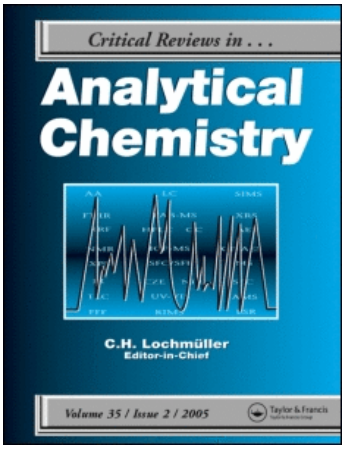

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Beata Godlewska-Żyłkiewicz

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Analytical Applications of Living Organisms for Preconcentration of Trace Metals and Their Speciation

Beata Godlewska-Żyłkiewicz

Institute of Chemistry, University of Białystok, 15-443 Białystok, Piłsudskiego 11/4, Poland, e-mail address: bgodlew@noc.uwb.edu.pl, tel. 48-85 7457588, fax. 048 85 7457581

ABSTRACT: Living organisms, naturally occurring in human environment, gained in the last decade, increasing interest in analytical chemistry. This review presents the application of bacteria, yeast, algae, and fungi for the preconcentration of heavy metals from environmental samples. The specific bonding sites on cell walls are responsible for the selective binding of different species of studied metals that permit the use of them as sorbents for speciation analysis purposes.

KEY WORDS: biosorbents, living organisms, immobilization of living organisms, preconcentration, speciation.

I. INTRODUCTION

The determination of metal ions at the trace level in environmental matrices often requires a preconcentration step. For this purpose solvent[1] and solid-phase[2,3] extraction, coprecipitation,[1,4] electrolysis (electrodeposition),[5] and other techniques are often applied. The use of solid phase extraction as a preconcentration method is simple, fast, and very effective in various procedures.

In the last decade living organisms such as algae, yeast, fungi, bacteria, and erythrocyte cells were studied for the purpose of trace metal preconcentration. The possible advantage of using microorganisms is that potentially the cell wall has many constituents that can be implicated in metal binding, including amine, hydroxyl, and carboxyl groups, phosphate, and sulfydryl groups. The uptake of heavy metals by biomass can take place by an active mode (independent on the metabolic activity) known as bioaccumulation or by passive mode (sorption and/or complexation) termed as biosorption. The capacity for biosorption depends on the type of microorganism, the element, and even on the oxidation state of the element.[6] These properties can be used not only for removing heavy metals from environmental matrices but also for speciation analysis.[6–18]

Recently, yeast are more widely used for this purpose owing to easy growth, nonhazard, great tolerance toward metals, and high binding capacity to the cell.[6,13,14,17–20] An abundant source of potentially metal-sorbing biomass are marine algae.[12,16,21–27] They are widely available and ecologically acceptable. Additionally, marine algae can be used as biosorbents in batch and flow procedures without intricate pretreatment such as, for example, immobilization. Also, fungi are easy to grow, produce high yields of biomass, and at the same time can be manipulated genetically and morphologically.[28] Both free and immobilized microorganisms could be used as biosorbents. However, generally for use in column applications biomass should be immobilized. Modification such as cross linking can improve the stability and mechanical properties of the microorganisms (e.g., algae, yeast).[17–19,23,25] It was observed that dead cells accumulate heavy metals to an equal or greater extent than living cells.[23,29–32] However, in some cases immobilization affects biological activity of microorganism and reduces its metal capacity.[23]

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The preconcentration by living organisms offers low analysis cost, because large quantities of waste microbiological biomass are produced in many industries such as citric acid biosynthesis and penicillin production. For this purpose the biomass may be also used to eliminate the problems of waste toxicity and nutrient requirements.

The different microorganisms were studied for preconcentration and speciation of trace metals, including: selenium,[6,9,12,32,33] arsenic,[13] mercury,[14,15,18] chromium,[11,17] copper,[21] cadmium,[22,32] lead,[23] gold,[29] beryllium,[30,32] and others. Atomic absorption spectrometry,[9,11,12,14,17,20,28–33] atomic emission spectrometry,[6,13,16,34] and anodic stripping voltammetry[35] have proven to be useful techniques for the determination of metals in these separation/preconcentration biological systems.

The literature on biological substrates for metal preconcentration was already reviewed by Camara and Madrid.[36] The number of studies on this subject is increasing in recent years; therefore, an updated review of the most relevant literature data on analytical application of living organisms in environmental, mainly speciation, analysis is presented in this article.

II. UPTAKE MECHANISM

The mechanism of accumulation involves the adsorption of metal ions at the cell wall or cell membrane via interaction with functional groups and/or transport into the cell with subsequent transformation. The major mechanisms responsible for metal biosorption may include ionic (electrostatic) interaction and complex formation between metal ions and biomass. However, some studies regarding the nature of bonding between metallic ions and cell walls explain adsorption of some metals, for example, Cu by algae[22] or mercury species by yeast[14] rather through covalent bonds then through the ion-exchange mechanism. That was assumed on the results that pH that affects the charge on the living cell surface only slight influences the uptake efficiency.

