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REVIEW

Micro-algal biosensors

Roberta Brayner · Alain Couté · Jacques Livage · Catherine Perrette · Clémence Sicard

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Abstract Fighting against water pollution requires the ability to detect pollutants for example herbicides or heavy metals. Micro-algae that live in marine and fresh water offer a versatile solution for the construction of novel biosensors. These photosynthetic microorganisms are very sensitive to changes in their environment, enabling the detection of traces of pollutants. Three groups of micro-algae are described in this paper: chlorophyta, cyanobacteria, and diatoms.

Keywords Biological samples · Biosensors · Fluorescence/luminescence · Metals/heavy metals · Pesticides/endocrine disruptors · Quality assurance/control

Introduction

Water pollution arising from industry and agriculture is becoming a major problem in developing countries, while in under-developed countries nearly 500 millions people do not have access to safe drinking water. Usual contaminants include both organic (detergents, insecticides, volatile compounds …) and inorganic (heavy metals, chemical wastes …) compounds. The objective of all water treatment processes is to remove existing pollutants or at least to reduce their concentration so that water becomes fit for its desired end-use. It is therefore very important nowadays to be able to control the nature and amount of contaminants in our environment. Two general analytical approaches are currently used for this purpose. They are based either on conventional physicochemical analyses or on biological assays. Although standard analytical techniques enable highly accurate detection and quantification of specific pollutants, exhaustive analyses are complex and costly. Moreover, they fail to provide data on the bioavailability of pollutants, their effects on living systems and their synergistic or antagonistic behavior in mixtures. As a partial response to these needs, biosensors have been developed and used in environmentally oriented bioassays.

A biosensor contains two parts: a *bioreceptor*, the biological sensing element, and a *transducer* that detects the biochemical signal and transforms it into an electrical or optical signal. These microelectronic devices enable rapid, accurate, and low-level detection of a variety of substances in body fluids, water, or air. The need for early-warning procedures to detect contaminants has prompted the development of cell-based sensors. The rapid and accurate evaluation of water toxicity is nowadays an important issue for environmental water safety. However, it is difficult to measure the toxicity of individual chemicals contained in water, because a wide variety of chemicals co-exist in environmental water, and a mixture may have even more complex toxicity. Bioassays seem to be one of the most

R. Brayner · C. Sicard
Université Paris Diderot (Paris 7), CNRS UMR 7086, Interfaces, Traitements, Organisation et Dynamique des Systèmes (ITODYS),
15 rue Jean de Baïf,
75205 Paris cedex 13, France

A. Couté · C. Perrette
Muséum National d'Histoire Naturelle, Département RDDM,
FRE 3206, USM 505, 57 rue Cuvier,
75005 Paris, France

J. Livage (✉) · C. Sicard
Collège de France,
11 place Marcelin Berthelot,
75005 Paris, France
e-mail: jacques.livage@upmc.fr

useful methods of measuring toxicity in environmental and industrial wastewaters. Many bioassays based on algae [1–4], bacteria [5], plant tissues [6], and animal cells [7] have been developed in recent years. In particular, micro-algae have been widely used for toxicity assays because of their high sensitivity and reproducibility. The use of micro-algae in the design of biosensors is a very recent topic in biotechnology. Several algal biosensors have been developed during the past decade in order to detect herbicides, volatile organic compounds (VOC), heavy metals and even chemical warfare agents [8]. In micro-algal biosensors the metabolic activity of the living organism is measured. Toxic substances in the surroundings of the cells have a large effect on their metabolic activity and this effect can be transformed into electrical or optical signals. One of the main target analytes of micro-algal biosensors is pesticides, a very broad term which includes herbicides, insecticides, and fungicides. Herbicides that target the enzyme acetolactate synthase (ALS) or acetohydroxy acid synthase (AHAS) are among the most widely used in the world. However, most scientific papers published during the past decade deal with PSII inhibitors. Therefore our review will focus on the analysis of photosynthesis quenching by these herbicides.

One of the main advantages of micro-algal biosensors is that frequent measurements can be made without extensive preparation of the sample. However, they usually have only low selectivity with regard to separate analyses. Only a global signal corresponding to a range of toxic substances is obtained. However, this global response is often more useful for assessment of water quality than the measurement of individual concentrations [9].

The micro-algal biosensors discussed in this review are summarized in Table 1.

Immobilization of micro-algae

One of the limiting steps in the development of whole-cell micro-algal biosensors is immobilization of the biomaterial in a matrix that prevents leaching without reducing the stability and activity of the cells. Most of the immobilization techniques rely on the use of organic supports for example poly(vinyl alcohol) (PVA) or polysulfone (PSU), which some algal strains may find toxic. More biocompatible supports include biopolymers such as calcium alginate. However they suffer from a lack of stability over time, precluding their use in long-term devices. Thus most appropriate entrapment matrices seem to be porous silica materials that are non-toxic to living cells and resist microbial attack. An in-depth review paper by Moreno-Garrido describes in detail the most recent advances realized in the immobilization of micro-algae. [1].

Gel entrapment is the most widely used technique for algal immobilization. It can be performed with synthetic polymers (acrylamide, photo-crosslinkable resins, polyurethanes), proteins (gelatin, collagen or egg white), or natural polysaccharides (agars, carrageenans or alginates).

To improve the stability of biological functions, various immobilization techniques have been proposed:

- microencapsulation within a permeable membrane [10];
- adsorption on to cellulose derivatives [11];
- gel entrapment [12, 13];
- reticulation in glutaraldehyde [14]; and
- co-reticulation in an albumin–glutaraldehyde matrix [15–17].

Among the gels, poly(vinyl alcohol) (PVA) is frequently used as matrix for immobilization of a variety of enzymes and cells [18]. A variant of this polymer uses styrylpyridinum

Table 1 The micro-algal biosensors discussed in this review

Strain	Classification	Inorganic/organic	Detection limit	Ref.
Chlorella vulgaris in alginate gel	Amperometric	Organic	2–3000 μmol dm^{-3}	[40]
Chlorella vulgaris	Amperometric	VOCs	1 μmol dm^{-3}	[41]
Dictyosphaerium chlorelloides	Optical	Organic	0.5 μmol L^{-1}	[42]
Chlorella vulgaris in silica micro-columns	Sequential elution and determination	Inorganic	0.5–4 μg L^{-1}	[43]
Chlorella vulgaris between two platinum electrodes	Conductometric	Inorganic	10 ppb	[44]
Chlorella vulgaris immobilized in BSA	Optical	Inorganic	1 ppb	[45, 46]
Dictyosphaerium chlorelloides in sol-gel silica matrix	Optical	Inorganic	0.6 mg L^{-1}	[47]
Synechococcus PCC 7942 immobilized in PVA-SbQ	Optical	Inorganic/organic	0.2 and 0.06 mmol L^{-1}	[53]
Synechococcus PCC7942	Optical	Inorganic	<5 mg L^{-1}	[60]
Anabaena torulosa immobilized on an oxygen electrode	Amperometric	Inorganic	0.4 mg L^{-1}	[61]
Thalassiosira rotula frustule	optical	Organic	10 ppm	[63, 64]

groups attached to the poly(vinyl alcohol) (PVA-SbQ) to cross-link the polymer chains under mild conditions without damaging the biological material to be entrapped [19]. *Synechococcus* sp. PCC 7942 entrapped in PVA-SbQ enabled efficient detection of herbicides [19]. In these photoelectrochemical cells, cyanobacteria were used to capture light energy and convert it into reduced species. The PSII acceptor DCBQ could be used as an electroactive mediator to transport electrons from the reduced species in the photosynthetic membrane to the working platinum electrode. A method to detect pollutants, for example diuron, that inhibit the photosynthetic electron flow in entrapped cyanobacteria was developed. The percentage inhibition could be measured after just 5 min of contact with the pollutant and 1 min of illumination, performance better than with bioluminescent cyanobacteria.

Micro-algal biosensors have to be regenerated after use, but most immobilization techniques irreversibly bind cells preventing the production of reusable biosensors. Several groups are trying to improve this process, but results are not yet very good and studies are still in progress. A recent publication suggests that a convenient method could be to use micro-algae coated with biocompatible magnetic nanoparticles (NdFeB). Living cells are attracted to the surface of a screen-printed electrode by a magnetic field. They can then be easily removed when the magnetic field is switched off [20].

Photosynthetically active whole cells within silica gel matrix

Silica is a biocompatible material very attractive for encapsulation of enzymes and whole cells. If a material is biocompatible it is neither cyto-toxic or geno-toxic, and neither are its eventual degradation products. Silica gels have numerous advantages as material host, for example mechanical and chemical stability and photo-transparency, which is sometimes required for photosynthetically active cells.

Photobiochemical hybrid materials have been obtained by immobilization of *Synechococcus* sp. PCC 6301 and PCC 7002 cyanobacteria within silica gels [21–24]. Even after 12 weeks, entrapped cells continue to autofluoresce, as proved by epifluorescence microscopy images (Fig. 1). This is indicative of the preservation of their photosynthetic apparatus and, therefore, their ability to photosynthesize. Both chlorophyll *a* and phycocyanin can be detected *via* characteristic reflectance bands. Chlorophyll a was detectable after 12 weeks for *Synechococcus* PCC 6301 yet the phycocyanin component had disappeared. For *Synechococcus* PCC 7002 this trend was reversed with the water-soluble phycocyanin remaining longer. These hybrid materials could be used as biosensors to detect traces of metals and herbicides by monitoring the photosynthetic activity of the cyanobacteria.

