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Production of Surface Active Organic Material and Reduced Sulfur Species During the Growth of Marine Diatom *Cylindrotheca closterium*

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Abstract: Electrochemical methods at the mercury electrode were used for monitoring production of surface active substances (SAS) and reduced sulfur species (RSS) during growth of marine diatom *Cylindrotheca closterium* isolated from the Adriatic Sea in the laboratory conditions. In the same culture samples, production of particulate and dissolved organic carbon (POC, DOC) was followed by high temperature catalytic oxidation method (HTCO). The culture growth curve obtained by microscopically counted phytoplankton cells showed an exponential growth phase that lasted 10 days, transition phase until 14 days and stationary phase until 21 days. In these time periods twofold increase of SAS and DOC was followed, while POC increased 41 times. Detail analyses of a.c. *out of phase* voltammetric curves recorded in original and in acidified phytoplankton culture samples indicate transformation of organic material during growth, from more anionic (negatively charged) to less anionic polymeric surface active material. In culture samples presence of non-volatile RSS were confirmed.

Keywords: a. c. voltammetry, surface active substances, phytoplankton exudates, *Cylindrotheca closterium*, DOC, POC, reduced sulfur species.

INTRODUCTION

IT is generally accepted that diatoms are the main producers of the mucilage polysaccharide matrix in the seawater.[1–3] The massive appearance of the mucilage in the northern Adriatic in summer of 2001 and 2002 was characterized by the domination of the epipelic diatom *Cylindrotheca closterium*.[4] A prominent contribution of this species to the mucilage-associated phytoplankton community was identified and published in many papers in the early nineties.[2,5,6] It was assumed that mucous production by this diatom is a triggering mechanism leading to marine snow formation followed by large aggregation. Several papers reported that entrapped *C. closterium* does not only preferentially grow in the aggregates but can also significantly contribute to the mucilage hyperproduction under certain physiological conditions.[7–9] These results suggest that diatoms inhabiting aggregates can contribute to cycling of carbon through extracellular polymeric substances (EPS) production especially under nutrient limitation. Specifically, in the northern Adriatic Sea diatoms can produce large amounts, up to 50 g m^{-3} of extracellular polysaccharides per month.[10] Chemical characterization of diatom EPS isolated from laboratory cultures revealed that they are predominantly polysaccharides that contain substantial amounts of uronic acid and sulphate residues,[11–13] and may contain proteins in the form of proteoglycans or glycoproteins.[14] Visualization of diatom EPS, particularly *C. closterium* EPS was recently done by AFM imaging.[15]

Cylindrotheca closterium belongs to a group of epipelic benthic diatoms. Benthic diatoms are the most common group of microphytobenthic algae inhabiting cohesive sediments.[7] Intensified cell polysaccharide secretion and cell lysis, followed by formation of surface active organosulfur species (mainly sulfopolysaccharides) were reported for predominantly benthic diatom macroaggregates, as obtained after sulfide poisoning experiments and exposure to anoxic conditions.[16,17] Surface active substances (SAS),

reported as important part of diatom exudates,[18] include a variety of organic substances (proteins, polysachaarides, lipids, humic type substances) which contain hydrophobic (e.g. fatty acid chains, aromatic rings, hydrocarbons) and hydrophilic functional groups (*e.g*. NH_2, COOH, OH, SH) and therefore have an important role in electrostatic and hydrophobic interactions that can be measured electrochemicaly by adsorption at the Hg electrode.[18–21]

Taking into account the importance of *C. closterium* which was regularly found with relative high frequencies in the northern Adriatic,[22] in this work production of surface active organic material was followed by electrochemical method of *out of phase* a.c. voltametry,[21] during the growth of the epipelic diatom *C. closterium* isolated from the Adriatic Sea in laboratory conditions. In the same samples, production of reduced sulfur species (RSS) which are shown to be one of important phytoplankton exudates,[20] was followed by cathodic stripping linear sweep voltammetry (CSLSV), while particulate and dissolved organic carbon (POC, DOC) was followed by high temperature catalytic oxidation method (HTCO, Pt/Si catalyst). The growth curve was monitored by microscopically counted phytoplankton cells.