Algae, yeast, or lyophilized bacteria act generally as ion exchange materials, and chemical species are retained on the external membrane of their cells. The adsorbed metal ions can be stripped of with acids prior to the determination. In this case, biosorption is an equilibrium process that is dependent on the pH of solution and concentrations of other ions in the sample.[16,20,31,37,38] The fast binding (3 to 5 min) of metal ions by organism cells suggests that these metals are concentrated through extracellular association. When a longer incubation period is required this suggests that the most active functional groups are inside the membrane. The mechanism of metal binding may, however, change according to the substrate and technique used for immobilization.

The more complex mechanism of adsorption on the bacterial surface has been suggested for gold.[26] At least two types of adsorption sites or two functional groups can take part in the binding of gold, and this differs with the pH of the medium. At low pH values the carbonyl and carboxylic groups could be responsible for the retention of gold by bacterial cells. At higher pH values the amino groups become deprotonated and the retention of gold is probably due to them. Two phase kinetics of the gold binding process could be explained by the fact that gold ions bind first to the functional groups with highest affinity and subsequently to groups with lower affinity.

In the procedure using living bacterial cells, chemical species of analyte can be retained by energetic process, which is assumed as more selective process. Many of the cellular homeostatic mechanisms are directly linked to solute transport because the uptake of chemical species requires in most cases metabolic energy and the transport processes can be regulated by the energy states of cells. One of the transport systems is a passive diffusion or alternatively adsorption/desorption where hydrophobic compounds from an external solution rapidly dissolve into the cytoplasmic membrane, diffuse across the membrane, and subsequently dissolve in the inside compartment of cells. The energy-dependent process is implied in the retention of low concentrations of gold by bacteria. For higher concentrations other retention mechanisms such as adsorption can also occur.[29] Lead accumulation by bacteria occurs by a combination of intracellular uptake and surface adsorption.[37]

The uptake of Se-Met follows a two-step process. A diffusion-controlled model can better de-

scribe the behavior of this selenocompound. In this case there is faster uptake process and a bigger maximum uptake capacity. The uptake of Se-U seems to take place be an alternative mechanism, in one-step kinetics. In this case the uptake mechanism requires an incubation period for as long as it depends on the growth rate of cells.

Mechanism of biosorption of chromate ions by erythrocytes is more complex.[11] It was assumed that chromate enters the erythrocytes via the anion-permeation channel, which is responsible for a fast exchange of Cl^- vs. HCO_3^-. The erythrocytes membrane has no transport channel for Cr(III).

For identification of functional groups as well as the number of sites responsible for metal binding to living organisms chemical modification techniques (e.g., estrification) as well as instrumental techniques as IR, X-ray, luminescence, and NMR spectroscopies were applied.[27]

III. BATCH PRECONCENTRATION PROCEDURES

Both free and immobilized microorganisms could be used as biosorbents. In the case of using free cells the procedures usually include several different steps. The general batch procedure requires mixing an analytical solution with 5 to 10 mg of microorganism, shaking the mixture, separating the supernatant from the leachate and determination of analyte in filtrate and/or resuspending the microorganism leachate in mineral acid for subsequent slurry GFAAS analysis. A potential disadvantage of such batch procedure is the need for multiple extraction for metals that have low partition coefficients.

Holcombe and co-workers performed a series of studies on the preconcentration of cadmium,[22] and copper[23] for ultratrace analysis using lyophilized algae biomass (*Stichococcus bacillaris)*. This microorganism can be effectively used for preconcentration of low cadmium concentration (<1 μg/L); however, low extraction efficiency of this algae limits its utility at higher concentrations. The advantage of using algae is that alkali and alkali-earth ions do not affect the adsorption of other metal ions. About 100-fold preconcentration factor was obtained for analysis of these metals in water, seawater, and river water. Algae *Spirulina major* were applied to the separation and preconcentration of Se(IV) and Se(VI) from water and river sediment and their subsequent determination by slurry GFAAS.[12]

The copper preconcentration by yeast (*Saccharomyces cerevisiae)* (10 mg) was performed in a cultivation medium containing 3.5 g L^{-1} diammonium hydrogen phosphate.[20] Authors have found that except nickel and vanadium other elements present in water (Al., Ca, Fe, Mg, Na, Pb, Zn) were not concentrated on yeast. About sevenfold copper preconcentration with reproducibility of 5.3% were obtained. The method was applied to the determination of copper in river water. Baker's yeast was also used for selective separation methylmercury and Hg^{2+},[14,18] selenium and antimony species,[6] and inorganic arsenic species.[13] Methylmercury and Hg^{2+} were determined in continuous flow by CV-AAS, while antimony and selenium by hydride generation atomic absorption spectrometry. The different mercury, selenium, and arsenic species were determined in spiked tap, river, irrigation water, and seawater.

