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Successful use of electrochemical nitrate sensor for bioremediation monitoring in microalgal cultures

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ABSTRACT

In this work, a copper-based electrochemical sensor, obtained using a single-side copper plate board commonly employed in printed circuit board technology, was proposed for the analysis of nitrate ions in microalgal cultures. The sensor with a three-electrodes configuration was fabricated by using a micro-milling machine. After the modification of the reference electrode with a silver/silver chloride paste and of the counter electrode with graphite ink, sensor performance was evaluated by assessing repeatability, reproducibility, stability, and selectivity using linear scan voltammetry to detect the reduction peak of nitrate ions. The produced sensor had a limit of detection of 5.2 ppm with a sensitivity of 0.776 μA ppm^{-1} and a relative standard deviation of 0.67 %. The sensor was then used to monitor the concentration of nitrates during the bioremediation by an indigenous microalgal culture in both synthetic and real wastewater. A linear correlation between the results obtained using both the standard ionic chromatography and the sensor was found ($R^2 > 0.99$). Thus, this work demonstrated the feasibility of using electrochemical sensors for real-time analysis in biological remediation processes. This potential application can be useful for optimizing microalgal use in wastewater treatment.

1. Introduction

Nitrate pollution is recurrent in natural waters and may derive from several sources, including point (such as wastewater effluents and intensive livestock farming) and diffuse (such as fertilizers, and degradation of soil organic matter) [1]. The main source of nitrate pollution originates from agriculture due to the use of fertilizers, with an estimated soil contamination of 32.74 kg ha^{-1} [2,3]. The consequences of this contamination may be hypoxia in the deep waters, as nitrate causes algal blooms, successively decomposed by bacteria, which require a high amount of oxygen. Nitrate also causes serious health problems for humans such as methemoglobinemia in infants and an increased risk of diabetes and thyroid disorders [4]. In the human body, it may be reduced to nitrite and nitrogen dioxide. While nitrite ions in the stomach's acidic environment are converted into nitrosamines (probable carcinogen compounds) nitrogen dioxide binds to hemoglobin preventing oxygen transport.

For all these reasons, attention is generally paid to the concentration of nitrate in groundwaters and wastewaters. According to the European Nitrates Directive, freshwaters and groundwaters are considered contaminated when nitrate concentration is above 50 mg L^{-1} [5]. Considering that this level is easily exceeded, the abatement of nitrate is one of the objectives of wastewater treatment plants, typically achieved during the tertiary treatment phase. For nitrate removal, several methods are employed such as chemical reduction [6], electrocoagulation [7], electrodialysis [8], or electrocatalytic reduction [9], each of them having strengths and drawbacks. The biological approach is a valid alternative to these methods, as it offers economic and environmental advantages, due to its selective removal capability for the complete nitrate elimination and formation of harmless products [10]. In particular, in recent years, the use of microalgae for enhancing nitrate abatement in wastewater treatment has been proposed [11]. Microalgae can effectively decrease the nitrate content by its preliminary assimilation, followed by subsequent reduction steps [12]. Microalgae can work synergistically with heterotrophic bacteria in wastewater treatment plants to achieve i) reduction of dissolved carbon; ii) decrease in nutrient content (nitrate, phosphate, ammonia, etc.); iii) cost savings, as the aeration costs are reduced due to the oxygen production by microalgae while performing photosynthesis.

Although the mechanism for nitrate removal using microalgae has

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been already proved, there is a lack of available instruments that assure a quick and accurate measurement of nitrate levels during the treatment. The most common methods to measure nitrate concentration are spectrophotometry and ionic chromatography. These methods have several drawbacks such as the need for skilled personnel and being time-consuming and challenging to be applied in *situ* and in real-time determinations. The availability of a technique which is fast and allows *in situ* and continuous analysis, would be of great importance to help refining the wastewater treatment process [13]. Electrochemical sensors offer a feasible solution to these problems. They can be powered with cheap instrumentation, have very small dimensions, and need small sample volumes (100–200 μL). Their operation is easy and can be remotely controlled due to the employed electrical signal output [14]. Because of their scientific interest, many researchers explored the use of electrochemical sensors for water/wastewater analysis, such as phosphate ions [15,16], heavy metals [17], pesticides [18,19], and other pollutants [20,21]. In an electrochemical sensor, the role of the active material is crucial as it determines its performance. In the case of sensors for nitrate ions (in which the electrochemical detection occurs by reducing nitrate to nitrite ions in acidic media [22,23]), different types of active materials were used such as noble metals (e.g., gold [24]), carbon-based materials (e.g. carbon nanotubes [25]), and metal oxides (e.g., zinc oxide [26]). Copper is one of the most investigated active materials because of its excellent properties towards nitrate reduction. Motaghedifard et al. have synthesized a multi-element electrode made of carbon nanotubes, copper and gold, finding that the presence of copper is essential to increase the electrode signal [27]. Zhenxing et al. have modified a platinum working electrode with copper nanoparticles obtaining excellent results in terms of sensitivity and stability [28]. A nitrate sensor based on Cu nanowires was proposed by Liang et al. obtaining a linear range from 0.5 to 375 ppm and a LOD of 0.08 ppm [29]. Interesting is the approach of Da Silva et al. that have recycled flat flexible cables to fabricate copper microneedle-based sensors able to detect nitrate ions in the range from 0.64 to 68.5 ppm with a LOD of 0.11 ppm [30].

In addition to its electrocatalytic properties, copper is an interesting active material because it has a lower cost compared to noble metals and it can be obtained by recycling electronic waste. In addition, copper-based sensors can easily be manufactured starting from copper plate boards and tools used in printed circuit board technology (PCB). The use of PCB manufacturing processes for electrochemical sensors has the advantage of creating compact, cost-effective, and mass-producible sensor platforms. In addition, this fabrication process enables miniaturization and integration of sensing elements with signal-processing circuitry on the same platform, which is an important aspect for the fabrication of low-cost, portable, and wearable sensors. PCB technology has been recently used to produce electrochemical sensors, but usually, the copper layer was not used directly as active material but was modified with other metals [31–35]. To the best of our knowledge, only in [36] the copper layer from PCB technology was directly used to fabricate sensor for zinc determination in blood serum. In this work, we have fabricated for the first time copper-based sensors using the PCB technology for nitrate ions. A three-electrodes configuration was created using a micro-milling machine. The sensors have a very low cost, less than 0.05 euro/electrode, and their fabrication can be easily extended for mass production. The sensor was used to quantify nitrate ions abatement by microalgae grown in synthetic and real wastewater. Overall, the results of this work were satisfying since the data obtained using the electrochemical sensor and the standard ionic chromatography detection method were consistent.

