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Abstract

Water pathways through the streambed actually determine driving, removal retention and uptake of particulate and dissolved matters. Much of these processes take place in the satured sediments below the surface and the banks, at the interface between surface and ground waters: the hyporheic zone. Intensity of hyporheic fluxes is expected to control the self-purification processes in sandy and gravelled beds. We are developing a non perturbing methodology based on in situ measurements by means of a biosensor based on interdigitated conductimetric electrodes in order to quantify the organic matter fluxes in hyporheic zone. Electrical conditions for electrode measurements were optimized (*f* 100 kHz, AC voltage 10 mV). A specific electronic board was designed to digitize the signal output. We developed on this microelectrode a proteinase K biosensor for protein determination. This paper presents the first results of the optimisation of the biosensor which was realised using different types of natural and polluted waters. Our experiments showed that .

Keywords

Hyporheic, protease, interdigitated electrodes, protein.

1. Introduction

The hyporheic zone (or hyporheon) is the porous habitat under a stream, bordered by the surface water and the true groundwater below. Along a simple stream riffle, there is a typical flow pattern in which surface water enters the hyporheon in a downwelling zone at the head of the riffle and retune at the tail of the riffle. This flow creates particular physicochemical conditions in hyporheon by mixing water with different physicochemical characteristics and enhancing microbial metabolic activity. Organic matter (OM) present in hyporheon is assimilated during this process (Boulton *et al*., 1998). Unfortunately the *in situ* determination of OM in this porous media is very difficult. Moreover the concept of ecological ambience, developed by Michel Lafont (2001), and the definition of functional units that it implies, requires the evaluation of polluting fluxes. The evaluation of these fluxes demands a metrology adapted to variable space-time situations and sometimes largely unforeseeable. Measurements with micro-sensors should be well adapted for monitoring processes of hyporheic assimilation through the continuous measurement of the content of OM. There are three types of organic compounds that could be used as a proxy to measure OM assimilation: lipids (10-40% of the oxidable OM), glucids (10-15%) and proteins (20-30%). The content in proteins seems to constitute a good indicator of the hyporheic activity (Namour, 1999). The general aim of our study is to measure hyporheic OM fluxes, which imply firstly to measure water flows in a porous medium and secondly to determine the concentration of the chosen compound with a specific field instrumentation. In this paper we present the first part of this study which objective was the design and the optimization of a biosensor able to measure protein in porous media, more particularly in hyporheon.

2. MATERIAL & METHODS

The study site is the stream La Chaudanne located near Lyon (France). The experimental station is a geomorphological unit composed by the pool-riffle-pool sequence (≈ 10 m long, ≈ 5 m wide). The device locations are mapped in Fig. 1.

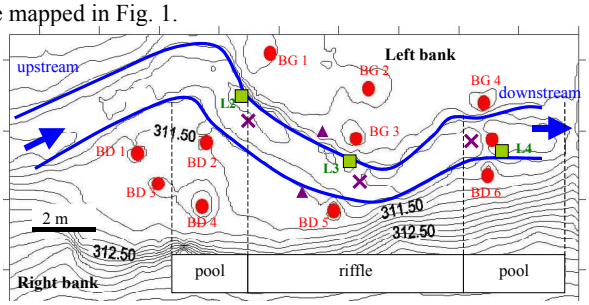

Figure 1. locations of the geomorphologic features and the different measurement devices. Solid lines delimit the streambed. ✕ Cluster of 3 micropiezometers in the streambed (15, 30 & 50 cm deep); BGx●: Piezometer in the banks; ■: Free surface water level measurement devices; ▲: Tensiometers.

This site exhibits contrasted discharge situations over a small distance and is equipped with accurate measurements of the water movement. Three piezometers profiles were installed in the banks, along the cross sectional directions of the geomorphic unit (Fig. 1). Four piezometers were driven in the left bank and six in the right bank. Three additional piezometers were placed in the water column, with pressure transducers to measure continuously the free surface water level. The water-flow measurements started at the end of January 2004. Three micropiezometers (*i.e.* 3 cm diameter external stainless steel tube with strainer and point at the bottom) were driven in the hyporheon.

