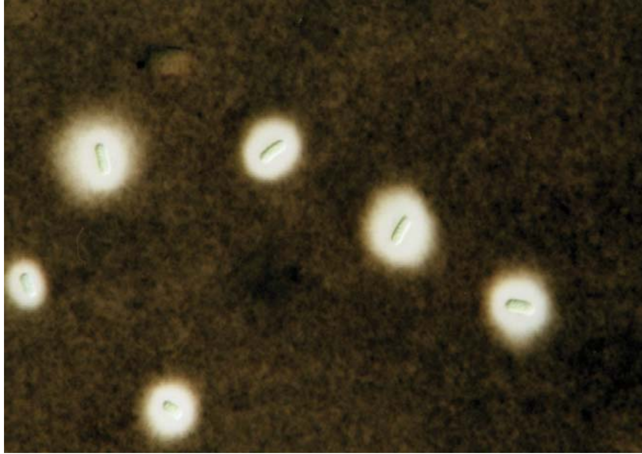

Figure 25: *Cyanothece* PE 14 : with India ink to evidence the capsule. Magnification 40x.

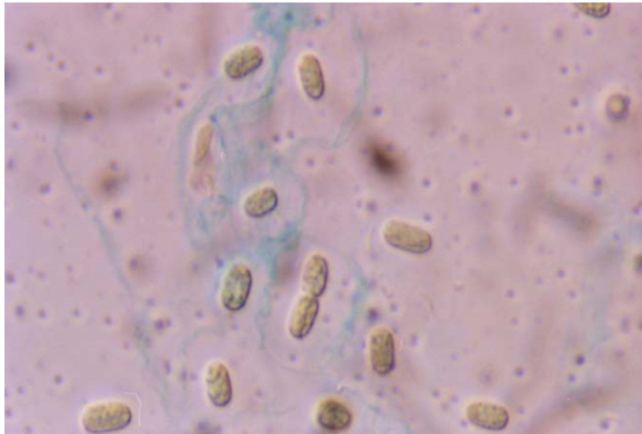

Figure 26: *Cyanothece* CE 4 : with Alcian blue to evidence the sites active in ionic exchanges. Magnification100x.

1.6 *Cyanothece* VI13

- Phylum : Cyanobacteria
- Subsection : I
- Order : Chroococcales
- Group : *Cyanothece*
- Strain : VI13
- Mol % G+C : 41 - 49
- Culture medium : A M A
- Isolated by : M.C.Margheri < M. Sarrica in 1995/96
- References: (De Philippis et al. 1998;M.Sarrica 1996).
- Sampling site : coast of Villasimius, Sardinia, Italy
- Habitat : hypersaline
- Photographs : figg. 27, 28, 29, 30.

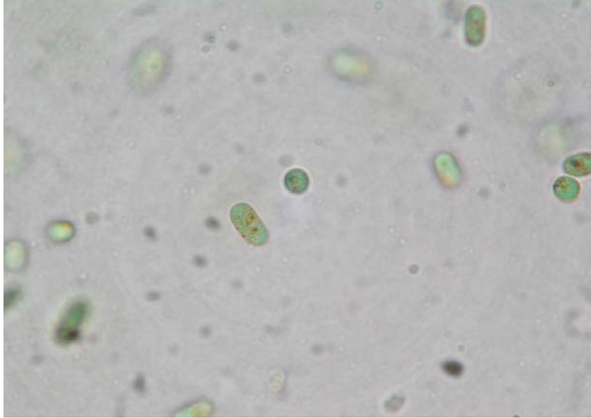

Figure 27: *Cyanothece* VI13 : Magnification 100x.

Figure 28: *Cyanothece* VI13 : with India ink to highlight the capsule. In this case the mass of polysaccharide produced is evidenced. Magnification 10x.

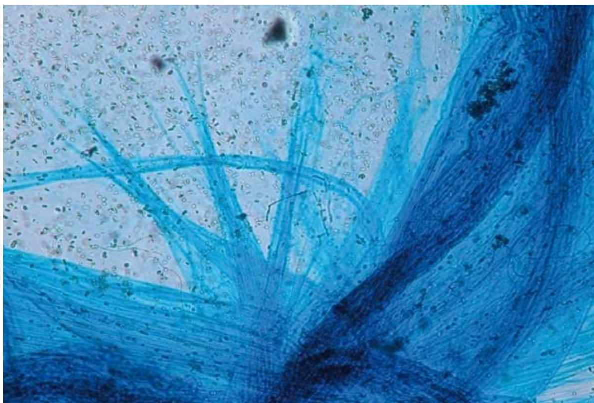

Figure 29: *Cyanothece* VI13 : with Alcian blue to evidence the sites active in ion exchanges. Strange structures, even separated from cells, due to the mass of polysaccharide are to be noticed. Magnification 10x.

Figure 30: *Cyanothece* VI13 : from this picture the gelatinous texture of the culture due to its richness in polysaccharide clearly appears.

1.7 *Cyanothece* VI 22

- Phylum : Cyanobacteria
- Subsection : I
- Order : Chroococcales
- Group : *Cyanothece*
- Strain : VI 22
- Mol % G+C : 41 - 49
- Culture medium : A M A
- Isolated by : M.C.Margheri < M. Sarrica in 1995/96
- References: (De Philippis et al. 1998;M.Sarrica 1996).
- Sampling site : coast of Villasimius, Sardinia, Italy
- Habitat : Hypersaline
- Photographs : figg. 31, 32, 33.

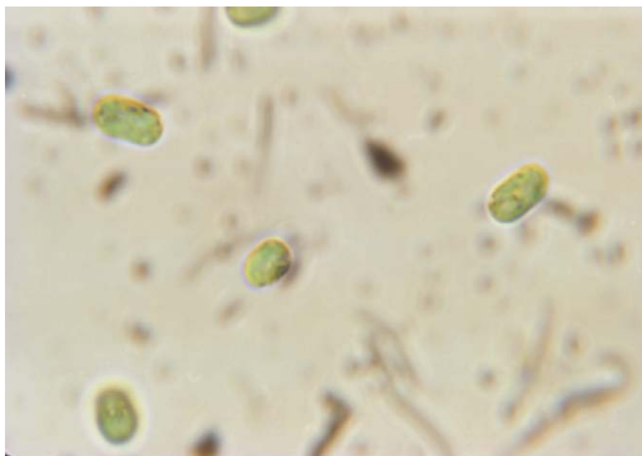

Figure 31: *Cyanothece* VI 22 Magnification 100x.

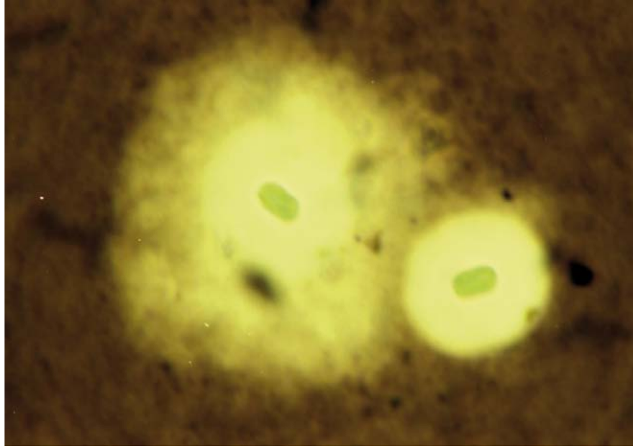

Figure 32: *Cyanothece* VI 22 with India ink. Magnification 40x with range extender 0.8x.

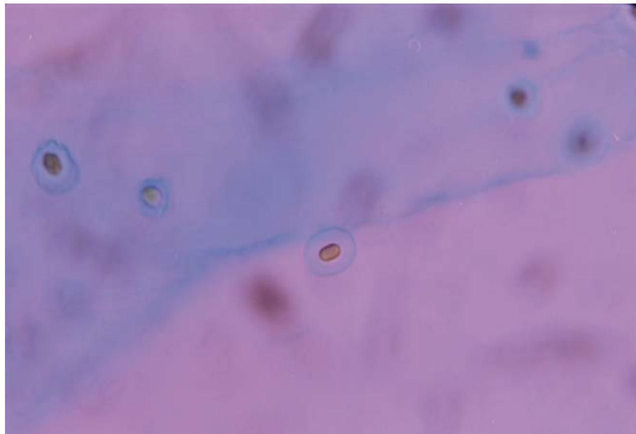

Figure 33: *Cyanothece* VI 22 with Alcian blue to highlight the capsule. Magnification 40x.

1.8 *Cyanothece* 16SOM2

- Phylum : Cyanobacteria
- Subsection : I
- Order : Chroococcales
- Group : *Cyanothece*
- Strain : 16SOM2
- Mol % G+C : 41 - 49
- Culture medium : A M A
- Isolated by : M.C.Margheri < M. Sarrica in1995/96
- References: (De Philippis et al. 1998;M.Sarrica 1996).
- Sampling site : Salt marshes, Getzira, Somalia
- Habitat : Hypersaline
- Photographs : figg. 34, 35, 36.

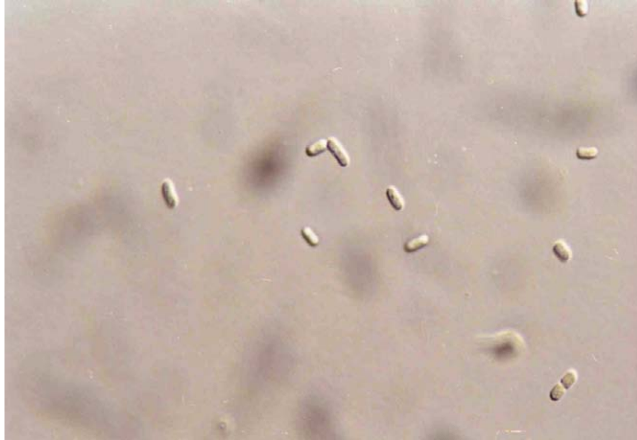

Figure 34: *Cyanothece* 16SOM2 Magnification 60x.

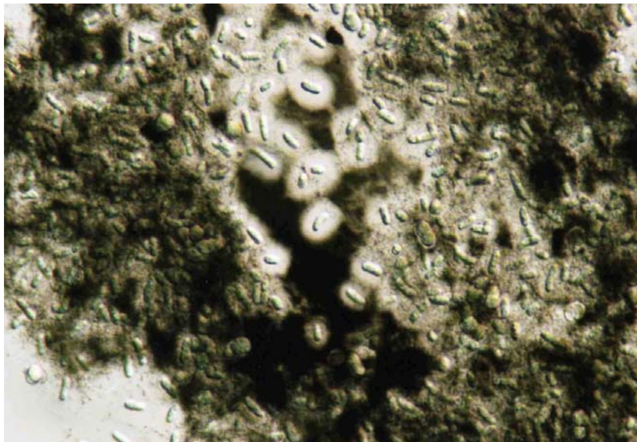

Figure 35: *Cyanothece* 16SOM2 with India ink. Magnification 60x.

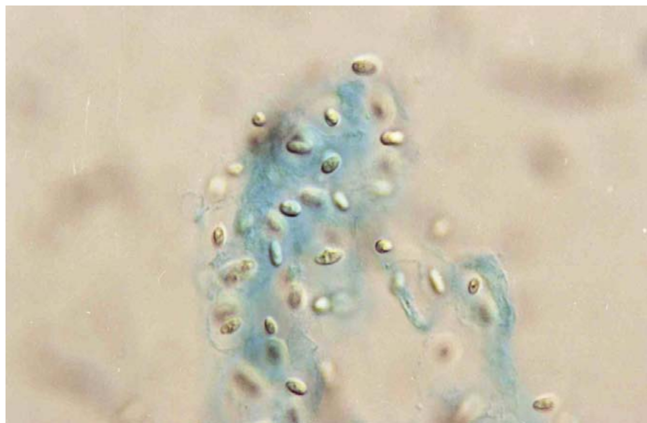

Figure 36: *Cyanothece* 16SOM2 with Alcian blue to highlight the capsule. Magnification 60x.

1.9 Cyanospira capsulata

- Phylum : Cyanobacteria
- Subsection : IV, heterocystous
- Order : *Nostocales*
- Genus : *Cyanospira*
- Strain : PCC 9502
- G+C (mol %) : 42.3
- Culture medium : $BG11_0$ + $NaHCO_3$ +Na_2CO_3
- Properties : nitrogen fixer, aerobic, heterocystous
- Isolated by: Sili C.
- References: (Florenzano et al. 1985)
- Sampling site : Lake Magadi, Kenya
- Habitat : Alkaline lake in the Eastern Rift Valley , Kenya
- Photographs : figg. 37, 38, 39.

Figure 37: *Cyanospira capsulata.* Magnification 100x. (Picture by C.Sili)

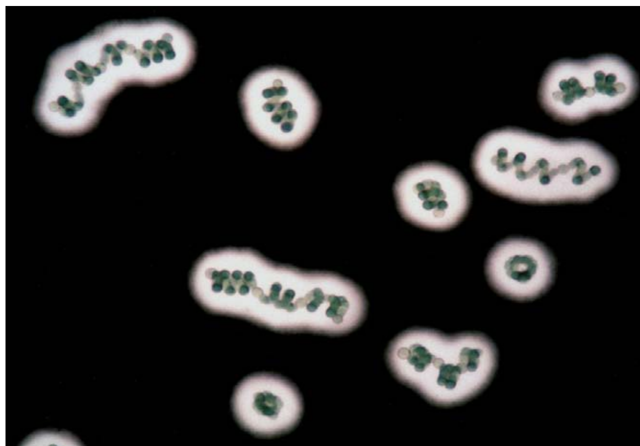

Figure 38: *Cyanospira capsulata* photographed at magnification 60x with India ink to evidence the polysaccharide enveloping the filaments. (Picture by C.Sili)

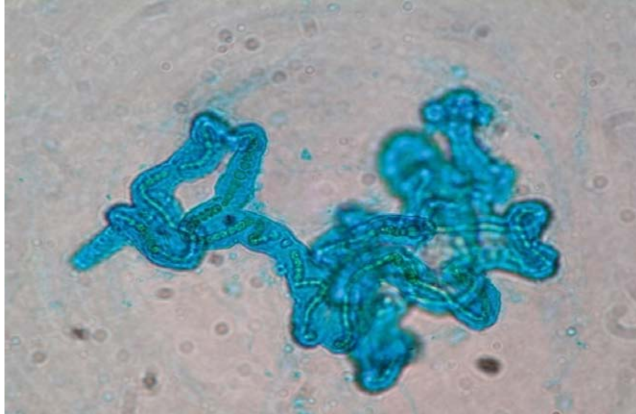

Figure 39: *Cyanospira capsulata* with Alcian blue to highlight the capsule. Magnification 60x.

1.10 Samples collected from the bloom in Lake Dian Chi

All the samples were collected from Lake Dian Chi, near Kun Ming, in the province of Yunnan, People's Republic of China. In all the samples the dominant organism was *Microcystis*. Hereafter the percent composition, referred to total cell number, of the species composing the bloom is reported (Tab. 5, Figg. 40, 41, 42, 43):

- Bloom of August 2007: The samples were collected from Lake Dian Chi and were tested directly without intermediate treatments.
- Bloom of July 2007: The samples were collected from Lake Dian Chi in July 2007, were dried under the sun on specially provided beds and then they were powdered and stocked in bottles.
- Bloom of August 2003: The samples were collected from Lake Dian Chi in August 2003 and, as the sample described above, were dried under the sun on specially provided beds, powdered and stocked in bottles.

Figure 40: Collection of the biomass from lake Dian Chi in August 2007.

Figure 41: Canal from which the bloom was collected. From the homogeneous colour on the surface of the canal it is possible to understand how thick the layers composing the bloom were.

Figure 42: View of Lake Dian Chi. From the colour of the water it is easy to verify that the whole surface of the lake is covered by the bloom.

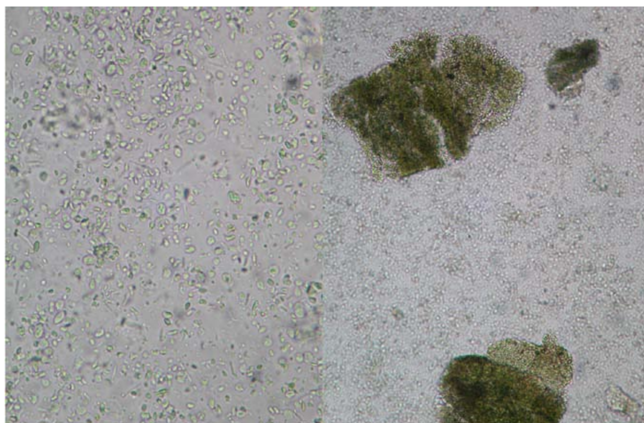

Figure 43: Bloom collected in Lake Dian Chi photographed at magnification 60x, left, and 40x, right.

2. Culture system

To obtain the culture volume necessary for laboratory experiments, about 300 ml for each trial at a concentration of 0.30-2.00 gL^{-1}, 1 mL of the maintenance culture of the strain provided by the collection of the Department of Agriculture Biotechnology of the University of Florence (Fig. 44) was diluted to 25 mL with fresh medium in 100-mL-Erlenmeyer flasks. The following steps were (Fig. 45):

- 25 mL of the culture were diluted to 50 ml with fresh medium in 100 mL Erlenmeyer flasks.
- 50 mL of the culture were diluted to 150 ml with fresh medium in 300 mL Erlenmeyer flasks.
- 150 mL of the culture were diluted to 400 ml with fresh medium in 1 L Erlenmeyer flasks.
- 400 mL of the culture were diluted to 700 ml with fresh medium in 2 L Erlenmeyer flasks.

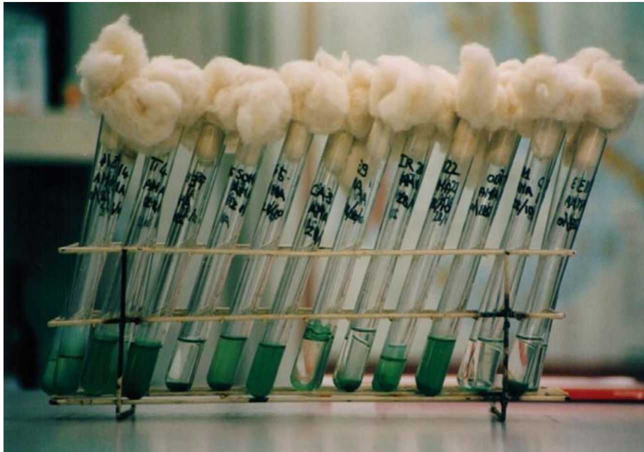

Figure 44: Some of the tubes used for maintenance of the cyanobacteria culture collection of the Department of Agriculture Biotechnology (DiBA) of the University of Florence (UNIFI).

The flasks were incubated, until biomass development, in an orbital shaker with temperature control and light regime set as follows:

- Platform rotations: 90 rpm (revolutions per minute), constant, to mix the culture and favour air-liquid exchanges,
- T: 30° C constant. This temperature was averagely good for all strains and allowed significant growth rates for all cultures during the whole experimentation,
- Light intensity: 100 μmol (photons) m^{-2} s^{-1} (photosynthetically active radiation = PAR) at the flask surface with light arriving from the top,
- The atmosphere in the incubation chamber was composed of air without CO_2 enrichment.

The physiological differences among the strains led to different growth and polysaccharide production rates that did not allow a standardization of culture period and of dilution schedule. To follow culture development it was then necessary to refer to dry weights. Cultures were also checked by light microscopy at least once a week to verify

Figure 45: Example of consecutive dilutions

their physiological state, the capsule production through India ink staining, and the possible presence of contaminants.

All the cultures have been observed by light microscopy before and after staining with India ink and Alcian blue, a dye that allows to detect the presence of acidic polysaccharides (LEV & SPICER 1964b;Reddy et al. 1996a;Scott & Dorling 1965a). The culture media used were:

- AMA : for *Cynothece* (see below)
- BG 11_0 : for *Nostoc* (see below)
- Zarrouk : for *Cyanospira capsulata* (see below)

Once the best strain was selected to continue the experiments, a fermenter was used to obtain the amount of biomass and polysaccharide needed.

