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Cell Suspension as a Model System for Electrochemical Analysis

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Cell suspensions of marine phytoplankton *Dunaliella tertiolecta* in 0.1 M NaCl solution are proposed as a suitable model monodispersed system (particle diameter 6–10 μm) for the calibration of electrochemical response in natural aquatic samples containing organic surface-active particles. The electrochemical analysis is performed by direct recording of chronoamperometric curves of oxygen reduction in suspensions using a fast dropping mercury electrode. The electrode acts as adhesion sensor. The adhesion of individual surface-active particles suspended in natural seawater, analogously to *Dunaliella tertiolecta* cells in model suspensions, results in well resolved attachment signals in amperometric curves. The calibration curve presents dependence of attachment frequency on cell densities in the concentration range 10^6 to 2.5×10^7 particles L^{-1} and it can be used for the determination of particle abundance in a seawater sample. The advantage of the electrochemical approach over more conventional methodologies for particle analysis is discussed.

Key words: attachment signals, dropping mercury electrode, *Dunaliella tertiolecta,* organic particle analysis.

INTRODUCTION

The standard way of characterization of marine organic matter in seawater and freshwater samples is either measurement of »dissolved« or »particulate« organic carbon (after filtration, 0.45 μm pore size),[1,2] or determina-

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tion of individual compounds or their classes after a complex pretreatment of the water sample.[3]

Difficulties in physico-chemical speciation of organic constituents of seawater stem in part from low concentrations involved (total organic carbon is 0.3–3 mg C L^{-1}) and from the complex nature of organic matter. The classes of compounds present range from the simplest hydrocarbons to the most complex biogeopolymers. Concentrations of carbohydrates, organic acids, proteins, lipids and other identified substances explain not more than 10% of the organic carbon in seawater.[4]

The electrochemical characterization of surfactant activity of organic constituents in seawater using a simple polarographic technique, first introduced by Zvonarić *et al.* (1973),[5,6] enabled identification of fluid surface-active particles,[7–9] a highly reactive class of organic particles that was not amenable to analysis by conventional methods. Surface-active particles could be detected electrochemically in the size range ≥ 1 μm. Their concentration in coastal and estuarine waters was estimated to be in the concentration range 10^6–10^9 particles L^{-1}.

In recent years, there has been a dramatic change in our knowledge of particulate matter in the ocean due to the discovery of new classes of highly abundant organic particles: colloids,[10,11] submicron particles,[12] and transparent microscopic particles[13] which had remained undetected by previous techniques. These range in size from submicron to hundreds of microns and, depending on the size range, their reported concentrations vary from 10^5 to 10^{14} particles L^{-1}. These particles play a major role in the ocean ecology and chemistry.

A major characteristic of a water sample containing colloidal particles is its intrinsic instability due to continuing aggregation processes and microbial activity. Consequently, sampling and sample processing should be shortned and simplified as much as possible.[14,15] The electrochemical particle analysis, being direct, rapid and simple, meets these requirements and also offers the possibility of single particle analysis. One of still unresolved problems is proper calibration of the electrochemical response. Here, we investigate a simple biological standard for calibration.

THE ELECTROCHEMICAL METHOD

The electrochemical technique employed is a modification of the widely used polarographic technique for measuring surface-active constituents in environmental aquatic samples.[16] The method is based on chronoamperometric measurement of the modification of the interfacial turbulence during oxygen reduction at a fast dropping mercury electrode in aqueous electro-

lyte solutions.[17–20] Attachment and adhesion of organic particles causes a transient increase in the interfacial turbulence, resulting in spike shaped attachment signals of individual particles.[21–25] The dropping mercury electrode has a fast growing and renewable surface, and the experiment can be repeated many times in the same environment.

EXPERIMENTAL

Cell Culture and Cell Suspensions

We used laboratory cultures of marine nanoflagellate *Dunaliella tertiolecta* Butcher (strain CCMP 1320), obtained from Provasoli-Guillard Center for Culture of Marine Phytoplankton, Bigelow Laboratory for Ocean Sciences, as a source of model particles. *Dunaliella tertiolecta* cells possess no cell wall, only a flexible outer membrane (Figure 1). The size of cells is in the range 6–10 μm.