2. Material and methods

2.1. Sensor fabrication and characterization

The manufacturing of the sensor unit, consisting of three coplanar electrodes (working electrode (WE), reference electrode (RE) and counter electrode (CE)) arranged onto a glass or polymer resin substrate, was achieved in house using a LPKF PorotMat S63 PCB milling machine. This micro-milling machine is widely employed for manufacturing prototype printed circuit boards (PCBs) by removing surface areas from a copper sheet laminated onto a non-conductive substrate to create electric signal pathways according to a pre-established geometry and layout. The application of a micro-milling process has been preferred in this research due to its versatility and flexibility, since it allows creating low-cost sensor prototypes with different geometries in short times, while achieving up to 1 μm tolerance with the use of precision micro-mills. A standard 0.2 mm thick copper plate for PCB was used. The whole plate is 1 mm thick. The electrode design was drawn using Fusion AutoDesk. The WE area has a circular shape with a diameter of 3 mm. From a single-side copper plate board, 112 sensors were obtained. Before being used, each sensor was polished with different grade emery paper, washed with distilled water and ultrasonically rinsed in pure acetone. After these treatments, the counter and reference electrodes were modified with graphite and silver/silver chloride pastes, respectively. Graphite ink was purchased from SunTronic (901970-250G) and consists of a suspension of graphite in isopropanol. The Ag/AgCl paste, purchased from Sigma Aldrich (C2130809D5), is a suspension in Butylglycol acetate and 2-(2-Butoxyethoxy)ethanol of a mixture of silver (60 %) and silver chloride (40 %). After pastes deposition, the sensor was cured in the air oven at 50°C for 1 h. The sensor was connected to the potentiostat using a slide connector with a 2 mm gap between pins (Figure S1A). Using a bi-adhesive layer, a disposable well-cell (Metrohm, DropSens) capable of containing 150 μl of the test solution (Figure S1B) was glued to the sensor.

Electrode morphology was analyzed through scanning electron microscopy (SEM), using a FEI FEG-ESEM (mod. QUANTA 200) equipped with a detector for Energy Dispersive Spectroscopy (EDS). X-ray diffraction (XRD) was carried out using a RIGAKU diffractometer (model: D-MAX 25600 HK). All the diffraction patterns were obtained in the 2θ range from 10° to 90° with a step of 0.004° and a measuring time of 0.067 s for each step, using the copper Kα radiation (λ = 1.54 Å). Diffraction patterns were analyzed by comparison with the ICDD database.

Electrochemical quantification of nitrate ions was performed using linear scan voltammetry (LSV) from −0.2 to –1V vs Ag/AgCl in a solution of Na_2SO_4 0.1 M. The effect of the scan rate was studied in the presence of 117 ppm of sodium nitrate. The quantification of nitrate ions is highly influenced by pH, acidic media is strongly recommended [37], thus it has been optimized in a range from 1 to 3 by adding sulfuric acid. The effect of nitrate concentration was studied in the range of 0.62–620 ppm. LSV was performed at 25 mV/sec. The intensity of the peak current was calculated after baseline subtraction. The limit of detection (LOD) was esteemed using the following equation [38]:

$$LOD = 3.3 * SD / S \quad (1)$$

where SD is the standard deviation of the blank (n = 15) and S is the slope of the calibration plot of the first linear range (at low concentration). The repeatability was studied by calibrating the same sensor 3 times in a raw. The reproducibility was verified by testing 5 different electrodes, while for stability the same electrode was tested for 21 days after storing it in air (tests were repeated every 7 days). The selectivity was studied towards different chemicals present in wastewater culture medium which composition is below reported. Each chemical was tested separately and at a concentration equal to the concentration present in the algal synthetic wastewater culture medium.

2.2. Algal growth and wastewater analysis

The microalgae *Chlorella* sp. CW2 was previously isolated from the active sludge coming from the Sicilian AMAP wastewater treatment

plant located in Balestrate (Palermo, Italy) [39]. The alga was grown in a synthetic wastewater medium, simulating an urban sewage, with the following composition: glucose 221.7 mg L^{-1}, $NaNO_3$ 160 mg L^{-1}, $CaCl_2{\cdot}2H_2O$ 165 mg L^{-1}, Na_2SO_4 63.4 mg L^{-1}, K_2HPO_4 56.4 mg L^{-1}, $NaHCO_3$ 200 mg L^{-1}, EDTA 4.14 mg L^{-1}, $FeCl_3{\cdot}6H_2O$ 3.15 mg L^{-1}, $CuSO_4{\cdot}5H_2O$ 0.01 mg L^{-1}, $ZnSO_4{\cdot}7H_2O$ 0.022 mg L^{-1}, $CoCl_2{\cdot}6H_2O$ 0.01 mg L^{-1}, $MnCl_2{\cdot}4H_2O$ 0.18 mg L^{-1}, $Na_2MoO_4{\cdot}2H_2O$ 0.006 mg L^{-1}. The initial pH of the synthetic wastewater was 8.4.

For some experiments, real river water collected from Fiume Oreto (Palermo, Italy) was employed as a growth medium with the addition of sodium nitrate up to a concentration of 200 mg L^{-1} of $NaNO_3$. The initial pH of the River Oreto water was 8.3. To start a new cultivation, 10 mL of a stock culture in a late-exponential phase was inoculated in 400 mL of fresh culture medium. All the cultivations were performed in a triplicate. The flasks containing the culture were placed in an oscillating incubator (Corning Lse) under a photon flux of 200 μmol m^{-2} s^{-1} at 27°C. Cellular concentration was measured over an interval of time by taking a sample of the suspension and reading its absorbance at 750 nm with a spectrophotometer (Cary 60 UV-Vis, Agilent Technologies). An experimental correlation between the dry weight (DW) and the optical absorbance at 750 nm was established as follows:

$$DW = 0.2315^{*} (Abs)_{750\ nm} + 0.0264 \quad (2)$$

The concentration of nitrate and other anions was measured using anionic chromatography (IC, Metrohm 882 Compact IC plus). A Metrosep A Supp 5 column was utilized with a flow rate of 0.7 mL min^{-1} of a solution containing 3.2 mM Na_2CO_3 and 1 mM $NaHCO_3$. A Metrosep C4–250/4.0 was employed for the cationic chromatography using a 1 mL min^{-1} of a H_3PO_4 5.5 mM aqueous solution. Before analysis, each sample was appropriately diluted, and when necessary, filtered through a Macherey-Nagel CHROMAFIX cartridge filled with C18 silica to eliminate any organic substances. The analysis of organic and inorganic carbon was assessed through an Elementar Enviro TOC.