Electrode design: The new biosensor consists of interdigitated electrodes (IDE) connected to an electronic board. The electrode device consists of two pairs of interdigitated electrodes (IDE) of platinum evaporated under vacuum, on a 30*10 mm glass substrate (manufactured by Institut für Chemo- and Biosensorik, Münster). With this system it is possible to perform differential conductimetric measurements without additional reference electrode. Their structure is schematically illustrated in Fig. 1. Each electrode pair forms a conductimetric cell.

Figure 1: Design of one pair of interdigitated electrodes and photograph of the transducer. The width (w) of the digits and the interdigitated distance (d) are both 10 µm. The digits are 900 µm long (l). Thus, the sensitive area of the device is about 1.6 mm^2.

Electronic board: This new biosensor consists of IDE connected to an electronic board specially designed for our application by PIMMA (Chaneins, France). The breadboard used for the conductivity measurement in laboratory is schematised in Fig. 2. The electrode geometry makes it possible to carry out a differential measurement between a working electrode and a reference electrode.

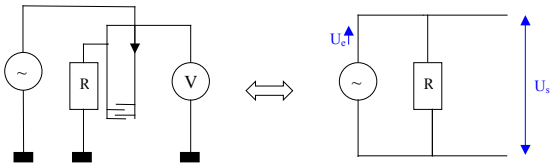

Figure 2: schematic wiring and equivalent circuit

The principle of measurement is simple: a high frequency generator sends an AC current of voltage U_{in} to the transmitting electrode. The receiving electrode returns a voltage U_{out} function of the resistance of the solution. Conductivity measurements were performed by applying to the IDEs an alternating current (AC). Indeed, using an AC of high frequency may involve risks of magnetic disturbances along wire.

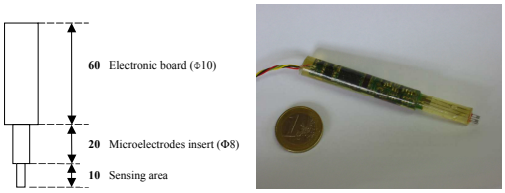

Figure 3: Biosensor design

To avoid these perturbations, the input signal is a direct current (9 V, battery LR61) converted by the electronic board into an AC. The output signal is digitized by the board and sent by wire to a data logger. A specific software acquisition, developed by PIMMA, saves the ratio output/input voltages with a variable time-step. The device is shown Fig. 3.

Choice of the protease: To detect protein, the enzymatic biosensor is based on protease catalyzing protein hydrolysis. Peptidic links are then broken and released amino acids increase the enzymatic membrane conductivity. The chosen enzyme is Proteinase K from *Tritirachium album*. It hydrolyses the proteins of all origins, in few hours, preferentially peptidic links located after hydrophobic amino acids. Its optimum pH is between 7.5 and 12.

Immobilisation of the enzyme on the micro-conductimetric structure: A mixture of Proteinase K and Bovine Serum Albumin (BSA) was co-reticulated with glutaraldehyde (GA), a bifunctional reagent reacting with the amine groups of the enzyme and BSA to give amide links. The procedure for enzymatic membrane preparation is: 1) solution A: phosphate buffer 1 mM, pH 7.5, 5% of glycerol; 2) Enzymatic membrane: 4 mg of protease mixed with 6 mg of BSA in 100 µl of solution A; 3) Deposition on the surface of the micro-conductimetric structure; 4) The structure is put in a saturated atmosphere of GA during an optimized time; 5) The sensor is then air dried during at least 30 minutes. This enzymatic membrane is put on one of the two interdigitated structures. On the second interdigitated structure (the reference) BSA replaces protease in the membrane.

Sample & assays: In order to validate the biosensor performances, we sampled freshwater from Rhône and Saône rivers (South-East of France) and one from a sewer (Aurignac, 10,000 inhabitants equivalent, low load activated sludge). All samples were refrigerated and brought back to the laboratory immediately after sampling. Protein contend were determined with the microBCA protein assay (Pierce, Micro BCA Protein Assay), briefly: to 1mL of sample add 1mL of working reagent, mix and incubate at 60°C for 60 min. measure absorbance at 562 nm. Samples were filtrated on glass fiber membrane according to EN 872 standard method. Dissolved nitrogen Kjeldhal and ammonium

were determined in accordance with the following standard methods: Kjeldahl EN 25663, ammonium NF T 90-015. DOC was determined according to

	Biosensor µS	MicroBCA mg/L	COD mg/L	Nkj mg/L
Aurignac (sewer water)	3,64	41,65	35,5	9,9
Rhône (river)	2,52	1,40	1,7	<0,5

ral et polluted waters used for biosensor assessment.