EXPERIMENTAL

Culture Growth

The diatom *C. closterium* (strain CCNA1) was isolated from a seawater sample collected at 12m depth at the off-shore station SJ108 (12º45'E, 44º45.4'N, November 13, 2006) in the northern Adriatic. The diatom was grown in conical flasks containing 100 mL f/2 medium.[23] Culture was incubated at a room temperature of 18 °C, 12 : 12 dark-light cycle and subcultured every 3–4 weeks. Growth rate was monitored by cells counting using a light microscope (Olympus BX51, 200 magnification) with haemacytometer.

SAS and Reduced Sulfur Species (RSS) Characterization

The surfactant activity of phytoplankton culture media was determined on the basis of capacity current measurements using a.c. voltammetry measurements in *out of phase* mode.[18,21,24] *Out-of-phase* measurements have found wide application in the study of organic substances with surface active properties in different natural water environments. Surface active substances (SAS) are accumulated at the hanging mercury drop electrode (HMDE) at a potential of –0.6V (vs. an Ag/AgCl reference electrode), by stirring, over different accumulation periods and the decrease of the capacity current (delta Δ*I*) was measured. The results for surfactant analysis are expressed in terms of equivalents of a calibration model substance, the nonionic surfactant Triton-X-100 (T-X-100). A rough characterization of unknown surface active material was then performed by comparison with selected model substances. If the surfactant content of the sample was at the saturation level of the method used, the sample was diluted with electrolyte (0.55 M NaCl) to reach the measurable region of the calibration curve. The ionic strength of the sample was kept constant throughout the dilution procedure.

RSS determination was performed as already described by cathodic stripping linear sweep voltammetry (CSLSV).[25,26] Measurements were conducted directly after sample preparation and again after purging the 50 mL solution with N_2 gas in acidified solution to determine the fraction of RSS present as volatile RSS which are purgable. RSS concentrations are expressed as equivalents of 3-mercaptopropionate (3-MPA), determined from a calibration (from 0 to 100 nM)[27] as appropriate for the concentration range observed in the cultures.

The oxidation-reduction processes of cadmium at a charged mercury electrode, partly or completely covered with an adsorbed layer of the organic substance, are studied by a.c. voltammetry, *in phase* measurements following work of Kozarac et *al*.[28] and Vojvodić et *al*. 1994.[29]

All electrochemical measurements were performed on an μ-Autolab analyser (Eco Chemie, Utrecht, the Netherlands) connected to a 663 VA Stand multimode system (Metrohm, Herisau, Switzerland) equipped with a static mercury drop working electrode (SMDE, surface area 0.52 mm^2). The reference electrode was an Ag/AgCl (3 M KCl). A platinum electrode served as the auxiliary electrode.

DOC-POC Determination

The DOC concentrations were analyzed in duplicate using the sensitive high temperature catalytic oxidation (HTCO) method on a TOC-V_{CPH} (Shimadzu) with platinum/silica catalyst (Elemental Microanalysis, UK) and non-dispersive infrared (NDIR) detector for CO_2 as recently described.[30] POC was analyzed with a solid sample module SSM-5000A associated with a TOC-V_{CPH} carbon analyzer, calibrated with glucose. Prior to analysis, filtration of the samples was performed on Whatman GF/F glass fiber filters, pore size 0.7 μm, previously combusted for 4h at 450 °C to separate POC and DOC fraction. The same filtration procedure was performed for measuring SAS in filtered culture samples.

RESULTS AND DISCUSSION

The growth curve for *C. closterium* showed an exponential growth phase that lasted 10 days, transition phase until 14 days and stationary phase until 21 days (Figure 1), similarly as already reported by other authors.[7] Total number of cells during growth changed from 2×10^5 to 1.5×10^6 cell mL^{-1}.