Robles and Aller studied living and dead bacteria (*Escherichia coli* and *Pseudomonas putida*) for gold,[29] beryllium,[30] and cadmium[31] preconcentration. Bacterial cells can be used as sequesters in different ways: as lyophilized cells in a batch technique or as living cells in a growth process. Living bacteria were cultivated in a TSB medium (tryptic soy broth) with metal ions, next bacterial suspension was centrifuged and washed. The lyophilized cells were added to the metal solution, then centrifuged and washed. In this case chemical species is retained on the external membrane of the cell. In the procedure using living cells chemical species of analyte can be retained by a more complex mechanism. The uptake process is based on the incorporation of an analyte by energetic process that is assumed as more selective process. The metal retained by living and lyophilized microorganisms was liberated by treating the biomass pellet with different concentrations of nitric or hydrochloric acid. The effect of several factors, such as pH, metal, and bacterial concentrations, as well as interferences from other metals were examined. The slurry sampling and platform

atomization were used for determination of these metals by GFAAS technique.

The preconcentration factor of all studied metals obtained for both bacteria types was 6.25, with the reproducibility less than 7% for gold and 4.5% for cadmium and beryllium. The enhancement factor (even 5000 times for Au, 10 times for Be, 17 times for Cd) depends not only on the preconcentration step but also due to the benefit of slurry atomization compared with conventional wall or platform atomization. The presence of cell pellet in the slurry acts as a matrix modifier for metals (mainly gold) determination due to the carbon formation that stabilizes the formation of metal atoms. From the analytical point of view the better results were obtained using the *E. coli* bacteria, although it required a rigid control of pH. There are some interferences from alkaline earth and transition metals salts. Immobilization of bacteria performed in silica gel did not increase the extraction efficiency. The procedure was applied for berylium[30] and cadmium[31] determination in environmental reference materials.

The same lyophilized bacteria were used to develop a preconcentration procedure for selenium prior slurry ETAAS determination.[33] However, in the case of that metal the best retention on lyophilized bacteria was obtained at pH = 3 for *E. coli*, and at pH = 1 for *P. putida*. Again nitric acid (3.5 mol L^{-1}) was used in order to liberate selenium from bacterial walls, and the same as above preconcentration factor (6.25) was obtained.

Bacteria *Pseudomonas putida* were also used in batch procedures for preconcentration of selenomethionine and selenourea[7] and selenocystamine[9] in spiked tap water samples.

IV. ON-LINE PRECONCENTRATION PROCEDURES

The immobilization of living organisms offers more advantages due to repeated use, easier regeneration for reusing in multiple adsorption-desorption cycles, and better mechanical stability. When immobilized the biosorbents behave as an ion-exchange resin and the removal of metals involves a conventional solid-liquid contact and separation process, different immobilization methods may be used, such as physical entrapment of microorganisms in polymeric material or covalent immobilization on solid surface. However, in some cases[16] dried dealginated brown seaweed (*Ecklonia maxima*) was just ground and packed into the column.

Ramelow and Harris have entrapped algal cells in an ethyl acrylate–ethylene glycol dimethacrylate copolymer.[39] The use of polyacrylamide was reported for immobilization of algae[40] and bacteria.[37] Others have employed silica gel for immobilization of algae,[23] bacteria,[29,30,32] and yeast.[18,41] The crosslinking technique was used to immobilize algae and led to the formation of stable cellular aggregates. Controlled pore glass that exhibits good mechanical properties was first silanized and then the bifunctional properties of glutaraldehyde were used to cross-link the microorganism to the glass surface.[25,38] Algae *Saragassum* sp. were embedded in polyethyleneimine resin, and the free amino groups of this matrix were further also cross linked with glutaraldehyde.[42]

In on-line procedures nondestructive recovery is required for the regeneration of the biomass and for its reuse in multiple biosorption-desorption cycles. As the elution of metal with mineral acids is proposed in most procedures, the concentration of acid solution (HCl, HNO_3) used for the stripping metals bound to the cell surface must be as low as possible. However, in some cases for complete recovery the addition of complexing agent, for example, thiourea[25] or CN^-[18] is necessary.

Holcombe[23] proposed the immobilization of lyophilized algae *Stichococcus bacillaris* in silica gel. Columns filled with this material were used for lead preconcentration. They obtained good precision associated with immobilization procedure (2.6%), while bigger differences were observed between different harvested batches of algae (the precision was equal 15%). The decrease of the capacity of the column was observed after 11 runs. This sacrifice in capacity is offset by the reusability of the immobilized algae and the increase in extraction efficiency using the column instead of the batch mode operation.

The immobilization of the algae reduced the available metal adsorption sites by approximately 40%. This reduction is caused by the loss of

exposed cell wall surface area where the metal adsorption likely occurs.[23] The similar effect (40% decrease in uptake compare to batch procedure) was observed when bacteria *Streptomyces rimosus* were immobilized on the column and used for zinc preconcentration.[43] However, others[44] stated that immobilization of *Rhizopus arrhizus* in polyvinyl formal do not diminish copper uptake level. In the study of Aksu et al.[45] the biosorption of cadmium (II) ions to dried flocs of *Cladophora crospata*, a kind of green algae, was investigated in a packed bed columns.