For the analysis of the media containing microalgal cells, cells were first removed by centrifugation (4500 rpm, 15 min, 4 °C). Subsequently Macherey-Nagel Chromafix C18 Cartridges were used to remove all the organic content from the solution, to avoid interferences in the electrochemical measurement.

3. Results and discussion

3.1. Sensor fabrication and characterization

The design of the copper-based sensor is shown in Fig. 1A. It consists of three electrodes (1 mm wide) separated by a gap of 2 mm. The central one is the WE while the CE is placed at the left of the WE. The optical image of the copper plate after the micro-milling process is shown in Fig. 1B. The final sensor, after paste deposition and curing, is shown in Fig. 1C.

The copper layer, after the cleaning process, was morphologically and chemically analyzed by SEM, EDS, and X-ray diffraction. The morphology of the electrode is typical of a surface treated with emery paper. Grooves of different depths, caused by the different grains of the used emery papers, are visible (Figure S2A). The layer consists of pure copper as demonstrated by EDS and X-ray diffraction. In both spectra (Figure S2B and E) only the copper peaks are present. In particular, as demonstrated by X-ray diffraction (Figure S2E), the film consists of polycrystalline Cu. The peaks were indexed using the card n. 4–836 of the International Centre of Diffraction Database which is related to the crystalline cubic form of metallic copper. The strong and sharp peaks indicate that the copper layer has a high crystalline order degree. Throughout the width at half height of the main peak located at 43.23°, and by using Sherrer's equation, the mean size of copper grain was calculated obtaining a value of 36.32 ± 2 nm.

Before the use for nitrate detection in microalgal suspension, the performance of the sensor was studied to quantify nitrate ions in a solution of 0.1 M Na_2SO_4. As above reported, solution pH influences the reduction of nitrate ions [37], thus its effect was studied in the range from 1 to 3. The results reported in Fig. 2A showed that a pH higher than 2 leads to an almost flat voltammogram, indicating that nitrate reduction is catalyzed in more acidic environments. Voltammogram recorded at pH 1 and 2 (Fig. 2A), showed a reduction peak current corresponding to the reduction of nitrate ions to nitrite ions, according to the reaction:

$$NO_3^- + 2H^+ + 2e^- = NO_2^- + H_2O \quad \text{(Reaction 1)}$$

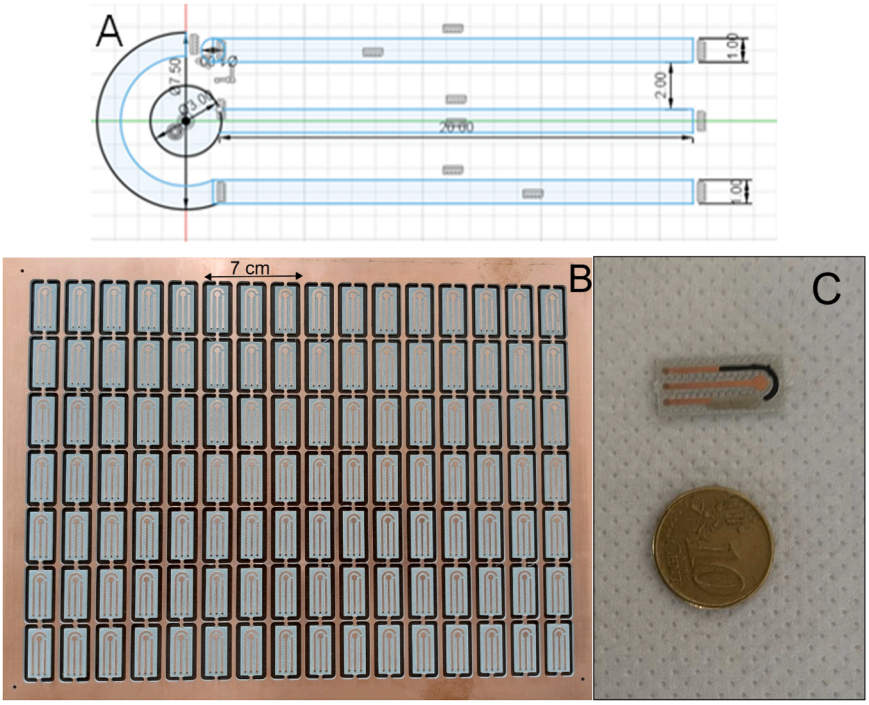

Fig. 1. A) Sensor design obtained using Autodesk Fusion, B) Copper plate after micro-milling process, C) Final sensor after the curing process.