3. Results & discussion

Electrode optimization: According to our equivalent circuit (Fig. 2) we selected the frequency, the input voltage and resistance as variables to optimize. The monitored answers are the signal linearity and sensitivity. The results of the experimental design indicate a positive effect on the linearity for frequency and tension, but negative alias between frequency and tension.

	Linearity			Sensibility		
f	0.0290	f & U_{in}	-0.026	-0.0002	f & R	0.00011
U_{in}	0.0250			0.00026		
R	0.0006			0.00001		

Table 1: results of the experimental design.

The sensitivity is proportional to the tension and inversely proportional to the frequency. We thus have an antagonism between linearity and sensitivity optimizations. That means that to work on narrow ranges of conductivity we should use low frequency to increase the sensitivity, as the linearity is not a problem in this case. On the other hand, for wider measurement ranges we have to choose high frequencies to optimize the linearity. This implies the possibility to modify the biosensor constants according to the considered applications. For the continuation of our project, considering the low amplitudes of the conductivity variations shown by the protein hydrolysis, we choose to work at a low frequency. So, after optimization, differential measurements were performed between measuring (with proteinase) and reference electrodes (without proteinase) with f=100 kHz and U_{in}= 10 mV in accordance to Steinschaden *et al*., (1997).

Biosensor optimization:

The enzymatic detection has been possible with the immobilization of the enzyme on the sensor surface by cross-linking of proteinase K with bovine serum albumin (BSA) in a saturated glutaraldehyde (GA) vapor. Firstly, a mixture of 4% of proteinase K and 6% of BSA was prepared in 20 mM phosphate buffer pH 7.5, and in a 10% of glycerol solution was added in the mixture. As a differential experimental set-up was used, we prepared two electrodes: the working one coated with the enzymatic membrane and the reference one with only a mixture of 10% BSA and 10% glycerol in phosphate buffer. With these concentrations of enzyme and BSA, a bad behavior of the enzymatic membrane was observed. After that, a two times dilution of the two types of membrane was tested to decrease the thickness of the membrane and thus to improve its adhesion on the sensor. After optimization, the best mixture was proteinase K: 2% and BSA: 3% in 20 mM phosphate buffer, pH 7.5, in a 10% glycerol solution and for the reference one 5% BSA in phosphate buffer, 10% in a glycerol solution. The time of immobilization with GA strongly intervenes in the quality of the response of the biosensor (Fig. 3). In fact, for short times, the links between protein amine and GA are not sufficient to stabilize the enzyme and to have a rather strong membrane. On the opposite, for too long times we have a strong attachment and enzyme can be inactive or the active site becomes inaccessible for the substrate. That is why, as we can see we obtain an optimum time for immobilization, about 20 min in GA vapour.

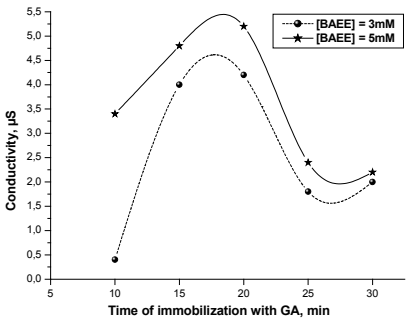

Fig. 3. Effect of time of immobilization with GA on the biosensor response for tow concentration of BAEE (Nalpha-Benzoyl-L-Arginine éthyl ester hydrochloride) a synthetic substrate.

The biosensor presents stability in laboratory above 30 days (Fig. 4) and a high reproducibility (zone b). Figure 4 displays the response of the electrodes in µS as a function of time. The graph presents two zones: zone *a* (8 hours) with a decreasing response due to the salting out of some enzymes not well adhering on the membrane; zone *b* where the response is quite stable for over one month.