Covalent immobilization of an algae,[25] yeast,[46] bacteria,[34,47] and fungi[38] on controlled pore glass (CPG) was performed. CPG is formed by phase separation of homogeneous borosilicate glass followed by dissociation of the boron-rich glass phase by strong acid (HNO_3), leaving a highly porous silica-rich glass. Six metals were preconcentrated on green algae *Selenestrum capricornutum*.[25] For copper, mercury, and lead the highest sensitivity was obtained in phosphate buffer at pH values of 7.5, 6.5, and 5.5, respectively, while for zinc, cobalt, and cadmium better results were obtained in TRIS buffer at pH values 7.5, 8.0, and 8.5, respectively. Elution with diluted 0.1 to 0.5 mol L^{-1} hydrochloric or nitric acid was performed, while for mercury additionally the 0.1 mol L^{-1} thiourea was added. The relative affinities of the different metal ions for *Selenestrum capricornutum* in decreasing order are Hg>Cu>Pb>Zn>Co>Mg. The enhancement factor as high as 4000 was obtained for copper, 1000 for zinc, and 500 for cadmium. Controlled-pore glass (CPG) and sand were compared for their effectiveness in the immobilization of the fungus *Penicillium notatum*.[38] In this case also glutaraldehyde was used as a bifunctional or a linking reagent for immobilization on solid matrix. BSA (bovine serum albumin) solution was passed through a column packed with glutaraldehyde treated CPG or sand in order to block the free aldehyde groups and to ensure that the retention of metal ions was a function of the cell, not the aldehyde group. It was found that metal adsorption varies from one support to another, as well as from the procedure (normal or ultrasonic) used for preparation of the *Penicillium* solution. The reported breakthrough capacities were higher for yeast[46] and bacteria[47] than fungi[38] (Table 1). Tap and mineral water samples were analyzed for zinc and copper using proposed method of preconcentration.

Yeast *(Sacharomyces cerevisiae)* immobilized on sepiolite (natural mineral) was used for the determination of Cu, Zn, Cd,[19] Fe, Ni[48] and chromium speciation[17] in water by the FAAS technique. The retained metals were eluted with 1 mol L^{-1} HCl. Two types of adsorption sites or two functional groups of the yeast cause the difference in metal binding to the surface with the pH of the medium. The optimum pH of metal solution was 6 for zinc and cadmium, 8 for copper, and 2 for Cr(III) ions. The differences in the breakthrough capacities for these metals were observed between yeast immobilized on sepiolite[19] and CPG[46] (Table 1). It may be due to a change in the structure of the yeast during the killing and immobilization process. The proposed method was applied for river and synthetic sea water.

Some seaweeds have been identified for the ion-exchange properties associated with their polysacharide content. These properties are particulary pronounced in brown algae.[16,24] Seki and Suzuki[24] showed that the biosorption of bivalent metal ions (cadmium and lead) to brown algae was due to bivalent binding to carboxylic groups on alginic acid in algae. At low pH values, the carboxyl groups are protonated, thus reducing the available sites for metal binding. Maximum, rapid binding of metals was obtained between pH 6 and 7. In this way also the speciation of chromium was possible, because anions CrO_4^{2-} were not retained at the column. The column capacity for each element is affected by the presence of others elements,[16] but is still enough for preconcentration of trace metals (Table 1). On-line preconcentration of Cd, Cr, Cu, and Pb using microcolumn packed with dealginated seaweed biomass was applied for analysis of lake water and synthetic sea water.

The ability of the zinc uptake by bacterial biomass (*Streptomyces rimosus*) in continuous mode has been shown by Addour et al.[35] The initial pretreatment of bacteria with sodium hydroxide effected in higher Zn (II) binding. However, the results obtained in continuous mode were worse than in the batch procedure. Biomass

TABLE 1
Examples of Flow Procedures for Metal Preconcentration on Biosorbents

Sorbent	Element	BC [µmol/g]	Preconcentration factor (initial sample volume)	Detection technique	Application	Remarks	Ref.
Yeast *Saccharomyces cerevisiae* immobilized on sepiolite, 0.2 g	Cu Zn Cd	74 128 95	50x (500 ml) 75x (750 ml) 75x (750 ml)	FAAS	River water, synthetic sea water samples	Elution with 10 ml 1 mol L^{-1} HCl, 20 runs**	19
Yeast *Saccharomyces cerevisiae* immobilized on CPG, 0.3 g	Cu Zn Cd Fe Pb	56 180 25 20 43	286* EF (5ml) 2000 250 750 125	FAAS	Sediment reference material (BCR 144)	Elution with 0.1 ml 0.1 mol L^{-1} HNO_3 for Cu, Zn; 0.5 mol L^{-1} HNO_3 for Pb, Cd, 4 mth**	46
Yeast *Saccharomyces cerevisiae* immobilized on sepiolite, 0.3 g	Cr	228	75 (750 ml)	FAAS	River water	Elution with 10 ml 1 mol L^{-1} HCl	17
Algae *Selenestrum capricornutum* immobilized on CPG, 0.1g	Cu Pb Zn Co Hg Cd	9.7 mol/g 11.45 8.36 2.52 1.6 11.7	4000* EF 50x (5 ml) 80 1000 50 167 500	FAAS	Standard solutions	Elution with 0.1 ml 0.5 mol L^{-1} HCl or HNO_3 Thiourea addition for Hg, Sampling rate 20/h, 3 mth**	25
Algae *Stichococcus bacillaris* immobilized on silica gel, 0.6 g	Pb	278	20x (60 ml)	FAAS	Standard solutions	Elution with 0.12 mol L^{-1} HCl 11 runs*	23
Bacteria *Sprulina platensis* immobilized on CPG, 0.02g	Cu Zn Cd Pb Fe	0.0035 ng/ml 0.0008 0.0011 0.0028 0.0017	667*EF (5 ml) 1000 125 100 900	FAAS	Reference material of sewage sludge (BCR 144)		47