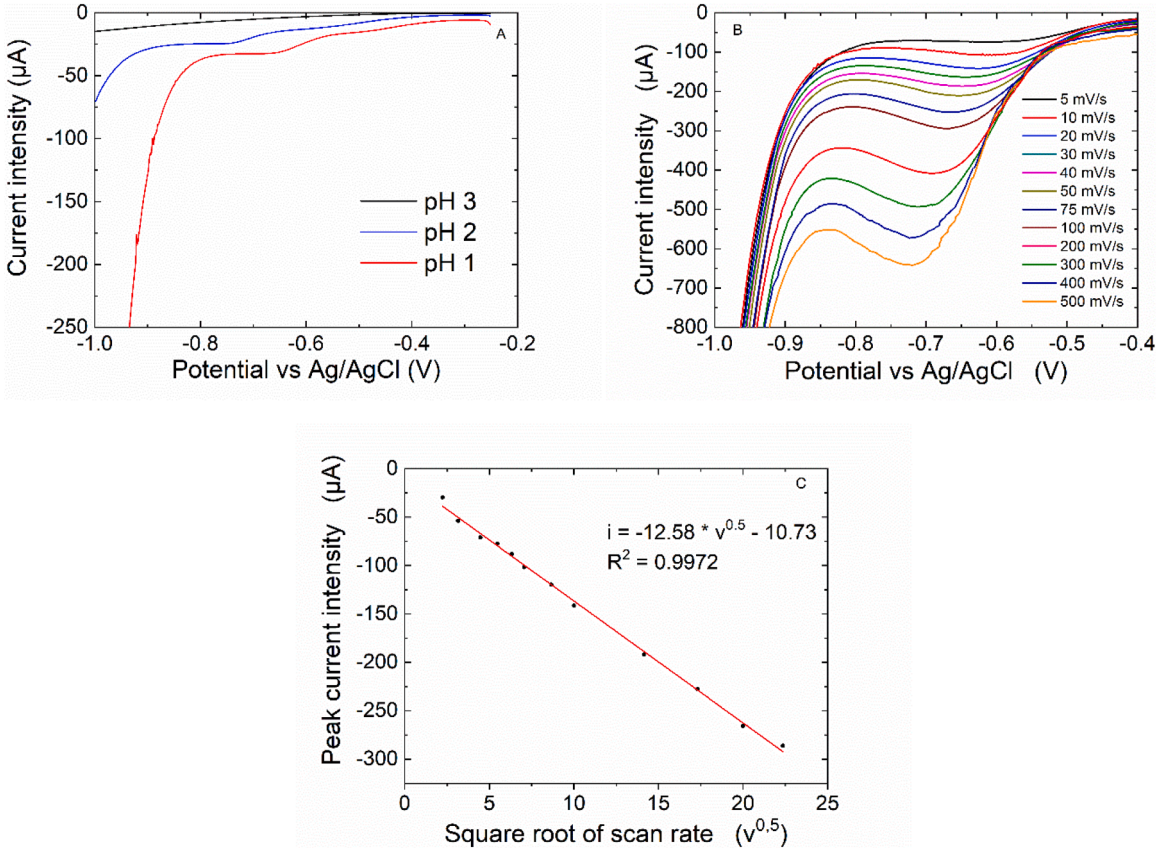

Fig. 2. A) Effect of pH on the reduction of 15.5 ppm of nitrate ions in a solution of Na_2SO_4 0.1 M with different amount of sulfuric acid. B) Effect of scan rate on reduction of 170 ppm of nitrate ions in a solution of Na_2SO_4 0.1 M pH 1, and C) Fitting of peak current vs square root of scan rate. All experiments were performed with a scan rate of 25 mV/s.

After baseline subtraction, the peak current densities were 4.2 μA (at pH=2) and 6.65 μA (at pH=1). Considering the better response of the sensor at pH= 1, the following experiments were performed at this value. Considering the highly acidic conditions, the stability of the reference electrode was monitored over time (Figure S3) measuring the open circuit potential vs a commercial Ag/AgCl reference electrode. For the entire duration of the test (40 h) the OCP value is stable with a mean value of −59.9 mV ± 0.6 mV suggesting a good stability of the home-made Ag/AgCl reference in acidic environment.

The effect of scan rate was studied in the range from 5 to 500 mV s^{-1} (Fig. 2B). The peak current increases with the scan rate and shifts towards more cathodic potentials. This behavior is a typical treat of quasi-irreversible reactions [40]. Nitrate reduction is controlled by the diffusion of the ions from the bulk of the solution to the electrode surface as suggested by the linear increase of the peak current intensity vs the square root of the scan rate (Fig. 2C). The irreversibility of the reaction was then confirmed by carrying out a cyclic voltammetry test in the presence of 15.5 ppm of nitrate (Figure S4) that showed only one reduction peak.

The sensor was calibrated in Na_2SO_4 0.1 M at pH 1 where nitrate concentration ranged from 0.62 to 620 ppm. Also, the Na_2SO_4 0.1 M solution without nitrate ions (blank) was tested. Fig. 3 shows the LSV curves, before baseline subtraction, from 0.62 to 6.2 ppm (Fig. 3A), from 6.2 to 62 ppm (Fig. 3B), and from 62 to 620 ppb (Fig. 3C).

The LSV curve of the blank presents only one small peak located at about −0.42 V vs Ag/AgCl due to the reduction of dissolved oxygen. This was verified with specific trials where a de-aerated solution was tested. When nitrate ions are spiked into the solution, a new peak appears at about −0.61 V vs Ag/AgCl due to the reduction of nitrate ions according to Reaction 1. Increasing nitrate concentration leads to an increase in the height of this peak. In Fig. 3D the corresponding calibration plot is depicted, showing two different linear ranges described by the equations reported in the graph. The presence of two linear ranges, with higher sensitivity at higher concentrations, is typical of the quantification of nitrate ions and in general of electrochemical sensors, as confirmed in [41–44].

To fully characterize the sensor, its repeatability and reproducibility were studied. For the repeatability test, the same sensor was tested 3 times in a row, varying the nitrate concentration in the first linear range (from 0.62 to 62 ppm). The corresponding calibration plots are shown in Fig. 4A. The behavior of the sensor is not changed, and in fact, a mean sensitivity of −0.786 μA ppm^{-1} (red line) with a standard deviation of 0.67 % was calculated. These results indicate an excellent repeatability of the sensor.

A similar approach was conducted to evaluate the sensor reproducibility by testing 5 different sensors (Fig. 4B). All sensors yielded a similar behavior, a mean sensitivity of −0.800 μA ppm^{-1} (red line) with a standard deviation of 7.9 % was obtained. Thus, it can be concluded that the electrode is highly repeatable and reproducible.

To study the stability over time, three electrodes were tested after a simple storage in air for 21 days. Every 7 days the stored sensors were tested performing an LSV test in the presence of 170 ppm of nitrate ions. The peak current intensity was measured to calculate the signal loss over time defined as

$$\%\_Signal\_Loss = (I_t - I_0)/I_0 \quad (3)$$

where I_0 is the peak current measured using an us-prepared sensor, while I_t is the peak current value measured after the different days of storage.