Sol–gel process for encapsulation of vegetal cells [25]

Silica matrixes are relatively inexpensive to synthesize and have interesting properties including optical transparency, biocompatibility and chemical inertness. Moreover, shape materials such as microspheres, fibers, or thin films can be easily processed from the precursor solution. Algal silica for instance could be deposited in front of the tip of an optical fiber to construct an optical biosensor. The optical

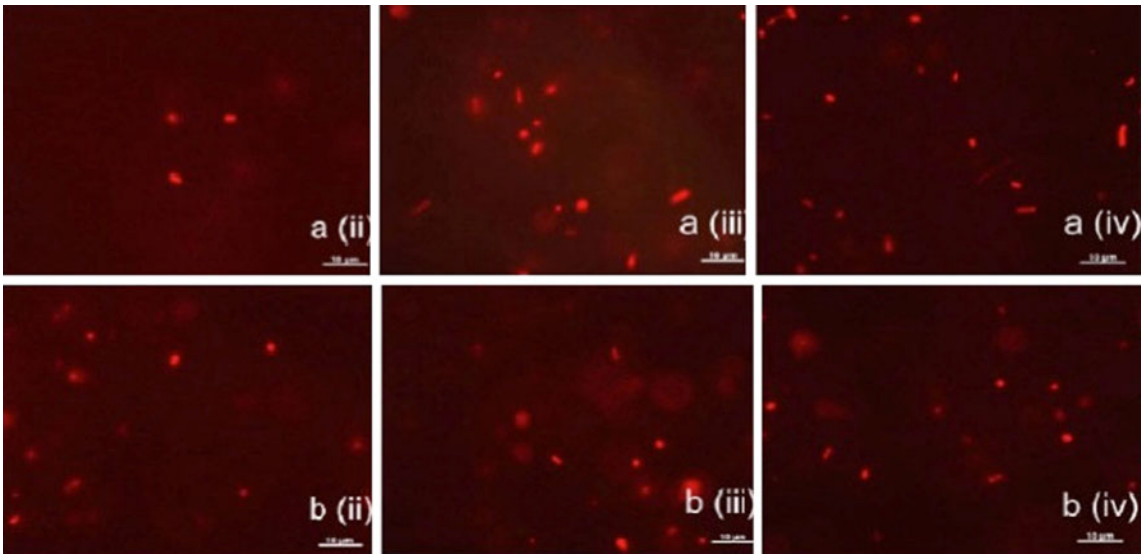

Fig. 1 Epifluorescence microscopy images of *Synechococcus* encapsulated within silica gel after (**ii**) one week, (**iii**) four weeks, (**iv**) 12 weeks, for strains **a** PCC 6301 and **b** PCC 7002. *Scale bar* represents 10 μm. (Reprinted from Ref. [22], Copyright (2010), with permission from Elsevier)

fiber would be used to send the excitation radiation to the algal cells and convey the fluorescence radiation up to a fluorimeter. Similarly, sol–gel films can be deposited on to an electrode for amperometric measurements.

The objective of this review is to highlight micro-algal biosensors based on three families of eukaryotic (green algae, diatoms) and prokaryotic (cyanobacteria) micro-algae. They are all photosynthetic, using CO_2 and water to make sugar, with oxygen as a by-product. Their characteristic color depends on the nature of their photo-synthetic pigments. Their metabolic activity is very sensitive to changes in the surrounding medium, enabling the bio-detection of water pollution.

Chlorophyta or green algae

Green algae are eukaryotic with their genetic material confined inside a nucleus surrounded by a membrane. Some green algae, called macro-algae are quite large, visible to the naked eye, whereas others, called micro-algae, are much smaller and not visible without a microscope. Only this second group will be discussed here.

The Chlorophyta thallus is organized into one of four patterns:

- The first, and the most important here, consists of isolated microorganisms constituted of a single cell separated from the outer medium by a membrane often protected by a cellulosic wall (Fig. 2). The wall thickness, morphology, and ornamentation are very diverse. A mucilaginous substance (mucus) of variable thickness produced by the cell separates it from the outside medium. This group includes *Chlorella vulgaris*, the green micro-alga most used for biosensor applications (Fig. 3). This micro-alga has solitary and spherical cells, with a distinct wall, one or two chloroplasts, and sometimes one pyrenoid.
- In the second group, green micro-algae form colonies made of several cells that may have different morphologies.

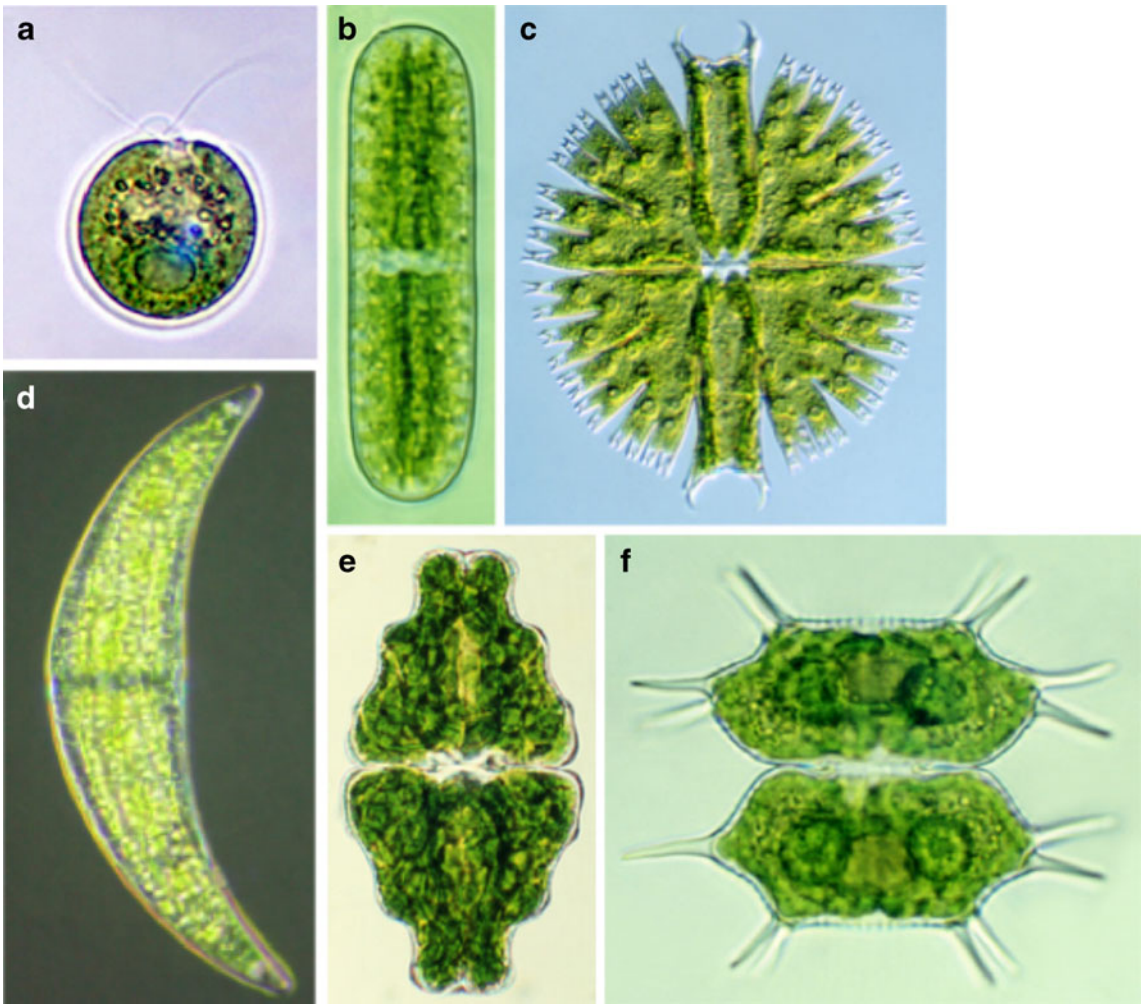

Fig. 2 Isolated green algae: **a** *Chlamydomonas*, **b** *Cylindrocystis*, **c** *Micrasterias*, **d** *Closterium*, **e** *Euastrum*, **f** *Xanthidium*

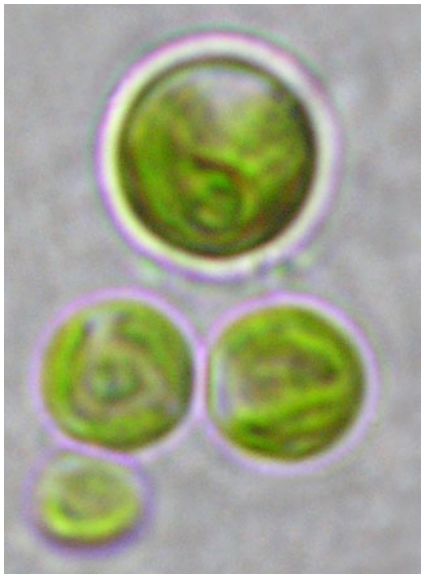

Fig. 3 Isolated green alga *Chlorella vulgaris*

- The third type assembles filamentous green micro-algae which are uniseriate (one file of cells), non branching, sometimes very short, possibly becoming pluriseriate thalli (with several axes) with branching and indeed even lamellar (frond) in form, some being visible with the naked eye.
- The last type of thallus organization corresponds to green micro-algae containing single wall, without septa. The cytoplasm contains numerous nuclei and chloroplasts. This kind of structure, which is named siphon, is uncommon in green micro-algae

Some micro-chlorophyta incorporate lipids, for example sporopollenin, in their cell wall or calcium carbonate precipitates inside their outer mucilage (Fig. 4a, b). This reinforcement of their envelope gave some of them the opportunity to resist fossilization, so they can be used as bio-climatic indicators. The genus *Botryococcus* (Fig. 4c), which produces hydrocarbons, has left life marks on earth for more than 500 million years.