A 3 L vessel thermo regulated at 28°C, illuminated by an incandescence light with an intensity of about 580 μmol (photons) $m^{-2} s^{-1}$ and bubbled with air was used. pH was maintained at 7.5 by CO_2 injection. Mixing was obtained by a three-bladed propeller set at 100 rpm during the biomass production phase and at 600 rpm, with a light intensity reduced at 300 μmol (photons) $m^{-2} s^{-1}$, in the polysaccharide production phase. This was aimed to slow the growth rate and mechanically increase the detachment of the outer polysaccharide layers, so favouring new polysaccharide synthesis. The inoculum consisted of 200 mL of culture at a cell concentration of about 500 mgL^{-1}. The culture medium used in the fermenter was $BG11_0$.

To obtain the amount of *Nostoc*, the strain selected for this part of the experimentation, necessary for industrial pilot plant experiments, 3L fermenters, 10 L bottles and raceway ponds with increasing volumes were used.

In the fermenter (Fig. 46) the culture was kept in active growth phase for 24 days, after which it was necessary to start again from a new inoculum. The system was set in semi-continuous regime with a harvest rate of 50% of the culture volume every 190 h (8 days). The harvested culture was replaced with fresh medium. To monitor the culture state two parameters were considered: pH and dissolved O_2: when the culture became unable to compensate the pH increase with CO_2 injection, the whole culture volume was renewed with fresh culture (Fig. 47).

10 L bottles: Six 10 L bottles were used as intermediate step between fermenters and raceway ponds. Each bottle was bubbled with air, illuminated with about 400 μmol (photons) $m^{-2}s^{-1}$ and incubated at 30°C (Fig. 48).

Raceway ponds: Three different types of ponds were used, differing only for their capacity: 3x30 L, 2x250 L, 1x500 L. All ponds were of the "raceway" type, made of two external curves and a central straight tract longitudinally divided by a separator to form two passages in which the culture can flow in opposite directions propelled by a paddle wheel worked by an electric engine. The depth of the culture in the ponds was no more than 0.1 m so that the light could penetrate down to the bottom. The ponds were made of polypropylene (moplen). Mixing of the culture was achieved by paddle wheels and illumination was of about 300 μmol (photons) $m^{-2} s^{-1}$, obtained with cold lamps. The ponds had no extra bubbling, so air arrived to the culture only from the mixing device.

The first step was to grow *Nostoc* in the fermenters up to a density of 2 g dry weight L^{-1}, then half of the culture was harvested and used as inoculum for one 10 L

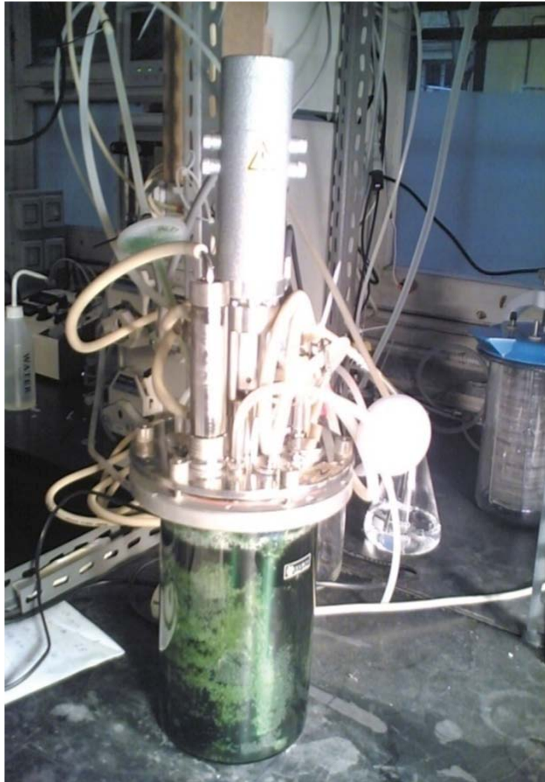

Figure 46: The 3 L fermenter containing *Nostoc* PCC7936

bottle, from which, once reached again the density of 2 gL^{-1}, half of the culture was harvested and used as inoculum for the second 10 L bottle, then, starting from the third bottle, all the culture volume was used as inoculum for the 30 L ponds (Fig. 49). The culture in the three 30 L ponds inoculated was grown for about 10 days until a 2 g dry weight L^{-1} was reached. The whole culture volume (about 90 L) was then used to inoculate two 250 L ponds (Fig. 50). Once a density of ca 1.5 g dry weight L^{-1} was reached,

the whole culture of one pond was used to inoculate a 500 L pond (Fig. 50), while the other was split in two to inoculate again both 250 L ponds. Once the dry weight of the culture in the 500 L pond reached 1 g L^{-1} the culture was considered ready for the experimentation as biosorbent material.

For the trials to be carried out in semi-pilot systems, the culture from the 500 L pond, once reached a dry weight of 1 g L^{-1}, was transferred into a decanter for 24 h (Fig. 51), from which the lower fraction of about 150 L was collected and transferred into a tank illuminated by a 1000-W-halogen lamp positioned at 500 mm from the culture surface to evaporate water while maintaining a viable culture. At the end of the process about 50 L of culture at a cell density of ca 10 g dry weight L^{-1} were obtained (Fig. 52).

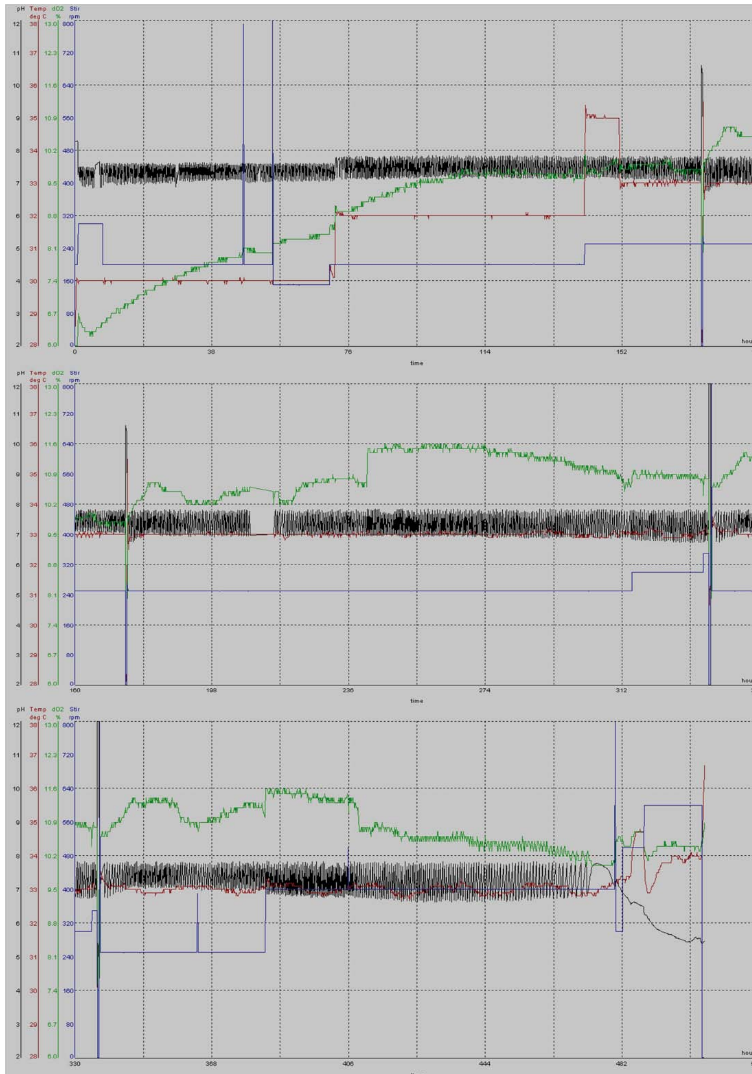

Figure 47: Graphs reporting the parameters of Nostoc PCC7936 culture: in black pH variation, in green dissolved oxygen, in red temperature and in blue the rpm of the engine that activates the propeller inside the vessel.

To obtain the necessary amount of the strain *Cyanothece* TI4 fermenters in the same culture conditions described for *Nostoc* were used, except for the culture medium, that was AMA, and temperature, kept at 35°C. Once reached ca 2 g L^{-1} the culture was boiled until 80% of water was evaporated. The residue was dialysed for 48h is dialysis tubes against deionized water, which was changed every 6 h to remove from the system the high concentration of salts present in the medium and concentrated during the boiling phase. At the end of this process, the biomass was taken out of the dialysis tubes and transferred into isopropyl alcohol in a ratio of 10 alcohol:1 biomass. A gel was formed easy to extract from the liquid fraction by heating it in a stove at 120°C until complete desiccation (about 24 h). Once dried, the sample was grinded and the powder stored in a freezer at -20°C.

Figure 48: 10 L bottles for *Nostoc* PCC7936 culture. The air injection tubes are evident

Figure 49: Raceway pond of 30 L volume used for *Nostoc* PCC7936 culture.

Figure 50: Raceway pond of 250 L and 500L volume used for *Nostoc* PCC7936 culture, illuminated by fluorescent lamps positioned above the ponds.

Figure 51: Decanter used to concentrate biomass. The valve at the top was used to let the medium flow away, while the valve at the bottom was used to collect the concentrated biomass.

Figure 52: Tank illuminated by a 1000-W halogen lamp to provide light to the culture and at the same time allow water evaporation to concentrate biomass.

2.1 A M A medium

Composition of the AMA (*Acqua di Mare Arricchita* = enriched seawater) culture medium (De Philippis, 1998):

Components	Concentrations (g L^{-1})
Artificial seawater salts	33.330
$NaNO_3$	1.500
Iron (III) ammonium citrate red	0.003
Citric acid	0.003
Na_2EDTA	0.0005
Micronutrient solution (Tab. 6)	0.5 mLL^{-1}
K_2HPO_4	0.040
$NaHCO_3$	0.100

To obtain the final medium, two solutions were prepared. One contained artificial seawater salts added with $NaNO_3$, Iron (III) ammonium citrate red, Citric acid, Na_2EDTA and micronutrient solution at the concentration indicated above in 980 mL of deionized water, with pH adjusted to 7.5 by using HCl 1 N or NaOH 1N. The second solution contained K_2HPO_4 and $NaHCO_3$ at the concentration reported above in 20 mL of deionized water. Both solutions were sterilised by autoclaving at 120 °C for 20 minutes and after cooling they were mixed in sterile conditions to obtain the final medium.

2.2 BG 11_0 medium

Composition of BG-11_0 culture medium (De Philippis, 2000):

Components	Concentrations (g L^{-1})
$K_2HPO_4 3H_2O$	0.040

$MgSO_4 7H_2O$	0.075
$CaCl_2 2H_2O$	0.036
Citric acid	0.006
Iron (III) ammonium citrate red	0.006
Na_2EDTA	0.001
Na_2CO_3	0.020
Micronutrient solution A5 + Co (Tab. 7)	1 mLL^{-1}

All the components were dissolved in 1000 mL of deionised water; pH was adjusted to 7.5 by adding HCl 1 N or NaOH 1 N. The medium was autoclaved at 120 °C for 20 minutes.

2.3 Zarrouk medium

Composition of Zarrouk medium (Vincenzini, 1990):

Components	Concentrations ($g L^{-1}$)
$NaHCO_3$	16.800
K_2HPO_4	0.500
K_2SO_4	1.000
$MgSO_4 * 7H_2O$	0.200
$CaCl_2 *2H_2O$	0.040
$FeSO_4 * 7H_2O$	0.010
Na_2EDTA	0.001
Micronutrient solution A5 + Co (Tab. 7)	1 mLL^{-1}

This medium resulted from 4 different solutions :

1. with $NaHCO_3$ and K_2HPO_4
2. with K_2SO_4, $MgSO_4 * 7H_2O$, $CaCl_2 *2H_2O$, $FeSO_4 * 7H_2O$
3. with $FeSO_4 * 7H_2O$, Na_2EDTA
4. micronutrients A5 + Co

All four the solutions were autoclaved at 120°C for 20 minutes. Once cooled, they were mixed in sterile conditions to obtain the final medium

Table 6: Micronutrient for AMA medium

Stock solution for micronutrients (De Philippis, 1998)
Stock solution indicates a solution where solutes are 100 times more concentrated than in the final medium; in this way , to obtain the desired concentration in final medium it is necessary to add 10 mL of stock solution to 1 L of medium.

Components	Concentrations ($g L^{-1}$)
$MnCl_2 4H_2O$	0.9000
$ZnSO_4 7H_2O$	0.1100
$CuSO_4 5H_2O$	0.0395
$Co(NO_3)_2 6H_2O$	0.0245

Table 7: Micronutrient for $BG11_0$ and Zarrouk media

Stock solution for micronutrients A5+Co(Vincenzini 1990)
Stock solution indicates a solution where solutes are 100 times more concentrated than in the final medium; in this way , to obtain the desired concentration in final medium it is necessary to add 10 mL of stock solution to 1 L of medium.

Components	Concentrations ($g\ L^{-1}$)
$H_3BO_3H_2O$	2.860
$MnCl_24H_2O$	1.810
$ZnSO_47H_2O$	0.220
$CuSO_4 5H_2O$	0.079
$Co(NO_3)_2 6H_2O$	0.049
$Na_2MoO_42H_2O$	0.390

3. Polysaccharide extraction system

For trials with polysaccharide alone, polysaccharide was extracted by centrifugation of the culture at 8000 rpm for 20 minutes, to separate the cell fraction from the supernatant rich in dissolved exopolysaccharides. The mixture of RPS obtained in this way was added with isopropyl alcohol in ratio of 3 alcohol:1 biomass and the mixture was let to rest for 2 h at 4°C. At the end of this step, a gelatinous mass, mainly composed of polysaccharide and easy to separate from the liquid fraction, was obtained. This gel was then transferred into pots successively heated to 120 °C in a stove till complete desiccation of the polysaccharide (ca 24 h).

Once they were dried, the polysaccharides were grinded to obtain a fine powder that was stored in a freezer at -20 °C to avoid the formation of condensation, re-hydration of polysaccharide and possible growth of moulds or other contaminants, that would have altered polysaccharide characteristics.

The isopropyl alcohol was used instead of the more efficient ethylic alcohol to reduce costs as, at the end of the polysaccharide extraction procedure, as much as 90% of isopropyl alcohol can be recuperated through distillation.

4. Confinement system

In the laboratory experiments, dialysis membrane made of regenerated natural cellulose containing glycerine as humectant agent were used to confine biomass (Medicell International Ltd, *Dialysis Tubing – Visking, size 2 and 9* MWCO – 12-14000 Daltons).

Two different diameters were used:

- For the dialysis phase: tubular membranes of 28.69 mm in diameter (size 9) containing ca 150 mL of culture each, with a surface/volume ratio of ca $1.1 cm^{-1}$ (Fig. 53).
- For the trial: tubular membranes of 14.30 mm in diameter (size 2) containing ca 50 mL of culture each, with a surface/volume ratio of ca $2.3\ cm^{-1}$.

The systems tested for biomass confinement during the wastewater treatment at industrial scale were three: two for continuous treatment, a column filled with quartz and a filter press, and one for treatment in batch, made of a dialysis cell (see below).

Figure 53 : Some of the dialysis membranes of tubular form and diameter of 28.60 mm, used in the laboratory during the dialysis phase, containing ca 150 mL of culture and at the end of the treatment.

5. Tests of biosorption in mono-metal and ternary systems

Strains selected for biosorption experiments in systems containing three metals were tested before in solutions containing only one metal or monometal. Aliquots of the cultures was confined in dialysis membrane made of regenerated natural cellulose containing glycerine as humectant agent, dialysed against deionized water for 12 hours and immersed in a 0.1 M solution of hydrochloric acid for about 30 minutes, to obtain negatively charged groups in the polysaccharide in protonated form. After this the culture was dialysed for 24 h against water in order to eliminate the salt possibly present in the culture medium; the pH of the culture was corrected with diluted HCl or NaOH solutions to a value of about 5. The culture volume prefixed (50 ml) was then dispersed in a 500-ml cylinder containing 450 mL of metal solution under continuous stirring (nickel, copper or chromium at the concentration, usually 10 mg L^{-1}, tested either in trials with only one metal or in trials with more metals at the same time). In order to determine the kinetic of the biosorption process, 5-mL samples were collected at fixed intervals, *i.e.* after 5, 15, 30 and 90 minutes for monometal solutions, and after 30 and 180 minutes for ternary solutions.

Biomass was then separated from the metal solution by centrifugation (10000 rpm for 10 minutes) and filtration on glass fiber (porosity 0.45 μm) for the successive determination of metal concentration by atomic absorption spectrometry.

6. Experiments on lake Dian Chi bloom

6.1 August 2007 bloom

Biomass composing the bloom that every years develops in Lake Dian Chi is a possible source of biosorbent material, available in huge amounts and at very low cost. Moreover, to reduce the trophic load of the lake would improve the ecosystem quality.

The bloom collected from the lake showed a concentration of about 14 gL^{-1} and was utilized for the trials without pre-treatments:

1. The bloom was subdivided in three 500 mL beakers for the experiment with Cu and three for the experiments with Cr(III).
2. Biomass was filtered after centrifugation to obtain lake water to be analysed.
3. To each biomass (500 mL) 1 mL of metal solution with Cu-Cr(III) at a concentration of 10000 ppm was added.
4. The first sample was collected after 3 h.
5. The second and last sample was collected after 7 h.
6. Each sample was centrifuged at 10000 rpm for 20 minutes and then filtered with 0.75-µm filters.

For the trials with confined biomass (biomass concentration ca 10 gL^{-1}):

1. 150 ml of fresh bloom were divided in three dialysis membranes (50 mL x3)
2. Each membrane with 50 mL of biomass were transferred into a beaker containing 450 mL of solution with Cr(III)-Cu at about 10 ppm
3. pH of biomass and metal solution was corrected
4. Biomass pH 7,5
5. Metal solution pH 4,5
6. Samplings were carried out after 6, 12, and 24 h.

Figure 54 : Dialysis membrane immersed in an acidic solution during a pre-treatment phase

For the experiments with fresh bloom pre-treated with acid (biomass concentration ca 14 g L^{-1}):

1. 400 mL of fresh bloom were placed in dialysis membrane size 9 (Medicell International Ltd, *Dialysis Tubing – Visking, size 9* MWCO – 14000 Daltons).
2. Biomass, confined in membranes, was immersed in 10L of distilled water to which 100 mL of HCl (36%) were added (Fig. 54). The treatment lasted 4 h.
3. At the end of the pre-treatment biomass was transferred from the acidic solution to 5L of distilled water for 6 h.
4. Biomass was then transferred again in 10L of distilled water and let to rest for 12 h.

5. Biomass pH was then adjusted to about 7 with diluted solutions of HCl or NaOH.
6. Metal solution containing Cu (trial A) and Cr(III) (trial B) at a concentration of ca 10 ppm were prepared and pH adjusted at 7.
7. For each trial 3 dialysis tubes size 2 containing 50 mL of pre-treated biomass each and 1 dialysis tube with 50 mL of distilled water as a blank were prepared and placed in 500 mL beakers containing 450 mL of metal solution (4 with Cu and 4 with Cr(III)).
8. Samples of the initial solution of both Cu and Cr were collected.
9. Samples during trials were collected after 6, 12 and 24 h.

6.2 July 2007 and August 2003 blooms

Bloom was dried under the sun for about 72 h by spreading a 1 cm layer on 2 m x 1 m beds lined with a synthetic cloth. Once dry, biomass was harvested and crushed to obtain a powder, useful for storage also for long time (Fig. 55).

To determine possible loss of efficiency due to the desiccation process or to prolonged storage, the trials described below were carried out using a powdered biomass just produced, in July 2007, and a powdered biomass produced in August 2003, then stored for 4 years.

In the first series of tests the bloom powder without pre-treatment was used and 1 g of powder was placed in beakers containing 500 mL of metal solution at ca 20 ppm.

The experimental procedure was:

Biomass density during the test = 2 g/L

1. Three 500 mL beakers for the test with Cu and three for the test with Cr(III), each containing 500 mL of metal solution at a concentration of 20 ppm.
2. In each beaker 1 g of bloom powder without pre-treatment was added.
3. Sampling were carried out after 3, 7 and 24 h.
4. Each sample was centrifuged at 8000 rpm for 40 minutes.