As showed in Fig. 4C, after 1 week of storage, the electrode lost about

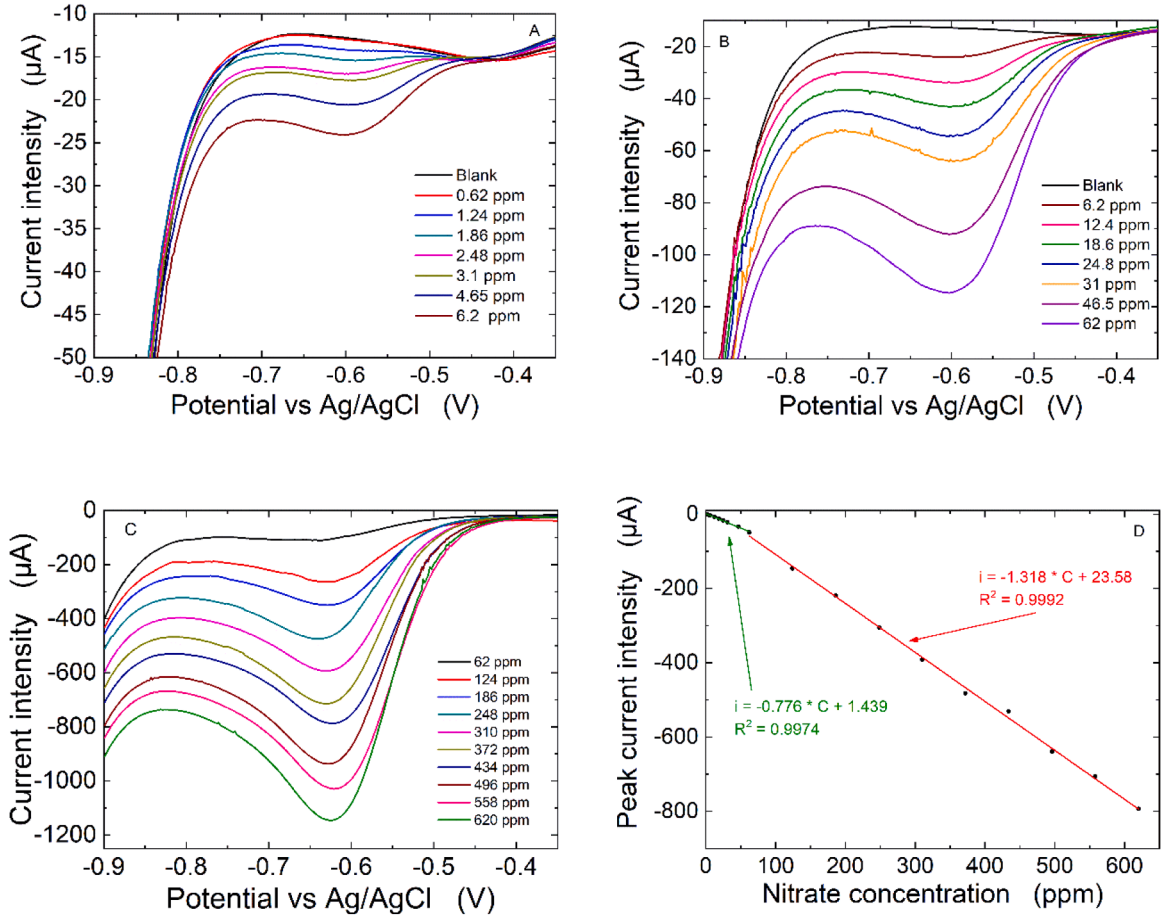

Fig. 3. LSV curves at increasing nitrate concentration: A) from 0 to 6.2 ppm, B) from 6.2 to 62 ppm and C) from 62 to 620 ppm. D) Corresponding calibration lines. Electrochemical tests were performed with a scan rate of 25 mV/s in a solution of Na_2SO_4 0.1 M pH 1.

6 % of the original signal; after 2 weeks the signal decreased by 10.3 % while after 3 weeks the signal dropped by 33.01 %. This signal decade overtime was probably due to the oxidation of copper in air. This was also confirmed by SEM and EDS analysis conducted on the chip before and after storage (Figure S2). The electrode morphology was not altered, but the EDS analysis showed the presence of the oxygen peak which suggested a surface oxidation of copper film. It can be concluded that the same electrode can be used for at least 7 days without any considerable signal loss. It is important to highlight that the decay of signal loss can be easily avoided by scratching the surface of the working area with emery paper before using or by stored the sensors in inert atmosphere.

The electrode selectivity was tested on different species. In particular, all the components present in the synthetic wastewater, used for microalgal cultivation, were selected as interfering agents. The complete synthetic wastewater was also tested (S-Wastewater) to evaluate its overall interference on nitrate detection. The effect of the interferences was evaluated by performing tests using a solution containing 170 ppm of nitrate ions and a solution containing the same amount of nitrate ions and the interfering agent. The current intensity was measured to calculate the following signal ratio

$$\%\_Signal\_Ratio = I_{NO3-}/I_{Int.} \quad (4)$$

where I_{NO3-} and I_{Int} are the peak current values without and with interferent species. A signal ratio close to 100 % indicates any effect of the interferent species on sensor performance. Fig. 4D shows that the different tested interfering agents, but also the complete wastewater, do not influence the ability of the sensor to detect nitrate ions. A maximum deviation of 4.8 % was in fact calculated. Only chloride ions have a modest effect on the sensor performance. In particular, a shift of the peak potential towards more cathodic value (from −672 mV to −745 mV) was measured, while current value appeared practically unchanged (Figure S5). This behavior is due to the dependence of the potential on the chloride concentration of the pseudo-reference electrode that is made using Ag/AgCl paste. Thus, it can be concluded that both the single components and the entire wastewater do not influence the sensor performance in nitrate reduction.

By comparing the proposed electrode with previous ones (Table S1), it can be observed that comparable performance was obtained. On the other hand, the proposed sensor is produced in a much easier, faster and cheap way.

3.2. *Microalgal growth and nitrate abatement*

The microalgal strain *Chlorella* sp. CW2 was firstly grown into a synthetic wastewater to monitor its growth and its nitrate abatement capacity. Fig. 5 shows the growth curve of the microalgal cultures that were performed in triplicate. Results are reported as concentration of microalgae per volume of suspension, measured by reading the absorbance at 750 nm and converting this data using the Eq. 2. This approach is effective when microalgal cells are thoroughly dispersed within the suspension. Under these conditions, there is a linear correlation between the optical absorbance and the concentration. In the medium simulating civil wastewater, an effective growth of microalgae was obtained, reaching a concentration after 14 days of culture of 0.35, 0.20, and 0.18 g L^{-1} respectively for Culture 1, Culture 2, and Culture 3 (Fig. 5 A). During the batch cultivation, nitrate concentration was monitored by using ionic chromatography. For the optimal performance of the ionic chromatography column, it was necessary the use of a Chromafix C18

