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Short Communication

Exploring the biocapacitance in M3C-based biosensors for the assessment of microbial activity and organic matter

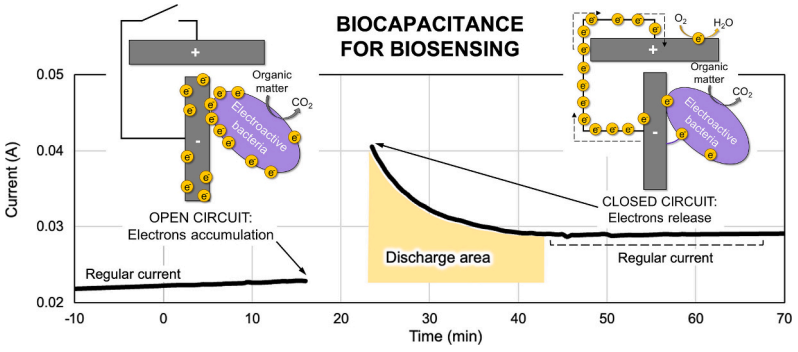
Marta Fernandez-Gatell [a,b], Xavier Sanchez-Vila [b,c], Jaume Puigagut [a,*]

[a] GEMMA - Environmental Engineering and Microbiology Research Group, Department of Civil and Environmental Engineering, Universitat Politècnica de Catalunya·BarcelonaTech (UPC), c/Jordi Girona 1-3, Building D1, 08034 Barcelona, Spain
[b] GHS - Dept. of Civil and Environmental Engineering, UPC, Jordi Girona 1-3, 08034 Barcelona, Spain
[c] Associated Unit: Hydrogeology Group (UPC-CSIC), Spain

HIGHLIGHTS

- Bioelectrochemical systems capacitance as a biosensing tool
- BES-based biosensor for environments with water content fluctuations
- BES-based biosensor for microbial activity, biomass, and organic matter content
- Low energy demand BES-based biosensor

GRAPHICAL ABSTRACT



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ABSTRACT

Reliable monitoring of microbial and water quality parameters in freshwater ecosystems (either natural or human-made) is of capital importance for improving both the management of water resources and the assessment of microbially-driven bio-geo-chemical processes. In this context, bioelectrochemical systems (BES), such as microbial three-cell electrodes (M3C), are very promising devices for their use as biosensors. However, current experiences on the use of BES-based devices for biosensing purposes are almost exclusively limited to water-saturated environments. This limitation hampers the use of this technology for a wider range of applications where the biosensor may work discontinuously (such as discontinuously saturated ecosystems). Discontinuous operation of M3C-based biosensors creates an electric current peak immediately after the reconnection of the system due to electron accumulation, in a process known as biocapacitance. The present work aimed at quantifying the bioindication potential of biocapacitance for the assessment of key ecosystem parameters such as microbial metabolic activity and biomass, as well as organic matter concentration. Significant linear regression coefficients ($R^2 > 0.9$) were found for all combinations of parameters tested. Moreover, for most of the ecological parameters assessed, an electric charge accumulation of 1–5 min (biocapacitance elapsed time) and discharge of 5 min was enough to get reliable information. In conclusion, we have demonstrated for the first time that

* Corresponding author at: Group of Environmental Engineering and Microbiology (GEMMA), Universitat Politècnica de Catalunya, BarcelonaTech, C/Jordi Girona 1-3, 08034 Barcelona, Spain.
E-mail address: jaume.puigagut@upc.edu (J. Puigagut).

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biocapacitance in M3C-based biosensors can be used as a proxy parameter for the assessment of microbial activity, microbial biomass and organic matter concentration in a model nature-based ecosystem.