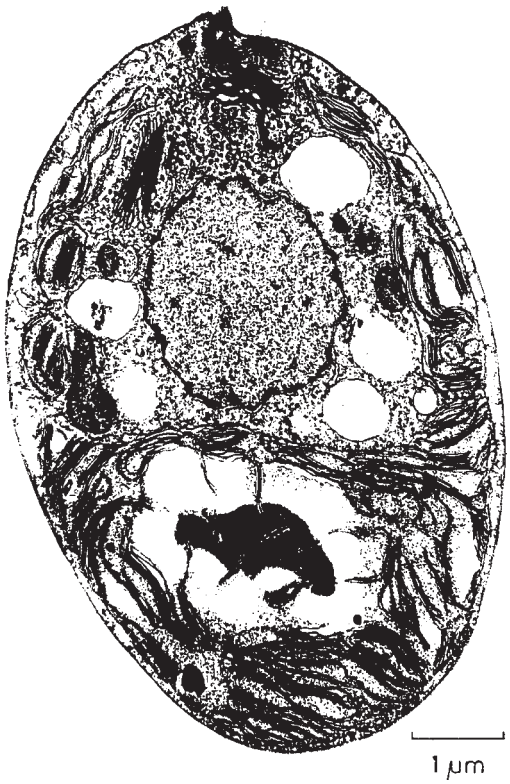

Figure 1. Electron micrograph of a thin section through a *Dunaliella tertiolecta* cell, showing the cell membrane and details of cell structure (magnification 18000 : 1). Courtesy of dr. Mercedes Wrischer.

Cells were grown in F-2 medium (composed of nutrient salts, essential trace metals and vitamins)[26] in batch cultures at ambient conditions. The medium was prepared by adding F-2 nutrients with Sterile Acrodisc (0.2 μm, Gelman Sciences) to sterilized seawater. Cell densities in culture, in stock and in the analyzed suspensions were counted using a light microscope (Hund Wetzlar H 500) with haemacytometer (Fuchs-Rosenthal, Fein-Optik Jena, Germany, Tiefe 0.2 mm).

After 6–8 days of growth, cell density in the medium reached up to $10^9\ L^{-1}$. Then, the cells were separated from the growth medium using mild centrifugation (1500 g, 5 minutes), the supernatant was carefully removed, and the loose pelet was washed with filtered seawater. This procedure was repeated 2 times to remove traces of the growth medium. The pelet was then resuspended in 2 ml of filtered seawater, which served as a stock suspension. Cell densities in stock suspensions were $(1–6) \times 10^{10}\ L^{-1}$.

Aliquotes of stock suspension were added with a micropipette to 25 ml of organic-free electrolyte (0.1 M NaCl) to prepare suspensions of given cell densities immediately before electrochemical measurement. pH in measured suspensions was 8.2 and was mantained constant by addition of 5×10^{-3} M $NaHCO_3$. Water used for the preparation of organic-free electrolyte or for dilution of seawater samples was ultra-pure MilliQ water. The purity of the system was controlled by measuring the polarographic maximum of oxygen reduction.

Electrochemical Measurement

Figure 2 shows a block scheme of the electrochemical measuring system. Chronoamperometric measurements were performed using a PAR 174A Polarographic Analyzer. The current-time curves (time dependence of the instantaneous current

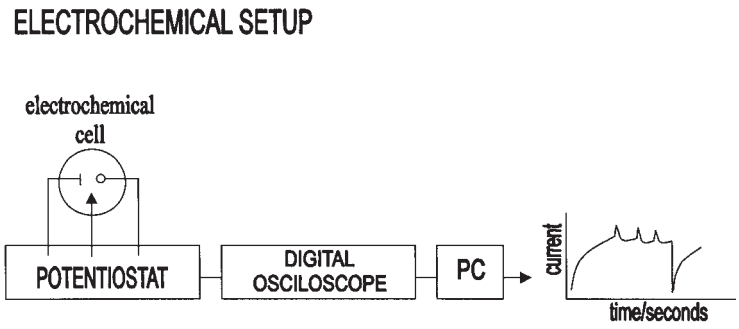

Figure 2. Block scheme of the electrochemical measuring system.

during one drop life)[27] at the constant potential –400 mV (*versus* an Ag/AgCl reference electrode as described below) were recorded and stored using a Nicolet 3091 digital oscilloscope connected to a PC.

Measurements were performed in a standard Methrom vessel with 25 ml of a freshly prepared cell suspension, thermostated at 20 ± 1 °C. The measured samples were saturated with air and the vessel was open to air throughout the experiments.

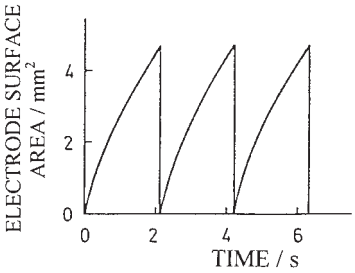

Figure 3. Periodic change of the surface area of the dropping mercury electrode used in this work.