Bacteria *Sprulina platensis* immobilized on CPG, 0.025 g	La Ce Nd	17 11 13	36 34 51	ICP-AES	$Ce(NO_3)_3$ $6H_2O$	Elution with 0.1 ml 1 mol L^{-1} HCl, sampling rate 24/h	34
Bacteria immobilized on silica gel, 0.025 g	Au		6.25× (25 ml)	Slurry GFAAS	Standard solutions		27
Escherichia coli,		0.0012–0.0025	2800*EF				
Pseudomonas putida		0.0005–0.0006	2000				
Fungi *Penicillium notatum* immobilized on CPG, 0.2 g	Fe Cu Zn Cd Pb	12 14 18 10 3	50× (5 ml) 29*EF 500 277 19 27	FAAS	Tap and mineral water	Elution with 0.1 ml 1 mol L^{-1} HNO_3, sampling rate 24/h	38
Fungi *Penicillium notatum* immobilized on sand, 0.2 g	Fe Cu Zn Cd Pb	10 30 16 1 23	40* 625 625 20 50	FAAS	Tap and mineral water	Elution with 0.1 ml 1 mol L^{-1} HNO_3	38
Dealginated seaweed *Ecklonia maxima*, 0.34g	Cd Cr Cu Pb	8 (4)# 33.7 (8.7) 26 (9.4) 12 (8.5)	5× (5 ml)	ICP-AES	Reference lake water, simulated sea water	Elution with 1 ml 1 mol L^{-1} HCl	16

Note: BC — breakthrough capacity; *EF enhancement factor — factor by which limit of detection decrease using preconcentration as opposed to direct injection; CPG — controlled porous glass; # — in parenthesis in the mixture.

regeneration with 0.1 mol L^{-1} HCl gave a 90% recovery of the adsorbed Zn (II).

V. SPECIATION

There is an increasing interest within scientific community for the development of methods that discriminate among different metals species. Speciation generally has been carried out using hyphenated techniques, mainly chromatographic methods (liquid and/or gas chromatography) or other related techniques together with specific sensitive detectors. The new concept that appeared in literature is to use the biological substrates for selective species preconcentration. In most cases the more toxic forms of an element as As(III), Se(IV), Sb(III), CH_3Hg^+ forms strong metal-thiol bonds with yeast and is sorbed by yeast, while the more charged species reminded in the solution (Table 2).

A. Chromium

It is well known that the two main oxidation states of chromium show different physiological and toxicological behavior.

The chromium speciation in water samples by flow method was evaluated using a microcolumn packed with dealginated seaweed biomass (*Ecklonia maxima*) that acts as a cation exchanger.[16] Chromium(III) at neutral pH was retained on the column while Cr(VI) (CrO_4^{2-} ions) remained in the solution, and this makes it possible to differentiate both these species. Chromium(III) was eluted from the column after a few minutes (250 to 350 s) by the injection of 0.5 ml 1 mol L^{-1} HCl. The removal of trivalent and hexavalent chromium by seaweed biosorbent was reported also by Kratochvil.[49]

The next flow procedure for the determination of Cr(III) and Cr(VI) in water was proposed by Bağ[17] and co-workers on yeast *Saccharomyces cerevisiae* immobilized on sepiolite (0.3 g). Chromium (III) was retained on the column at pH = 2 and eluted with 10 ml of 1 mol L^{-1} HCl. Chromium (VI) was determined as a difference between total chromium and Cr(III). The reduction of Cr(VI) to Cr(III) was performed by the addition of 0.5 ml concentrated H_2SO_4 and 0.5 ml of ethanol. As described earlier,[19] the retention of other metal cations on yeast occurred at different pH (6 to 8) and led to separate Cr(III) ions. For 750 ml of the water sample the 75-fold preconcentration factor was obtained. Very good precision (0.2%) and a low detection limit for Cr(III) (94 ng mL^{-1}) was obtained by the FAAS method. Also, cyanobacterium (*Spirulina platensis)* was used for chromium speciation in river and sea water samples.[50]

Human erythrocytes in physiological solutions have the ability of fast uptake of dissolved chromates. It was assumed that chromate enters the erythrocytes via the anion-permeation channel, which is responsible for a fast exchange of Cl^- vs. HCO_3^-. The erythrocyte membrane has no transport channel for Cr (III). Neidhart et al.[11] used human erythrocytes under physiological conditions for the selective determination of chromate in the presence of Cr (III). The red blood cells were separated for pellet and supernatant by centrifugation. Ca-alginate was used to immobilize erythrocytes in beds of 2 to 3 mm diameter, which show a remarkable resistance against mechanical attack. The best results were achieved for 60 min incubation time at pH = 6.5. Under these conditions less than 0.5% of Cr (III) ions that are adsorbed on the cell membranes were found in the erythrocyte pellet. After Cr(VI) sampling, the Ca-alginate was dissolved in citrate solution, erythrocytes were separated by centrifugation, hemolyzed with Triton X-100,® and the chromium content was measured by the GFAAS technique. Immobilization and mechanical stabilization of the cells by gel entrapment did not affect their biological activity against chromate.