In the second series of tests the bloom was re-hydrated, confined and used without pre-treatment.

Biomass density during the test = 20 g/L

For each metal the following procedure was adopted:

1. 4 g of powdered bloom were re-hydrated in 200 mL of distilled water, so as to have 1 g of biomass/50 mL of volume.
2. 150 mL of re-hydrated bloom were divided in three dialysis membranes
- (50 mL x 3).
3. Each membrane with 50 mL of biomass was placed in a beaker containing 450mL of metal solution at about 10 ppm.
4. pH of the biomass suspension and of the metal solution were not corrected.
5. Biomass pH 7,5
6. Metal solution pH 4,5
7. Samples were collected after 6, 12 and 24 h.

For the third series of experiments, besides biomass re-hydration, an acidic treatment was performed, in the same way as for biomasses previously tested.

Biomass density during the test = 20 g/L

For both metals the following procedure was adopted:

Figure 55: Top: Spreading of the bloom on a bed under the sun. The summer temperature above 35°C and the altitude above 2000 m above sea level allowed complete desiccation of biomass in 72 h, that was then ready for crushing. Bottom: the final powder obtained from the bloom, ready for storage or use.

1. 4 g of powdered bloom were re-hydrated in 200 mL of distilled water, so as to have 1 g of biomass/50 mL of volume.
2. 400 mL of re-hydrated bloom were placed in dialysis membranes.
3. The biomass confined in the membranes was immersed for 4 h in 10L of distilled water, to which 100 mL of HCl (36%) were added.
4. After the 4 hours the biomass was taken away from the acidic solution and placed in 5 L of distilled water for 6 h.
5. The biomass was then transferred in 20 L of distilled water for another 12 h.
6. Biomass pH was corrected to about 7 with diluted solutions of HCl or NaOH.
7. pH of the metal solutions, Cu and Cr(III), were corrected to a value of 7.
8. For each test three dialysis tubes containing 50 mL of pre-treated biomass each and 1 dialysis tube with 50 mL of distilled water as a blank were placed in 500 mL beakers containing 450 mL of metal solution each (4 with Cu and 4 with Cr (III)).
9. A sample of the original solution of both Cu and Cr was collected.
10. During the test, samples were collected after 6, 12 and 24 h.

7. Tests for Cr(VI) biosorption

7.1 Wastewater used in the experiments

In all the tests carried out, both in the laboratory and at the company, washing waters of metal parts which had undergone a galvanic chromium-plating, were used. Differently to the solutions prepared in the laboratory, these washing waters have a varying composition both for chemical composition and for the percentage of each constituent; they were very heterogeneous poly-metal solutions. To try to limit the variables in the tests, the water from a washing tank with a concentration of about 10400 ppm of Cr(VI) was used after dilution with distilled water, until a concentration suitable for the experimentation was obtained, as it will be described for each test.

7.2 Biosorption tests for strain selection

Figure 56: Left: System of dialysis: in the picture 300mL of biomass in 10 L of deionized water. Right: Example of cylinders during the removal experiments.

The initial screening of the strains listed above, to find the best one to be used for chromate biosorption at the industrial scale, was carried out as follows:

- For each cyanobacterial strain ca 300 mL of biomass were collected from the growing culture to be used in the tests (ca 150 mL) and for dry weight determination (ca 150 mL).
- All the 300 mL of culture were placed for 18 h in dialysis against ca 60 L of deionized water to eliminate the salts of the culture medium. The culture, confined within the dialysis membrane, was immersed in 20L of deionized mixed by a magnetic stirrer for ca 6 h, after which the entire volume of water was substituted. This operation was repeated three times (Fig. 56).
- At the end of the dialysis process, the culture was placed in a beaker and the pH was adjusted to about 7 with HCl 1 N or NaOH 1 N.
- After this, 150 mL of culture were subdivided in three aliquots of 50 mL, each of which was confined in a dialysis tube. The three dialysis tubes containing the treated biomass were immersed for 48 h in 500 mL cylinders containing 450 mL of wastewater previously diluted with water to obtain a Cr(VI) concentration of about 10 mgL^{-1}.
- During the immersion of the biomass two samples of the metal solution were collected at time zero (T_0) and after 48 h.
- At the end of the experiments Cr(VI) concentration in the solution was determined spectrophotometrically using a colorimetric method and total chromium was determined by atomic absorption spectrometry.

For the experiments pyrex glass 500 mL cylinders were subdivided in groups of four and contained 450 mL of diluted wastewater at the same concentration and pH (Fig. 56). Three out of the four cylinders contained the wastewater and 50 mL of confined biomass and the fourth contained the metal solution and 50 mL of deionized water at pH 7 confined within the same system of the tested biomass, used as a blank for evaluation of the removal not due to biomass.

7.3 Laboratory tests of Cr(VI) biosorption with Nostoc PCC7936

After the initial tests the strain *Nostoc* PCC7936 was further investigated to verify its possibility of industrial use. With this aim, several trials were carried out to determine the optimal parameters of utilization for scaling up. In these experiments the efficiency of *Nostoc* PCC7936 was verified by using the culture directly from the fermentor, without pre-treatments:

1. About 300 mL of culture at a density >1 g/L were collected from the fermentor and subdivided in two aliquots of 150 mL each: one to be used in the tests and one to determine dry weight (used as a parameter for determination of the specific biosorption).
2. The 150 mL for the trials was subdivided in three parts of 50 mL each and confined in dialysis membrane with a cut-off at 16000 Dalton and diameter 28,6 mm.
3. The three dialysis tubes containing 50 mL of culture each were immersed in 500-ml cylinders, each containing 450 mL of industrial wastewater, supplied by a galvanic company, diluted with deionized water to a concentration of Cr(VI) about 20 ppm. Wastewater was mixed by means of magnetic stirrers.
4. For each trial, besides the three tubes containing biomass, a fourth one containing only deionized water was used as a control to determine the effects of the experimental system on chromate solution.
5. After 48 h of immersion of the dialysis tube in the diluted wastewater an aliquot of ca 100 mL for each of the four cylinders was collected to determine the content of Cr(VI), Cr(III) and total Cr.

Even if the tests without pre-treatment are, for economic reasons, the way preferred by industries, to evaluate if the pre-treatments would have increased the efficiency of biosorption in such an amount to justify its use, tests with an acidic pre-treatment were carried out, based on the literature data on treatment of wastewaters rich in Cr(VI) with biomasses (Volesky, 2003).

In this case, the experimental procedure was the same previously described, but the four dialysis tubes, three containing biomass and one containing deionized water, were subjected to a dialysis pre-treatment against deionized water for 24 h and against 3% HCl for 6 h, to remove the salts contained in the culture medium. At the end of the treatment with hydrochloric acid, the dialysis tubes were directly used in the cylinders containing diluted wastewater. The dialysis tube containing deionized water was treated as biomass with the aim to have a control also in the pre-treatment phase to evidence possible interferences in the trial due to the experimental system.

Once obtained the dialysis tubes containing the pre-treated biomass and the blank, the trial continued as previously described at points 3, 4 and 5 of the experimental procedure.

Nostoc PCC 7936 biomass is composed by a cellular fraction and a polysaccharide fraction with very different chemico-physical characteristics. To determine which of these two components plays a key role in the biosorption and bioreduction processes of chromium (VI), specific trials were carried out. The summary of the experiments is reported in table 8.

In this case biomass was centrifuged at 7000 g for 15 minutes to separate the polysaccharide released in the medium (RPS- Released PolySaccharide) and then obtain a biomass composed almost exclusively by the cellular fraction, to be tested as previously described at points 1-5. The centrifuged biomass was resuspended in deionized water before its transfer in the dialysis tubes.

Also in this case the effect of acidic pre-treatment was investigated, by subjecting the centrifuged biomass to the treatment previously described.

Besides optimising the efficiency of biomass with pre-treatments the investigations aimed at understanding the necessity to have viable biomass during the biosorption processes.

These processes, as already described, are not of physiological origin but of chemical nature, between the cell surface, either membranes, or cell wall or capsule, and the medium in which the cell is immersed. Post mortem cell degeneration creates alterations of these structures that makes them completely different from those in the viable cells.

The necessity to have or not viable cells is a crucial point in view of an industrial application, since the answer to this question determines the choice to be done for storage and supplying of the necessary provisions.

To investigate this, experiments with autoclaved *Nostoc* PCC7936 biomass were carried out. Also in this case the experimental procedure previously described at points 1-5 was followed for biomass. Trials with and without acidic pre-treatment, as previously described, were also carried out (Tab. 8).

Table 8: Summary of the parameters used in the experiments of Cr(VI) biosorption carried out in laboratory with *Nostoc* strain PCC7936 (X= done, O= not done).

Trial	Concentration of biomass in g/L	ppm of Cr (VI) present in the wastewater used	centrifugation	autoclaving	Dialysis against demineralized H_2O and HCl 3%
A	2,28	20,27	O	O	O
B	4,21	20,27	X	O	O
C	6,5	17,32	X	X	O
D	0,93	18,15	O	O	X
E	10,49	14,39	X	O	X
F	5,73	17,58	X	X	X

At the end of the experiments *Nostoc* PCC7936 polysaccharide was tested alone:

- 1.400 g of polysaccharide extracted, lyophilized and stored in powder at -20°C were placed for 12 h in 200 mL of deionized water for re-hydration.
- 30 ml of the solution containing polysaccharide were subdivided in 3 aliquots of 10 mL to determine dry weight.
- 150 mL of the solution containing the polysaccharide were divided in three dialysis tubes “size 2”, placing in each tube 50 mL of solution with 0.35 g of dry polysaccharide (the concentration value was determined in a second time on the basis of dry weight of the solution containing the polysaccharide).
- The three dialysis tubes, each containing 50 mL of polysaccharide, and one for blank containing 50 mL of deionized water were placed in dialysis against 0.3% HCl for 6 h.
- At the end of the pre-treatment, the three dialysis tubes and the blank tube were placed each in a 500 mL cyclinder containing 450 mL of Cr(VI) solution at a concentration of 12.12 ppm.
- Samples were collected after 6, 12, 24, 48 and 90 h since the start of the experiment.

7.4 Cr(VI) biosorption experiments with Nostoc PCC7936 in pilot plant in the firm.

7.4.1 Experiments for treatment in continuous: PVC column and system with filterpress

The plants described in this paragraph were designed and realised by our research group. Patenting is under evaluations, so a detailed and exhaustive description is not possible. Minimal information for their understanding will be provided.

PVC columns, designed and realised purposely for the experimentation, were positioned in a vertical way. At the top a manometer was placed to measure the inside pressure, while a laterally-positioned rotary electro-pump guaranteed the circulation within the system of a flux directed from top to bottom. At the beginning a flux of culture loaded the column with biomass and, in the following step, wastewater containing chromium was circulated for the biosorption experiment. The methodology followed was:

- The column was loaded with 3-mm quartz grains for 50% of its height.
- For 6 h 30 L of *Nostoc* PCC7936 culture, concentrated according to the procedure previously described, were circulated.
- Then the column was loaded for the remaining 50% with quartz grains of the same granulometry of the others.
- The same *Nostoc* culture was circulated again until the liquid coming out from the column was no longer green (about 12 h).
- At the end of the column loading operation, the biomass at its interior was conditioned by circulating a 3% HCl solution for 30 minutes and then letting to rest for 3 h.
- After this pre-treatment 30 L of wastewater with a Cr(VI) concentration of 10.4 ppm was circulated inside the column.
- The experiment was monitored by sampling every 24 h.
- After the first 48 h strain *Cyanothece* TI4 was added from the top of the column. Since the addition of this second strain the trial continued for another 100 h.

The operations described above have been possible thanks to the help of a series of valves that allowed to circulate the culture, the acid, water and wastewater, as described in figures 57 and 58.

The filter press (Figg. 59, 60, 61) is made of a series of filters spaced by separators kept together thanks to a press with a screw piston. A rotary pump, linked to the internal space of the press, allowed the circulation of solutions through the filters.

The experimentations was carried out as follows:

- At the start water was circulated inside the filter press to verify its holding.
- Then 30 L of concentrated *Nostoc* PCC7936 culture were circulated according to the procedure previously reported, to load the filters with biomass. The end of this phase was established, as for the column, from the observation of the colour of the liquid coming out of the system (it must become colourless) or at the limit of the 2 bar pressure inside the press chamber. The pressure inside the filter chamber progressively increases with the increase of the biomass kept by the filters. This phase lasts about 24 h.
- At the end of this phase a 3% HCl solution was circulated inside the filters for 30 minutes to pre-treated the biomass.
- At the end of 30 minutes the acid was discharged from the filter press and the system was let to rest for 3 h to allow acid action.
- At the end of the 3 h deionized water was circulated to eliminated the acid residues.

Two experiments in a row were carried out in the filter press without intermediate treatment to regenerate the biomass:

- In the first, 30 L of wastewater with a Cr(VI) concentration of 10.40 ppm were treated.
- In the second, 30 L of wastewater with a Cr(VI) concentration of 112.30 ppm were treated .
- In both cases the 30 L were circulated inside the filter press while samplings were carried out every 24 h for 3 days (first experiment), and 4 days (second experiment). Between the two experiments there were 24 h, during which the biomass on the filters was not subjected to any regeneration treatment.

7.4.2 Treatment in batch: system with dialysis cell

The dialysis cell (Figg. 62, 63) was designed to emulate, on a higher scale, the dialysis tubes used during laboratory experiments. To this aim, a moplen frame with windows closed with a dialysis membrane on one side was realised. The cell had, in the top side, opposite to the windows, an anchorage system that allowed its placement on the rim of a tank, also purposely realised to facilitate the immersion and recovery operations (fig. 62, detail). The experimental procedure was the following:
- The cell was filled with 33 L of *Nostoc* PCC7936 culture at about 15.5 g d.wt/L, mixed inside the cell by air, bubbled through a tube placed at the bottom of the cell.
- The cell was immersed in 30 L of 3% HCl for 24 h and then used for the biosorption experiment against 100 L of wastewater with 23.05 ppm of Cr(VI).
- Samples were collected every 8 h for determination of Cr(VI), Cr(III) and total Cr.

Figure 57: Panoramic view of the system realised with a PVC column. It is possible to see the column and the tanks linked to it by a rotary pump allowing the circulation of the different solutions inside the system. The blue valves placed along the circuit allowed column loading, recirculation of the culture and of wastewater, besides the discharge at the end of the experiment. Top: Detail of the PVC column. It is worth noting the green part, at the bottom, loaded with biomass and the light grey part, at the top, made of quartz added in a second time and still deprived of biomass.

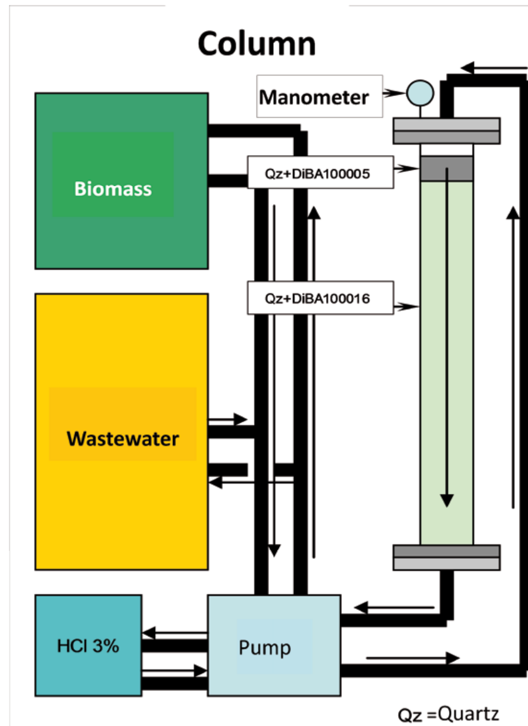

Figure 58: Column experimental system functional scheme. The system is composed of 5 parts: three tanks, a rotary pump and a column loaded with quartz at variable granulometry. The system is designed to allow separate links between the column and each tank, in order to be able to load the column, at the beginning of the trial, with biomass and, after, to allow biomass exposition to acidic solution for pre-treatment and then to metal solution for the biosorption experiment.

Figure 59: Panoramic view of the filter press. The filter press is at the top, while beneath there is the rotary pump and under the pump the tank.

Figure 60: Details of the filter press. Left: detail of the inlet tube, at the left, recognizable by the yellow colour of the Cr(VI) solution at its interior, and the outlet tube of the filter press, white, through which the solution flows after reaction with biomass. Right: panoramic view of the filter press. In foreground the control unit and in the background inlet and outlet tubes of the filter press.

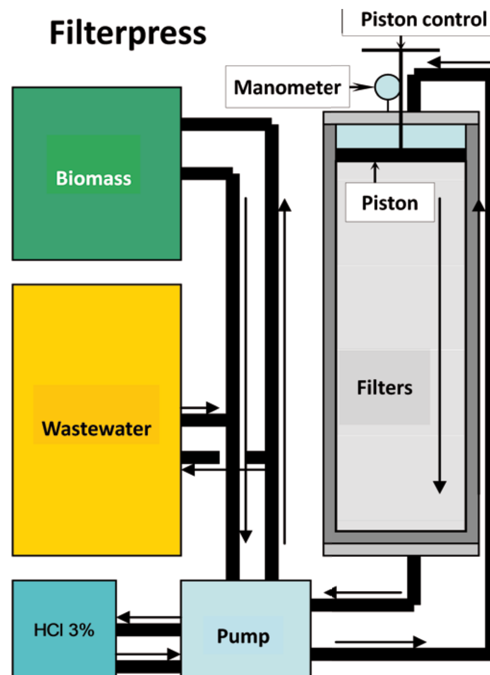

Figure 61: Scheme of functioning of the experimental system with filter press. The system is composed by 5 parts: three tanks, one rotary pump and one filter press made of a battery of filters with <2 μm porosity. The system is designed to allow separate links between the filter press and each tank, in order to be able to load the filters with biomass at the beginning of the trial, and, after, to expose the biomass to acidic solution for pre-treatment and then to the metal solution for the biosorption experiment.

Figure 62: Dialysis cell inside the tank used for the experiments. Detail: Experimental tank with the dialysis cell hanged at its right edge.

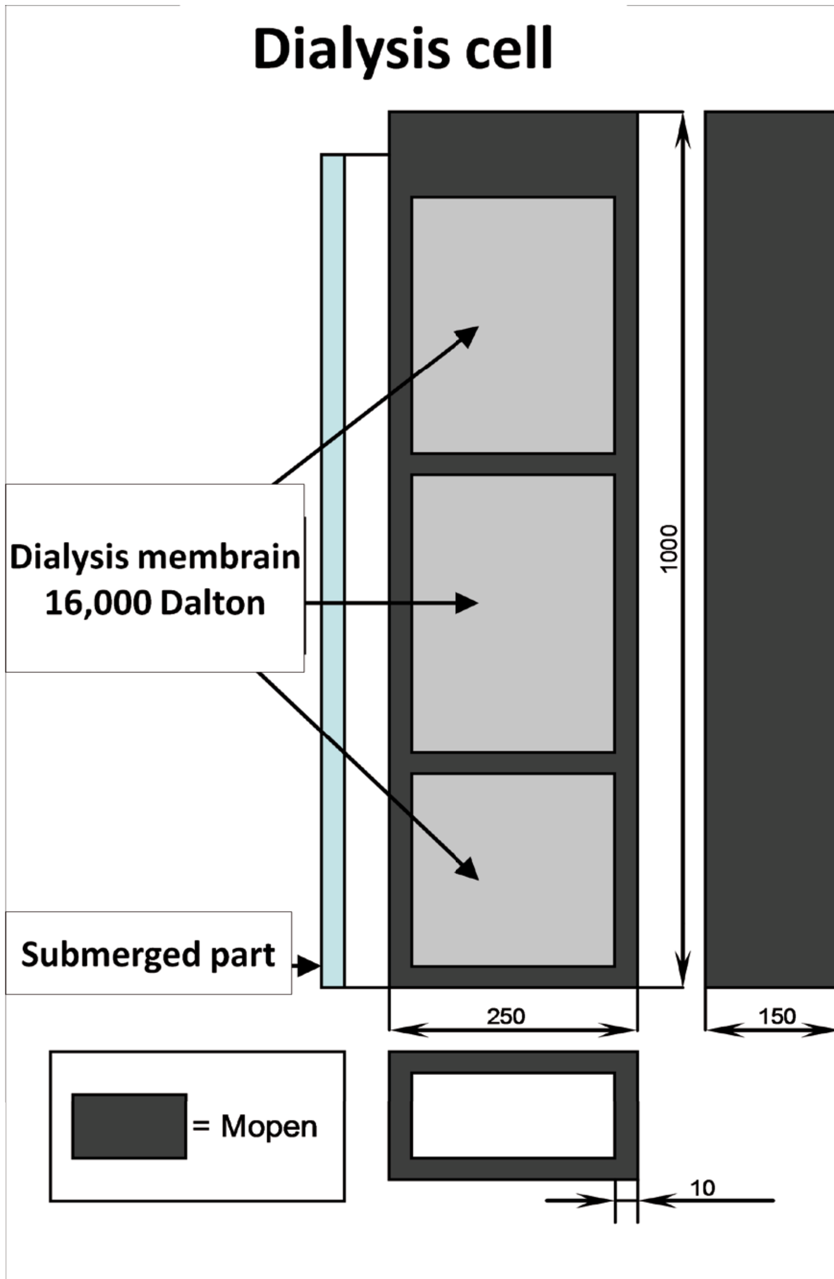

Figure 63: Assonometric view of the cell. Proportions are not respected. This cell was designed to emulate, on a higher scale, the dialysis tubes used in laboratory experiments. To this aim, a moplen frame with, on one side, windows closed with a dialysis membrane with a 16000 -Dalton cut-off was realised. The cell had, in the top side, opposite to the windows, an anchorage system that allowed its placement on the edge of a tank, also purposely realised to facilitate the immersion and recovery operations.