1. Introduction

Microbial communities are major quality drivers responsible for carbon and nutrient turnover in natural and human-made ecosystems (Sofi et al., 2016). Their presence and activity are especially relevant in soils and lentic ecosystems, such as wetlands, peatlands, or marshes, since they represent large organic carbon reservoirs and are one of the most significant natural sources of methane emissions (IPCC, 2021). Microbial communities also play an important role in the water cycle. Accordingly, the organic matter removal in wastewater treatment systems (WWTS) is driven by microorganisms and their performance is directly linked with the treatment efficiency (García et al., 2010; Perujo et al., 2017; Truu et al., 2009). Among WWTS, nature-based solutions (NBS) are particularly interesting since their energy demand is very low and they are easy to operate and maintain. In these systems, the treatment is driven by a combination of natural biological, chemical, and physical processes. Besides, these systems are usually installed in rural areas or small communities where land availability is not a limiting factor. Therefore, reliable and quasi-real-time monitoring of microbial and water quality parameters in freshwater natural or human-made ecosystems is key for improving both the management of water resources and the assessment of microbially-driven biogeochemical processes. Despite their relevance, the vast majority of techniques currently used to assess parameters indicative of water quality or microbial characterization bear some drawbacks. Among them, the techniques involve sample extraction and processing, the use of reagents, expensive materials and machinery, and complex protocols that require qualified personnel or cannot be applied for real-time monitoring (Faulwetter et al., 2009). In this context, there is a clear need for accurate alternative methods that allow a fast, low-priced, and non-invasive determination of microbial activity and water quality parameters.

Bioelectrochemical systems are devices that gathered attention for their use as biosensors in recent years. Their functioning mainly relies on electroactive bacteria (EAB). EAB grow as a biofilm directly on a conductive material that is used as an electron acceptor or donor. Hence, EABs can produce a low-power electric signal indicative of their metabolic activity (Abrevaya et al., 2015a, 2015b; Olias and Di Lorenzo, 2021). Furthermore, plenty of bacterial genera, found all over ecosystems, have been described with electroactive properties (Lovley and Holmes, 2021). Therefore, unlike conventional biosensors, BES-based ones are self-grown with bacteria that are already in the environment, are self-maintained, and so there is no need for a transducer (Prévoteau and Rabaey, 2017). Most BES-based biosensor research has been performed with Microbial Fuel Cells (MFCs) for organic matter and toxicity assessment (Kumar et al., 2022). However, one of their main drawbacks is that the current produced depends on the EAB's activity and other factors that can alter the internal resistance or the cathode performance. Consequently, the signal produced can be unstable, and the sensor is less reproducible and accurate than conventional ones (Prévoteau and Rabaey, 2017). Microbial three-electrode cells (M3Cs) are modified MFCs in which a reference electrode and a potentiostat are used to poise and control a pre-set potential at the anode (the working electrode). In this case, the current produced is only dependent on the microbial processes taking place at the anode, and, therefore, the M3C signal is more accurate and stable than that produced in MFC-based biosensors (Grattieri et al., 2017; Prévoteau and Rabaey, 2017). The presence of the potentiostat represents an additional improvement over MFC-based sensors, as it allows the application of multiple electrochemical techniques that can deeper characterize the biosensor functioning. M3C-based biosensors investigation is still in its infancy; so far, they have been used for organic matter and toxicity assessment and, recently, also for microbial activity (Fernandez-Gatell et al., 2022; Modin and Wilén, 2012).

To date, the research performed with BES-based biosensors has been carried out in water-saturated environments. However, both in natural ecosystems and in several types of NBS, the water level and humidity are variable in time. Natural ecosystems combine periods of rainfall or high humidity with dry periods. Also, several types of NBS (vertical or tidal flow treatment wetlands, green walls, or managed aquifer recharge) are fed discontinuously, forcing flooded and drained phases that create anaerobic and aerobic conditions, respectively. This operation favours different metabolic pathways which, in turn, allows higher mineralization of some contaminants and nutrients. Therefore, it is clear that if BES-based biosensors have to be applied in real environments, they should also be proven functional in a discontinuous operation where the electric circuit may experience phases of connection/disconnection due to cathode exposure to air. Further, this discontinuous operation of a BES circuit would reduce the biosensor's power consumption, which is also beneficial for their actual application.

The hypothesis of using the discontinuous operation of M3Cs for biosensing function is based on the electric capacitance of electroactive biofilms. A bioelectrochemical system operated with an intermittent polarization undergoes phases of open-circuit (OC) conditions and those with closed-circuit (CC). During the former, even though the electrons cannot flow, the substrate is still oxidized, and cellular metabolism continues. This phenomenon is produced due to different electron-storage mechanisms' of EABs (ter Heijne et al., 2021). When the circuit is closed again, all the electrons accumulated in OC conditions are released into the circuit, creating a current peak (Fig. 1). The peak magnitude relies on a combination of EAB's metabolic activity and the electrons' release rate from the storage compounds (ter Heijne et al., 2021).