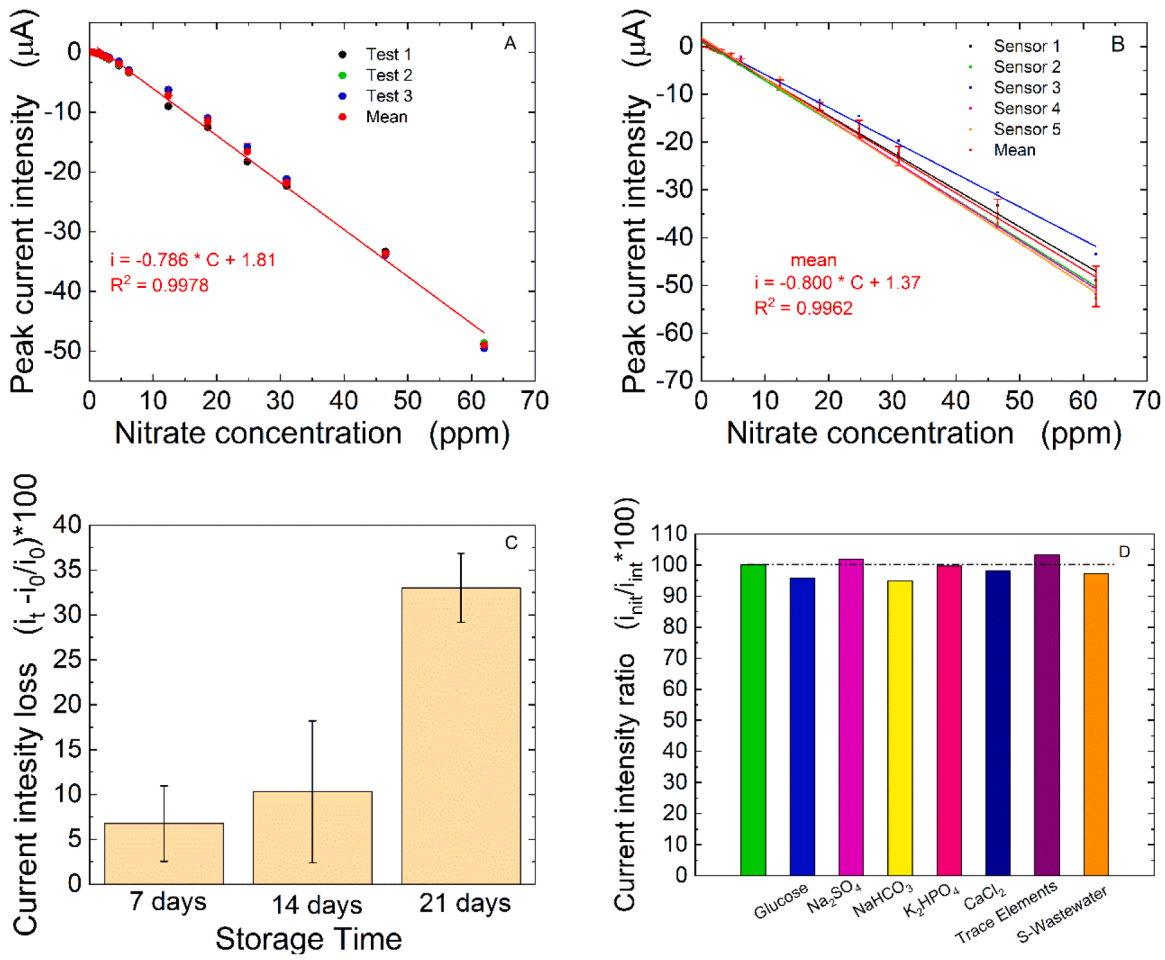

Fig. 4. A) Repeatability, B) Reproducibility, C) Stability and D) Selectivity of the sensor. All electrochemical measurements were performed with a scan rate of 25 mV/s in a solution of Na_2SO_4 0.1 M pH 1.

silica column to remove the organic content of the solution, which may damage the anionic column used for the analysis. Each chromatographic analysis took approximately 35 min and the solution had to be diluted according to the manufacturer's specifications. Results showed that the cells can effectively decrease the concentration of nitrate, bringing it below the threshold level of 50 mg L^{-1} after day 2 for Culture 1, day 6 for Culture 2, and day 3 for Culture 3 (Fig. 5B). Variations among culture replicates are typical of the microbiological cultures performed in batch cultivation mode.

During the microalgae growth, the nitrate content was also measured by the electrochemical sensor. The microalgal suspension was acidified with sulfuric acid and treated to remove the microalgae and the organic carbon as above explained. The removal of algal cells was necessary because they precipitated on the electrode surface causing biofouling. Before testing the algal suspension, each sensor was calibrated using the blank solution. Then, to quantify nitrate ions, different volumes of microalgae growth medium were diluted in the same blank solution. Fig. 5C and D shows the LSV curves reordered for Culture 2 after 2 and 7 days of cultivation. For each dilution ratio, the height of the peak current decreases with time (from day 2 to day 7 in Fig. 5C and D), confirming the decrease in nitrate concentration. The quantitative analysis of the nitrate concentration of Culture 2 over time is resumed in Fig. 6. Fig. 6 also shows a direct comparison between the nitrate concentration measured by using ionic chromatography and the proposed sensor. The results of the two methods have a linear correlation with an $R^2 > 0.99$ and a regression slope very close to 1, confirming that the proposed sensor can be successfully used to quantify nitrate ions instead of the more complex ionic chromatography. The same tests were also carried out to analyze the growth medium of the other cultures obtaining similar results (Figure S6).

To validate the sensor for real samples, the same experiments above discussed were carried out using real wastewater collected from the Oreto River (Palermo, Italy). The water was collected and before starting the experiment its content of ions and organic carbon was assessed by using ionic chromatography and elemental analysis as above explained. As shown in Table 1, the Oreto water has the characteristic freshwater ionic content but a low nutrient availability for algae growth if compared to synthetic wastewater.

Considering the low content of nitrate ions in Oreto water, 200 ppm of sodium nitrate were added to simulate a contamination of the river. This contaminated water was used for growing the microalgae *Chlorella* sp. CW2 using the same procedure described above. Fig. 7 shows the growth curve (A) and the nitrate abatement measured using ionic chromatography (B).