Green algae are autotrophic photosynthetic microorganisms. They can synthesize food directly from carbon dioxide and water to produce sugars, releasing oxygen as a waste product. The actual chemical equation which takes place is the reaction between carbon dioxide and water, catalyzed by sunlight, to produce glucose and a waste product, oxygen:

$$6CO_2 + 6H_2O \rightarrow C_6H_{12}O_6 + 6O_2$$

The glucose sugar is either directly used as an energy source for metabolism and growth or polymerized to form starch that is stored in granules located inside chloroplasts. The waste oxygen is excreted into the atmosphere, where it is used by plants and animals for respiration.

Sunlight energy is absorbed by the photosynthetic pigments (chlorophyll a and b) and by other pigments, for example carotenes and xanthophylls. The pigments, responsible for the typical green color are located on the thylakoids, lamellar structures situated inside chloroplasts.

Photosynthesis takes place in organelles called chloroplasts. Chlorophyll molecules are specifically arranged in and around photosystems that are embedded in the thylakoid membranes of chloroplasts. The function of the reaction center, chlorophyll, is to use the energy absorbed by and transferred to it from the other chlorophyll pigments in the photosystems to undergo a charge separation, a specific redox reaction in which the chlorophyll donates an electron into a series of molecular intermediates forming an electron transport chain. Water provides a source of electrons.

Detection of pollutants and herbicides

Green micro-algae are the main component of phytoplankton populations. Because they can survive under environmental conditions that could be harmful to other microorganisms, they have been used in the development of biosensors that can respond to critical changes in aquatic ecosystems.

Evaluation of the toxicity of water is an important issue for environmental water safety. Herbicides, for instance, are widely used in agriculture for crop protection but they cannot be easily removed from the soil. After application

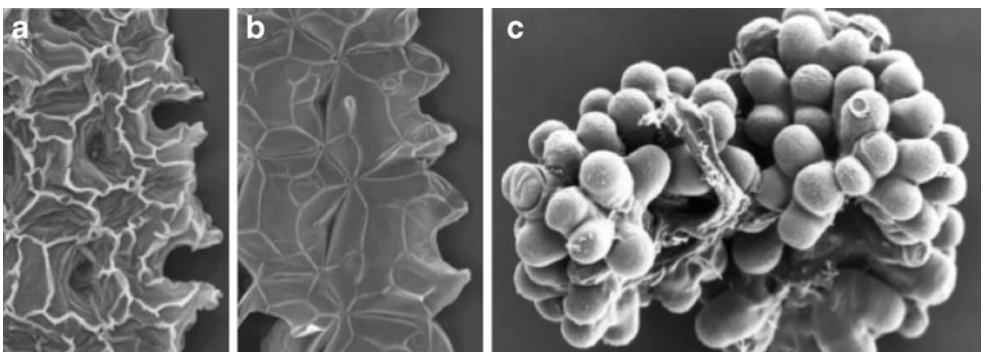

Fig. 4 Cell walls of two species of *Pediastrum* (**a**, **b**) and of *Botryococcus* (**c**)

they can, therefore, be found in soil, ground and surface waters, and tissues and body fluids. For safety reasons, only very small traces of herbicides should remain in water. The "European Water Act of 1980" document, states that the concentration of herbicides in water must be below 0.1 or 0.5 μg L^{-1} for any individual or total herbicide class, respectively [3]. There is, therefore, growing interest in the development of rapid inexpensive assays to screen for the presence of herbicides in the environment. However, classical physicochemical analysis of herbicides requires a sample clean up step, followed by liquid or gas chromatography and tandem mass spectrometric detection. Biosensors should lead to new possibilities for the development of automatic, rapid, and direct analytical methods. They avoid sample pretreatment or require minimum sample preparation, enabling on-site field monitoring. Bioassays are among the most useful methods for determination of the toxicity of environmental and industrial wastewaters. Many bioassays based on micro-algae have been developed in recent years [6, 26, 27]. They are highly sensitive and more reproducible than those based on physical or chemical analysis.

The green alga *Chlorella vulgaris* is usually selected for making biosensors because of its greater stability in producing biological signals. The chlorophyll fluorescence emitted from its photosynthetic activity enables detection of pesticides [28], and inhibition of its alkaline phosphatase and esterase activity enables the determination of heavy metals [29] and organophosphorus insecticides [30]

Whole-cell biosensors based either on chlorophyll fluorescence or enzyme (phosphatase and esterase) inhibition have been constructed for real-time detection and on-line monitoring. Results show that these devices are sensitive to heavy metals and pesticides. The system enables the cells to operate in their natural environment, which favors long-term stability and reflects the mechanism of toxic action of ecological interest [31].

Fluorescence of the photosynthetic system

The first algal biosensors were based on the photosynthetic activity commonly observed in all plants. In such biosensors the photosynthetic activity of the living cells is modified by the presence of pollutants. This effect can be transformed into electrical or optical signals. Optical sensors are based on the fluorescence of chlorophyll contained in chloroplasts [32–36] whereas amperometric sensors follow the evolution of photosynthetic oxygen with a Clark electrode [37–39].

Algae-based biosensors currently use the chlorophyll fluorescence as the measurable signal. Light absorption takes place in the so-called antenna pigments in the thylakoid membrane. The energy is transferred to the reaction centre of the photosystems and is used by the organisms for ATP production. Variations in the fluorescence of chlorophyll in the presence of toxic substances can be measured and correlated with pollutant concentration.

Photosynthesis is initiated in photosystem II (PSII), in which water is split into protons and electrons with release of molecular oxygen. Protons are used to create a potential gradient across the thylakoid membrane, enabling the production of ATP from ADP and phosphate. Electrons enter the electron transport chain where they proceed, through a series of transport molecules and proteins, to photosystem I (PSI). The excited electrons are finally used to produce reducing equivalents in the form of NADPH, which are used in the Calvin cycle for carbon fixation and other metabolic reactions. Biosensing applications are based on the total or partial inhibition of this electron transfer, because of the presence of chemical or physico-chemical environmental conditions reacting either by direct binding to the reaction center complexes or by changing the equilibrium of the local environmental chemistry. Pollutants or herbicides in contact with Photosystem II (PSII), inhibit the transport of electrons from the primary acceptor Q_A to the secondary quinone Q_B along the photosynthetic chain and partially or fully block the electron transfer. This inhibition results in variation of PSII fluorescence emission that can be monitored by fluorescence analysis. Herbicides targeting PSII belong to a variety of chemical classes, for example triazines, triazinones, ureas, biscarbamates, dinitrophenols, and cyanophenols, to name only a few.

Fiber optic biosensors for herbicide analysis have been developed using three different micro-algae, *Dictyosphaerium chlorelloides* (D.c.), *Scenedesmus* sp. (S.s.), and *Scenedesmus intermedius* (S.i.). The micro-algae were immobilized in a sodium silicate sol–gel matrix to preserve their biological activity. The increase in the amount of chlorophyll fluorescence was used to quantify thee herbicides that inhibit the photosynthesis at PSII, for example triazines (atrazine, simazine, terbuthylazine) and urea-based herbicide (linuron). The best results, i.e. the lowest detection limits, the broadest dynamic calibration range, accurate response, and reversibility, were obtained with *Dictyosphaerium chlorelloides*.