A fast dropping mercury electrode with drop life 2.0 s, flow rate 6.03 mg s^{-1} and maximum surface area 4.57 mm^2 (Figure 3) was used with the Ag/AgCl electrode as a reference in the three-electrode configuration. The reference electrode was separated from the measured suspension by a ceramic frit and its potential in 0.1 M NaCl was + 2 mV *vs*. calomel electrode (1 M KCl).

RESULTS AND DISCUSSION

All experiments were performed using 0.1 M NaCl as supporting electrolyte because the electrochemical literature[28] contains reliable physical and chemical information about the mercury electrode/0.1 M NaCl interface. Besides, in 0.1 M NaCl solutions, the streaming maximum of oxygen reduction is well pronounced and has been studied before.[20] At a potential of –400 mV, the charge density of the hydrophobic mercury surface is +3.8 μC cm^{-2}, and the interfacial tension is close to electrocapillary maximum.[29] Under such conditions at the interface, hydrophobic and electrostatic attractions are expected to prevail in the adhesion phenomena of natural organic particles.

Marine microorganism *Dunaliella tertiolecta* was chosen as model particle because of its suitable size and membrane properties. It is easily available, simple to grow in batch culture, and it forms stable suspensions of single cells that approach characteristics of a monodispersed system. Concentrations of cells in suspensions can be dosed and measured precisely. Suspensions are easy to prepare by adding aliquots of stock cell suspension to the electrolyte solution immediately prior to the measurements.

Figure 4a shows current-time curves of oxygen reduction in a seawater sample from Northern Adriatic.[30] Characteristic electrical signals appearing as sharp spikes on *I-t* curves are a result of random attachment of surface-active particles onto the electrodes. Attachment signals appear at irregular intervals and with different amplitudes.

To analyze the recorded current-time curves in terms of particle abundance in the sample, we conducted a series of calibrating experiments using

suspensions of *Dunaliella tertiolecta* cells under identical experimental conditions.

A typical response of a *Dunaliella tertiolecta* cell suspension is given in Figure 4b. The cell concentration was 6.8×10^6 L^{-1}. The attachment signals of similar shape and amplitude appearing on *I-t* curves are a result of random attachment of individual cells. In Figure 5, we present examples of *I-t* curves recorded over one drop life with a higher time resolution for better observation of the attachment signals of individual cells. The attachment signals are well defined, they appear single or in a sequence separated from one another, and are very similar in shape. Their amplitudes vary between 1.2 and 1.8 μA and duration between 100 and 140 ms. It has been established in previous studies performed in the laboratory that the sharp increase of current in each attachment signal reflects the initial molecular

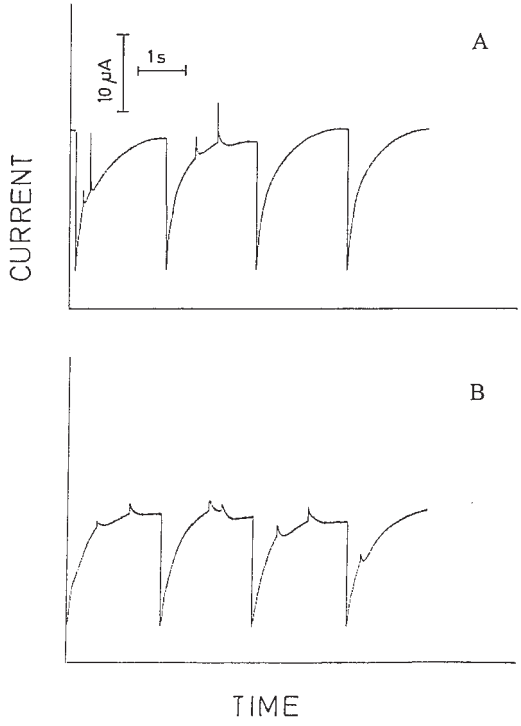

Figure 4. Current-time curves of oxygen reduction recorded in an Adriatic seawater sample, North Adriatic, station 103,[30] depth 10 m, February 27, 1998, (A); and in cell suspension of 6.8×10^6 cells L^{-1} *Dunaliella tertiolecta* in 0.1 M NaCl (B).
Actual recordings: potential –400 mV, time resolution 10 ms per point. Prior to measurement the seawater sample was diluted (1:5) with MilliQ water to reach the ionic strength of a 0.1 M NaCl solution.