This work aimed at determining the feasibility of using an M3C-based biosensor under discontinuous operation for the assessment of (i) microbial metabolic activity, (ii) biomass content, and (iii) the organic matter concentration. To the authors' knowledge, this is the first attempt to use the biocapacitance phenomenon of BES as a bioindication tool. Note that, due to the "proof-of-concept" nature of the present work, and in spite of the significance of characterizing natural ecosystems, this investigation is only focused on NBS-type setup for its simplicity. Demonstrating the suitability of using an M3C-based biosensor in discontinuous mode will open the possibility of developing a cost-effective, self-maintained and accurate biosensor that can be applied in discontinuously saturated environments to assess ecosystems' quality-related parameters.

2. Materials and methods

2.1. Systems design and operation

To this purpose, four reactors (described in Fernandez-Gatell et al., 2022) were fed with decreasing organic matter concentrations to create a suite of microbial activity and biomass conditions. The reactors aimed to simulate different situations that can be found in a shallow treatment wetland.

Each reactor (Fig. 2) consisted of a PVC cylinder (30 cm height, 15 cm diameter) of 3.8 L of effective volume. On each one there were 23 plastic mesh sockets filled with granitic gravel (4/8 mm) and a bioelectrochemical system (BES) as a biosensor. The BES used was a microbial three-electrode cell (M3C) which was placed at the centre of the reactor and had a working electrode posed potential of 0.3 V (VS Ag/AgCl). The M3C was controlled by means of a potentiostat (NEV4,

NanoElectra). The anode (working electrode of the M3C) consisted of a plastic mesh socket filled with an electron collector of stainless-steel mesh (grade A316L) of 46 cm^2 filled with recycled granular graphite (>98 % C). Total anode volume was 251 cm^3 and the projected surface, 5.3 cm^2. The cathode (counter electrode) consisted of a 72 cm^2 projected surface stain-less-steel mesh box filled with graphite rods (>99 % purity, Alfa Aesar). Finally, the reference electrode, placed near the anode, was a commercial Ag/AgCl reference electrode ((RE-6 Ag/AgCl, 3 M NaCl) ProSense). To guarantee a good homogenization, the water within each reactor was continuously recirculated using a peristaltic pump at a rate of 3.2 L h^{-1}.

Systems were fed in a semi-continuous mode with a mixture of real and synthetic wastewater. The real wastewater was collected at the beginning of the experiment directly from the Barcelona city sewage system, underwent a settling process of 3 h and frozen at −20 °C until the day it needed to be used. The synthetic wastewater was prepared as recommended by Nopens et al. (2001). Fresh wastewater was pumped to the reactors every 2 to 4 days through a peristaltic pump (Damova MP-3035-6M) at a flow rate of approx. 3 L h^{-1}, while the water within the reactor was removed at the same time through an effluent pipe. Moreover, each reactor received a different concentration of the substrate (displayed in Table 1) to force diverse microbial activity, biomass, and organic content conditions.

2.2. Microbial activity, biomass, and organic matter concentration assessment

Microbial activity tests, biomass and organic matter assessment were performed all on the same day.

Microbial activity was assessed with two different techniques: (i) hydrolytic enzymatic activity, using the fluorescein diacetate (FDA) hydrolysis method, and (ii) ATP content. In the former, the hydrolytic enzymatic activity is assessed by means of the color development of the fluorescein when hydrolytic enzymes cut the fluorescein diacetate molecules. To perform a test, three gravel cores of a given system were taken and placed in a reactor. Then, a reaction solution of FDA 10 μM was added and the agitation turned on to ensure a good homogenization during the test. After 30 min of incubation, a 2 mL sample was taken to read the absorbance at 490 nm with a quartz cuvette and a spectrophotometer. The absorbance readings were transformed to fluorescein concentration through a calibration curve and were normalized to the amount of gravel used in each test.