8. Incineration of the exhausted biomass

The experimentation with strain *Nostoc* PCC7936 ended with the biosorption trials in pilot plant, but, on the basis of that experience, the work continued with the screening of new strains, new ways of application, pre-treatment, recovery of the biomass, etc. This part of the experimentation is, however, presently covered by confidence agreement with the company that funded the research. The company agreed to publish in this thesis the data obtained from incineration of bacterial biomass at the end of the new biosorption experiments.

At the end of a trial in which a chromate rich wastewater was treated, a fraction of the biomass used for the treatment was harvested with the aim to evaluate the possibility of metal recovery. To this purpose, the biomass was incinerated through a thermal treatment in muffle kiln at about 800 °C for about 6 h.

At the end of this treatment there was a huge reduction in volume, useful in a perspective of a possible necessity to store these ashes rich in metal as a special waste. The colour of the ashes was a light green, index of a significant presence of chromium oxides (Fig. 64).

Figure 64: Biomass incinerated after biosorption. The green colour is due to the abundance of chromium oxides

9. Analytical systems

To monitor the growth of the culture used for the trials and determine its effective state at the moment of the experiments, aliquots of the cultures were periodically collected and analysed through chemical and colorimetric methods to estimate the temporal variation of protein content and total and soluble carbohydrate concentration.

As a reference parameter for evaluation concerning the capability of removal, dry weight of the culture and protein content were used, because in a balanced growth protein content of a culture is proportional to the amount of biomass present.

9.1 Dry weight determination

To determine the dry weight of a biomass used in an experiment the following procedure was adopted:

1. Placement of membranes of 47mm-diameter and 0,45 μm porosity in a stove at 110°C for 3 h.
2. At the end of the 3 h, transfer of the membranes in a desiccator until cooling at room temperature.
3. Weighing of each membrane separately.
4. Filtration of 5 ml of biomass (Fig. 65) on each membrane.
5. Reintroduction of the membrane used for filtration in the stove at 110°C until they reach constant weight (about 5 h).
6. Weighing of each membrane after desiccation.
7. Calculation of biomass weight in the 5 ml of culture filtered by subtracting the weight of the membrane before filtration from that of the membrane + biomass (Fig. 66).

In the biosorption experiments dry weight was carried out on biomass after the dialysis treatment. With this system the error due to the presence of salts in the culture medium, especially for strains growing in 33 g/L salinity medium as *Cyanothece*, was reduced.

Another method to determine dry weight was adopted when cell density was so high to make filtration difficult. A known volume of culture was transferred into a beaker of known weight, that was then placed in a stove to allow water evaporation. Knowing the initial volume the weight of the salts was estimated from the weight of the salts contained in the same volume of fresh medium. The net weight of the culture expressed in g/L was obtained by subtracting the beaker weight + salt weight from the biomass + salts + beaker weight after biomass desiccation. Operatively, 50 mL of culture were poured into a 50 mL beaker and placed in a stove at 120 °C for 24 h. Once the biomass was dried the beaker was weighed, washed from biomass, dried and weighed again. The difference between the two weights, in grams, represented the weight of the 50 mL of culture.

Figure 65: System for biomass filtration.

Figure 66: Filters after filtration of 5 mL of biomass

9.2 Total chromium determination

To detect the amount of metals in the solutions, before and after contact with cyanobacterial biomass, an atomic absorption spectrometer equipped with an air/acetylene flame atomiser was used (Fig.67).

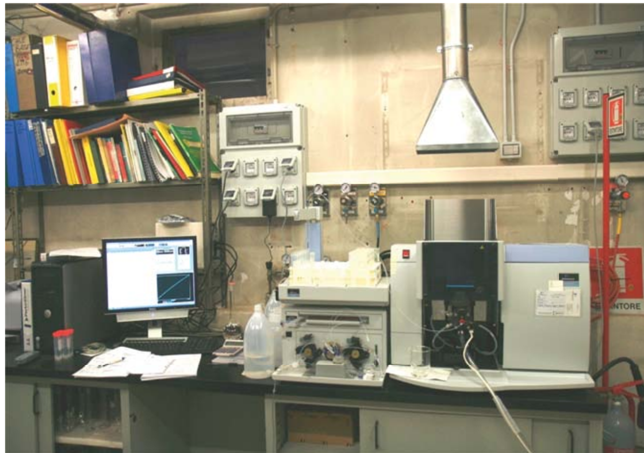

Figure 67: Perkin Elmer Analist 400 atomic absorption spectrometer.

9.3 Cr(VI) and Cr(III) determination

To determine Cr(VI) a colorimetric method based on diphenylcarbohydrazide was used, and the reaction product read at 525 nm with a spectrophotometer, setting a

blank for calibration with a sample before reagent addition (ASTM Manual of Water and Environmental Technology, D1687-92). Each sample was read in triple. Chromium (III) was determined by difference between total chromium, determined by atomic absorption, and Cr(VI) determined as described above.

9.4 Complete analysis of wastewater

To have a complete analysis of the wastewater provided by a galvanic industry, and to evaluate the results of the second experiment with the filter press a detailed metal composition analysis was performed at the ARPAT laboratory of Piombino (LI - Italy).

The initial wastewater and that at the end of the trial were analysed by an ICP-mass to obtained data not only concerning Cr (VI), Cr (III) and total Cr, but also the presence and variation of other metals such as: Fe, Cu, Zn, As, Cd, Hg, Ni, Pb.

9.5 COD determination

At the end of some of the experiments, to evaluate the amount of organic substance in solution, COD was analysed according to the EPA 410.4 method, in which the oxidizable organic compounds reduce dichromate (orange) to Cr(III) (green) allowing determination of the remaining chromate by means of a UV-visible spectrophotometer at 420 nm.

9.6 Ash speciation

While investigating the possibility to recover the metal in the most effective way, ashes were analysed to determine its main constituents. To this end, ashes were observed by scanning electron microscopy. Aliquots of the ashes were let to dry on vessels in a desiccator to eliminate humidity. In the scanning electron microscope (SEM) a very thin electron bundle with an energy up to 30 keV was focused on the surface of the sample inside the microscope and a scan in form of a succession of parallel lines was taken. Some phenomena occur at the surface submitted to the electron impact, among which the most important for electron microscopy are:

1. the emission of secondary electrons with an energy of few dozens eV;
2. the re-emission or reflection of electrons at high energy, or back-diffused, belonging to the primary ray.

In particular, the secondary electrons were used to create images magnified up to 200000 times and resolved up to 5 nm, as, thanks to their low energy, they arrive from the surface layer of the sample, while the primary electrons are used to identify the presence of different compounds within an heterogeneous sample, as the intensity with which they emerge are a direct function of the mean atomic number of the substance subjected to the primary ray.

The chemical analysis (microanalysis) in the SEM was realised by measuring the energy and distribution of the X-ray intensity generated by the electronic bundle on the sample through an EDS (Energy Dispersion Spectrometry) dispersion energy detector. The analysis produced can be of the area that is magnified in that moment, or, stopping the scan of the electronic bundle, of a point of interest on the surface of the sample (microanalysis).

10. Elaboration of results

10.1 Specific removal determination

Specific removal determination: in the literature two main systems are reported for specific biosorption (q) operated by a certain biomass against a certain metal, both based on the amount of biosorbed metal per mass unit of biomaterial tested (Volesky, 2003). The substantial difference between the two techniques is in the way in which the bioabsorption reaction is evaluated:

- It is possible to evaluate biosorption, usually evaluating the efficiency of the biosorption systems, from an atomic-molecular point of view, by the number of mmol of metal removed per mass unit of the biosorbent material.
- Biosorption can be evaluated from an applied point of view, considering the mg of metal removed from a solution per mass unit of the biosorbent material. Sometimes for scaling up and in plants where large volumes of wastewater are treated using huge amounts of biomass, this formula is readapt by increasing the unit of measurement used.

In terms of dimensions this means:

- In case 1 :
  q = moles (mmol) of metal removed $\cdot$ g^{-1} of biomass used
- In case 2 :
  q = mg of metal removed $\cdot$ g^{-1} of biomass used

As a mass unit grams of dry biomass are usually adopted but, in certain cases, and overall for base research, mg of proteins or carbohydrate can be used for a more accurate investigation and to explain more deeply a specific result.

To study the electro-chemical and adsorption processes, besides determination of specific removal, it is important to quantify the ion charges at the base of the metal biosorption reactions.

This was made by multiplying the number of mmol of metal removed by the ionic valence of the metal under examination, and dividing this result by the grams of dry biomass used, then obtaining the number of functional charges per biomass unit.

The unit of measurement of the charges, for this type of investigation, is Faraday (F), not to be confused with Farad (also indicated by the letter "F"), that is a unit of measurement of electric capacity. One Faraday is equal to the charge of an electron mole: $F = N_a \cdot e$

As the Faraday is one mole of charges it is more useful to the type of investigation carried out in this thesis, than to the more diffused Coulomb (C). It is, however, to remember that:

$1\ F = 9{,}6 \cdot 10^4$ Coulomb

In some cases mFaraday will be used:

$1\ mF = 1 \cdot 10^{-3}\ F$

In the case of Cr(VI) two types of specific removal, which will be specified every time to avoid confusion, were used:

- $q_{(Cr(VI))}$ = mg of Cr(VI) removed per g of dry biomass. It is not correct to call it biosorption as the Cr(VI) removed is usually partly adsorbed by biomass and partly reduced to Cr(III).

- $q_{(tot)}$ = mg of total Cr removed per g of dry biomass. In this case we refer to the total amount of chromium in the solution, either Cr(VI) or Cr(III).

10.2 Comparison of biosorption in mono- and multi- metal systems

In the experiments carried out with solutions containing only one metal specific removal was determined both as mg of metal removed per g of dry biomass and as mmol of metal removed per g of dry biomass.

Moreover, the mFaraday per gram of dry biomass necessary to each biosorbent for the biosorption of each single metal have been determined

For the experiments carried out with multi-metal solutions, the same specific removal parameters were determined for each metal, but besides comparing the variation of q for different metals, and with the respective q in mono-metal solutions, a comparison, for each biomass, between the maximum q obtained with mono-metal solution and the maximum q for multi-metal solutions, calculated as the sum of q for each metals was performed. Then, for multi-metal solutions two types of specific removal (q) were used:

- $q_{(metal)}$, determined for each of the metals contained in the solution.
- $q_{max} = q_{(metal\ 1)} + q_{(metal\ 2)} + \ldots + q_{(metal\ n)}$, obtained from the sum of the specific removal calculated for each metal contained in the multi-metal solution.

It was then possible to determine if, in absolute value, the biomass in multi-metal solution removes more or less metal compared to the same biomass operating in mono-metal solutions.

Moreover, the same type of comparison was carried out between mFaraday that the biomasses were able to adsorb in mono- and multi-metal solutions, to evidence also in this case the possible phenomena of inhibition or stimulation of biomass in different systems.

10.3 Analysis of results

All the laboratory experiments were carried out in triple with at least one control and each single sample was read in triple or quintuple or more, according to the complexity of the sample reading. For the experiments carried out at semi-pilot plants it was not possible to repeat the trials thrice but the differences among the three systems used were analysed to confirm the data observed.

Where possible, standard deviation has been reported, expressed as percent of the mean results (RSD%), either for the single reading or for the results obtained from the mean reading. When this was not possible the mean value calculated on at least three samples has been reported.

Moreover, to determine the significance of the results, comparisons were evaluated by Student's t-test.

Chapter 3
Results

1. Biosorption tests in mono-metal and ternary systems

The first series of tests carried out on cyanobacterial strains, seven *Cyanothece* ET5, TI4, CE4, VI13, VI22, PE14, 16SOM2, *Nostoc* PCC7936, and *Cyanospira capsulata*, showed that all these biomasses have a good biosorbent power, compared to the literature data (Volesky B. 2003), with a mean value between 30 and 60 mg of metal removed per gram of dry biomass (mg/g(DW)) and a maximum of more than 200 mg/g (DW) for strain *Cyanothece* 16SOM2 with a 10 ppm copper solution.

1.1 Specific removal from mono-metal solutions

As to the capability of specific removal (q) of the tested strains in mono-metal solution, biomasses generally showed a different adsorption efficiency according to the metal tested, in decreasing order Cu>Cr>Ni. This is evident from the average removal for the single metals, considering specific removal expressed both as mg of metal removed per gram of dry biomass and as mmol of metals removed per gram of dry biomass. If, instead, the mFaraday that biomasses can accumulate as ion charge are considered, biomasses appear to adsorb a higher number of charges with chromium solutions (Fig. 68).

For specific removal the highest values were obtained with chromium solutions using *Cyanothece* 16Som2, CE4 and TI4, corresponding to ca 196, 95 and 67 mg Cr(III) removed/ g dry weight, respectively (Fig.69, Tab 9).

With copper solutions, the strains that showed the highest biosorptions were *Cyanothece* 16SOM2, ET5 and *Cyanospira capsulata*, able to sequester ca 201, 143 and 113 mg of metal per gram of dry weight, respectively. The results with nickel evidenced a lower removal efficiency compared to the other metals tested, with maximum values of ca 30.6, 19 and 13.4 mg of metal per gram of dry weight for strains *Cyanothece* ET 5, *Cyanospira capsulata* and *Cyanothece* PE 14, respectively.

Considering mmol of metal removed per gram of biomass the picture is similar to that just exposed, as it is evident comparing figure 69 with figure 70. Slight differences appear for strains *Cyanothece* 16SOM2 and VI22, as the millimoles of chromium removed were higher than those of copper, differently from what emerged considering the data expressed in mg of metal.

Significant differences emerge when considering the data expressed in mFaraday, as it is clear from the data reported in figure 71, compared with those in figures 69 and 70.

To calculate the ion charges adsorbed by biomasses it is useful for the study of biosorption and, overall, in the cases when, as in this thesis, the comparison is among elements with different ionic valence, in this case biosorption of bivalent ions, as Cu and

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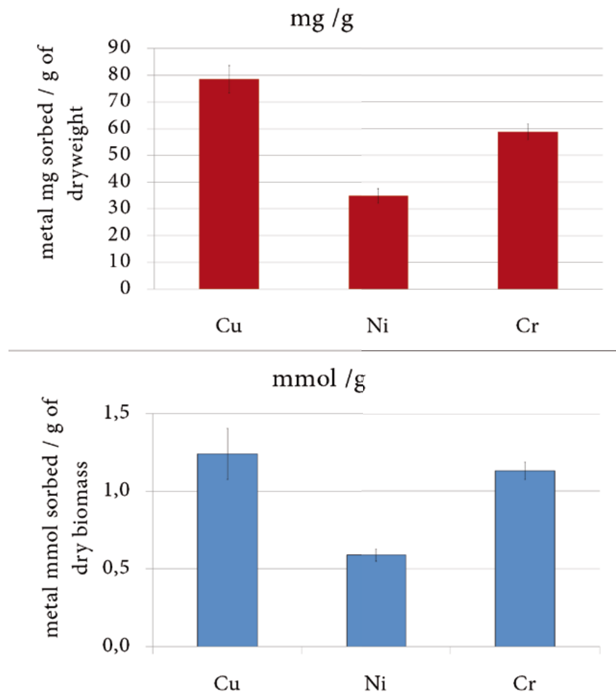
Ni in water solution, with biosorption of a trivalent ion, as Cr(III). In particular, it is worth noting how the strain *Cyanothece* 16SOM2 with chromium adsorbs about twice the charges than with copper. Comparing biosorption of strains *Cyanothece* VI13 and VI22, expressed in mmol of metal /g dry biomass, with copper and chromium, the specific removals appear similar, while analysing the same biosorption expressed in mFaraday the differences are much more evident, reaching nearly 1 mF of difference between the two metals, and indicate how many charges in excess have to be adsorbed with the same number of metal atoms adsorbed.

1.2 Kinetic of removal in mono-metal systems

The biosorption tests carried out with mono-metal solutions showed a rapid kinetic, reaching saturation after a time of contact biomass-metal solution of about 30-40 minutes (Figg. 72, 73, 74). This velocity of metal sequestration is in accordance with the hypothesis of adsorption phenomena and not of physiological uptake. In fact, already during the first 5 minutes of exposure an average of 58% of chromium and copper and 50% of nickel ions were removed. After 15 minutes, removal reached values of about 83, 75 and 68% for chromium, copper and nickel, respectively. In the following 15 minutes the system reached equilibrium, indicating that the maximum specific removal possible with those metal concentrations, culture density and temperature had been reached.



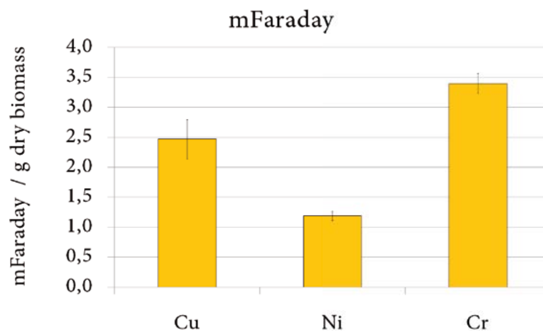


Figure 68: Top and center: Average specific removal (q) obtained in the tests carried out with solutions containing one metal at a time, expressed as mg/g (top) and mmol/g (middle). Bottom: average mFaraday value used for biosorption.

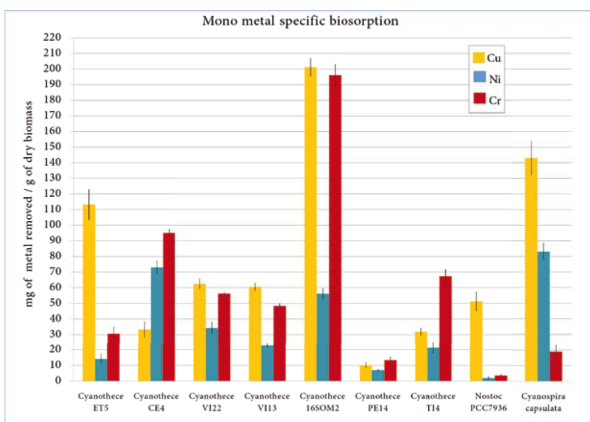


Figure 69: Specific removal (q) obtained in the experiments carried out with solutions containing one metal at a time. In this case q is expressed as mg of removed metal / g of dry biomass.