Changes in the concentration of SAS produced during the growth of the *C. closterium* are presented in Figure 2. The largest increase of the SAS was recorded in the exponential growth phase when SAS increased from 0.157 to 0.327 mg L^{-1}. In these samples there was no difference in SAS between original non-filtered and filtered samples, except in sample analyzed after 10 days of incubation when maximum of exponential growth was recorded (Figure 1) and then maximal concentration of SAS (0.327 mg L^{-1} eq. T-X-100) was measured in the filtered samples. In the same samples largest concentrations of DOC as well POC were also recorded (see later Figure 5). Between 10–15 days, in so called transition phase (Figure 1) SAS was always higher in the filtered samples, while towards to the end of the experiment, higher values were recorded in the non-filtered fraction. Such results indicate that during the exponential growth probably small and more hydrophobic molecules of SAS were in the dissolved phase, which after removal of the particulate fraction were more pronounced at the Hg surface. On the other hand, observed effect was discussed as a consequence of the filtration and conformational changes that may occur on large macromolecules, which may lead to an increase in surfactant activity of the filtrate.[18,31] Later during the growth a significant percentage of more hydrophobic substances were more associated with particulate organic matter fraction.

The characteristic a.c. *out of phase* voltammetric curves which reveal the suppression of the capacity current in comparison to the capacity current of the pure electrolyte (0.55 M NaCl) due to adsorption of unknown SAS, are presented in Figure 3 for original, non-filtered and filtered samples. A rough characterization of SAS, performed by comparing the shapes and intensities of the electrochemical responses with those obtained with different model substances[16–18,21,29,32–34] indicate prevalence of large macromolecular molecules of mostly humic (beginning of the experiment) and polysaccharide type compounds (xanthan and dextran, with maximal evidence in the exponential phase and later), visible through appearance of wide and flat desorption peaks around –1.5 V. The more pronounced peak was recorded in filtered samples during exponential phase, while later these peaks were more expressed in non-filtered fraction, indicating their association with more hydrophobic organic material mainly in the dissolved, and later during the growth, in the particulate phase.

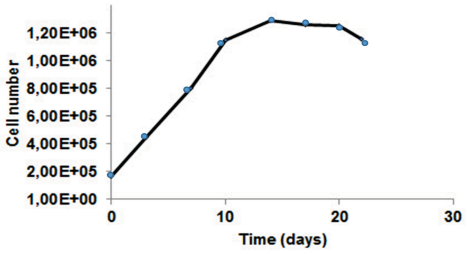

Figure 1. Growth of the *C. closterium* in complete f/2 medium.[23]

According to Magaletti et *al*. 2004,[12] *C. fusiformis* produces at least three polysaccharides and two of them have different monosaccharide compositions as well as molecular weights. In conclusion, the same authors suggest presence of a mixture of different polysaccharides produced at different stages of algal *C. fusiformis* growth. Similarly in experiment here, during the growth, different adsorption effects and shapes of the a.c *out of phase* voltammetric curves were recorded. The shapes of the voltammograms change from those usually obtained for humic type substances (Figure. 3a–c) to those which resemble superimposed effects of more hydrophobic (lipophilic) substances (Figures 3d–f).[33,35] Experiment in which the culture's pH in different stage of the growth was changed by addition of HCl, show how the SAS concentration in culture samples increased upon acidification to pH 2, in the both filtered and non-filtered samples. Such results can be considered as an indication of the presence of negatively charged polymeric SAS.[18] Namely, as previously discussed, in acidic solution, the negative charges of the polyelectrolytes are neutralized, and more neutral and adsorbable organic substances could be formed. Changes of the culture pH in different stage of the growth indicate transformation of organic material from more anionic (negatively charged) which prevail in the exponential phase to almost neutral and recorded at maximum of exponential growth in filtered phase, and less anionic polymeric surface active material in the stationary phase. In addition, organic matter transformation was proved by studying electrode processes of Cd(II) (data not given) using a.c. voltammetry (*in phase mode)*, which are shown to be very sensitive (inhibited) by formation of less (compact) or more porous layer on the Hg surface in the presence of different SAS.[28,29,33] In our experiment for the first 3 days, there was no influence on the Cd reduction process, while later during the growth inhibition became more pronounced, indicating already

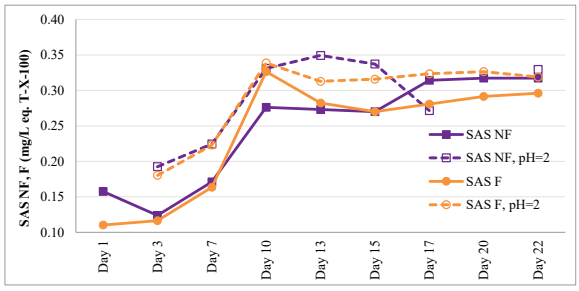

Figure 2. SAS monitored by a.c. *out of phase* voltammetry during growth of *C. closterium* in original (pH 8) and acidified (pH 2) non-filtered (NF) and filtered samples (F).