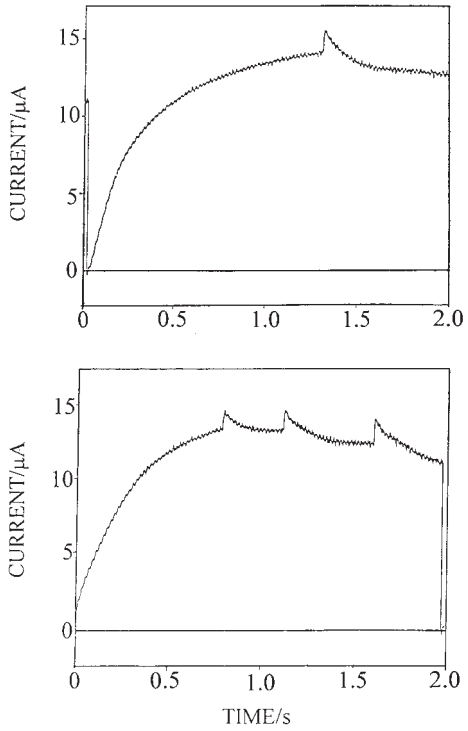

Figure 5. Examples of current-time curves recorded with time resolution of 2 ms per point in the suspension of 4.6×10^6 cells L^{-1} in 0.1 M NaCl.

contact between the cell and the mercury surface, while its slow decay follows the spreading of the cell material over the surface after the cell membrane rupture.[24,31,32]

For the series of increasing cell densities in the range from 2.3×10^6 to 3.1×10^8 L^{-1}, the frequency of appearance of attachment signals is expressed as the number of attachment signals per drop life and plotted as a function of cell density (Figure 6). The attachment frequencies were presented as mean values with standard deviation, obtained by analyzing 30 *I-t* curves. The values of standard deviations reflect the stochastic nature of the process.[32] In the concentration range where the mean frequency of attachment signals increased proportionally, the individual attachment signals are well resolved and do not overlap with one another. This concentration range, 2.3×10^6 to 10^8 L^{-1}, is best suited for the construction of a calibration curve. With further increase of cell concentration in the suspension, the situation arises where at a given time instant there is more than one cell attaching to the electrode surface, resulting in a sporadic overlap of attachment signals

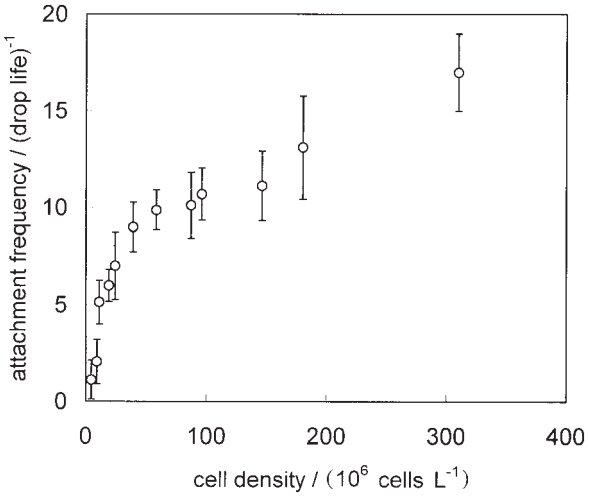

Figure 6. Dependence of mean attachment frequency on cell densities in the suspensions of *Dunaliella tertiolecta* cells in the concentration range 2.3×10^6 to 3.1×10^8 cells L^{-1}.

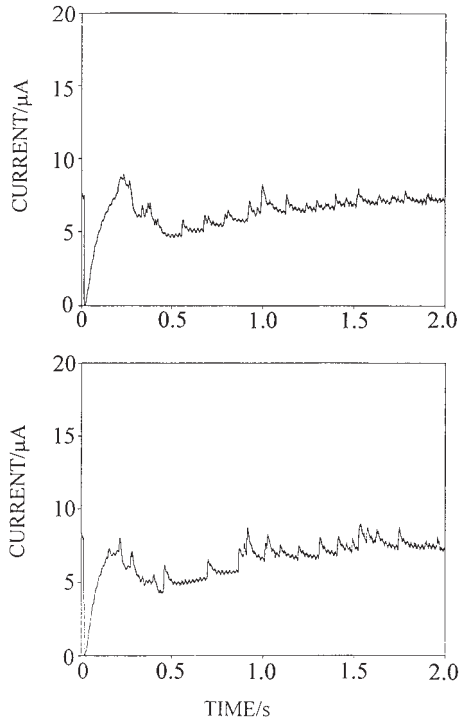

Figure 7. Typical current-time curves recorded in the suspension of 3.1×10^8 cells L^{-1} in 0.1 M NaCl; time resolution 2 ms per point.

or a significant reduction of the electrode free surface area.[32] Counting of individual attachment signals becomes difficult when the attachment frequency approaches 20 and this represents the limit of measurable range (Figure 7).