For chromium speciation natural erythrocytes and erythrocyte ghosts were also used.[10] The ghost is modified erythrocytes: hypotonically lysed then filled with the desired media and resealed by warming. The experiments showed that erythrocytes filled with ascorbate or cysteine as intracellular reductants, like human erythrocytes, are able to accumulate chromate nearly completely. Ghost filled, for example, with KCl show only a small accumulation of chromium. The optimum pH (pH = 6) value was almost the same as in the former experiments. Accumulation by cysteine ghost is

TABLE 2
Application of Biosorbents for Speciation Analysis

Species	Biosorbent	Adsorption conditions	Stripping	Sample	Detection technique	Ref.
Cr(III)/Cr(VI)	Human erythrocytes immobilized in Ca-alginate beds	pH 6.5, 1 h, 37°C for Cr(VI)		Standard solutions	GFAAS	11
Cr(III)/Cr(VI)	Dealginated seaweed *Ecklonia maxima* (0.34 g), column	pH 6.5-7, sample flow 0.8 ml/min for Cr(III)	Cr(VI) in effluent, 0.5 ml 1 mol L^{-1} HCl for Cr(III)	Water reference material, seawater samples	ICP-MS	16
Cr(III)/Cr(VI)	Yeast *Saccharomyces cerevisiae* (0.3 g), immobilized on sepiolite	pH 2, sample flow 3ml/min for Cr(III), Cr(VI) by difference between total Cr and Cr(III) in reduced sample	1mol L^{-1} HCl for Cr(III)	River water and spiked samples	FAAS	17
Se(IV)/Se(VI)	Yeast *Saccharomyces cerevisiae* (0.2 g)	pH 7, 0.5 h, 37°C for Se(IV)	Se(VI) in supernatant	Water samples	HG AAS for Se(VI) ICP-MS for Se(IV)	6
Seleno-methionine SeMet, Selenourea SeU	Living bacteria *Pseudomonas putida*	pH 6.5, 1.3 h, 30°C in glucose medium for SeMetpH 6.5, 12 h, 30°C in glucose for SeU	3.6 mol L^{-1} HNO_3	Standard solutions	Slurry ETAAS, Pd as MM	7
Selenocystamine	Bacteria *Pseudomonas putida,* (0.006 g) - living - lyophilized	pH 7.5, 20 h, 30°C pH 7.5, 1 h, 30°C	3.5 mol L^{-1} HNO_3	Spiked water samples	Slurry ETAAS, Pd as MM	9

TABLE 2 (continued)
Application of Biosorbents for Speciation Analysis

Species	Biosorbent	Adsorption conditions	Stripping	Sample	Detection	Ref.
Se(IV)/Se(VI)	Algae S*pirulina major,* washed with 1% HCl (0.01 g)	pH 5, 2 min, room temp., for Se (VI) pH 1, 3 min, room temp., for Se(IV)		Sediment and water	Slurry GFAAS	12
Hg(II)/CH_3Hg^+	Yeast *Saccharomyces cerevisiae* (0.2 g)	pH 7, 30 min, 37°C in HEPES buffer for CH_3Hg^+	Hg(II) in supernatant	Water samples	Slurry HG-CV-AAS for CH_3Hg^+, CV-AAS for Hg(II)	14
Hg(II)/CH_3Hg^+	Yeast *Saccharomyces cerevisiae* immobilized on silica gel (0.05 g),	pH 7	0.02 mol L^{-1} HCl for CH_3Hg^+, 0.8 mol L^{-1} CN^- for Hg(II)	Spiked sea water samples	CV-AAS	18
Sb(III)/Sb(V)	Yeast *Saccharomyces cerevisiae* (0.2 g)	pH 10, 0.5 h, 60°C for Sb(III)	Sb(V) in supernatant	Water samples	HG AAS	6
As(III)/As(V)	Yeast *Saccharomyces cerevisiae* (0.25 g)	pH 7, 0.5 h, 60°C in oxalic acid for As(III)	As(V) in supernatant	Spiked natural waters	Slurry HG-ICP-AES, 0.002% Triton X-100	13

faster than by erythrocytes, as a result of the higher cell concentration. These experiments also confirm that an intracellular reduction to a product that cannot leave the cell also takes place in the ghost. Then the uptake of chromate and other oxoanions is based both on the accumulation by the cell membrane and intracellular reaction processes.

B. Selenium

The toxicity and significance of selenium in the environment depends on its concentration and chemical form. In many environmental matrices this element appears as inorganic selenium (selenite SeO_3^{2-} or Se(IV), and selenate SeO_4^{2-} or Se(VI)), which are the most environmentally mobile and biogeochemically important valence states of selenium. However, other oxidation states of selenium (-II, 0) are found more frequently in biological systems as selenoamino acids, selenomethionine, selenocysteine, and others. The inorganic selenium (selenite and selenate) is more toxic than organic species, and Se(IV) is more toxic than Se(VI).