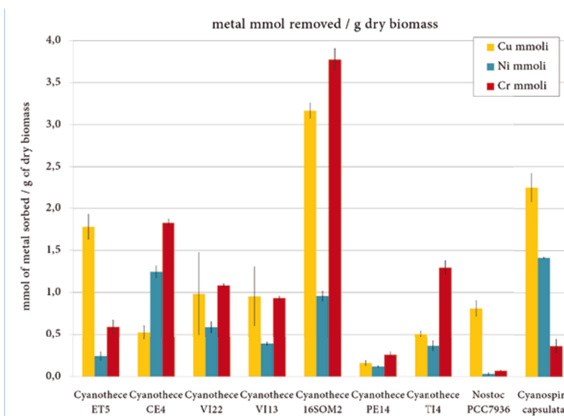


Figure 70: Specific removal (q) obtained in the experiments carried out with solutions containing one metal at a time. In this case q is expressed as mmol of removed metal / g of dry biomass.

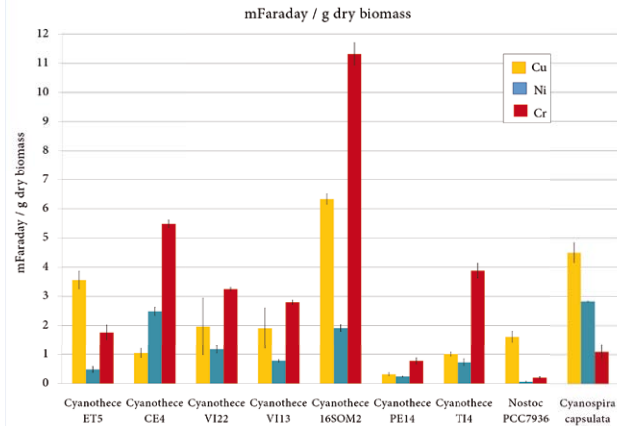


Figure 71: mFaraday adsorbed by 1 g of biomass during biosorption in the experiments carried out with solutions containing one metal at a time.

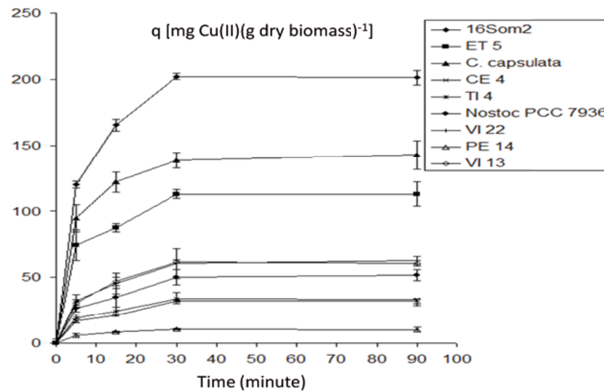


Figure 72: Kinetic of removal of copper ions operated by the cyanobacterial biomasses tested (T=20°C, metal concentration in solution 10 ppm, biomass concentration ca ~0,5 g/L).

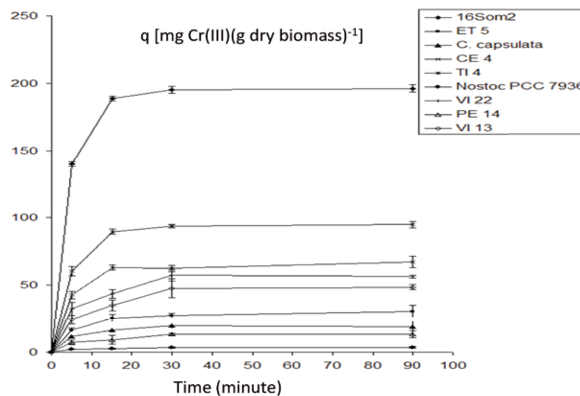


Figure 73: Kinetic of removal of chromium ions operated by the cyanobacterial biomasses tested (T=20°C, metal concentration in solution 10 ppm, biomass concentration ca ~0,5 g/L).

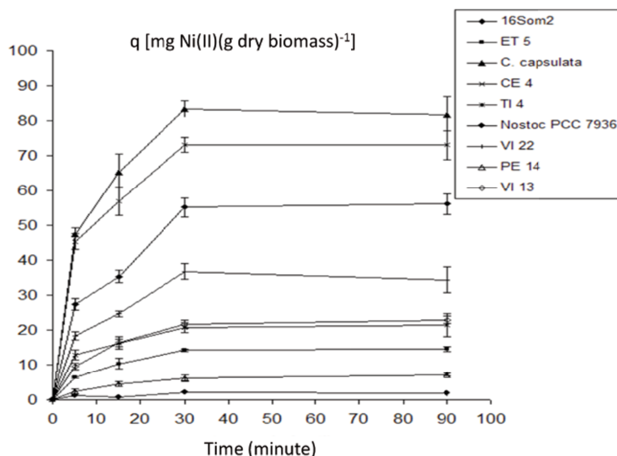


Figure 74: Kinetic of removal of nickel ions operated by the cyanobacterial biomasses tested (T=20°C, metal concentration in solution 10 ppm, biomass concentration ca ~0,5 g/L).

1.3 Specific removal from multi-metal solutions

The experiments carried out with multi-metal solutions showed that specific biosorption was generally lower than that obtained with mono-metal solutions. Exceptions were the strains *Cyanothece* TI 4 and PE 14, which were able to remove more nickel in the multi-metal than in the mono-metal solution. In table 10 the results obtained during the biomass screening phase with the multi-metal solution containing Cu, Cr (III) and Ni are reported, while in figures 76, 77, 78 the bar charts originating from the same data are represented. It is possible to observe:

- Specific removal (q) expressed in mg of metal removed per g of dry biomass, figure 76.
- Specific removal (q) expressed in mmol of metal removed per g of dry biomass, figure 77.
- mFaraday of ion charges adsorbed by 1 g of dry biomass during the biosorption tests, figure 78.

Table 10: Specific removal (q) of the nine cyanobacterial strains in multi-metal systems, expressed as mg of metal removed per gram of dry weight.

	Cu^{2+}	Ni^{2+}	Cr^{3+}
Cyanothece ET5	56,4±6,5	12,0±2,0	17,0±2,0
Cyanospira capsulata	65,0±7,6	25,0±2,4	15,0±2,0
Cyanothece CE4	17,0±1,5	23,0±3,1	47,0±3,4
Cyanothece Vi22	16,0±1,0	13,0±2,6	19,0±3,05
Nostoc PCC7936	51,3±2,0	1,5±1,0	1,2±0,6
Cyanothece VI13	35,4±2,0	0,1±0,0	10,4±0,7
Cyanothece 16SOM2	181,0±5,0	2,5±0,1	98,7±7,0
Cyanothece PE14	8,42±0,9	11,2±0,9	4,13±0,4
Cyanothece TI4	24,3±3,9	36,4±4,4	43,5±2,6

Comparing the results obtained for each metal in the mono-metal system with those obtained in these experiments, where multi-metal solutions were used, it appears that the specific removal for a single metal always decreased in the presence of other metal species in solutions, except for strains *Cyanothece* TI 4 and PE 14, which showed, in the case of the nickel ion, an increased removal of about 70 and 60%, respectively, while for other ions the decrease of sorbent power varied from 0.2 to 95.6 % (Fig. 75).

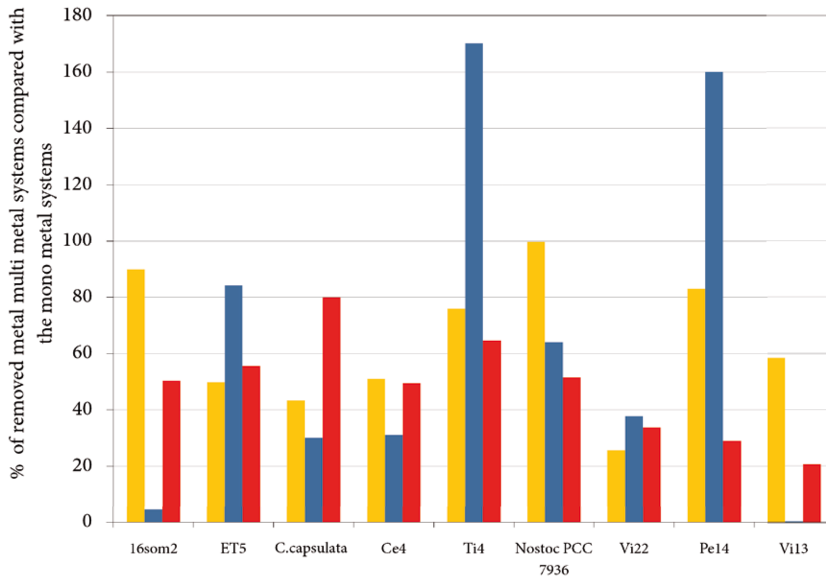


Figure 75: Removal of copper (yellow bars), nickel (blue bars) and chromium (red bars) in three-metal systems, expressed as percentage of removal of the single metal compared to the removal in mono-metal systems.

Ion Cr^{3+} biosorption, which was always lower in muti-metal than in non-metal solutions, showed higher specific removal values with strains *Cyanothece* 16SOM2, CE 4, TI 4, able to remove about 98, 43 and 47 mg Cr(III)/g dry biomass (Tab. 10, Fig. 75). *Cyanospira capsulata*, *Cyanothece* TI 4 and ET 5 had a specific removal equal to 34.4 and 55.5% of that shown with solutions containing only chromium. The reduction in removal of the other strains was from 78.6 to 50.5%.

The higher values of copper biosorption from ternary solutions, equal to 181.0, 55.4 and 65.0 mg of metal removed per gram of dry weight, were obtained with strains *Cyanothece* 16SOM2, ET 5 and *Cyanospira capsulate*, respectively.

The contemporary presence of Ni^{2+} and Cr^{3+} ions was always antagonistic to Cu^{2+}, except for strain *Nostoc* PCC 7936, which adsorbed similar amounts of Cu^{2+} ions in mono-metal and poly-metal solutions.

Experiments showed a lower affinity of biomasses for nickel. The higher removal values were about 36.4, 24 and 25 mg/g, reached by strains *Cyanothece* TI 4, CE 4 and *Cyanospira capsulate*, respectively. The only strain to have a higher affinity for nickel was *Cyanothece* PE 14. The relation between metals was of antagonistic type, except for strains *Cyanothece* PE 14 and TI 4. In this case, there was an increased total removal

capability of about 60 and 70%, respectively, compared to the systems with only one ion type.

There are no significant differences comparing specific removal expressed in mg of metal per g of dry biomass (Fig. 76) with those expressed in mmol (Fig. 77), but, when comparing these with the mFaraday adsorbed by biomass, some differences emerge. The main one is for strain *Cyanothece* 16SOM2, where a similar amount of charges was removed for copper and chromium, while a difference of about 1 mmol of adsorbed metal was noticed (Fig. 78).

To compare the biosorption results obtained using biomasses to treat solutions with one and more than one metal, the best specific biosorption obtained for each biomass with single metal solutions was compared to the sum of specific removals obtained by the same biomass with multi-metal solutions (Figg. 79, 80, 81). Data obtained in this way evidenced three types of responses concerning the total biosorbent power of biomasses:

- the contemporary presence of three metal ions in solution led to a decreased biosorbent capability compared to the best result obtained in the mono-metal system (antagonism);
- the contemporary presence of three metal ions in solution did not show any influence on the total removal capability observed in the mono-metal systems (non-interaction);
- the contemporary presence of three metal ions in solution led to an increased removal capability (synergism).

In the case of *Cyanospira capsulata* and *Cyanothece* ET5 and CE4 a negative interaction was observed, as the total number of metal ions removed was higher in the mono-metal system, that is 2.2, 1.8 and 1.8 mmol of metal removed /g in mono-metal systems, compared to 1.7, 1.4 and 1.6 mmol/g, respectively, in ternary systems (Figg. 81, 82). *Nostoc* PCC7936 and *Cyanothece* VI13 and VI22, instead, did not show significant variations in terms of removal capability, maintaining the same values observed in mono-metal solutions also in the ternary system, being then ascribable to the second type of responses, non-interaction (Fig. 82). Finally, strains *Cyanothece* 16Som2, TI4 and PE14 were more efficient in sequestering metals from ternary solutions than from mono-metal solutions, indicating a positive interaction among metals that amplified biomass biosorbent power (Fig. 82). The antagonistic, synergic or non-interactive behaviour was determined by comparing the differences described above by T-test with a significance value of $p<0.05$.

It is worth noting that mFaraday adsorbed by strain 16SOM2 in chromium biosorption as a single metal and the sum of the mFaraday adsorbed when removing the three metals from the multi-metal solutions were very similar, while comparing the same data expressed as mmol or mg of metal removed per g of dry biomass showed a higher efficiency of biosorption in multi-metal solutions.

1.4 Kinetic of removal in multi-metal systems

Kinetic of metal removal by the tested biomasses did not evidence differences between mono-metal and multi-metal systems, and after 30 minutes the system had reached an equilibrium (Fig. 83, 84, 85).

The analyses performed after 24 h of contact between biomass and metal solutions did not show significant differences compared to those after 30 minutes

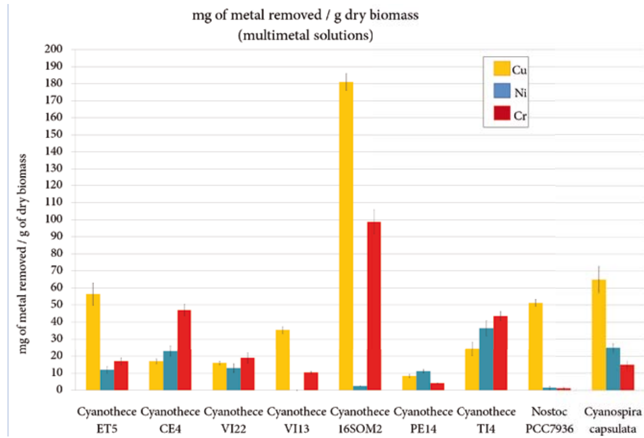


Figure 76: Specific removal, expressed as mg of metal removed /g of dry biomass, determined in the experiments with multi-metal solutions.

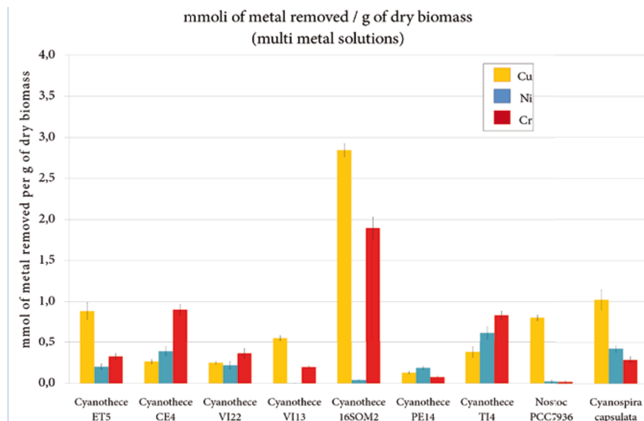


Figure 77: Specific removal, expressed as mmol of metal removed /g of dry biomass, determined in the experiments with multi-metal solutions.

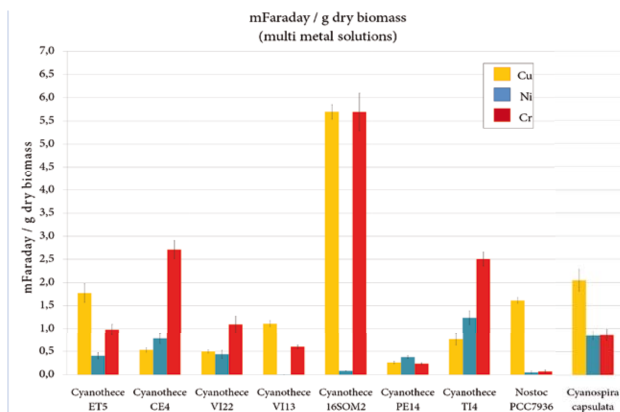


Figure 78: Charges adsorbed by biomass, expressed as mFaraday / g of dry biomass, determined in the experiments with multi-metal solutions.

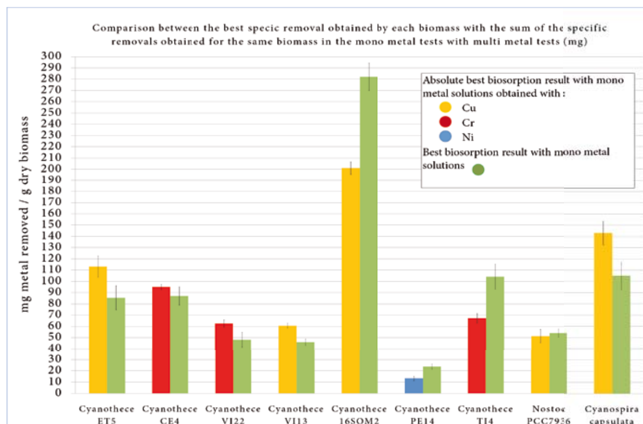


Figure 79: Comparison between the best specific removal obtained by each biomass, expressed as mg of metal removed /g of dry biomass, with the sum of the specific removals obtained for the same biomass in the biosorption tests with multi-metal solutions. In green the bars derived from the sum of specific removals obtained in multi-metal solutions. The other bars are of the colour attributed to the metal with which the best removal in mono-metal solution was obtained: yellow for copper, blue for nickel and red for chromium.

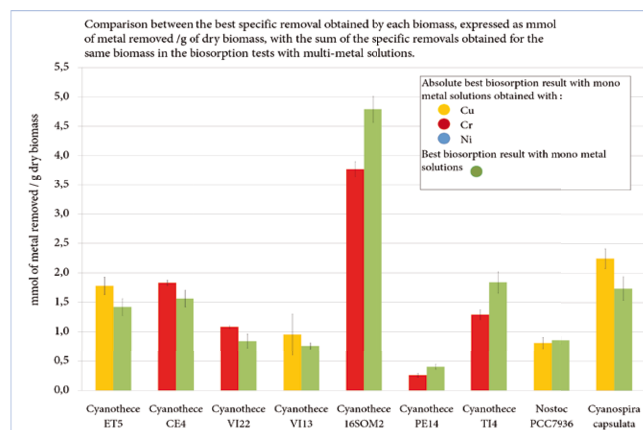


Figure 80: Comparison between the best specific removal obtained by each biomass, expressed as mmol of metal removed /g of dry biomass, with the sum of the specific removals obtained for the same biomass in the biosorption tests with multi-metal solutions. In green the bars derived from the sum of specific removals obtained in multi-metal solutions. The other bars are of the colour attributed to the metal with which the best removal in mono-metal solution was obtained: yellow for copper, blue for nickel and red for chromium.

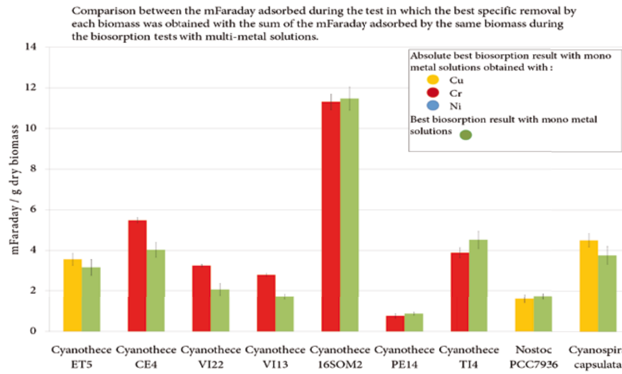


Figure 81: Comparison between the mFaraday adsorbed during the test in which the best specific removal by each biomass was obtained with the sum of the mFaraday adsorbed by the same biomass during the biosorption tests with multi-metal solutions. In green the bars derived from the sum of specific removals obtained in multi-metal solutions. The other bars are of the colour attributed to the metal with which the best removal in mono-metal solution was obtained: yellow for copper, blue for nickel and red for chromium.

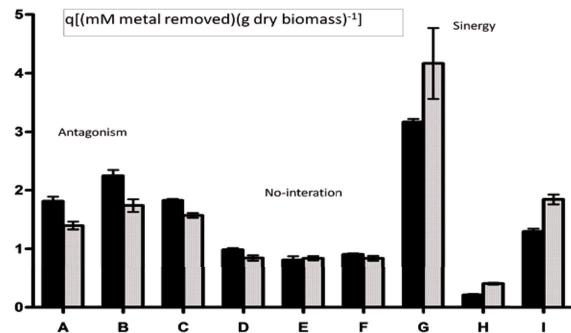


Figure 82: Comparison between the mmol of metals removed, in mono-metal (black bars) and ternary (grey bars) solutions, by strains Cyanothece ET 5 (A), Cyanospira capsulata (B), Cyanothece CE4 (C), Cyanothece VI22(D), Nostoc PCC 7936 (E), Cyanothece VI13 (F), Cyanothece 16SOM2(G), Cyanothece PE14 (H) and Cyanothece TI 4 (I).