noted changes in the SAS that is adsorbed on the Hg surface. According to previously published results[28,29,33] such behaviour indicate formation of more porous layer at the beginning of the experiment which usually are typical for prevailing fulvic (humic) material, and more compact layer as is usually observed with polysaccharide dextran type compounds, which formation in our case is assuming later during the growth of the culture. Similarly, transformation from more to less acidic organic material was reported for simulated phytoplankton bloom experiment with mixture of natural phytoplankton from the northern Adriatic,[36] where diatoms were dominant phytoplankton group and polysaccharides were recognized as the major surface active material formed during the experiment.[37] By AFM imaging, changes in the organic matter structure mainly associated with fibrile EPS were also noticed during the *C. closterium* growth, and major production of fibrile EPS was noticed in the stationary phase.[15]

In almost all studied samples (F and NF) after 7th day of the growth, a presence of non-volatile reduced sulfur species (RSS) were confirmed by recording cathodic stripping linear sweep voltammetry curves with typical reduction peak at around –0.6 V (Figure 4).[25,38] An indication for RSS presence was also a small shoulder visible at around –0.7 V (*vs.* Ag/AgCl) in a.c. *out of phase* voltametric curves recorded after 30, and 120 s of accumulation at –0.6 V (Figure 3e). The RSS peak become more pronounced during the growth, reaching its maximum in the stationary phase.