Photo-amperometry

A compact and disposable biosensor for rapid toxicity testing has been developed using the green micro-alga *Chlorella vulgaris.* Micro-algae were entrapped in an alginate gel or a poly-ion complex membrane immobilized directly on the surface of a transparent ITO (indium tin oxide) electrode (Fig. 5) [40]. The oxygen generated

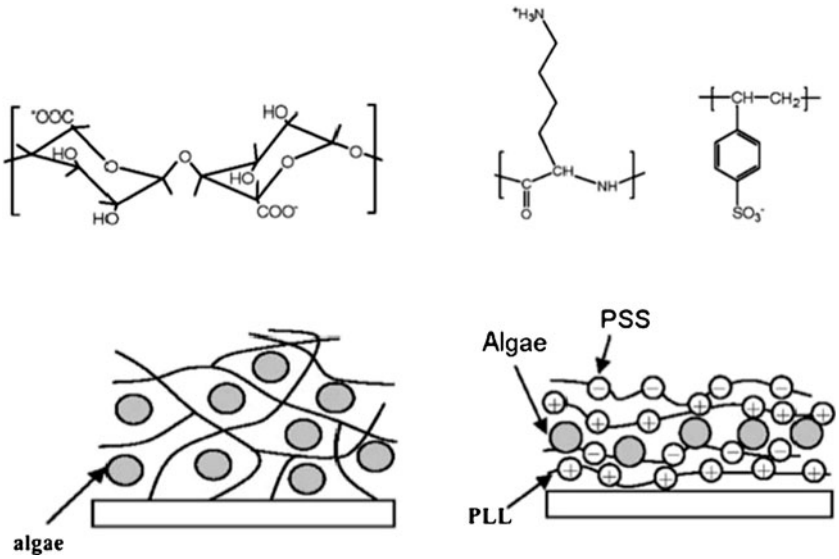

Fig. 5 Molecular structures and schematic illustrations of membranes immobilizing algae on an ITO electrode (Reprinted from Ref. [40], Copyright (2010), with permission from Elsevier)

photosynthetically by these immobilized micro-algae was monitored amperometrically. The response of the algal biosensor was tested with four toxic compounds: atrazine (6-chloro-*N*-ethyl-*N*-isopropyl-1,3,5-triazine-2,4-diamine), DCMU (3-(3,4-dichlorophenyl)-1,1-diethylurea), toluene, and benzene. A good correlation was obtained between results from these amperometric measurements and those obtained from a conventional standard growth test. The main advantages of this new biosensor are that it is much smaller, less expensive, and its assay time is much shorter (≤200 s) than other conventional algal biosensors based on Clark electrodes.

Gas biosensors for volatile organic compounds (VOC)

Most biosensors based on enzymes or microorganisms are effective in aqueous solutions only. It would be useful to make biosensors operating in the gas phase that can be used as a warning device to monitor the quality of the atmosphere in a workplace. This is required for VOC, for instance. Volatile organic compounds are often used in the form of vapor or aerosol for dry-cleaning and in municipal treatment plants. They also affect the photosynthetic activity of *Chlorella vulgaris* micro-algae. Biosensors capable of responding to toxic VOC such as perchloroethylene have been developed to determine volatile organic compounds (VOC) in the form of aerosols [41]. They were based on *Chlorella vulgaris* cells immobilized on the membrane of an oxygen Clark electrode. They measure the oxygen produced by the photosynthetic activity of micro-algae under light in the presence of atmospheric carbon dioxide. Because the algal membrane is designed to work in the atmosphere. The biosensor is kept inside a controlled atmosphere chamber and a water reservoir is fixed to the electrode body to provide humidity to enable the algal cells to operate properly. This method is based on modification of the photosynthetic activity in the presence of the analyte used in the form of an aerosol. Spraying a toxic compound (perchloroethylene) into the controlled atmosphere chamber, leads to a modification of the oxygen produced under illumination, and this can be detected with a Clark electrode (Fig. 6).

Improved specificity [42]

Algal biosensors usually have good sensitivity but a rather poor specificity. A new genetic approach was thus developed to increase the specificity of micro-algal biosensors. This method is based on the joint use of two different genotypes to detect a given pollutant. Experiments

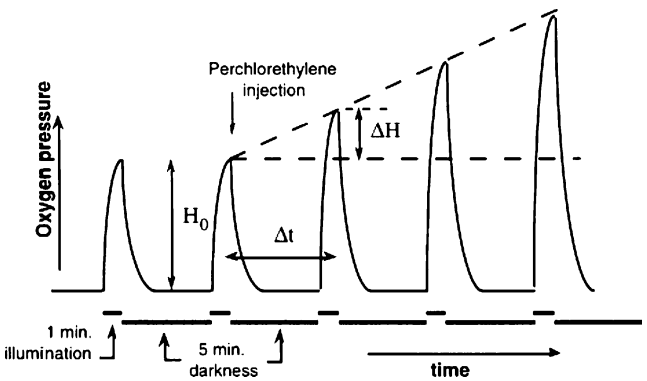

Fig. 6 Typical responses of an algal biosensor to perchloroethylene aerosols (Reprinted from Ref.[41], Copyright (2010), with permission from Elsevier)

have been performed to detect TNT (2,4,6-trinitrotoluene), a well-known explosive that readily enters groundwater supplies where it can have harmful effects on all life forms. Several methods have been developed to detect TNT; most are based on expensive chemical analyses, for example high-performance liquid chromatography (HPLC), gas chromatography–mass spectrometry (GC–MS), or even immunoassays, but none is based on living organisms.

In this micro-algae-based biosensor, two genotypes of the green micro-alga *Dictyosphaerium chlorelloides* have been used simultaneously: a sensitive one to obtain sensitivity (wild-type DcG1wt) and a TNT-resistant mutant to obtain specificity. Inhibition of the chlorophyll *a* fluorescence of PSII by TNT was used as the biological signal. In such algal biosensors the biological response of wild-sensitive cells decreases in the presence of contaminants; isolated specific mutants for which the decrease in biological response is smaller work as controls. This low cost algal TNT biosensor is less sensitive than immunoassay sensing systems (detection limit 0.5 mg L^{-1}) but its time response is five time faster (<3 min).

Metal speciation

Micro-algae are known to have strong affinity for metal ions. They are able to accumulate large amounts of metal from their environment and are, therefore, currently used as biosorbents for metal pre-concentration before titration with analytical devices. After desorption, metals are analyzed by conventional physicochemical methods, for example optical absorption or plasma emission spectroscopy [2]. Binding of metals to algal surface occurs in living and dead algae. It seems to be not only a simple sorption process, but also involves exchange of Ca^{2+} ions.

Many metals, for example chromium, cobalt, copper, cadmium, or mercury, can be fixed by micro-algae. Among these metals, mercury is one of the most toxic, because of his tendency to react with organic molecules giving strong C–Hg bonds. It is therefore especially important to be able to detect traces of this metal in water. CH_3Hg^+ and Hg^{2+} are the most significant mercury species in aquatic media, the first, CH_3Hg^+, is especially dangerous because of its ready diffusion through biological membranes. Interesting results have been obtained recently using the green micro-alga *Chlorella vulgaris* immobilized on a solid substrate (silica gel) [43]. Mercury speciation was performed in micro-columns packed with this silica-algae composite. Hg^{2+} and CH_3Hg^+ have been shown to be efficiently retained within the column. The efficiency of uptake for both species at pH 3 was higher than 97%. They can then be sequentially eluted at lower pH by adding HCl. One of the main advantages of using micro-algae is that the retention capacity remains unaltered for three weeks at 0 °C. Mercury species can therefore be stored until analysis, avoiding problems associated with maintaining species integrity in aqueous solutions. *Chlorella vulgaris* on silica gel is, therefore, a low-cost promising alternative to conventional water sampling for mercury analysis.

Alkaline phosphatase activity

Enzyme sensors have been widely developed. They are mainly based on inhibition of purified enzymes. However enzyme purification may be quite expensive and it would be better to keep them in their natural environment to ensure a longer lifetime. Whole-cell biosensors are more suitable for meeting all the requirements for environmental surveillance [28]. They are more resistant than those using purified enzymes. Their enzymes and cofactors are hosted in an environment optimized by nature.

The alkaline phosphatase activity (APA) of the micro-alga *Chlorella vulgaris* is inhibited in the presence of heavy metals, a property that can be used for sensing. A biosensor was therefore constructed to detect heavy metals from inhibition of alkaline phosphatase (AP) present on the external membrane of *Chlorella vulgaris* [44]. Micro-algae are placed between two platinum interdigitated electrodes, which form the transducer. Conductometric measurements are based on a differential measurement between a working electrode (on which the active culture is deposited) and a reference electrode (on which an inactive culture is deposited). The APA algal activity is measured via the change in conductivity. Metal ion concentrations below 10 ppb can then be detected. These biosensors are not specific for a single metal, but they provide a global response to the presence of heavy metals and can be regarded as an early warning system [30].

Micro-algae can also be trapped within a polymer or a gel layer, but such matrices can form a diffusion barrier that restricts the accessibility of the substrate or inhibitors of algal cells. A better solution would be to immobilize micro-algae on self-assembled monolayers (SAMs) of alkanethiolate deposited between Pt electrodes (Fig. 7). The bioreceptor in this case is linked to the transducers by a covalent link. It is free in the reaction medium instead of being trapped in a matrix. Such chemical immobilization enables direct contact between enzymes and the substrate so that all the substrate comes into contact with the enzymes. This conductimetric biosensor was developed for the of metal ions. Published results show that it is sensitive to the presence of Cd^{2+} ions with a detection limit of 1 ppb [44]. Other enzymatic activity, for example esterase activity, could be used with similar biosensors for the identification of pollutants in the natural environment.