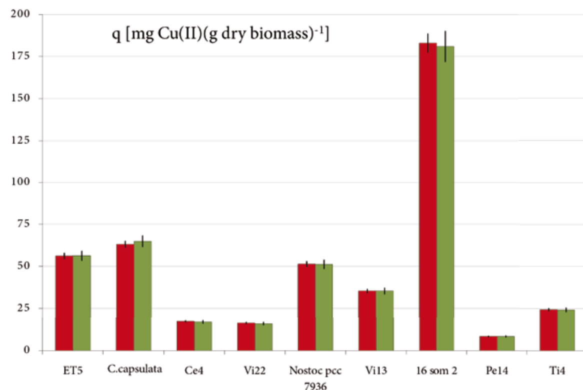


Figure 83: Copper removal by cyanobacterial cultures after 30 (red bars) and 180 (green bars) minutes of contact with solutions containing the tested metals (Cu, Ni, Cr).

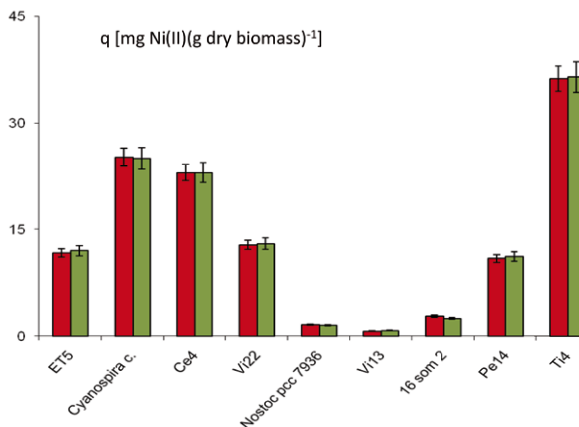


Figure 84: Nickel removal by cyanobacterial cultures after 30 (red bars) and 180 (green bars) minutes of contact with solutions containing the tested metals (Cu, Ni, Cr).

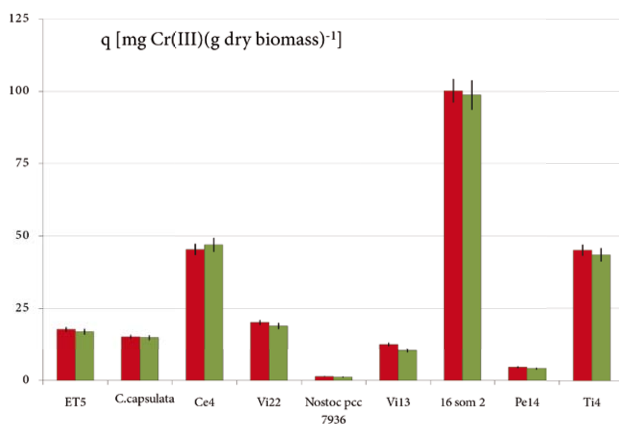


Figura 85: Chromium removal by cyanobacterial cultures after 30 (red bars) and 180 (green bars) minutes of contact with solutions containing the tested metals (Cu, Ni, Cr).

2. Tests with Lake Dian Chi bloom

The whole picture of the results is reported in tables 11 and 12, while the removal kinetics are reported in figures 86, 87, 88.

The trials carried out with the bloom form the Chinese Lake Dian Chi showed that the biomass utilised has a quite low biosorbent power, comparable to that reported in the literature (Volesky B.,2003). The mean biosorption was around 5 mg of metal removed per g of biomass and the maximum was 8.7 mg/g for Cr^{3+} removal.

The first series of trials carried out, named "fresh bloom" as the bloom used was directly collected from the lake in August 2007, with freely dispersed biomass (without

confinement systems) showed a rather low biosorption, with 0.87 and 1.34 mg of metal removed per gram of biomass in the tests with copper and chromium, respectively.

Trials A, B, C, D, using still the fresh bloom collected in August 2007, differently from the first series of experiments, were performed with an acidic pre-treated and confined biomass (trials A and B) or with confined biomass without pre-treatment (trials C and D).

Trials E, F and G were performed on dehydrated and crushed bloom biomass collected in July 2007. Trial E was carried out on confined biomass, trial F on confined pre-treated biomass and trial G on dispersed biomass.

For trials H, I, J, K and L biomass from a bloom collected in August 2003, dehydrated and crushed, was used. Also in this case a trial was carried out with free biomass (trial H), one with confined biomass (trial I), and finally three were carried out with pre-treatments: one with a final pH of 7 (trial J), one with a final pH of 5 (trial K) and one with a final pH of 4 (trial L).

The best result was obtained with free biomass without pre-treatment (trial G), in which biomass showed a biosorption power of 8.7 mg of Cr(III) removed per gram of dry biomass. The best result for copper was, instead, obtained in trial C, with a specific removal of 7.59 mg of Cu(II) per g of dry biomass.

These trials were carried out during my stay at the Institute of Hydrobiology of the China Academy of Sciences in the town of WuHan, China, and at their detachment by the Lake Dian Chi near the town of Kun Ming, China. The laboratories, of very good international level, however, were not completely equipped for this kind of experimentation, and this has led to some mishaps that determined the loss of some data such as in the case of trials F and H with copper solution and trial J with chromium. For these trials the value "n.d." is to be intended as a lack of the result rather than as a lack of response during the experiments.

It is currently in progress an experimentations aimed at completing the picture of the results.

Table 11: Summary of the results of the trials carried out on Lake Dian Chi blooms for Cu^{2+} biosorption.

	Specific removal Cu (II)	
Test	Result	RSD%
Fresh bloom	0,870	0,065
A	2,750	0,229
C	7,590	0,588
E	2,630	0,054
F	n.d.	
G	5,190	0,266
H	n.d.	
I	4,188	0,249
J	2,161	0,096
K	3,737	0,261
L	0,831	0,147

Table 12: Summary of the results of the trials carried out on Lake Dian Chi blooms for Cr^{3+} biosorption.

Results of removal tests for Cr (III)		
Test	Result	RSD%
Fresh bloom	1,344	0,035
B	2,378	0,087
D	5,688	0,027
E	3,708	0,225
F	0,405	0,254
G	8,733	0,157
H	6,743	0,559
I	4,882	0,184
J	n.d.	
K	1,357	0,051
L	0,728	0,030

2.1 Kinetics of removal

In six trials periodic samples were collected in order to determine the kinetic of removal. Compared to several cases reported in the literature (Volesky B., 2003; De Philippis R., 2003, Micheletti E., 2007), the first result emerging from the analysis of graphs, reported in figures 86, 87, 88, is the failure to achieve the equilibrium after 24 h, except in trial I. In all the cases, after a first period lasting from 3 to 12 h, there was a slow of the growth velocity of biosorbent power, which, in nearly all the cases, continued to grow even quite markedly, as in trial G.

In the case of trial I, the lowering of the biosorption power was not due to a release of the metal from the biomass in the solution, but instead to a reduction of the metal in the blank used to calculate the specific removal (Fig. 87).

In particular, from figure 86, reporting the kinetic of biosorption in trials G and H, it is evident how in trial G after 24 h the equilibrium was still not reached. In trial H, instead, the specific removal increased a lot in the first 3 h, then its slope decreased during the following 21 h. Figure 87 reports the kinetics of biosorption for trials I and J. In trial I there was a decrease of biosorption after 6 h till the end of the test, after 24 h. As it emerges from table 13 this was not due to a real decrease in specific removal with time, but rather to an increased removal by the experimental system. In trial J there was a trend similar to that reported for trial H, with a rapid increase in specific removal in the first 7 h and then a slow decrease in velocity for the successive hours, though the equilibrium was not reached after the 24 h of the test. In figure 88 the kinetics of biosorption for trials K and L show that trial K had a different behaviour compared to previous trials, because the decrease in the velocity of removal occurred after 24 h from the beginning of the test. In trial L, though the specific removal values were very low, the kinetic showed that, even if a reduction in the velocity of removal occurred, this was still increasing after 24 h, especially for the test with Cu(II) solutions.

Table 13 shows an example of file produced after every test, in which the results for every cylinder, or beaker, are reported, together with the average, standard deviation, and specific removal value calculated on the basis of the volumes of solution and the grams of biomass used in the test. In the specific case of trial I, it is worth noting that, even if the amount of chromium and copper is always decreasing, in the different cylinders, the lowering of metal concentration in the blank leads to a decrease of specific removal.

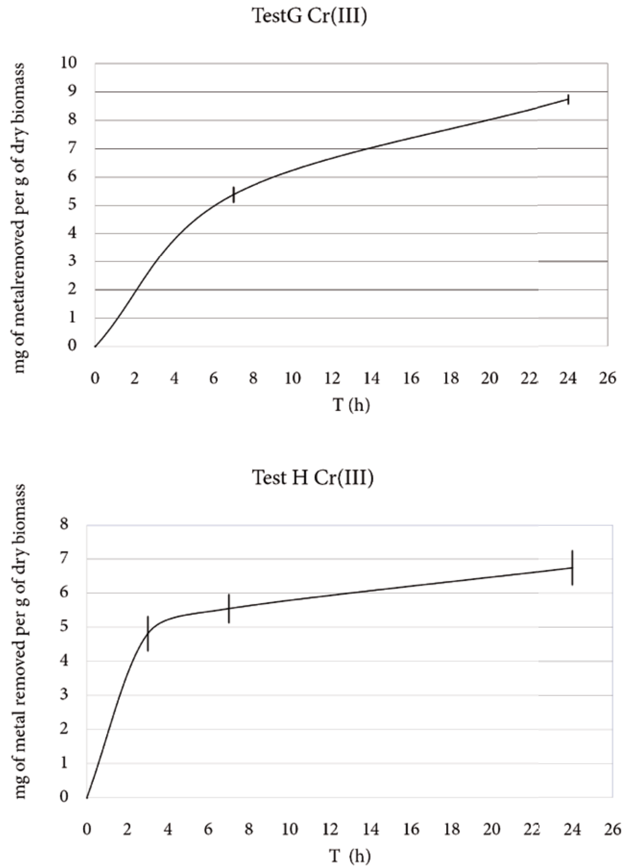


Figure 86: Kinetic of removal in trials G and H.

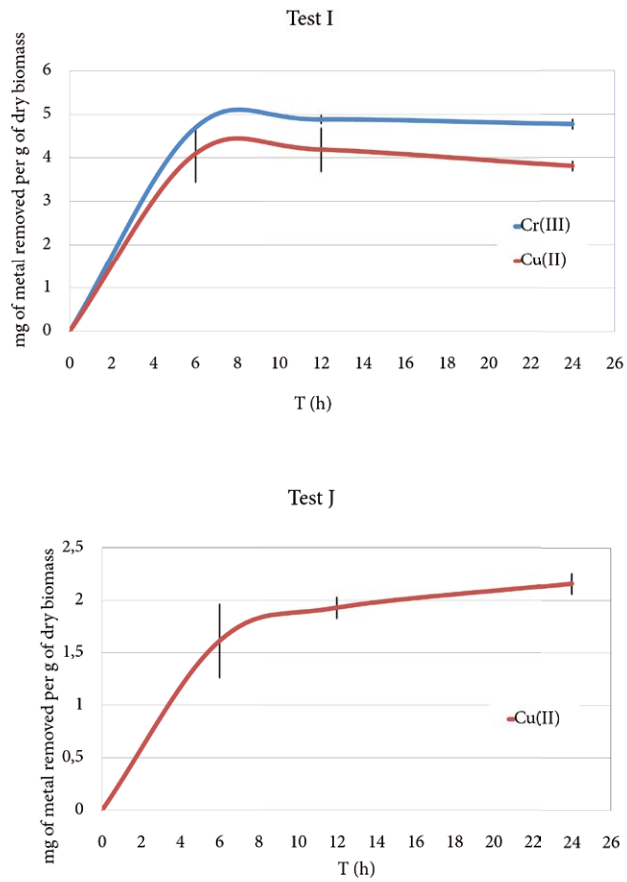


Figure 87: Kinetic of removal in trials I and J.

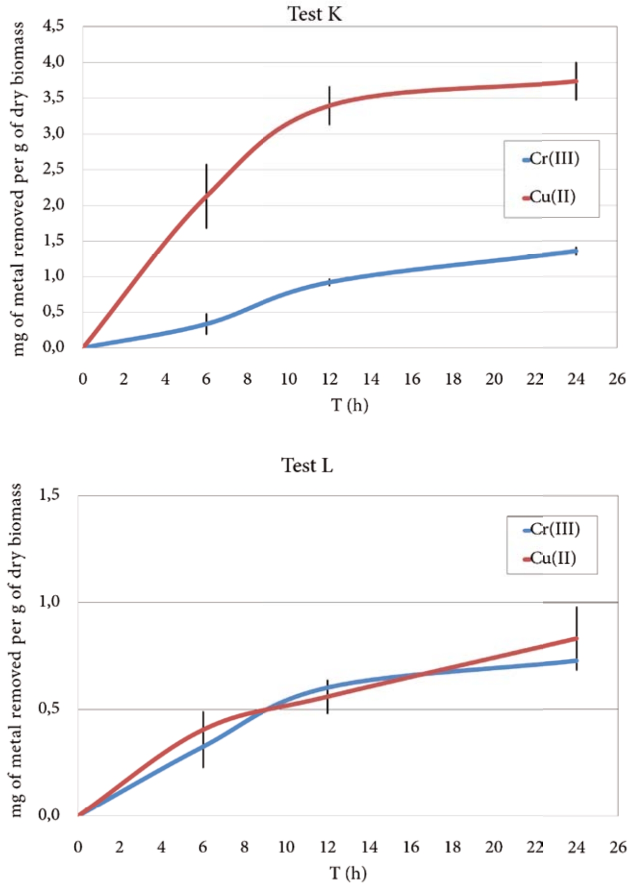


Figure 88: Kinetic of removal in trials K and L.

3. Cr(VI) biosorption experiments

3.1 Wastewater used in the experiments

The heavy metal content of washing water used in the experiments was determined at the Piombino (LI) laboratories of ARPAT.

The average percent composition of the wastewater used (Fig. 89) was :

- Cr(VI) : 89.71 %
- Cr(III) : 8.97 %
- Others : 1.32 % (Zn: 0.518%; Cu: 0.402%; Ni: 0.330%; Fe: 0.050%; Pb: 0,019%; Cd:<0,001%; Hg:<0,001%; As:<0,001%)
- 1<pH<3.5 according to dilution.

Table 13: Results for every cylinder, or beaker, including average, standard deviation, and specific removal value calculated on the basis of the volumes of solution and the grams of biomass used in the test. In the specific case of trial I, it is worth noting that, even if the amount of chromium and copper was always decreasing in the different cylinders, the lowering of metal concentration in the blank leads to a decrease of specific removal.

Trial I Cr(III)			
Cylinder \ sampling	6h	12h	24h
1	1,190	0,727	0,422
2	1,166	0,695	0,055
3	1,105	0,393	0,036
Average	1,154	0,605	0,171
RSD%	0,044	0,184	0,218
Blank	10,541	10,370	9,716
Specific removal (mg metal removed/g dry biomass)	4,694±0,022	4,882±0,092	4,773±0,109

Trial I Cu(II)			
Cylinder \ sampling	6h	12h	24h
1	3,791	2,414	2,506
2	3,132	3,288	2,553
3	2,489	2,435	2,350
Average	3,137	2,712	2,470
RSD%	0,651	0,499	0,106
Blank	11,322	11,088	10,082
Specific removal (mg metal removed/g dry biomass)	4,093±0,325	4,188±0,249	3,806±0,053

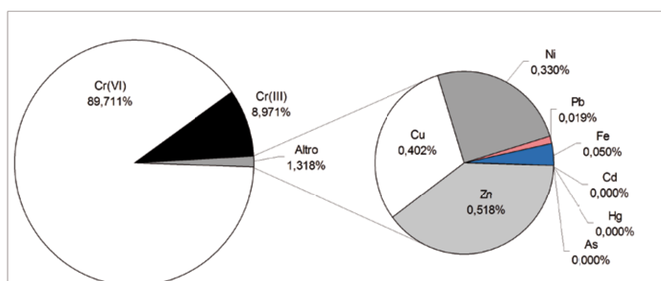


Figure 89: Average percent composition of the washing water used both in laboratory experiments and in industrial scaling up trials.

3.2 Biosorption experiments for strain selection

Compared to the results obtained with bivalent, Cu^{2+}, Ni^{2+}, or trivalent, Cr^{3+}, cations, those obtained with the anion chromate, CrO_4^{2-}, evidenced a lower specific removal,

though similar to the values present in the literature, with a mean biosorption value of 9.5 mg of Cr(VI) removed per g of dry biomass (Volesky, 2003).

Strain *Cyanothece* CE4 was the best with a specific removal of 18 mg Cr(VI) per g of dry biomass, while the worse was strain *Cyanothece* VI22 with a value of 0.51 mg Cr(VI) per g of dry biomass. Results are summarized in table 14.

3.3 Cr(VI) biosorption laboratory experiments with *Nostoc* PCC 7936

Nostoc PCC 7936 showed a removal and conversion capability equal to zero in the laboratory experiments carried out with biomass without pre-treatment (trial A), with centrifuged biomass (trial B) and with biomass centrifuged and autoclaved (trial C) (tab. 15). In trial D, where biomass was only pre-treated with acid, chromium (VI) disappeared in an amount equal to 8.25 mg per gram of dry biomass. To this value corresponded an actual reduction of total chromium, that is a reduction of chromium both in trivalent and in hexavalent form (VI+III), of 7.86 mg per gram of biomass dry weight, while 0.39 mg of chromium (VI) were reduced to Cr(III).

In trial E, where biomass was centrifuged to separate RPS before the acid pre-treatment, the amount of Cr(VI) removed and/or reduced was of 7.97 mg of chromium (VI) per gram of dry biomass. In this case total chromium (VI+III) removed was of 4.39 mg per gram of dry biomass, while 3.58 mg of chromium (VI) per gram of dry biomass were reduced to chromium (III).

Trial F, where biomass was centrifuged, autoclaved and submitted to acidic pre-treatment, showed that the chromium (VI) removed and reduced was 11.63 mg per gram of dry biomass, of which total chromium (VI+III) removed was 6.31 mg per gram of dry biomass and chromium (VI) reduced 3.63 mg per gram of dry biomass (Tab 15, Fig 90).

In the experiments carried out with the polysaccharide alone there was no biosorption. The results obtained after 90 h of contact between polysaccharide and chromium solution showed that, starting from a solution containing chromium at a concentration of 12.12 ppm, in all three the cylinders containing polysaccharide, the concentration of chromium was the same as in that containing water (blank):

- T_0 = 12.12 ppm
- Sampling after 90h, cylinder 1: 10.32 ppm
- Sampling after 90h, cylinder 2: 10.44 ppm
- Sampling after 90h, cylinder 3: 10.75 ppm
- Sampling after 90h, cylinder B: 10.68 ppm

Table 14: Results of CrO_4^{2-} biosorption experiments carried out with strains *Cyanothece* ET5, TI4, PE14, VI22, CE4, *Nostoc* PCC7936 and *Cyanospira Capsulata*.