In order to obtain information about the probabilities of appearance of a given number of signals during a drop-life, detailed analyses of attachment signals were performed in the sequence of *I-t* curves. Figure 8 shows the frequency distribution for three representative cell concentrations, 2.4×10^6, 9.5×10^6 and 2.4×10^7 L^{-1}. The range of the numbers of signals appearing

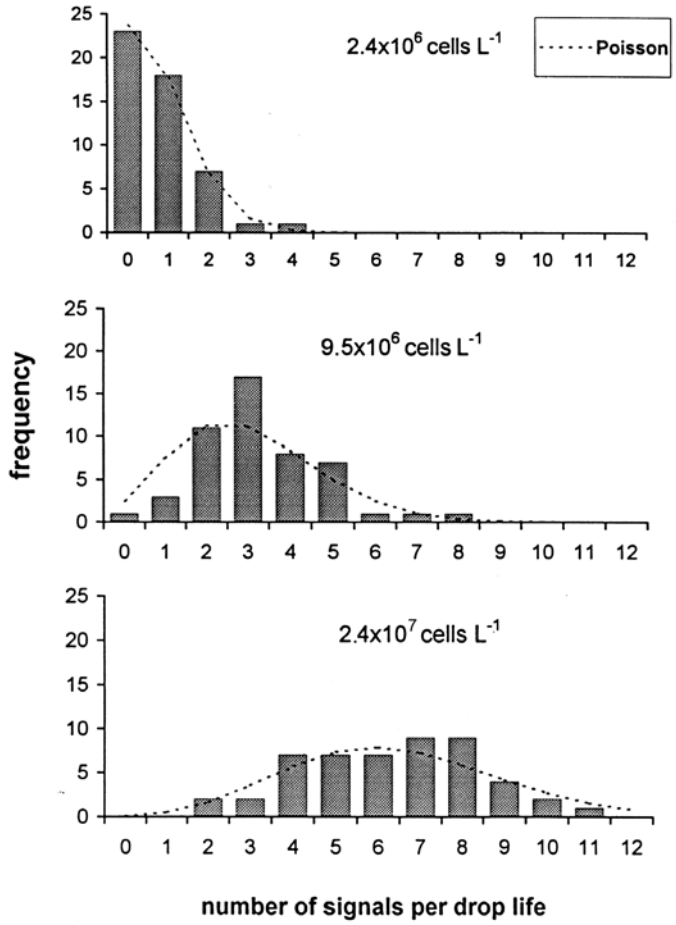

Figure 8. Frequency dependence of the number of attachment signals per *I-t* curve recorded at –400 mV in suspensions of *Dunaliella tertiolecta* cells for 60 *I-t* curves analyzed in 0.1 M NaCl solutions. Cell densities were 2.4×10^6, 9.5×10^6 and 2.4×10^7 cells L^{-1}.

over a drop-life increases with increasing cell density. A hypothesis test (χ^2-test) has been introduced to decide how reasonable it is to assume that a Poisson probability model fits particular data sets. With respect to the fact that χ^2-approximation is adequate if no expected frequency is less than five[33] and by taking into account the fact that data sets were used to estimate Poisson's means, the χ^2-distributions against which the statistics are to be tested have 1, 3 and 5 degrees of freedom, respectively, for the three cell densities considered. The values obtained for the χ^2-test statistic are 0.034, 6.728 and 3.998, meaning that significance probabilities are 0.85, 0.08 and 0.54, respectively. Therefore, we can conclude that there is little evidence to reject the null hypothesis that data may be fitted by Poisson's distribution,[33] which is typical of rare events.

To obtain the best results, knowing the nature of the process, the experiments were repeated by counting attachment signals on 100 *I-t* curves for each cell density. The results are plotted in Figure 9. This calibration curve can then be used for the determination of concentrations of surface-active particles in natural seawater samples, as illustrated below.