ATP content was measured with QuenchGone21™ Wastewater Advanced (QG21Wa) commercial kit and the PhotonMaster™ Luminometer (LuminUltra Technologies). For this purpose, a gravel core was taken from the system and emptied to mix the gravel before taking a small amount (approx. 2 g). The rest of the gravel was used to perform the biomass analysis. The test was performed following the kit instructions with a few grams of the mixed gravel. After the test, the gravel used was dried and weighed. Results were determined in RLU units (relative light units) which were converted to ATP concentration through a Standard Calibration procedure given by the kit. Finally, attached growth ATP (cATP) content was calculated according to the manual and normalized to the gravel weight.

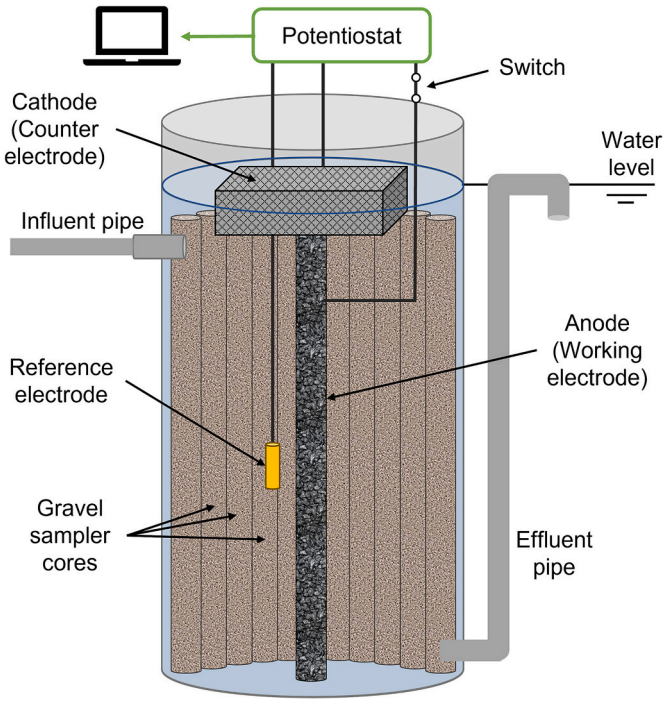

Fig. 2. Scheme of one of the reactors.
Adapted from Fernandez-Gatell et al. (2022).

Table 1
Average influent organic matter concentration (in chemical oxygen demand, COD) of each system.

System	Influent COD (mg O_2 eq. L^{-1}) (AVG ± SD)
1	316.1 ± 73.4
2	158.9 ± 39.7
3	83.6 ± 27.7
4	55.0 ± 21.8

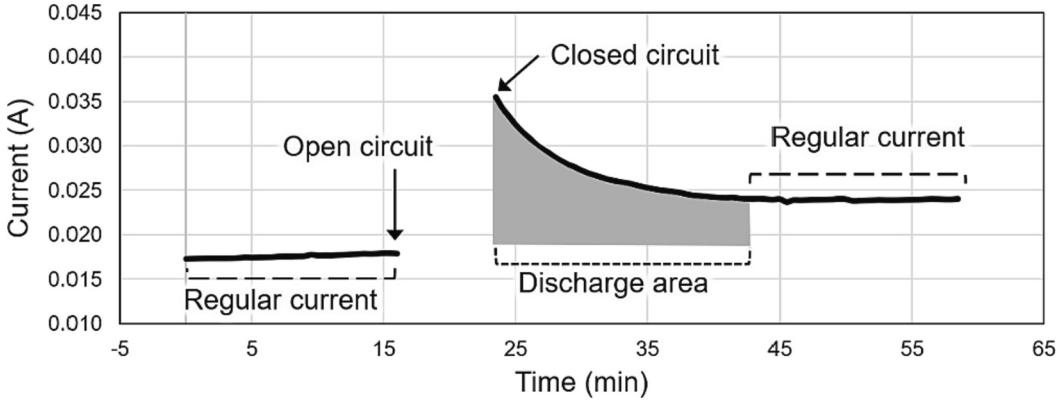

Fig. 1. Example graph of a peak current after the biocapacitation. When the circuit is opened, the electrons cannot flow, but they accumulate in the surrounding area, creating a gap in the electric signal. When the circuit closes, all the electrons accumulated are suddenly released, creating a peak of electric current. The area under the peak until the regular current is reached again is called the discharge area (shaded in the figure).