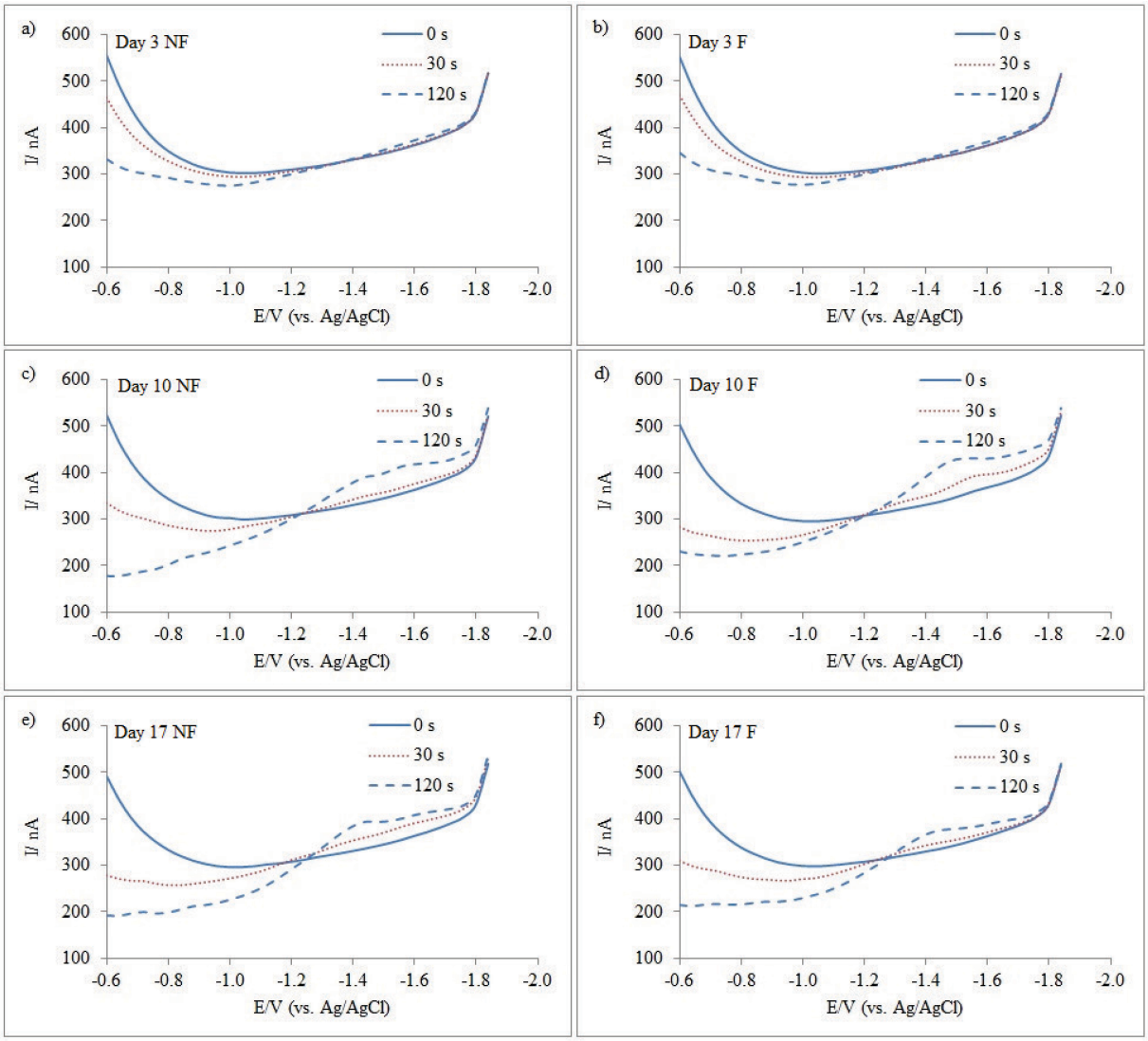

Figure 3. A.C. *out of phase* voltammetric curves recorded in non-filtered (NF) and filtered (F) aliquots of *C. closterium* during different stage of growth. Different accumulation times with stirring at –0.6 V (*vs*. Ag/AgCl: solid line – 0 s; dotted – 30 s; dashed – 120 s.

From recorded peak, concentration of RSS was calculated as equivalent to concentration of thio-compounds represented here by 3-mercaptopropionate (3-MPA),[27] and its concentration ranged from 30 to 80 nM. 3-MPA was chosen as one of the alternative standard because of its electrochemical similarity with the RSS recorded peak, i.e. the appearance of the half-wave potential and the behavior in the acidification and purging experiment which is usual procedure for RSS characterization.[25,26,38] Furthermore, such type of organosulfur compounds are known to be generated as exudates in phytoplankton cultures and/or transformed from the originally produced sulfur compounds, even diatoms are not known as the main producers of sulfur.[24,38] Similar and higher concentration of organo-sulfur species was already reported to be produced in the model phytoplankton cultures including diatoms[24,38] as well as surface seawater and mucilage samples where their presence were associated with EPS, increased surfactant activity (stickiness) and stabilization of mucous material in the seawater column.[16,17,24]

According to DOC results (Figure 5), DOC was minor fraction in total organic C in the studied culture samples, by changing its mass percentage from 40 % at the beginning of the experiment to around 10 % in stationary phase (days 17–22). Such low DOC content is in accordance with already reported values for *C. closterium* as well for other diatom species.[7] DOC concentration ranges from 2.639 mg L^{-1}, to maximum values of 5.060 mg L^{-1} in stationary phase (20th day of growth). In the same time POC ranges from 1.31 to 54.21 mg L^{-1}, indicating 41-fold increase (Figure 5). Statistically significant (P = 0.0003) strong correlation (R = 0.93; 9 pairs) was obtained for SAS(NF) - POC pair in comparison with SAS(F) – DOC pair (R = 0.81; P = 0.008), indicating that hydrophobic material in average is predominantly associated with particulate fraction.

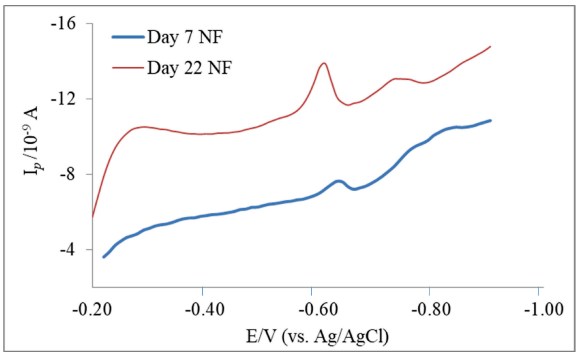

Figure 4. Cathodic striping linear sweep voltammetry curves recorded in non filtered aliquots of *C. closterium* in 7th and 22rd days of growth. Experimental conditions: accumulation with stirring at –0.2 V *vs*. Ag/AgCl for 120 s, san rate 100 mV s^{-1}.