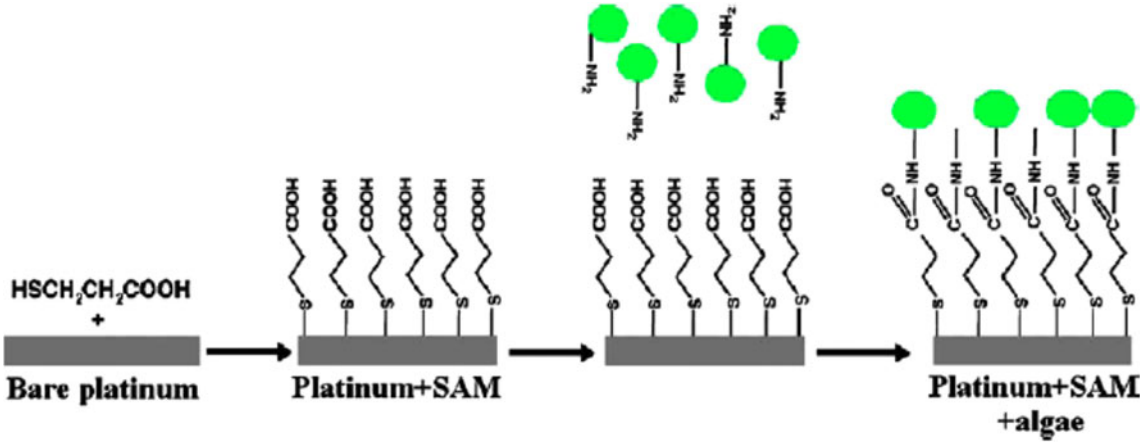

Fig. 7 Immobilization of algal cells on a Pt electrode modified by SAMs (Reprinted from Ref. [44], Copyright (2010), with permission from Springer)

Optical biosensors based on inhibition of algal APA by heavy metals (Cd^{2+}, Pb^{2+}) were developed by C. Durrieu et al. [45]. Alkaline phosphatase (AP) activity was determined either via optical or conductivity measurements [45]. For example, whole cells of *Chlorella vulgaris* were immobilized inside bovine serum albumin (BSA) membranes cross-linked with glutaraldehyde. APA in the presence of cadmium ions was measured. It was shown that conductimetric biosensors using immobilized algae seemed to be more sensitive than bioassays in the detection of low levels of cadmium ions (the detection limit for the first experiments was 1 ppb Cd^{2+}) [46].

APA membrane phosphatase measurements can be done on whole cells, without any extraction step. Therefore they provide an interesting method for detection of metals adsorbed on the surface of the cell.

Copper sulfate is one of the algicides most commonly used to treat surface waters from lakes, reservoirs and other water supplies. It is also currently used as an insecticide in agriculture. However, Cu^{2+} ions enter the food chain, and act as a primary factor of species selection, significantly reducing biodiversity. It is therefore important to develop a screening method for detection of copper in water. This was achieved in a recent study in which the freshwater green algae *Dictyosphaerium chlorelloides* were encapsulated in a sol–gel silica matrix. The toxic effects of Cu(II) were examined by pulse amplitude modulation (PAM) [47]. With this method, two quantities, Fm′ and qN, seem to be especially sensitive for performing Cu field screening in water. Fm′ is the maximum fluorescence yield reached during last saturation pulse with an illuminated sample and qN is the coefficient of no photochemical quenching. The selectivity of this cell biosensor was enhanced by comparing responses of wild-type Cu-sensitive and Cu-resistant mutant algal cultures [42, 48]. A detection limit of 0.6 mg L^{-1} was observed, low enough for determination of Cu concentrations exceeding regulatory levels (2 mg L^{-1}).

Functional biohybrid materials based on micro-algae tissue have been used as an active phase in the development of sensors for heavy-metal ions (e.g. Pb^{2+}, Cu^{2+}, and Cd^{2+}) in aqueous solutions. Two different micro-algae (*Chlorella vulgaris* and *Anabaena* sp. PCC7120), have been used. Attempts to encapsulate living algal cells were unsuccessful but sol–gel silica behaves well as a support for cell growth and proliferation. This system was useful for entrapping dead lyophilized algae preserving their ability to uptake heavy-metal ions from aqueous solutions. On the basis of this property, electrochemical sensors using algal/sol–gel biohybrids as active phases were developed [49].

Blue-green micro-algae (cyanobacteria)

Cyanophyta, blue-green algae, or cyanobacteria are prokaryotic photosynthetic microorganisms. Their genetic material is not enclosed by a membrane and remains diffuse in the central part of the cell.

Three patterns characterize the thallus organization of these micro-algae. The first is unicellular individuals with a single cell protected from the outer medium by a wall. The membrane wall can be coated or not by a mucilaginous sheath (Fig. 8a). The second pattern is colonies made of many cells with different disposition without close contact, inside common mucilage with diverse morphology (Fig. 8b, c). The third type assembles simple filamentous structures, uniseriate (arranged in one row) or pluriseriate (branched or not) coated with mucilage of various thickness (Fig. 8d, e). In this type and according to ecological conditions, special cells may appear in peculiar genera or species, for example heterocytes, that are able to reduce atmospheric nitrogen (Fig. 9, arrows),

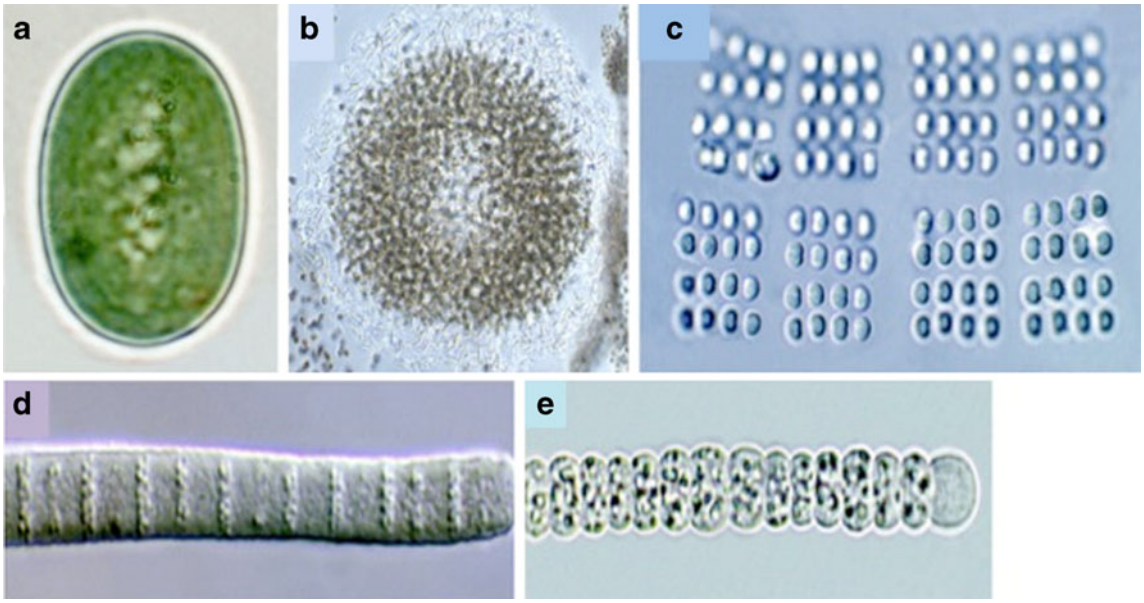

Fig. 8 Cyanobacteria: **a** *Cyanothece*, **b** *Microcystis*, **c** *Merismopedia*, **d** *Phormidium*, **e** *Trichodesmium*

or akinetes, bulky cells with thick walls, filled with stored carbohydrate and fat, that play the role of resistant cells.

Chlorophyll *a* is responsible for the photosynthetic properties of cyanobacteria. The first substance synthesized via photosynthesis is a glucose polymer called glycogen, which is stored in granules dispersed in the cytoplasm. However, chlorophyll *a* absorbs efficiently in the red part of the spectrum, a wavelength range which is completely absorbed below 10-m depths in water. Therefore phycobiliproteins, which do not exist in green micro-algae (red and blue pigments) are also involved in the photosynthetic process. They absorb light in the green and orange spectral region and transfer energy to chlorophyll *a*, which can then participate in its light-harvesting function. These pigments are located on thylakoids—lamellar structures located along the cell wall.

Pollutants—herbicides

Wild-type cyanobacteria were among the first organisms to be used for developing whole-cell biosensors for environmental monitoring. These phototrophic organisms are ecologically important and therefore ideally suited to monitoring of compounds such as herbicides that inhibit the photosynthetic activity of the organisms. Immobilized

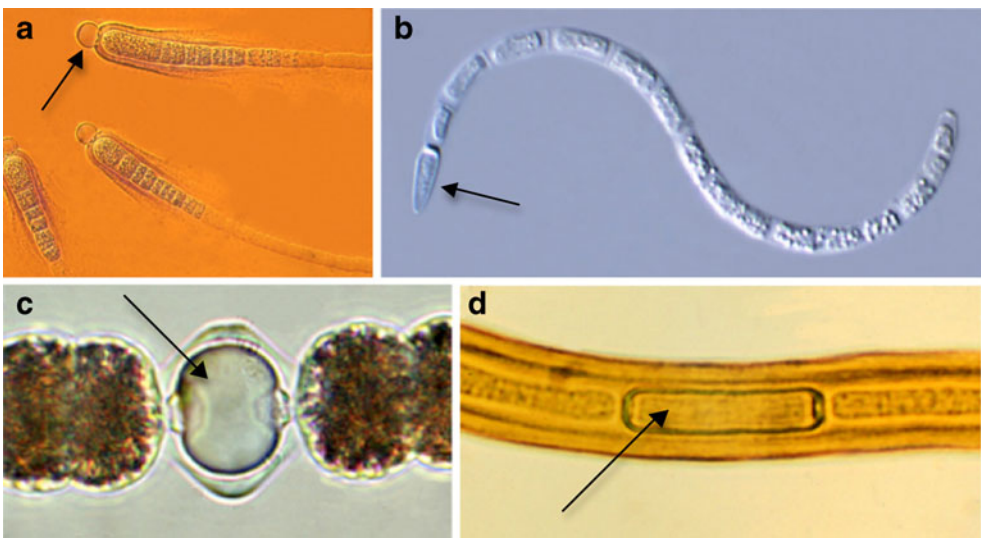

Fig. 9 **a** *Gloeotrichia*, **b** *Cylindrospermopsis*, **c** *Anabaena*, **d** *Scytonema*

cyanobacteria (*Synechocystis* and *Synechococcus*) can also be used as biosensors for detection of water toxicity because of their versatile metabolism, for example photosynthetic activity (thylakoids membranes in vegetative cells), respiration, fermentation, and nitrogen fixation (heterocyt cells). Amperometric biosensors based on cyanobacteria have also been used for detection of phytotoxic pollutants [50, 51]. However, they are not very stable and robust.