The biomass content was analyzed as the volatile solids attached to the gravel. A bulk of approx. 25 g of gravel from a gravel socket was picked up, mixed with a volume of autoclaved saline phosphate buffer solution and shacked for 3 h at 100 rpm to detach the solids from the gravel following the procedure described by Weber and Legge (2010). Then, the solids were analyzed following the Standard Methods for the examination of water and wastewater (APHA-AWWA-WEF, 2012). After the test, the gravel used was dried and weighted to normalize the volatile solids results.

Finally, organic matter content of the wastewater that was within the reactor was analyzed with the colorimetric closed reflux method described in the Standard Methods (APHA-AWWA-WEF, 2012).

2.3. Bioindication with the M3C biocapacitance

Simultaneously to the reactors' sampling for the aforementioned tests, the M3C was disconnected to provoke the biocapacitance of the anode. The electric current was monitored continuously (every 30 s) with the potentiostat. Current peaks generated after the disconnection and reconnection of the M3C were easily identified visually. Once the final point of the discharge peak was identified, its cumulative electric charge (it is, the discharge area of a peak) was calculated. To do so, the recorded electric current was multiplied by the elapsed time between two electric current measurements. Cumulative electric charge was calculated at each minute of the discharge area and to a maximum of 20 min (the highest time that a current peak lasted).

Statistical analyses were performed with IBM SPSS Statistics (v.23).

3. Results and discussion

The discharge area (Fig. 1), measured in cumulated electric charge (in Coulomb), is considered to be the area under the peak of electric current produced after the M3C re-connection (closing the circuit) to some reference value reached at steady state conditions, and is representative of the electroactive bacteria activity. The actual time required to release all electrons accumulated in the surroundings of the electroactive biofilm and the conductive material depends on the number of electrons that had piled up, as well as on the release rate of the electrons from the storage compartments.

To assess the bioindication potential of biocapacitance, linear regression analyses were performed between the metabolic activity of electroactive bacteria, represented as the cumulative electric charge already indicated, and the results from the soil microbial community and the water quality. Note that, as stated above, the time required to fully discharge the electrons accumulated in the anode, can be different for each system and each charge-discharge cycle. For the linear regression, the complete discharge area was considered for all the tests carried out, regardless of the time that they lasted. Soil microbial community was characterized by (i) its metabolic activity, which was determined through the ATP content and the hydrolytic enzymatic activity-FDA, and (ii) the biomass content, assessed with the concentration of volatile solids suspended (VSS) of the soil sample. The water quality was evaluated using the influent COD (chemical oxygen demand) as a proxy for the organic matter concentration.

Linear regression analyses (Fig. 3) showed statistically significant results for all the parameters tested, with R^2 coefficients above 90 %. Best results were obtained with the organic matter assessment, with an R^2 of 0.998, $p < 0.001$, and a standard error of 6.19 mg L^{-1}. Nevertheless, microbial activity determined with the ATP content also showed a remarkable significant regression coefficient with $R^2 = 0.959$ ($p = 0.021$), with a standard error of 13.34 ng cATP g^{-1}gravel. Similar results have been reported by Sim et al. (2018), who obtained $R^2 > 0.9$ for both acetate concentration and wastewater COD assessment with different charging times. Regarding continuous operation, Modin and Wilén (2012), and Yuan and Kim (2017), reported R^2 coefficients for acetate detection up to 0.99 in both cases. Results on microbial activity and biomass are also in accordance with previous experiments using the continuous operation of BES (Fernandez-Gatell et al., 2022; Zhang and Angelidaki, 2011).