The number of attachment signals obtained by analyzing *I-t* curves, as exemplified in Figure 4a, was 43 for 100 *I-t* curves. According to the calibration curve, the corresponding concentration of surface-active particles is 1.5×10^6 L^{-1}. As the measured sample was diluted 1 : 5, the concentration in the original undiluted seawater is then 7.5×10^6 L^{-1}. This concentration refers to surface-active particles in the size range ≥ 1 μm. With the simple electro-

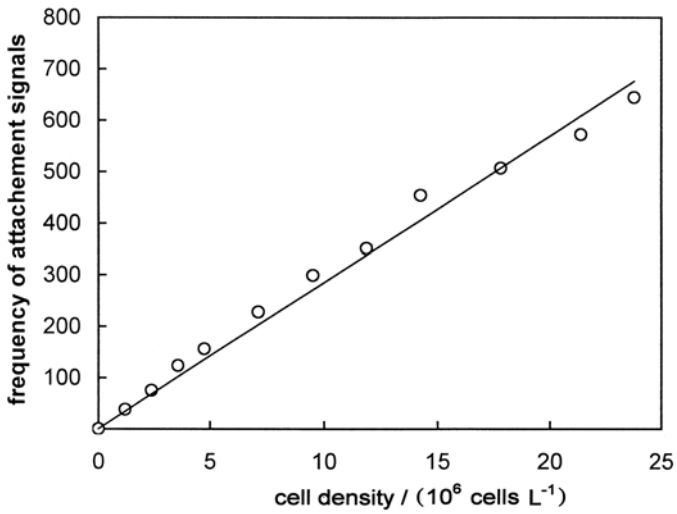

Figure 9. The calibration curve: frequency of attachment signals on 100 consecutive *I-t vs.* cell density in suspensions of *Dunaliella tertiolecta* cells.

chemical equipment used, the attachment signals for smaller particles (≤ 1 μm) cannot be distinguished from the instrumental noise.[21,24,34,35]

The optimum measurement range under the experimental conditions used in this work is 2×10^6 to 10^8 particles L^{-1}. This range can be extended to lower concentrations by prolonged recording of *I-t* curves (duration of analysis > 5 minutes) and also to higher particle concentrations by appropriate dilution with 0.1 M NaCl solution. For field analysis when a large number of samples is involved, a procedure for direct measurements in undiluted seawater can be easily adopted.

GENERAL CONCLUSIONS

The unique advantages of the electrochemical approach over impedance volume measurements, fluorescence flow cytometry and microscopic observation of single particles generally used in characterization of aquatic particles[14] are:

– the possibility to selectively analyze aqueous samples directly without any pretreatment,
– the inexpensive equipment and simple manipulation, and
– simple model system for calibration.

The method is suitable for selective analysis of surface-active organic particles in the size range 1–100 μm[16] and the concentration range 10^5–10^7 particles L^{-1}.

In future investigations, the *Dunaliella tertiolecta* model system can be used to characterize the size of particles (according to the amplitude of attachment signals)[34] and also the sticking characteristics of natural particles. Moreover, it would be possible to develop more sophisticated methods of signal treatment and to use the electrochemical method for a number of industrial applications, such as to control the purity of water for specific purposes.[36,37]

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SAŽETAK

Suspenzija stanica kao modelni sustav za elektrokemijsku analizu

Solveg Kovač, Romina Kraus, Sunčana Geček i Vera Žutić

Suspenzija stanica morskog fitoplanktona *Dunaliella tertiolecta* u vodenoj otopini NaCl (c = 0,1 mol dm^{-1}) predlaže se kao prikladan monodisperzni modelni sustav (veličina čestica 6–10 μm) za kalibraciju elektrokemijskog odziva u uzorcima prirodnih voda koje sadržavaju organske površinski aktivne čestice. Elektrokemijska analiza izvodi se izravnim snimanjem kronoamperometrijskih krivulja redukcije kisika u suspenzijama, rabeći brzokapajuću živinu elektrodu, koja predstavlja adhezijski senzor. Adhezija pojedinačnih površinski aktivnih čestica suspendiranih u morskoj vodi, kao i stanica *Dunaliella tertiolecta* prisutnih u modelnim suspenzijama, dovodi do dobro razlučenih signala prianjanja na amperometrijskim krivuljama. Kalibracijska krivulja, koja prikazuje ovisnost učestalosti signala prianjanja o gustoći stanica u rasponu brojnosnih koncentracija stanica od 10^6 do 2.5×10^7 L^{-1} može se iskoristiti za određivanje koncentracije čestica u uzorcima morske vode. Prodiskutirane su prednosti elektrokemijskog pristupa pred uobičajenim metodama analize čestica.