To overcome these disadvantages, microbial cells can be genetically modified by introduction of a "reporter gene" to connect the initial biological interaction of the tested chemical or physical event to an easily recordable output signal (e.g. light). The most commonly used reporter proteins are β-galactosidase, green-fluorescent protein (GFP) and luciferase.

Shao et al. described a bioluminescent *Synechocystis* PCC 6803-derived reporter strain used to monitor the correlation between cyanobacterial activity and the presence of herbicides [52]. To make this reporter strain they introduced a construct made of the constitutive *tac* promoter fused to *luc* (the gene encoding firefly luciferase) and *luxAB* (encoding the bacterial luciferase) into the cyanobacterial cells by means of an integrative vector. In this case *Synechocystis* PCC 6803 reporter strain is enabled for assessment of the bioavailability and effects of various herbicides (diuron, atrazine, propazine, and simazine) on the cyanobacterial cell. The sensitivity of the system for the analytes (determined as EC_{50} values) reached the low mg L^{-1} level. The optimum pH for assay of bioluminescence was found to be 6.5. This may be for one of two reasons: either this pH supports the optimum physiology of the cyanobacterial biosensor or it is the pH at which cyanobacteria are most permeable to the luciferin substrate. Bioluminescence measurements are quite fast (10 s) but a minimum exposure time of 30 min is required to reach these values. In this case, the tested herbicides specifically reduce the energy state of the cells by blocking the electron flow in PSII, resulting in inhibition of photosynthesis. With regard to environmental relevance, cyanobacterial biosensors are sensitive to herbicides at the parts-per-million level, which is appropriate for detecting residues in groundwater or soil. In addition, these biosensors provide information on the bioavailability of the herbicide in environmental samples. Although phenoxy acid herbicides are less toxic than other herbicides, they are highly soluble, enabling easy determination of EC_{20} and EC_{50}. Compared with the green alga *Selenastrum capricornutum*, the cyanobacterial biosensor is more sensitive to glyphosate in terms of reaction time and sensitivity.

Whole-cell luminescent cyanobacterial biosensors seem to be more simple, rapid, accurate, and economical than other methods, for example photosystem-based whole-cell and tissue biosensors, in detection of the toxicity of herbicides. They could also be used to indicate the type of herbicide and possibly its potential mode of action. The disadvantage of this method is the need for knowledge of genetic tools. To circumvent this limitation, the photosynthetic activity of cyanobacteria in the presence of herbicides was followed by photoelectrochemical [53] and amperometric measurements [54]. Herbicides interact with the process of photosynthesis and are generally inhibitors of PSII-dependent electron flow. However, the major inconvenience of this method is the very short time in which free biological material is available.

Detection of heavy metals

Some organisms carrying luminescent reporter genes fused to metal ion-inducible promoters may be used as biosensors for detection of bioavailable heavy metal ions in environmental samples. Mercury ion biosensors have been constructed by fusing the *lux* genes of *Vibrio fischeri* bacteria to the mercury resistance operon of Tn*21* [55, 56]. Biosensors have also been designed for specific responses to copper, nickel, zinc, chromate, and thallium ions by fusion of the *lux* genes to a number of metal ion-responsive promoters from *Alcaligenes eutrophus* [57]. Use of the gene expression driven by the *smt* operator/promoter of the cyanobacterium *Synechococcus* sp. for detection of metal ions in aquatic environments has also been proposed [58]. The *smt* locus of *Synechococcus* sp. consists of the prokaryotic metallothionein gene, *smtA* (absent in green algae and diatoms), and a divergently transcribed gene encoding a repressor of *smtA* transcription, designated *smtB*. The transcription of *smtA* is induced in the presence of trace metal cations (cadmium, zinc, copper, mercury, cobalt, and nickel) [59, 60]. To determine the sensitivity of *Synechococcus* PCC 7942 (pJLE23) light emission to traces of heavy metal cations, cultures were incubated in the presence of different concentrations and luminescence was monitored over 5 h [60]. *Synechococcus* PCC 7942 (pJLE23) was able to detect cations such as Zn^{2+} in aqueous solutions at levels well below the World Health Organization recommended maximum for drinking water (5 mg L^{-1}, 80 μmol L^{-1}).

To improve the reproducibility of toxicological tests in the presence of heavy metals, cyanobacteria can be immobilized in different matrices. The cyanobacterium *Anabaena torulosa* was immobilized on an oxygen electrode by use of a poly(2-hydroxylethyl methacrylate) matrix [61]. The behavior of this microorganism in the presence of lead was monitored by measuring changes of photosynthetic oxygen release. In this case, the EC_{50} of the cyanobacteria for lead was 0.4 mg L^{-1}.

Diatoms

Diatoms, Diatomophyceae or Bacillariophyceae are the most prominent micro-algae in oceans and fresh waters. They are eukaryotic microorganisms, with the genetic material confined to the cell nucleus. These photosynthetic algae play a critical role in carbon cycling, fixing carbon dioxide (CO_2) and releasing oxygen. Almost 25% of all organic carbon fixation on the planet (transformation of carbon dioxide and water into sugars, using light energy) is performed by diatoms. They synthesize chlorophylls a and c, xanthophylls (amongst them diatoxanthin), and carotenoids (which are responsible for their typical yellow color). The pigmentary sites are located within chloroplasts (photosynthetic apparatus) thylakoids. The first substance produced during the photosynthetic process is a polymer of glucose called chrysolaminarin (named also leucosin and chrysose), together with lipids. It appears like large refringent grey colored granules.

Diatoms also cycle silicon, the second-most abundant element, after oxygen, in the earth's crust. Silica rocks slowly dissolve in water giving silicic acid $Si(OH)_4$ which is used to build an outer envelope, called a frustule, made of silica and organic matter synthesized by the diatom cell. This rigid shell is formed from two valves joined together by a series of silica bands linked along the margins. Frustule size may range from 1 μm to a few millimeters. These inorganic shells have an incredible variety of sophisticated shapes. Their dimensions and ornamentation are the first taxonomic characteristics used to distinguish genus and species. The frustule of centric diatoms has axial symmetry whereas pennate diatoms have an elongated frustule with bilateral symmetry (Fig. 10).

They are all unicellular but some species appear filamentous because of connection of the cells through their apices via mucilage they synthesize or, sometimes, because of one or several erected appendices which reinforce cell coupling (Fig. 11). After sedimentation on ocean or lakes bottoms, frustules progressively transform via compaction and dehydration into a kind of rock called diatomite or diatom earth. This porous material is widely used nowadays to filter beer, wine, and swimming pool water, or as an additive to paints and polymers. The dynamite invented by Alfred Nobel was obtained by impregnation of porous diatomite with very sensitive liquid explosives, for example nitroglycerin.

Because of their porous frustule, the biosensing properties of diatoms are based both on the metabolic properties of the cell and the physical properties of the silica shell. The low cost and wide distribution of diatoms make them good candidates in the field of nanotechnology.

Diatom biological index (BDI)

Diatoms are very sensitive to climate-induced changes in lake and river conditions. Their siliceous cell walls can be well preserved in sediments. Therefore the study of diatom fossils can be an important tool in the reconstruction of paleoclimatic conditions. They have left life marks on the earth for nearly 200 millions years. Past changes in climate can be inferred from changes in species abundance within a