Strain	% Cr (VI) removed	Specific removal mg Cr(VI)/g biomass
ET 5	2,63±0,10	12,25±0,50
TI 4	1,43±0,10	6,50±0,23
PE 14	2,17±0,20	9,75±0,80
VI22	2,66±0,30	0,51±0,30
CE4	4,05±0,50	18,00±0,80
PCC7936	2,50±0,01	10,87±0,09
C. capsulata	1,77±0,01	8,00±0,10

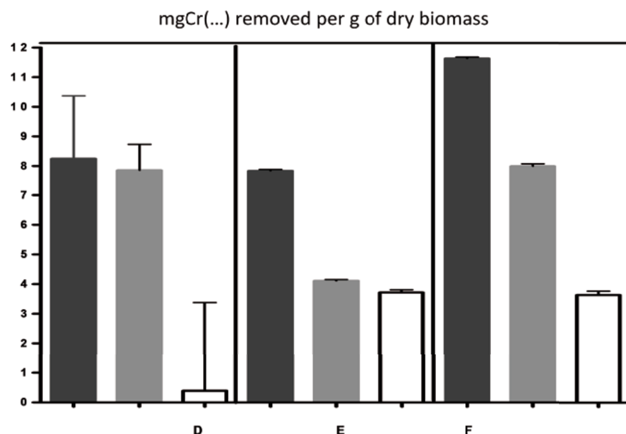


Figure 90: Results of experiments D, E and F, carried out in the laboratory. Experiments A, B and C are not reported as they give nil response. In black, Cr(VI) removed/reduced, in grey total Cr, in white Cr(III) found in the cylinders at the end of the tests, expressed as mg of metal removed per gram of dry biomass.

Table 15: Summary table of the results obtained during the laboratory experiments. Top: summary of the parameters adopted in the tests. Bottom: results obtained (X=done, O= not done).

Trial	Biomass concentration g/l	ppm of Cr (VI) present in the wastewater used	centrifugation	autoclaving	dialysis against demineralized H_2O and 3% HCl
A	2,28	20,27	O	O	O
B	4,21	20,27	X	O	O
C	6,5	17,32	X	X	O
D	0,93	18,15	O	O	X
E	10,49	14,39	X	O	X
F	5,73	17,58	X	X	X

Trial	Cr(VI) removed per mass unit (mg metal removed/g dry biomass)	total Cr removed per mass unit (mg metal removed/g dry biomass)	Cr(VI) reduced to Cr(III) per mass unit (mg metal reduced/g dry biomass)
A	0	0	0
B	0	0	0
C	0	0	0
D	8,25	7,86	0,39
E	7,97	4,39	3,58
F	11,63	6,31	3,63

3.4 Cr(VI) biosorption experiments with Nostoc PCC7936 in pilot plants at the firm.

In the trials carried out with column system, among the systems in continuous, a decrease of Cr(VI) concentration from 10.64 ppm to 0.48 ppm was observed already after 24 h, while in the same time Cr(III) increased from 0 at t_0 to 9.48 ppm and total chromium was almost constant, going from 10.64 ppm at t_0 to 10.32 ppm after 24h, indicating that an almost complete conversion of Cr(VI) to Cr(II) occurred. Then, strain *Cyanothece* TI4 was added after 48 h from the top of the column. At the time at which strain *Cyanothece* TI4 was added, abatement of Cr(III) started and continued keeping pace with that of total Cr, bringing the two values from 10 to 3.6 ppm in 100 h, with a linear decrease (Tab. 16, Fig. 91).

The first experiment carried out with the filter press has shown an almost complete abatement of Cr(VI), which decreased from 10.40 ppm at time t_0 to 0.44 ppm after 72 h, while at the same time Cr(III) increased from 0 to 13.76 ppm and total chromium from 10.40 to 14.24 ppm. The increase in Cr(III) and total chromium in solution was due to the release of chromium from the pump impeller, which was used to recirculate wastewater, due this latter acidic pH (Tab. 17, Fig. 92).

In the second experiment with the filter press, carried out with a wastewater 10 times more concentrated, the initial Cr(VI) concentration of 112.25 ppm decreased at 12.13 ppm after the first 24 h, to 0.4 ppm after 72 h and to 0.03 ppm after 96 h. Cr(III) increased in an inversely proportional way, going from 0.05 ppm at t_0 to 53.10 ppm after 48 h, decreasing then to 47.12 after 96 h. Total Cr decreased from the initial concentration of 112.30 ppm to 47.12 ppm after 24 h, then it increased again to 53.5 ppm after 48h and decreased again to 48.30 ppm after 96 h (Tab. 17, Fig. 93).

Results of the complete analysis of the wastewater used in the experiments with the filter press at t_0 and after 96 h are reported in table 18 and figure 94.

The batch treatment, carried out with a dialysis cell, showed a parallel decrease of Cr(VI) and total Cr, in both cases, from the initial concentration of 23 ppm to 16 ppm after 72 h. In the same time Cr(III) increased from 0.15 to 0.70 ppm (Tab. 19, Fig. 95)

Table 16: Results of the experiments carried out with confined biomass in a column filled with quartz grains and biomass of *Nostoc* PCC7936 and *Cyanothece* TI4.

Column experiment	Concentrations		
Sampling time h	Cr(VI)	Cr(III)	Total Cr
0h	10,64	0.00	10,64
24h	0,48	9,84	10,32
96h	0,45	6,15	6,60
144h	0,10	3,56	3,66

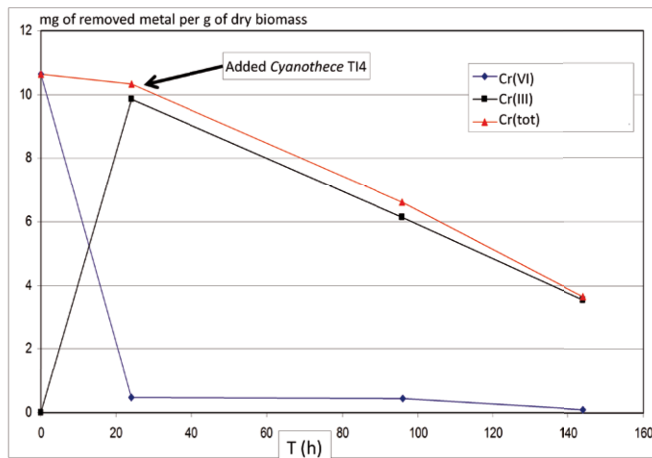


Figure 91: Progress of the removal/reduction experiment with a column filled with quartz grains and Nostoc PCC7936 biomass. After 48 h from the start of the experiment biomass of the strain Cyanothece TI4 was also added.

Table 17: Results of the experiments carried out by using a filter press containing biomass of the strain *Nostoc* PCC7936.

1st experiment with filter press	Concentrations		
Sample	Cr (VI) (ppm)	Cr (III) (ppm)	Total Cr (ppm)
0h	10,40	0.00	10,40
24h	0,72	13,52	14,24
48h	0,72	12,12	12,84
72h	0,44	13,76	14,20
2nd experiment with filter press	(see fig.93)		
Sample	Cr (VI) (ppm)	Cr (III) (ppm)	Total Cr (ppm)
0h	112,25	0,05	112,30
24h	12,13	35.00	47,12
48h	0,40	53,1	53,50
72h	1,25	51,50	52,75
96h	0,03	48,27	48,30

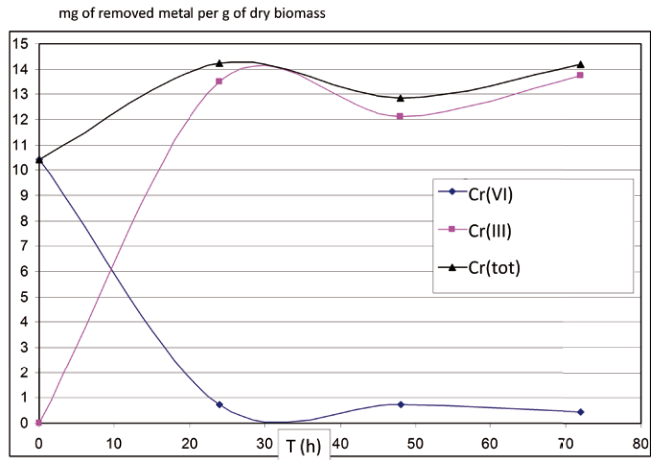


Figure 92: Progress of the first experiment with the filter press containing Nostoc PCC7936 biomass.

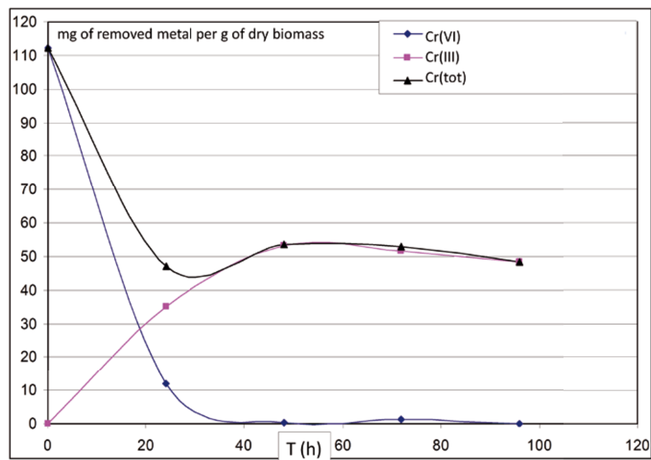


Figure 93: Progress of the second experiment with the filter press containing Nostoc PCC7936 biomass.

Table 18: Results of the complete analysis of wastewater before (T_0) and after biosorption treatment (96 h). (Analysis by ARPAT)

	Concentrations	
	T_0 (ppm)	96h (ppm)
Cr (VI)	112,2500	0,0300
Cr (III)	0,0500	48,2700
Cu	0,3000	1,3000
Zn	0,5000	7,0000
As	<0,0200	<0,0200
Cd	0,0090	0,0070
Hg	<0,0005	<0,0005
Ni	0,9600	1,0000
Pb	<0,0200	0,5000
Fe	0,0400	88,2000
COD	9,4000	147,0000

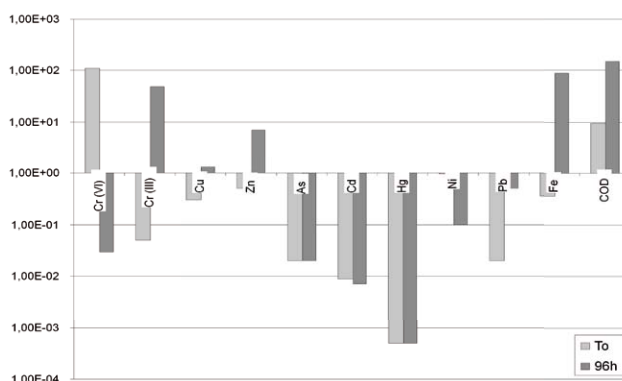


Figure 94: Comparison between the complete analyses of wastewater carried out at time T0 and after 96 h (values are referred to a logarithmic scale and are expressed in ppm). It is worth noting that at the end of the 96 h Cr(III) in solution had significantly increased, as well as iron and zinc, indicating that the components of the galvanized sheet-iron superficially treated with chromium had disintegrated because of the pH of wastewater. Moreover, COD increased due to the possible presence of polysaccharide released by the cyanobacterial culture.

Table 19: Results of the experiments with the dialysis cell containing Nostoc PCC7936 biomass.

Dialysis cell experiment	Concentrations		
Sample	Cr(VI) (ppm)	Cr(III) (ppm)	Total Cr (ppm)
0h	23,05	0,15	23,20
48h	15,85	0,30	16,15
72h	13,50	0,70	14,20

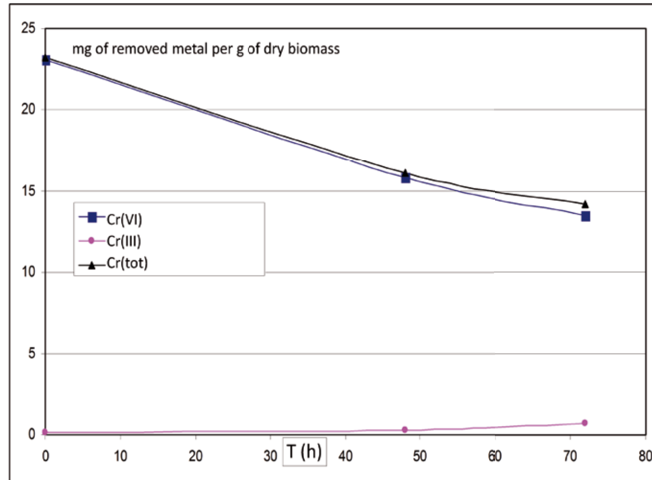


Figure 95: Progress of the experiment with the dialysis cell containing Nostoc PCC7936 biomass.

4. Analysis of the ashes obtained from biomass after biosorption

The results here exposed were obtained also during another experimentation with bacteria different from Nostoc, but carried out in a similar way, to evaluate the possibility of recovery of the adsorbed metals from microbial biomass.

Ashes, after incineration in the muffle kiln, appeared of a light green colour, characteristic of the chromium oxide (Fig. 96). Ashes were analysed for their composition with a technique described in the Materials and Methods section.

Figure 96 : Ashes obtained after the treatment of the microbial biomass in muffle kiln.

In figure 97 it is possible to observe the area used for analysis, taken in the top picture in a microphotograph made by using the re-emission or reflection of high energy

electrons, also named back-diffuse, belonging to the primary ray. With this technique it is possible to identify the elements present. At the bottom it is possible to observe a microphotograph of the same area taken by using the emission of secondary low energy electrons, technique used to have photos with a resolution up to 5 nm.

In figure 99 a detail of figure 97 is reported, evidenced by a red frame. This detail was observed by using the same techniques described above. In figure 98, in the bottom photograph, the points where the dot-like analyses were carried out are indicated. The points were chosen as they responded in a different way to the primary electrons, as it is possible to see from the different colour shown in the top photograph.

The results obtained, expressed as percentage of weight (Wt%) and number of atoms (At%), show how the chromium content in the ashes was of about 92% in weight of the ashes themselves (Figg. 98, 100).

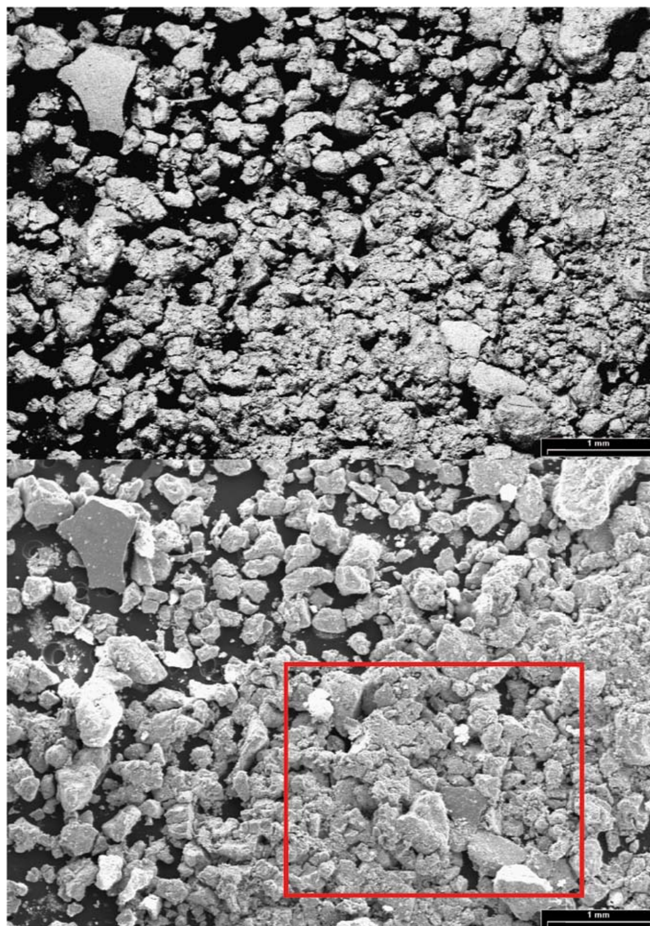


Figure 97: Microphotograph taken at SEM of the ashes of microbial biomass loaded with the removed metal obtained from the muffle kiln. Top: microphotographs taken by utilising the re-emission or reflection of high energy electron belonging to the primary ray, useful to the identification of the substances present. Bottom: microphotograph of the same area, taken by utilising the emission of secondary low energy electrons, technique used to take pictures with a resolution up to 5 nm. In red the magnified area in figure 98.

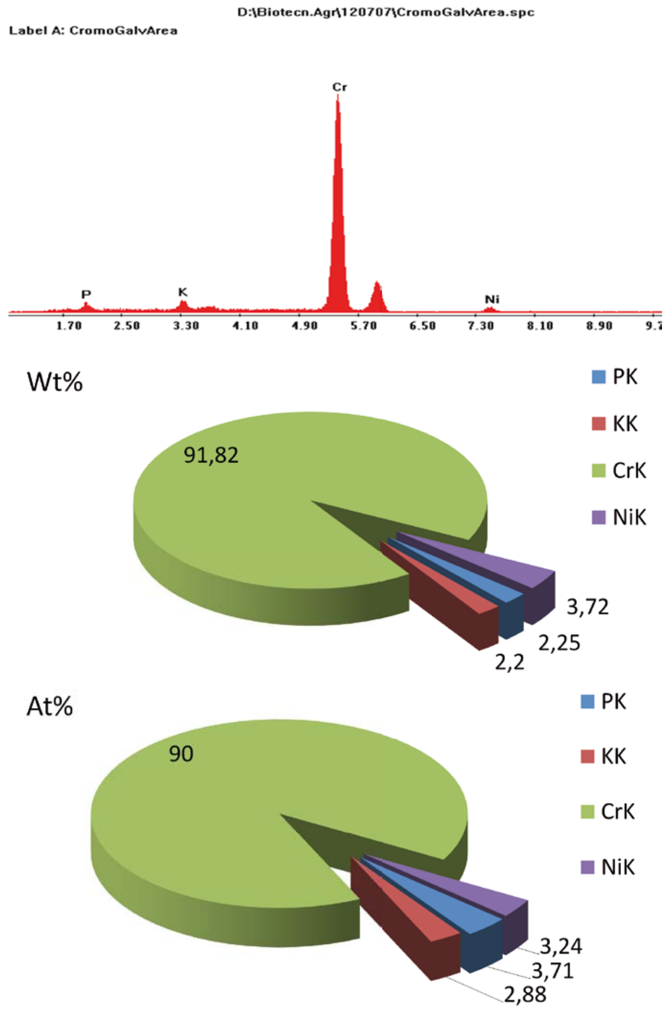
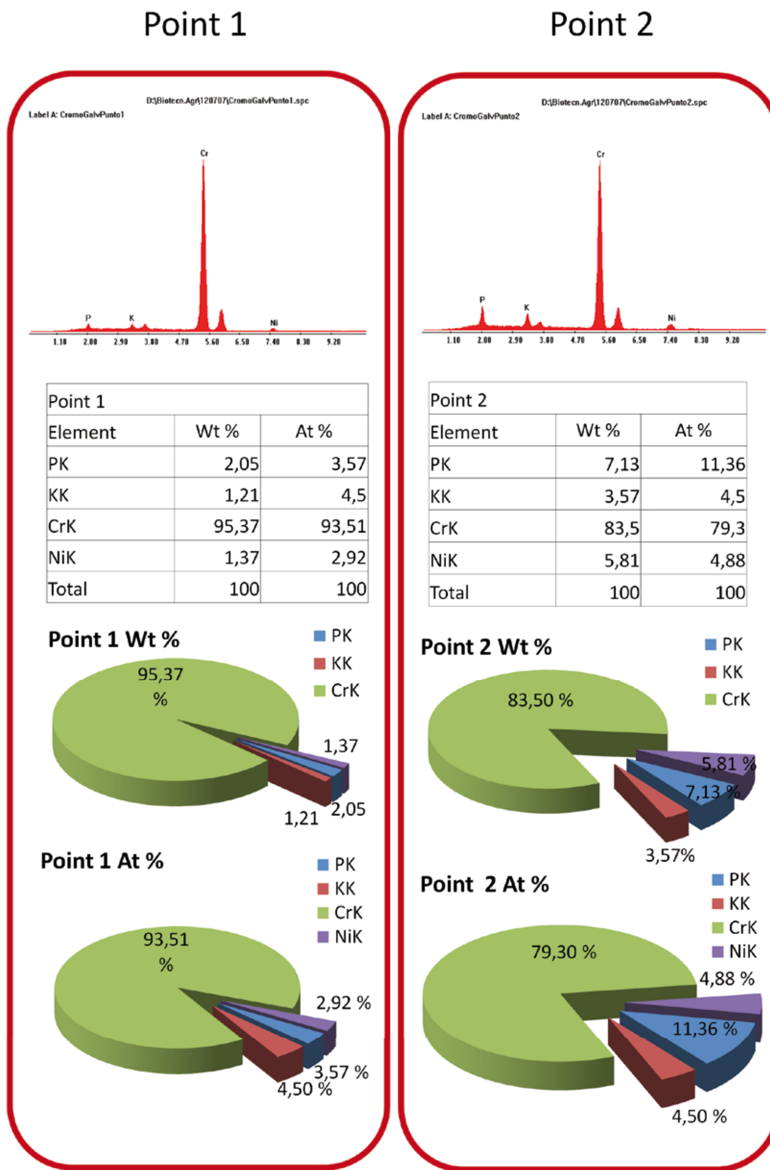


Figure 98: Results of SEM – EDS analysis (top) and percent composition of the analysed ashes for weight (Wt%) and number of atoms (At%). The letter "K" near the element analysed refers to the energetic level at which the quantitative determination was performed.