The microalgae were able to grow and reduce nitrate content also when using Oreto water. Respect to synthetic sewage, a different trend was observed. In particular, the growth curve (Fig. 7A) shows an irregular development due to the formation, during the culture, of microalgal floccules, which prevented a linear correlation between absorbance and concentration. A picture of the cultures with granules formation is shown in Figure S7. The formation of granules during the bioremediation of wastewater is a typical phenomenon occurring for example in wastewater treatment plants and it does not interfere with the efficiency of the treatment, as shown before [45]. In fact, even in these conditions, the nitrate concentration was reduced under the threshold after 24, 12 and 16 days for Culture 1, 2 and 3, respectively

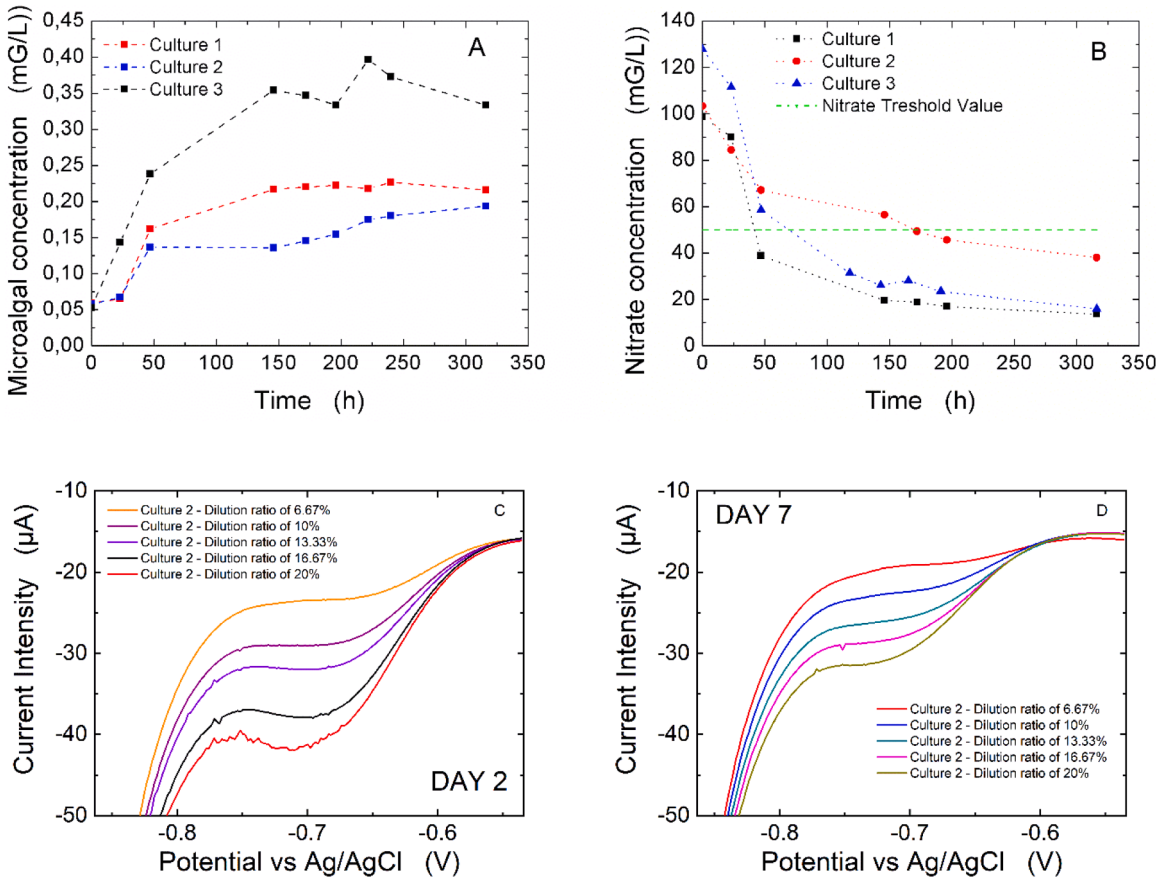

Fig. 5. (A) Growth of *Chlorella* sp. CW2 in synthetic sewage. (B) Nitrate bioremediation evaluated by ionic chromatography. (C-D) Nitrate ions detection using the electrochemical sensor in synthetic sewage after 2 days and D) 7 days of incubation with *Chlorella* sp. CW2. All electrochemical measurements were performed with a scan rate of 25 mV/s in a solution of Na_2SO_4 0.1 M pH 1.

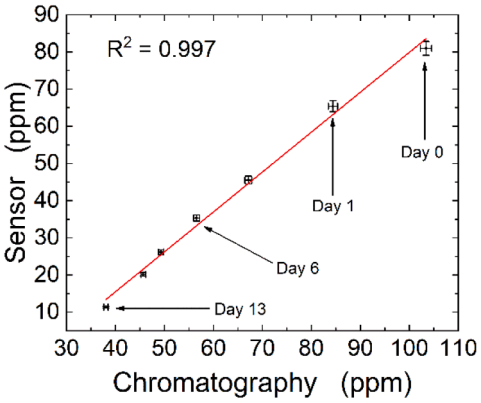

Fig. 6. Correlation between nitrate concentration measured with standard ionic chromatography and the proposed sensor in simulated wastewater.

Table 1
Analysis of the Oreto Water. (TOC: Total Organic Carbon; TIC: Total Inorganic Carbon).

Concentration [mg L^{-1}]			
Cl^-	23.2	NH_4^+	2.4
NO_3^-	7.4	Mg^{2+}	4.9
SO_4^{2-}	30.9	Ca^{2+}	12.7
PO_4^{3-}	0.3	**TOC**	9.6
F^-	0.1	**TIC**	62.7
Na^+	7.0		

(Fig. 7B). In Oreto water, a slower culture growth, and consequently removal of nitrate, compared to those cultivated in synthetic wastewater was obtained. This is due to the different compositions of the two employed media. During the microalgae growth in Oreto water, the electrochemical sensor was used to monitor the decrease of nitrate ions. Fig. 7C-D showed that, even in this real medium, the electrochemical sensor can monitor the reduction of nitrate ions by *Chlorella* sp. CW2. Also in this case, a linear correlation, Fig. 8, was found between the concentration measured using by ionic chromatography and the proposed sensor ($R^2 > 0.99$ and a regression slope very close to 1). Similar results were obtained for the other cultures (Figure S8). Thus, it can be concluded that the matrix of real wastewater, such as river water, does not interfere with the ability of the sensor to quantify nitrate ions.

It is worth pointing out that the applicability of the sensors in real wastewater is generally limited because of factors such as interference, reproducibility, stability, sensitivity and selectivity. In our case, the biggest challenge in using the sensor for real-world applications is the possibility of interference from unidentified species that may be found in wastewater from unknown sources. Even if, in the case of sources with low concentration of salts, such as the river water tested here, the full functioning of the sensor is assured. By the way, these limitations can be overcome with the implementation of a microfluidic system able to mix the real wastewater with a diluent acidic solution to reduce the concentration of other contaminants and thus the possible interference.