Camara et al.[6] evaluated the procedure for the selective accumulation of selenium inorganic species by baker's yeast in water samples. The behavior of Se(VI) is completely different from that of Se(IV). Selenium(IV) was adsorbed from aqueous solutions by yeast nearly independently of pH from 3 to 10, which suggests the covalent nature of interactions in this process. Under these conditions (pH = 7, temp. 37ºC, incubation time 30 min, 0.7 g of yeast) Se(VI) was not adsorbed by biomass, and its determination was performed in liquid phase. During these investigations it was also found that the selenium uptake occurs along with conversion of Se(IV) into other selenium species, probably Se(-II) as selenomethionine or selenocysteine. These compounds do not generate hydrogen selenide under the same experimental conditions as Se(IV) does, so its determination by hydride generation atomic absorption spectrometry (HG AAS) was impossible. Therefore, the use of ICP-MS technique was necessary for selenium determination.

Another batch procedure was proposed for selenium speciation in natural waters and rivers sediment samples.[12] In this case algae *(Spirulina major)* was used for the separation and the preconcentration of Se(IV) and Se(VI). The algae was washed initially with 1% HCl for 10 min. Selenium (VI) was selectively sequestered by algae at pH = 5, while Se (IV) remained in the supernatant. The subsequent preconcentration of Se(IV) by algae was carried out at pH = 1. Under these conditions its adsorption recovery was 96.4%. However, it was found that the recovery of selenium by algae is dependent on the absolute amounts of selenium and the total mass of selenium compounds cannot exceed 1.7 μg. The determination by slurry sampling graphite furnace atomic absorption spectrometry was performed.

Living and lyophilized (dead) bacteria (*Pseudomonas putida)* were used for the preconcentration of selenocystamine (Se-Cystm)[9] or selenomethionine (Se-Met) and selenourea (Se-U)[7,8] prior to the determination of selenium in biomass pellet by slurry ETAAS. The highest Se-Cystm retention on biomass occurs in glucose solution, and the uptake increases with the seeding density of the bacterial cells. Growth time of bacteria is an important parameter in the uptake by living cells. It was found that after growth time longer than 20 h the signal of selenium has been stable. There is different uptake mechanism by living and dead cells. In the case of lyophilized cells chemical species are retained on the external membrane of the cell. When living cells are used for preconcentration, the mechanism is more complicated due to additional incorporation of analyte into the cell by energetic process during the growth of the cells. The Se-Cystm was better retained (about 100%) by living bacteria. The detection limit of Se-Cystm after preconcentration procedure is as low as 0.1 ng mL^{-1}. The procedure was applied to the selective determination of Se-Cystm in natural water spiked with different selenium species.[9]

Bacteria *P. putida* was also used for selective preconcentration of selenomethionine (Se-Met) and selenourea (Se-U).[7] The living bacterial cells were cultivated in glucose solution at pH = 5 with Se-Met (for 1.3 h) or with Se-U (for 12 h). The

nine times higher glucose concentration, almost five times higher seeding density of cells, and nine times longer growth time were necessary for effective preconcentration of Se-U compare to Se-Met. The retention percentages of both compounds were in the range 27 to 42%, depending on the initial concentration of analyte. The Se-biomass pellet was treated with 3.6 mol L^{-1} nitric acid solution and analyzed by slurry ETAAS in the presence of palladium modifier. The concentration of selenium in the sample was evaluated from calibration graph prepared by treating selenium standards in the same way as samples via bacterial preconcentration. Under these conditions other selenium species as Se(VI), Se-cystamine and Se-ethionine were not retained in biomass, the small retention of Se-cystine, and Se(IV) was observed. These results could be explained on the basis of steric effect, because the two most effectively retained analytes Se-Met and Se-U are the two smallest molecules of those studied.[7]

A promising application of erythrocyte ghosts for the specific accumulation of selenite was proposed by Neidhard et al.[10] Natural erythrocytes are not able to accumulate different oxoanions except of chromate.[11] However, erythrocytes ghosts filled with the right intracellular reagents that only react with selenite can specifically accumulate selenite (but the uptake efficiency was less than 60%).

C. Mercury

Different types of bacteria (*Pseudomonas putida* and *Escherichia coli)* were tested for the selective preconcentration of inorganic mercury species: Hg (I) and Hg (II).[15,32] The uptake of these species depends on the solution pH and the bacteria cells type. Best retention pH for both mercury ions and bacteria were the following Hg(II): pH = 1 (*E.coli*) and 3 (*P. putida*), and Hg(I) pH = 4 (*E. coli*) and 8 (*P. putida*).

To separate methylmercury and Hg(II) the baker's yeast cells were used successfully.[14] CH_3Hg^+ is completely sequestered by the cell within a wide pH range (3 to 7) and independently on temperature, incubation time, amount of biomass, and analyte. Only a high concentration of chloride ions reduces the efficiency of methylmercury retention due to the high toxicity of $HgCl_2$ and a competition for yeast cells by CH_3Hg and $HgCl_2$. Mercury(II) has a low affinity for yeast cells, and only 20% retention was observed at pH = 4, and less than 5% retention at pH = 7. The mercury concentration in each phase were determined in continuous flow by CV-AAS. The slight pH dependence in the biosampling of mercury species suggests that these compounds might interact with yeast ligands through covalent bonds. In this article the authors proposed the uptake mechanism through transformations. The method was applied to the determination of CH_3Hg^+ and Hg(II) in spiked water samples.