Figure 99: Microphotograph taken with SEM of the ashes of microbial biomass loaded with the removed metal obtained from the muffle kiln. Top: microphotographs taken by utilising the re-emission or reflection of high energy electron belonging to the primary ray, useful to the identification of the substances present. Bottom: microphotograph of the same area, taken by utilising the emission of secondary low energy electrons, technique used to take pictures with a resolution up to 5 nm. In this picture the two points in which the dot-like analysis were carried out are indicated. The points were chosen as they responded in a different way to the primary electrons, as it is possible to see from the different colour shown in the top photograph.



Point 1		
Element	Wt %	At %
PK	2,05	3,57
KK	1,21	4,5
CrK	95,37	93,51
NiK	1,37	2,92
Total	100	100

Point 2		
Element	Wt %	At %
PK	7,13	11,36
KK	3,57	4,5
CrK	83,5	79,3
NiK	5,81	4,88
Total	100	100

Figure 100: Results of the dot-like analyses from two points, 1 and 2, carried out with SEM–EDS on the ashes obtained from the muffle kiln of microbial biomass loaded with the removed metal.

Chapter 4
Discussion

1. Biosorption experiments in mono-and multi-metal systems

In the first series of trials a more or less high biosorption capability was found in all the tested cyanobacterial strains. There was, moreover, a linear trend in the biosorption process, that was completed in a single phase when biomass was used both against mono-metal and multi-metal solutions. The results of these biosorption trials showed that metal sequestering from solutions operated by biomass increased very rapidly at the beginning, arrived to a stabilisation after a few time, and reached the equilibrium in about 30 minutes when biomass was freely dispersed and in about 2 h with confined biomasses. Volesky (2003) reports that the reaching of the equilibrium in the biosorption processes does not lead to a static equilibrium condition, but to a dynamic equilibrium between the quantity of ions captured and released.

At T_0 biomass has many free receptors and therefore more ions are bonded than released. Approaching the equilibrium, the adsorbed and released ions tend to become equal, leading to a progressive slow of adsorption increase until a complete equilibrium between the surface phase of the adsorbing substance and the solute is reached. Therefore the surface of the sorbent material exposed to the solution to be treated is fundamental to determine the adsorption potentiality, as adsorption is a process occurring exclusively at the liquid-sorbent interphase. In fact, the characteristics of the surface exposed to the solute, such as porosity, density, charge density, number of active ionic sites, and possible conformational changes, are responsible for the metal adsorption capability. So, a good material for biosorption needs to have specific chemico-physical characteristics at the surface in contact with the solute and high surface/volume ratio to have a large contact area in a reduced space. Therefore, bacteria, with their favourable surface/volume ratio appear as an optimal choice. In particular, cyanobacteria, as those tested in the experiments, has the ability to synthesize polysaccharide that, with its characteristics of polarity, density and solubility appears as an optimal material, because the contact surface with solute is virtually expanded to infinity. Actually, polysaccharide features like density, solubility, possibility of conformational changes and density of active ionic sites at the surface in contact with the solute are strain-specific and, in the presence of metal, lead to different responses, though with similar trends. The results of the screening carried out with the strains used in the first series of experiments, indicate that there is not an absolute "best" strain, as the "best" strain changes according to the metal tested. In the mono-metal solutions biomasses showed different affinity and a general adsorption efficiency decreasing in the order Cu>Cr>Ni. The strains most effective in removing copper ions, Cyanothece sp. 16SOM2, ET 5 and Cyanospira capsulate, were able to sequester 201, 143 and 113 mg of metal per gram of dry weight,

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respectively. For chromium, the highest biosorption values were reached by *Cyanothece* sp. 16SOM2, CE4 and TI 4, with a specific removal about 196, 95 and 67 mg Cr(III) per gram of dry biomass, respectively. Cyanobacterial biomasses showed a lower affinity for nickel compared to the other tested metals, as the highest specific removal obtained were 73, 83 and 56 mg of Ni removed per gram of dry biomass, reached by strains *Cyanothece* sp. CE 4, *Cyanospira capsulata* and *Cyanothece* sp. 16SOM2, respectively.

In the mono-metal systems, all cyanobacterial strains, except C. capsulata, showed a lower affinity for nickel, as it was already observed with other biomasses and sorbents (Gloaguen, 1996; Saeed, 2005). The relatively low capability of removing Ni(II) by all the cyanobacterial biomasses tested apparently contradicts the hypothesis that the higher the electronegativity of an element the higher its affinity for negatively charged sorbents (Singh, 2000). In fact, in accordance with electronegativity of the three metals, 1.91, 1.90 and 1.66 (Pauling scale) for Ni, Cu and Cr, respectively, the expected affinity of the biomasses should have been Ni>Cu>Cr.

It is, however, known that Ni^{2+} can form in water highly stable aqueous complexes (Uudsemaa, 2004; Tangkawanit, 2005) and this can make nickel unable to take the place of the proton bound to the active site of the biosorbent. The affinity scale of the biomasses used against metals in the biosorption tests, is therefore brought back to the values found in this work: Cu > Cr > Ni.

The results obtained in the experiments with multi-metal solutions can be subdivided in three main groups. Comparing the sum of the specific removals of the single metals in the multi-metal solution trials, expressed as mmol of metal removed per gram of dry biomass, with the specific removal obtained in the trials with mono-metal solution, three types of answers can be distinguished:

1) The contemporary presence of more than one metal species has caused a decrease in the total mmol of metal removed per mass unit, in the three-metal system compared to that with a single metal (antagonism);
2) The three metal species in solution did not influenced the capability of biomass to remove metal ions (non-interaction);
3) The contemporary presence of three metals in solution has determined a global increase of the removal capability of the biosorbent (synergism).

With the strains *Cyanospira capsulata*, *Cyanothece* ET5 and CE4 an antagonistic effect was observed, as the total metal ions removed were 1.73, 1.42 and 1.57 mmol g^{-1}, lower than the values obtained with mono-metal solutions (2.25, 1.81, and 1.82 mmol g^{-1} respectively). Strains *Nostoc* PCC 7936 and *Cyanothece* VI 13 and VI 22 has shown total removal values substantially similar to those obtained in the mono-metal systems, while the strains *Cyanothece* 16SOM2, TI 4 and PE 14 has shown a positive interaction, being able to remove a higher amount of metal ions in the multi-metal system than in the mono-metal one.

Always due to the strain-specific features of the biosorbent material a certain degree of selectivity, more or less marked, was observed. Of the seven unicellular and the two filamentous cyanobacteria used in the experiments of this thesis work, few strains were characterised by a high affinity and selectivity for some of the metals tested. For example, the cyanobacterium *Nostoc* PCC7936 has shown a high affinity for Cu^{2+} ions: in the mono-metal system copper was removed in higher amounts than the other two metal tested; in the ternary solutions, moreover, this preference was translated in a high selectivity, as copper was the unique metal removed in significant amounts from the solution. Also strain *Cyanothece* ET 5 evidenced a high affinity and selectivity for Cu^{2+}

ions. Finally, strain *Cyanothece* 16SOM2 has shown a high affinity and selectivity towards two metals, chromium and copper, removed in amounts much higher than nickel, probably owing to the reasons previously described.

As two metals in bivalent form (Cu^{2+}, Ni^{2+}) and one in trivalent form (Cr^{3+}) were used, besides comparing specific removals in mg and mmol of metal removed per g of dry biomass, in the experiments with mono and multi-metal solutions the biosorption process was investigated from the point of view of the number of adsorbed charges.

The results obtained were very interesting, as they showed that in the trials with three-metal solutions, the lowest efficiency of removal of chromium with respect to copper was compensated, sometimes, by an equal or higher charge adsorption. In the most evident case, *Cyanothece* 16SOM2 with the ternary solution, from the specific removal calculated in mg and mmol, copper resulted to be adsorbed in a more efficient way than chromium, with a difference of about $80 mg g^{-1}$ and $1 mmol g^{-1}$. Considering the adsorbed charges in the same experiment, it emerged that both for copper and chromium removal the amount of charges adsorbed was the same.

If we compare the sum of mFaraday adsorbed per mass unit during the removal of the three metals in multi-metal solution with the single mFaraday adsorbed per mass unit in the mono-metal solutions, the results obtained are rather different from those of the specific removal calculated in mg or mmol of metal removed per gram of dry biomass. Always with strain *Cyanothece* 16SOM2, for example, a difference of about 80 mg g^{-1} and 1 mmol g^{-1} was found between the sum of the specific removal in multi-metal system and the best specific removal found with Cr(III) solution in the single-metal system. This difference was equalised considering the mFaraday adsorbed in the two processes. The limit of charges that can be adsorbed by this strain appears to be 11.2 mF. The reason why this maximum value is not reached with all the metals has to be searched in the strain-specific affinity for the single metals.

2. Tests with the bloom of Lake Dian Chi

In the attempt to reduce the operational costs of biosorption the possibility to free the companies from biomass production by recovering it from blooms, often occurring in the water bodies all around the world, was investigated.

To do this we collaborated with a Chinese research group, located in WuHan, which studies since many years the evolution of blooms in lakes around the town of Kung Ming, in the South of China, and has a long time collaboration with our research group.

In this thesis, thanks to a stay of a couple of months in China during summer 2007, the first data obtained from the experiments of biosorption carried out with the bloom biomass are reported. These data have to be considered not as the results of a well-tested experimental system, but as data providing preliminary information on the possible use of the bloom as biosorbent material and indications on the goodness of the experimental design.

It is clear from all the experiments that biosorption occurs, but which is the actual potentiality of this technique is not as much clear. It was verified that, probably because of the excessive amount of biomass used, the kinetic of the reaction was unable to reach the equilibrium within 24 h from the start of the experiment. Possibly this was also responsible of the low specific removal values observed.

However, the removal occurred and it was independent from the state of the biomass; in fact, different biomasses were used in the experiments (fresh biomass, just harvested

from Lake Dian Chi and dried biomasses stored for one month or for four years), always obtaining a certain bioremoval. The fact that removal was independent from the biomass state is important as, in view of an industrial use, desiccation and storage of biomass, also for long time, would be fundamental to reduce transportation costs and to preserve biomass supplies for the periods of the year in which there are no blooms.

Moreover, the biomasses that were subjected to an acidic pre-treatment, very useful in experiments with biomasses from pure cultures (Paperi, 2006), showed lower biosorption values compared to non pre-treated biomasses. This phenomenon could be explained by the observation that in the bloom, made of cell aggregates included in a polysaccharide matrix, the acidic treatment could cause the destructuring of the biosorbent material, with a consequent sharp decrease in the biomass biosorbent capability.

3. Cr(VI)biosorption

3.1 Laboratory experiments

The first part of the experimentation was aimed to determine the capability of polysaccharide producing cyanobacterial strains to adsorb heavy metals. In the second part of the thesis, a possible industrial application of the selected strains was tested. The strains were experimented with in biosorption trials with industrial wastewaters containing chromium, in trivalent and hexavalent form, provided by a galvanic industry.

Industrial wastewaters, as it emerged from the analyses, are not mono-metal solutions, but they contain several metals at very different concentrations. Moreover, Cr in these solutions is usually present as CrO_4^{2-} or $Cr_2O_7^{2-}$, both anionic and not cationic forms, as those of the metals tested in the preliminary experiments.

In the first experiments, biomasses were able to remove Cr(VI) from solutions, after an acidic pre-treatment of the biomass, but with specific values lower than those observed for single cations, e.g. for Cr^{3+} solutions, with a mean removal of 9.5 mg of metal per gram of biomass. In the second series of experiments, carried out with strain *Nostoc* PCC7936, it was verified that Cr(VI) removal was not due to direct adsorption phenomena but to a two-step process, the first of which is an oxidation of biomass and the second is an adsorption of the Cr(III) produced during the first step.

When the cyanobacterial polysaccharide was separated from the biomass, the Cr(VI) removed was actually reduced to Cr(III) and not adsorbed, as it happened in trial "E" (tab. 9, fig. 95), while when polysaccharide was present, the removal of Cr(VI) occurred at the same time of total Cr removal (see trial "D"). In trial "F", carried out with cells lysed by autoclaving, it was observed that the presence of cellular components in the medium has led to an increase in Cr(VI) removal, reduced to Cr(III), and to an increased removal of total Cr. To verify these findings, a trial using only polysaccharide was carried out and confirmed the expected results: there was no reduction of chromium from hexavalent to trivalent and no adsorption.

To summarise, from the experiments carried out it emerges that Cr(VI) is not directly adsorbed by cyanobacterial biomass, but it is firstly reduced to Cr(III), oxidizing the cell fraction of biomass, and then, in the reduced form, it is adsorbed by the polysaccharide present in the biomass, that is unable to reduce chromate.

Nostoc PCC7936 was chosen, among the tested strains, for these trials and for the scaling up, owing to a combination of factors, such as its capability to remove chromi-

um, both total and Cr(VI), its easiness of cultivation, growth rate and low-cost culture medium. All these factors are of great importance for scaling up, which requires large amounts of culture in short times.

Strain *Cyanothece* TI4 was chosen for the same reasons as Nostoc, because it shows a high growth rate, a low cost culture medium and is able to produce large polysaccharide amounts in a few time.

3.2 Scaling up experiments

The experiments aimed at verifying the possibility of an industrial application have shown, in all the three systems tested, very good biosorption results, besides confirming the data obtained in the laboratory, both for specific removal and the possibility of future applications.

The laboratory trials have demonstrated that the reduction of chromium from (VI) to (III) is due to the cell matrix, independently from viability conditions, while the presence/absence of polysaccharide plays a fundamental role in the removal of chromium (III) produced during the reduction of Cr(VI).

In the trials carried out at a scale larger than the laboratory it was also possible to observe that the systems used for treatment, e.g. dialysis cell, or pre-treatments that do not involve the removal of the polysaccharidic fraction, lead to a similar removal of both Cr(VI) and total Cr, while when polysaccharide is removed from the culture by centrifugation or filtration, e.g. with the quartz-filled column, Cr(VI) is reduced to Cr(III), but total Cr is not abated.

An intermediate situation was found with the filter press, as the cardboard filters at its interior are able to keep a higher amount of the polysaccharidic fraction, as compared to the quartz grains of the column.

In these trials in pilot plant very rapid removal kinetics has been evidenced. The equilibrium was reached in about 24 h for reduction of Cr(VI) to Cr(III), while for removal of total chromium kinetics were slower and more than 7 days were necessary to reach the equilibrium.

The column filled with quartz grains, though conceptually similar to the filter press as both systems operate by filters filled with culture before wastewater treatment, was easier to use and its operational cost was lower. However, some limits emerged in the metal recovery phase, as it resulted difficult to separate ashes from the quartz grains. This problem did not occur with the filter press because of the cellulose filters, that can be burned together with biomass.

The addition of a second strain allowed not only to abate total chromium content in the wastewater treated but also to verify the possibility to successfully use more strains in the same system, allowing optimisation of the biosorption process according to the needs. In fact, starting from the addition of the second strain, total chromium content started to decrease leaving unaltered Cr(VI) content previously abated by *Nostoc*.

Two consecutive trials were carried out with the filter press without changing the biomass with the double objective to verify the efficiency loss by biomass during time and the volume of wastewater that could be treated with the same biomass. Results were excellent for both aspects, as a nearly complete reduction of Cr(VI) was achieved in about 24 h in both trials.

This result is even more interesting considering that in the first trial, owing to a wrong choice of the pump to be used, the amount of chromium dissolved increased because it was released from the mechanical components that were not designed to work

at pH around 2, while in the second trial, the amount of chromium was 10 times higher than in the first trial. Moreover, from the end of the first trial and the start of the second four days elapsed with biomass inside the filter press, without undergoing any regeneration treatment.

With the dialysis cell, as expected from the laboratory experiments, the response was very different compared to the trials with the other two systems, the column and the filter press. In this case, total chromium and Cr(VI) decreased at the same time and much slower than in the other trials. This phenomenon could be due, as described before, to the confinement system itself, because, being made of a dialysis membrane separating metal solution from the cell suspension, it could cause a slowed interaction between Cr(VI) and biomass. This situation of mediated contact between Cr(VI) and biomass has slowed the process of Cr(VI) reduction, allowing polysaccharide to adsorb Cr(III) at the same time of its production. This relation between reduction ad adsorption was probably favoured by the structure of the system, different from the quartz-filled column and the filter press, that did not submit biomass to wastewater fluxes. These fluxes favour the contact between chromate and biomass, but, on the other hand, can carry away part of the polysaccharide, an important component for Cr(III) biosorption.

In general, all the experiments carried out have produced good results not only in terms of specific removal of Cr(VI), Cr(III) and total chromium, but also by evidencing the peculiarity of each system, that will be useful in order to chose the most suitable system for industrial application, as a function of the operational needs, that cannot always be standardised.

4. Metal recovery

One of the main aspects of this thesis work was to verify the possibility to use biosorption in the industrial sector of wastewater treatment. The chemico-physical systems presently used by industries are conceptually old, based on ion exchange or evaporation, but also extremely reliable and optimised, and therefore with low management costs. Biosorption, a new and yet unapplied technology, needs to fulfil several issues from the management and economic point of view to result more convenient compared to conventional systems.

For this reason, besides selecting for the experimentation strains easy and economic to cultivate, the potentiality of a microbial bloom was investigated as well as the possibility to recover adsorbed metals with low cost technologies allowing the reuse of the metal without additional treatments and costs.

A system to recover metal different from chemical desorption that, besides being expensive, releases the recover metal in solution, was then adopted: direct combustion of biomass containing the metal at the end of the biosorption process.

This operation allowed to obtain ashes that, with equal metal concentrations, has a highly reduced volume with respect to the common sludge coming out from the galvanic waste treatment plants, making transportation and storage easier. Moreover, these metal-rich ashes, that in our case were constituted by over 90% in weight of chromium, could be directly used in the blast furnace to produce special steels, like stainless steel, that contains 11-30% of chromium.

Chapter 5
Conclusions

Basic research is fundamental for science evolution and for society improvement, but it is also important, and at the base of economical and social development, to translate the results of basic research into everyday reality.

The idea of this thesis was conceived in this frame, with the aim of experimenting the possibility of translate biosorption from laboratory experimentation to a technology utilisable by industry.

The first part of the experimentations has shown that the specific characteristics of each cyanobacterial strain tested are reflected on its adsorption power, which determines different responses according to the tested metal both in monometal and ternary systems.

The trials carried out in a galvanic factory, operating in the chromium-plating sector, has evidenced that microbial biomasses can be effectively used for biosorption of Cr(VI), as it appeared from the very interesting adsorption results obtained in three different pilot plants, two conducted in continuous and one in batch.

As a first attempt to solve the problem of making biosorption competitive in terms of yield, costs, operational easiness, reliability and possibility of integration in the existing plants, as compared to the conventional chemico-physical systems, the possibility to use as biosorbent material biomasses obtained from the cyanobacterial blooms that are increasingly affecting many lakes and seas worldwide was experimented with. The good results obtained with this biosorbent material show that this could be the right direction.

Moreover, the possibility to recuperate the metal adsorbed by cyanobacterial biomasses, by means of a simple combustion producing ashes made for 90% of the removed metal, was demonstrated.

To conclude, from the whole set of experiments it turned out that the use of cyanobacteria, or other microorganisms, for industrial biosoprtion is an actual and not only hypothetic possibility, even though many aspects that need further investigation are still present. In particular, several application problems due to the integration of the "biosorption technology" in the reality of factories, where usually there is the request to have a finished product, which is better and less expensive than the product already in use.

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