4. Conclusion

A copper-based electrochemical sensor for nitrate quantification fabricated using PCB technology was proposed. Sensor was obtained

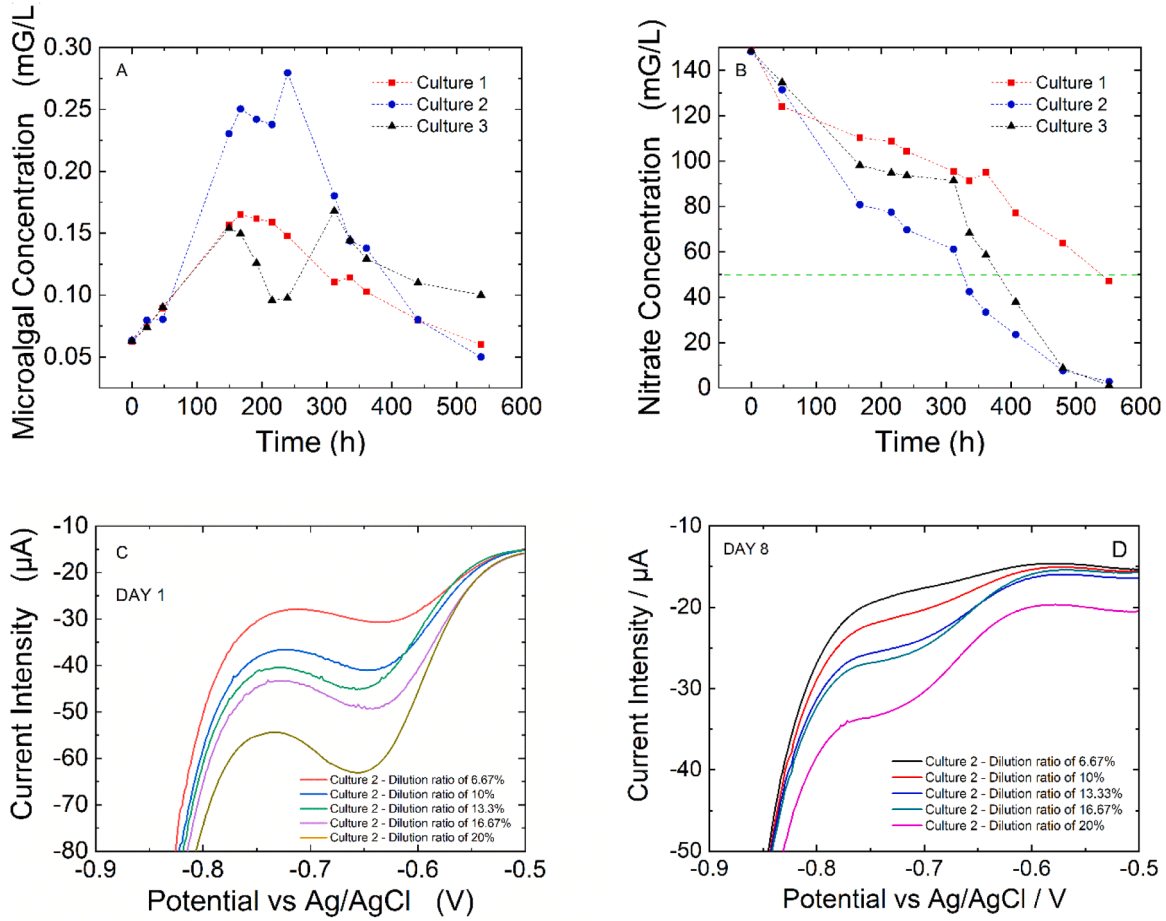

Fig. 7. (A) Growth of *Chlorella* sp. CW2 in the Oreto River water supplemented with $NaNO_3$. (B) Nitrate bioremediation evaluated by ionic chromatography. (C-D) Nitrate ions detection using the electrochemical sensor in Oreto River water after C) 1 day and D) 8 days of incubation with *Chlorella* sp. CW2. All electrochemical measurements were performed with a scan rate of 25 mV/s in a solution of Na_2SO_4 0.1 M pH1.

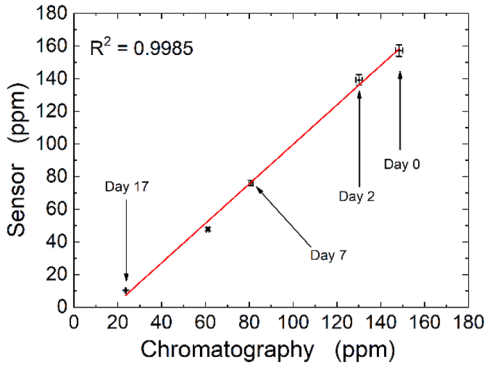

Fig. 8. Correlation between nitrate concentration measured with standard ionic chromatography and the proposed sensor in the river water.

with a three electrode-configuration and fully characterized in a solution of Na_2SO_4 0.1 M using LSV as electrochemical technique. A LOD of 5.2 ppm with a very high sensitivity of 0.776 µA ppm^{-1} and a relative standard deviation of 0.67 % was found. The sensor is characterized of high reproducibility and repeatability and shows a very high selectivity towards nitrate ions. The sensor was used to monitor the biological remediation of nitrate during the microalgal culture. Both synthetic solution and real river water were used to investigate the effect of different matrices in the sensor performance. To validate the method, the results of the proposed sensor were back-compared with a standard ionic chromatography analysis. A linear correlation between the two methods ($R^2 > 0.99$) was found. Although the ability of the sensor to properly function in the tested growth medium and in a real river water, there are still limitations in its applicability for real-world purposes especially because of the possible presence of unknown interfering species which may alter the performance of the sensor. Future research efforts should try to investigate this aspect with the implementation of a microfluidic system able to reduce the possible interference by diluting the real wastewater. Thus, this work demonstrates the possible development of easy-to-use devices capable of real-time monitoring of nutrient reduction by microalgal cultures and therefore represents a significant step towards microalgal industrial utilization for wastewater remediation.

CRediT authorship contribution statement

Caputo Giuseppe: Data curation, Investigation, Writing – review & editing. **Aiello Giuseppe:** Writing – review & editing, Validation, Software, Resources. **Cosenza Alessandro:** Methodology, Formal analysis, Data curation, Conceptualization. **Moukri Nadia:** Resources, Formal analysis, Data curation, Conceptualization. **Spinoso Alice:** Investigation, Formal analysis, Data curation. **Inguanta Rosalinda:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Scargiali Francesca:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Lima Serena:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation,

Conceptualization. **Patella Bernardo:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.snb.2025.137463.

Data availability

Data will be made available on request.

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