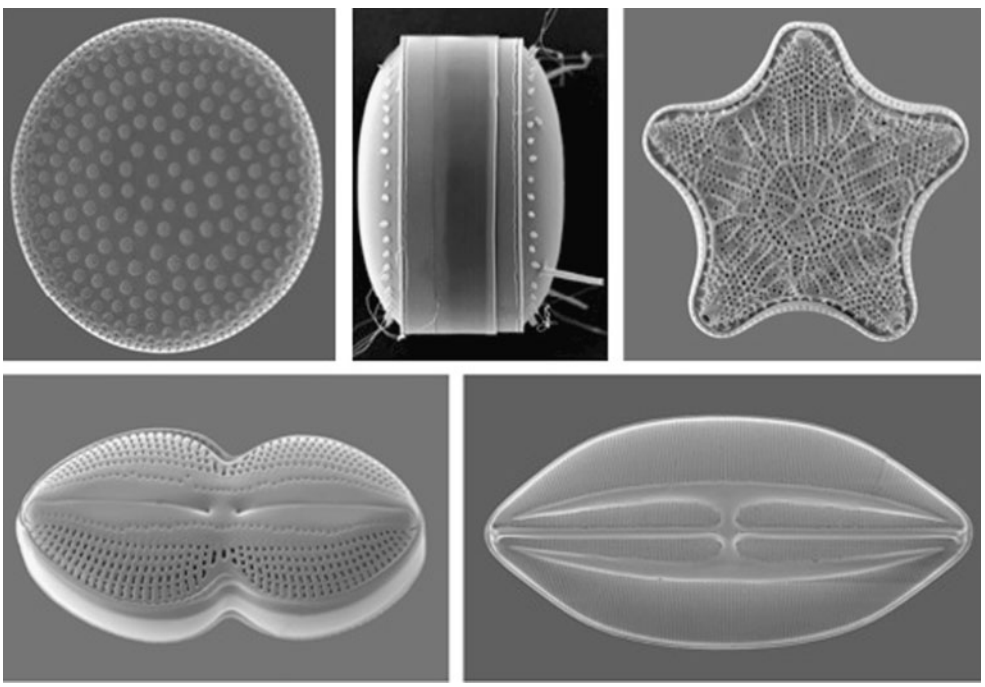

Fig. 10 Centric and pennate diatoms

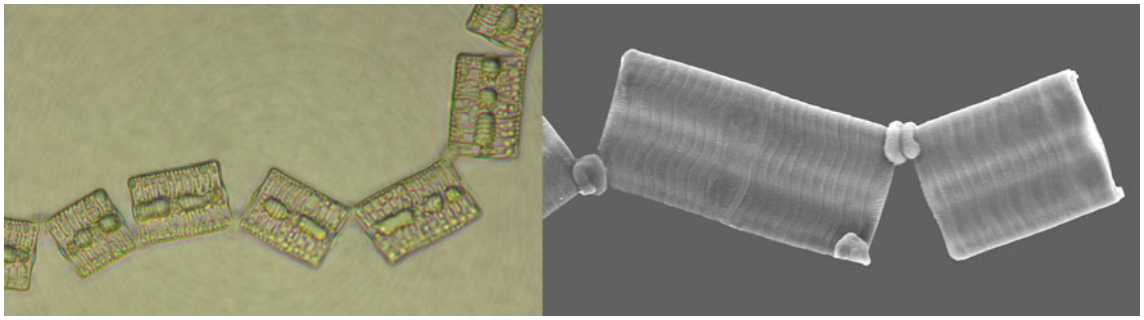

Fig. 11 Filamentous diatoms

sediment core, because the ecological requirements are well known for several "indicator" species. These species are indicative of several variables, which depend on a combination of primary factors (precipitation, solar output, wind strength) and secondary factors (upwelling, erosion).

As a primary producer, diatoms are key organisms in the water ecosystem. They live throughout the illuminated zone of every ocean, and in freshwater streams and lakes. Any water anywhere on the globe contains many different species of diatom living in an assemblage, or community. Because they are very sensitive to organic pollution from agriculture or industry, analysis of the diatom population enables evaluation of the extent of pollution and eutrophication of streams and rivers. It can be used as a simple biological method for monitoring water quality which avoids the need for more sophisticated physicochemical analysis. Freshwater diatoms are regarded as reliable indicators of the trophic status of rivers and lakes. In recent decades, several indicator indices have been developed for assessment of trophic conditions throughout Europe. A biological diatom index (BDI) has been established as a biological indicator of the quality of aquatic environments. It is based on the abundance of several hundred of diatom species, with different sensitivity to pollution, listed in a database. This is now a standardized method used routinely in France to monitor watercourse quality [62]. Scientists have also found that diatoms are sensitive to highly toxic metal contaminants, for example cadmium. This suggests that diatoms could also behave as a model bio-indicator of metal contamination of a watercourse.

Diatom-based sensors

Hierarchical porous structure

Diatom frustules have a remarkable porous structure, with a hierarchical distribution of pores from the nano to the micro-scale. They can be regarded as ready-made 3D nano-devices. More than 10^5 different species of diatom have been described. They can be easily cultured, giving large quantities of genetically controlled silica frustules. These three-dimensional silica shells could, therefore, provide the foundation for novel electronic devices, for example gas sensors that would be able to detect pollution faster and more efficiently than conventional devices.

An interesting use of diatom frustules will be in the field of optical micro-sensors for volatile substances. A key feature for an optical transducer, which should be sensitive to vapors and gases, is a large surface area in order to provide a very effective interaction with several adsorbates. The dimensions of the diatom pores are just in the nanometer range, so many volatile substances (solvents, hydrocarbons, etc …), and even pure gases, can penetrate and condense within the pores. The hierarchical porosity of diatoms enables intimate mixing between the analytical gas sample and the detector, enabling effective monitoring of biomolecular interactions (Fig. 12).

Silica is known to have photoluminescent properties in the visible range, approximately 2.2 eV, arising from defects in the Si–O network. Similar photoluminescence (PL) emission in the yellow region is also observed for silica diatom frustules. This luminescence activity is related to surface-oxygen stoichiometric defects. It can therefore be affected by even small modifications of the surrounding gas environment. Gas molecules are adsorbed on these defects leading to a change in the density of luminescent states. Photoluminescence (PL) emission can, therefore, be quenched or enhanced by the presence of gases. Gas detection at low concentrations is highly sensitive and a detection limit as low as 50 ppb could be obtained for diatom frustules with highest specific surface area.

Recent studies performed with the silica skeleton of marine diatoms *Thalassiosira rotula* show that photoluminescence (PL) depends strongly on the surrounding environment. Silica frustules have a broad emission band in the visible region, centered at approximately 2.26 eV, whose full width at half maximum is 600 meV. On exposure to NO_2, a decrease of the PL signal intensity is observed. The PL signal is quenched because the electrophilic properties of NO_2 can attract electrons from the silica substrate. Significant variation of PL intensity, even at low

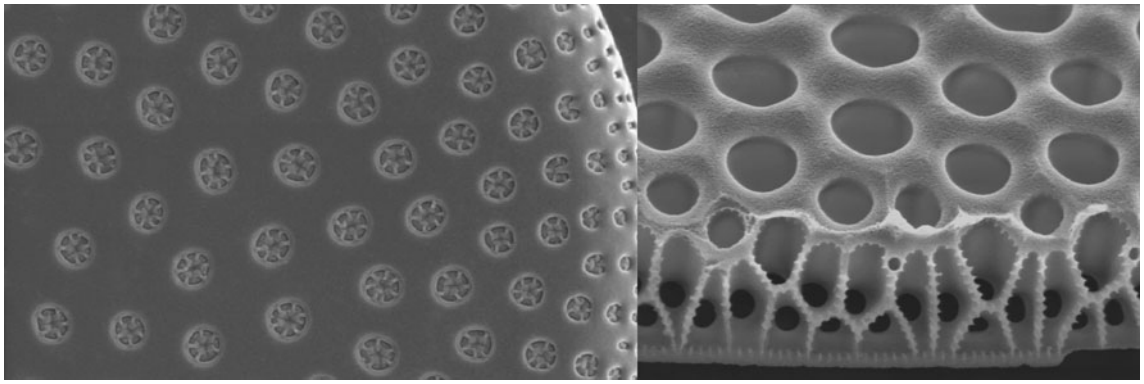

Fig. 12 Porous structure of diatom frustules

(sub-ppm) NO_2 concentrations (indicated as [NO_2]), has been observed for *Thalassiosira rotula* frustules (Fig. 13), and saturation of the quenching effect occurs at NO_2 concentrations of the order of 10 ppm [63, 64]. Both optical intensity and peak positions are affected by gases and organic vapors. Depending on the electronegativity and polarizing ability, some substances, for example acetone or ethanol, quench the luminescence whereas others (e.g. pyridine) effectively enhance it. These phenomena enable discrimination between different substances and were exploited to create the first photoluminescence gas-sensing devices based on diatoms [65]. These naturally occurring organisms are thus good candidates as optical sensing materials for toxic gas detection or air pollution monitoring.

The photoluminescent properties of silica frustules can be modified by introducing foreign elements in the culture medium. Germanium for instance can be metabolically inserted into the frustule biosilica of *Pinnularia* sp. Some Si atoms are replaced by tetravalent Ge and germanium-doped frustules have both photoluminescent and electroluminescent properties in the blue range, between 450 and 480 nm [66, 67]. Titanium also has been metabolically inserted into silica frustules by use of a two-stage cell-cultivation technique, leading to the formation of a semiconducting TiO_2 coating [68]. In this case, conductivity measurements can be used to control the amount of foreign gases such as NO_2. The modified frustule behaves like a micro-electrode (Fig. 14)

Diatom frustules are made of amorphous hydrated silica $SiO_2.nH_2O$. Reactive hydroxyl groups, Si–OH, enable chemical modification of the surface and subsequent functionalization of the silica shells. Recent work has demonstrated that the frustule surface of *Coscinodiscus wailesii*, a diatom species with radial symmetric valves, can be chemically modified and covalently linked to different kinds of bioprobe, acting as a functional support in the realization of fluorescent biosensors. Antibodies have been grafted on to the frustules of the marine diatom *Coscinodiscus concinnus*. Fluorescence measurements show that these antibodies, even when linked to the amorphous silica surface of diatom microshells, still efficiently recognize their antigens. The specific antibody–antigen recognition is