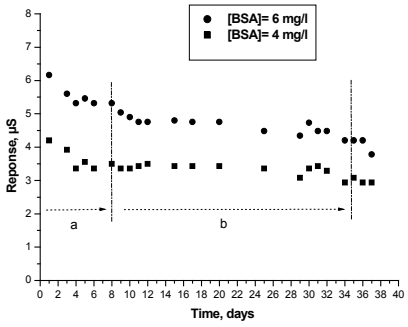

Fig. 4. Study of the biosensor stability

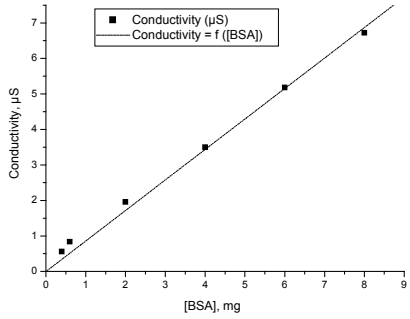

Fig.5: Dependencies of biosensor response (µS/cm) on BSA concentration

Calibration curve: The linearity for BSA determination varies from 0.4 to 8 mg/l with a sensitivity about 0.88 µS/mg BSA (Fig. 5). This range of response is in adequacy with the actual values of protein concentrations in the hyporheic zone (Namour, 1999). This biosensor displays a larger detection range than the amperometric one: 0.25 to 2.5 mg/l of BSA (Sarkar, 2000).

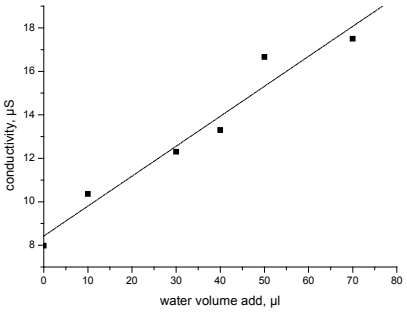

Fig. 6: Test of activation or inhibition effect of sewer water from Aurignac.

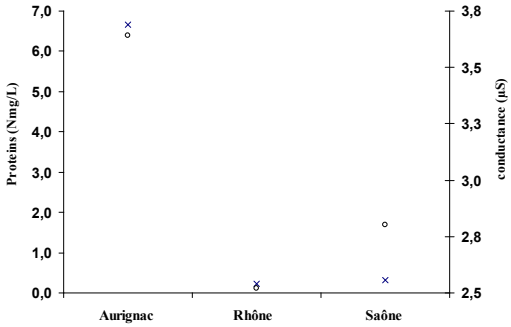

Fig.7: Comparison of the sensor response to values given by MicroBCA method, with ○ proteins (Nmg/L) and X conductance (µS)

Analyze of river and sewer waters: Firstly, we have tested the biosensor response of addition of increasing volumes of sewer water in a solution of 8 mg/l BSA to check possible inhibiting or enhancing effects by some molecules in the sewer water. We obtained a line with a good coefficient of determination equal to 0,97625 (Fig.6) and we can conclude that there is not a significant inhibition or activation effect on the proteinase K activity. The biosensor results and the BCA protein assay showed that the two methods are in good agreement: the water with the highest concentration of proteins is the sewer water (50.8 mg/l) then the water of the river "Saône" (0.7 mg/l) and finally "Rhône" (0.6 mg/l). Dissolved nitrogen Kjeldhal determination gave the same pattern for the river waters and a lower result for sewer water, indicating a protein over-estimation by BCA method, undoubtedly due to the presence of reducing compounds like sugars. So BSA seems not to be a relevant standard for the protein determination in freshwater, perhaps due to some differences between BSA and natural proteins (size and composition) changing the hydrolytic activity. That is why we project to establish a calibration curve with some freshwater standard for a more rigorous determination of protein concentrations in the river.

4. Conclusion

In this paper we have shown: the feasibility of using a biosensor based on IDE in order to measure protein content in water and so estimate OM concentration. The concentration range for detection of the reference protein (BSA) with the elaborated biosensor is between 0.4 to 8 mg/L, which is in adequacy with values of the real concentrations of proteins in water of the hyporheic zone.

The next steps will be: to build a specific calibration procedure for freshwater proteins; to evaluate the same biosensor but with a proteinase photo-immobilization in PVA/SBQ polymer (to choose the best immobilization mode); then to go to the field experimentations. In order to fully validate the biosensor for protein measurements, validation tests will be set up in our experimental site. Firstly in an experimental flume to assess sensor stability and reproducibility and secondly in the micro-piezometers in river La Chaudanne, in order to observe the resistance of the biosensor in natural conditions of use.

5. Acknowledgements

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