A comparison of SAS and DOC values was made for all studied culture samples in relation to model substances representatives for surface active organic matter composition of natural samples.[18] So called, the normalized surfactant activity value NSA = [SAS (eq. T-X-100) / DOC] for different model substances are reported to be as follows: Triton-X-100 (1.54), oleic acid (2.70), protein (here albumin) (0.20), fulvic acid (0.17), polysaccharides: dextran T-500 (0.20) and xanthan (0.04)[18,21]. In our culture samples NSA varies between 0.04 at the beginning of the growth to 0.08 when culture reached maximum at the exponential growth, and is slightly decreasing in the stationary phase (from 0.073 to 0.066).

Obtained NSA values are in the range typical for fulvic (humic) and polysaccharide (xanthan) type of organic material, what is in accordance with our previously made conclusion on prevailing SAS material indicated by a.c. curve shapes, and inhibition effect on the Cd(II) reduction process. Also obtained values are in agreement with the old data reported for the NSA in the northern Adriatic samples [18] and some experiments with mixed diatom cultures where it was found by fractionation on XAD-8 resin that during the growth significant increase of hydrophobic neutral components may occur, in contrast to the beginning of the exponential phase, in which hydrophilic compounds were main excretion products.[39]

However it is interesting that the similar normalized surfactant activity around 0.082 can be calculated for the previously characterized ambient northern Adriatic seawater collected during mucilage appearance in the summer of 2002, as well as for samples enriched with marine benthic diatoms isolated from the Venice Lagoon.[17] In the same samples RSS were measured in range between several nM to 500 nM.[16,17,38] More recent SAS and DOC data reported for surface samples collected at the station 101 in the northern Adriatic in spring and early summer of 2011, also indicate the presence of the same type of organic material.[24]

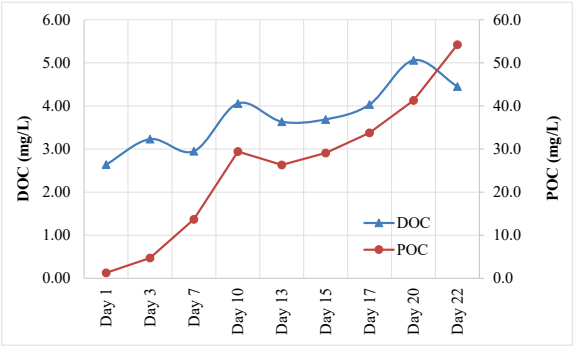

Figure 5. Changes in DOC and POC concentrations during the growth of the *C. closterium*.

CONCLUSION

In this work a simple, fast, non-destructive and well established a.c. voltammetric method at the Hg electrode, developed within our group, was applied for monitoring of organic matter with surface active properties during the growth of the phytoplankton culture *Cylindrotheca closterium.* A rough characterization of the SAS, performed by comparing the shapes and intensities of the electrochemical responses with those obtained for different model substances indicate prevalence of mainly humic (beginning of the experiment) and polysaccharide type compounds (xanthan and dextran, most evident in the exponential phase and later). A comparison of SAS and measured DOC concentration, which ratio is defined as the normalized surfactant activity (NSA), indicate the same type of surface active organic material that is produced during the growth experiment with the *C. closterium* and organic material that is previously characterized in the Adriatic seawater samples with similar levels of organosulfur species (RSS), collected during the mucilage appearance. The same type of the organic material was also characterized in the enriched marine benthic diatom sample isolated from the Venice Lagoon in 2000. Results from this study clearly prove already recognized great potential of electrochemistry at the Hg electrode for fast and simple qualitative and quantitative water sample analyses, especially efficient for rough characterization and tracing of naturally occurring organic material with surface active properties and sulfur content. So far replacement for smooth and reproducible renewable surface of the Hg electrode, based on both faradaic and non-faradaic processes in model measurements and water quality monitoring have not yet been found.

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