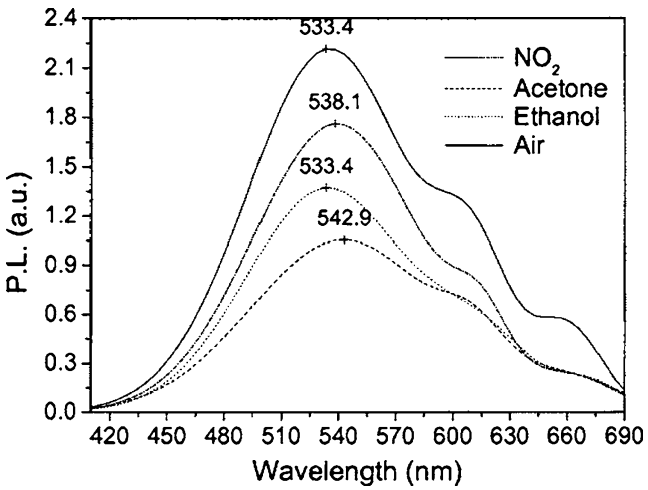

Fig. 13 Photoluminescence spectra of diatom frustules in the presence of several gases (Reprinted from Ref. [64], Copyright (2010), with permission from the American Institute of Physics)

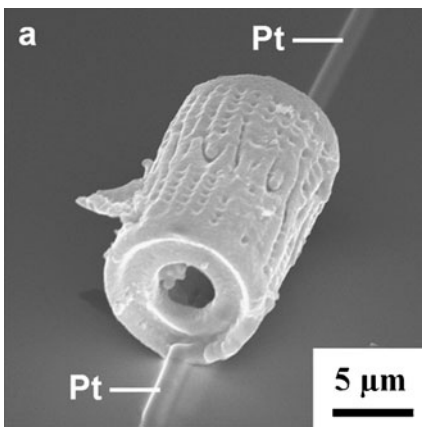

Fig. 14 Micro-electrode made with a diatom frustule (Reprinted from Ref. [68], Copyright (2010), with permission from the American Chemical Society)

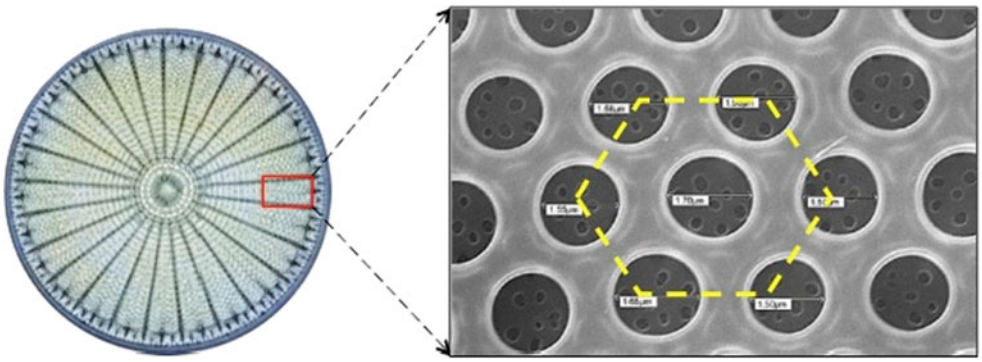

Fig. 15 Diatoms behave like photonic crystals because of the periodic distribution of pores within the silica frustule (Reprinted from Ref. [75], Copyright (2010), with permission from Springer)

revealed by changes in the photoluminescence emission of diatoms frustules [69–71].

Diatom frustules could also be used as templates for the fabrication of nanostructured materials. Silica shells can be chemically converted into other oxide materials without losing their 3D nanostructure. In such a process, currently called BaSIC (bioclastic and shape-preserving inorganic conversion), silica has been converted into a new composition *via* a shape-preserving gas–silica displacement reaction. The silica shell, for instance, can be transformed into MgO on heating in magnesium vapor at 900 °C for 4 h [72]. Many other nanostructured oxide materials (TiO_2, ZrO_2, $BaTiO_3$) have thus been synthesized [73, 74]. Silica can even be reduced to porous silicon, leading to new possibilities in micro-electronics. Such a synergistic combination of biological nanostructures with synthetic chemical functionalization could lead to a large number of 3D micro/nanostructures with chemistry and properties that can be designed for sensing applications.

Diatom frustules as photonic crystals

Some diatoms behave like "living opals"—they have iridescent properties arising from their peculiar porous structure. Pores are not randomly distributed among the silica shell; they sometimes form a regular periodic network with a mean separation distance of approximately 0.5 μm. They therefore have a periodic distribution of low (holes) and high (silica) dielectric constant materials with lattice dimensions close to the wavelength of visible light. Two different patterns have been found in *Coscinodiscus granii*, a hexagonal array of pores with a large lattice in the valve and a square array with a small lattice in the girdle (Fig. 15) [75].

Diatom frustules can therefore be described as "living photonic crystals". Strong interactions may occur between light and matter. Light in "photonic crystals" behaves like electrons in semi-conducting materials. As a result diatom frustules have iridescence like that of opals. Silica frustules absorb light mainly in the blue region, a property that protects diatoms against excessive irradiation and enhance their photosynthetic behavior [76]. This is because of a specific absorption arising from the periodic distribution of pores within the silica frustule that behaves as a photonic crystal slab waveguide. These diatomic nano-structures can be used in photonic micro-sensors for detection of volatile substances. The capillary condensation of organic vapors inside the pores leads to an increase in the average refractive index, which can be detected with several optical techniques [77].

Diatom-based sensors for rapid label-free electrochemical detection of cardiovascular biomarkers have been

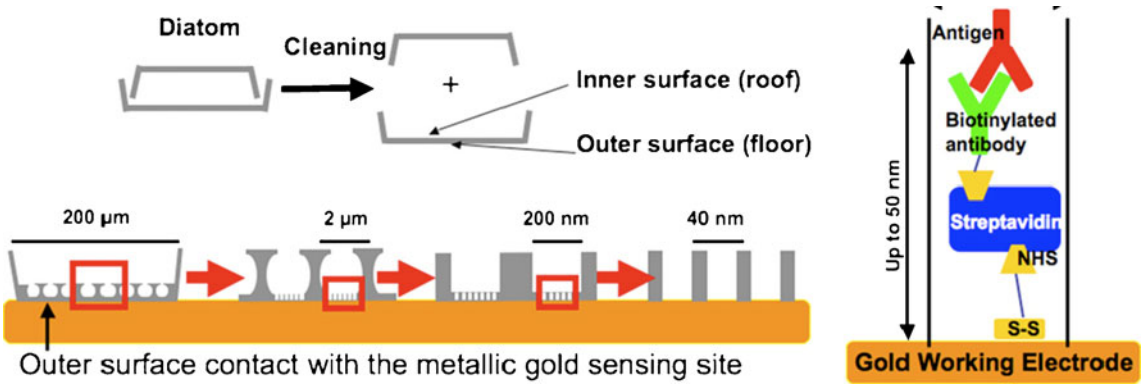

Fig. 16 **a** Schematic model of frustule pore architecture for a diatom placed on a gold working electrode. **b** Schematic diagram showing the antibody–antigen binding inside a nanowell (Reprinted from Ref. [78], Copyright (2010), with permission from Elsevier)

described [78]. The biosensor is made of an array of gold nano-electrodes deposited on a silicon chip. Each sensor is overlaid by a diatom frustule that provides a large density of nanowells. Their porous structure enhances the diffusion of biospecies enabling real control of the "molecular traffic". Inflammatory markers in human blood have been detected at low pg mL^{-1} levels, sensitivity sufficient to detect patients at risk of cardiovascular disease (Fig. 16).

Conclusion

This paper shows that micro-algae can be used as biosensors to detect pollutants such as herbicides, heavy metals, and volatile organic compounds in the ppb range. These biosensors are highly sensitive and more reproducible than those based on physical or chemical analyses. One of the main advantages of these micro-algal biosensors is that frequent measurements can be made without extensive preparation of the sample. They can also be selective, e.g. the chlorophyll fluorescence emitted from the photosynthetic activity enables detection of herbicides whereas inhibition of alkaline phosphatase and esterase enables determination of heavy metals and organophosphorus insecticides. Various sensing device can be made. Optical sensors are based on the fluorescence of chlorophyll contained in chloroplasts whereas amperometric sensors follow evolution of photosynthetic oxygen with a Clark electrode.

However, a limiting step in the development of these biosensors is immobilization of the biomaterial in a matrix, to avoid leaching, without reducing the stability and activity of the cells. Many different matrices are used, for example polysaccharides (alginates), polymers (PVA, PSU), silica … Among the matrices investigated, silica prepared by the sol–gel process has numerous advantages, for example mechanical and chemical stability. Its optical transparency is, moreover, a requirement for photosynthetically active cells.

It has been estimated that 200,000–800,000 species of micro-algae exist, of which only approximately 35,000 have been described. There is, therefore, huge potential for further development. There has recently been much progress in identifying relevant genes and pathways in micro-algae, and powerful techniques have been developed for genetic engineering. Collectively, the progress that has been realized in these areas is rapidly advancing our ability to genetically optimize the production of targeted products, for example biofuels. The applications of micro-algae are not limited to biosensors. Micro-algae can also be used to produce nanoparticles with well-controlled size and shape [79–82] and even biofuels to replace fossil energy. [83, 84]

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