The baker's yeast immobilized on silica gel were applied for separation of inorganic and organic mercury species from sea water.[18,41] Both mercury forms were retained simultaneously in neutral medium on the biosorbent, the inorganic form in the silica gel and the organic in the yeast. The efficiency of uptake for both species was higher than 95% over the pH range 2 to 12. The separation was carried out by selective sequential elution methylmercury with 0.02 mol L^{-1} HCl and Hg(II) ions with 0.8 mol L^{-1} CN^-. The procedure gave good results for collecting the mercury species in the field. The advantages of such a method are avoiding problems with preservation of the analyte, liquid transport, contamination, and loss of analyte.[41]

D. Antimony

Antimony is considered to be a pollutant of priority interest the by Environmental Protection Agency. It is known, that Sb(III) is more toxic than Sb(V). So far only one paper was devoted to separation of antimony species on living organisms[6].

Antimony species were incubated with 0.2 g baker's yeast at 60°C for 30 min and the supernatant was separated. Under these conditions Sb(III) was completely bound to the yeast, whereas Sb(V) remained in the solution. Sb(V) behavior was independent of the experimental conditions of the solution tested. The affinity of Sb(III) for yeast cells increased with increasing pH, incubation

time, temperature, and amount of yeast. This suggests that Sb(III) interacts with yeast ligands through an ion-exchange mechanism. The antimony(III) signal from HG-AAS was observed that suggests that this form does not undergo any transformation in the uptake process. Yeast cells allow the specific sampling of Sb(III) with the advantage that this is not only the most toxic oxidation state, but also the less common species.[6]

E. Arsenic

The determination of As(III) in environmental and biological samples is of particular interest because arsenite is 10 times more toxic than arsenate and 70 times more toxic than the methylated species. Organic compounds of arsenic are usually less toxic and compounds such as arsenobetaine and arsenocholine are tolerated by living organisms.

The baker's yeast *Saccharomyces cerevisiae* (0.25 g) was used in a batch procedure for the separation of As(III) and As(V).[13] As(III) showed a high affinity for the yeast cells and was almost completely retained by the biomass, while As(V) remained in the supernatant. It was found that the uptake mechanism depends on the medium of the solution, the best adsorption was in oxalic acid. Only a slight dependence of As(III) binding on pH was observed. That suggests that yeast cells may interact with the analyte through covalent binding. That observation confirms that As(III) is toxic primarily to living organisms because it acts like a metal, forming strong metal-thiol bonds. A preconcentration factor of 7 calculated as the ratio of initial to final volume was achieved. Arsenic determination was performed in liquid phase for As(V) and in slurry for As(III) by HG-ICP-AES. The speciation of inorganic As in different kinds of natural waters was studied.

VI. CONCLUSIONS

The ability of living organisms to incorporate metals into their structure became a useful property used in many analytical and environmental applications for the purposes of trace metals preconcentration. Biomass technologies offer an attractive alternative for the decontamination of waste waters from heavy metals. Numerous studies in the literature were focused on the identification of functional groups or active sites responsible for metal binding by living organisms. The infrared and X-ray spectroscopy, luminescence and NMR techniques were applied for these purposes,[27] but up to now there is no complete knowledge on the mechanism of biosorption. The examples of other natural materials used as sorbents can be found in the literature, such as chitosan/chitin,[51] clay, peat moss,[52] moss, tea leaves,[53] bark/tannin–rich materials and others,[54] but they are not subject of this review.

The selective bonding of different forms of metal by a cell's constituents was a promising solution for speciation analysis. In this way the naturally occurring speciation of trace elements in studied samples is not disturbed, and excellent results were obtained. Although the first work on this subject appeared more than 10 years ago, only several procedures were described, and not many papers were published. When using living organisms (bacteria, yeast, fungi) close cooperation of chemists and biologists is necessary, due to requirements of specific knowledge, competence, and experience in the work with biological material. To do effective research in this field, one has to be a multidisciplinary and work must not be carried out in isolation.This is probably the main limitation for the rapid development of such procedures for analytical purposes.

The fundamental papers on slurry analysis were published in the beginning of 1980s. Most applications were focused on soil and sediment analysis.[55] The special usefulness of slurry sampling in batch procedures has been confirmed for the determination of metals incorporated in living cells by spectrometric techniques. However, the global trends for the development of automation can also be observed in this field, as the use of flow procedures results in better precision and reproducibility, easy operation, and low time consumption. In this case the direct contact with biological material is avoided, and the same biosorbent can be used several times in multiple cycles. As was shown in the flow procedures, the

columns filled with such sorbents can be used even for 3 to 4 months. Some of the immobilization procedures are well described and reproducible results of this step were obtained.

The above-described procedures are applied mainly for the preconcentration of metals from different water samples. Only a few papers deal with the analysis of other samples such as sediment reference material[12,46] or inorganic salts.[34] Despite these limitations, it is still a very interesting and promising field of science and it could be used effectively for speciation analysis.

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