The results of this experiment demonstrated the potential use of biocapacitance as an indication tool. One of the main highlights of these findings is that BES-based biosensors could be widened to include all types of ecosystems in which water-saturation conditions change over time and imposes discontinuous operation of BES systems. As mentioned above, different types of nature-based solutions for water treatment work under different hydraulic conditions; also, in natural ecosystems or

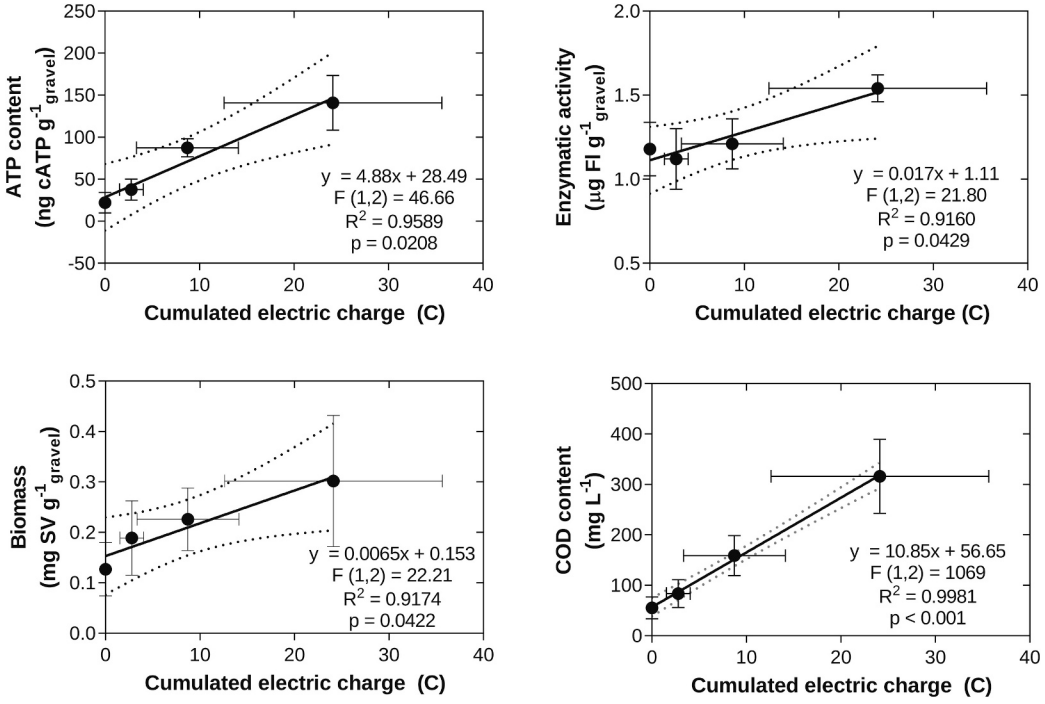

Fig. 3. Linear regression results between the cumulated electric charge of the complete discharge area (in C) produced by the M3C-based biosensor and the parameters assessed from soil microorganisms and water quality: (i) metabolic activity (ATP content and hydrolytic enzymatic activity); (ii) biomass (volatile solids suspended) and (iii) organic matter concentration (COD content). *Note*: standard deviation of cumulated electric charge is the product of six independent charge/discharge cycles for each system; alternatively, standard deviation of each assessed parameter is also the product of six independent tests.

agricultural fields, the water content of the soil fluctuates. Moreover, the energy consumption of the biosensor itself can be lowered since the time that the potentiostat is controlling the anode potential will be also shortened during discontinuous operation. On the counterpart, the discharge area needs to be calculated, which will increase the data processing requirements of the biosensor. This implies that the current of the biosensors has to be recorded continuously, the initial and end points of the current peak identified, and, finally, the discharge area calculated. A possible solution could be to use an external data processor. In this case, the biosensor would send the electric current signal continuously to the cloud where it can be stored and computed. Further, the time used to calculate the discharge area can be shortened. With this approach, not only the calculation needs are reduced but also the energy consumption is optimized. In our experiment, discharge areas lasted for an average of 8 min, yet they ranged between 1 and 20 min.

In order to determine the minimum time needed to still obtain reliable results, linear regression analyses were performed at each minute of the discharge area from 1 to 20 min of discharge elapsed time. In Fig. 4, the regression coefficient for each regression analysis is displayed over time for each parameter assessed. Each point represents the regression coefficient obtained between the cumulated electric charge at a determined minute of the discharge time (between 1 and 20) and the parameter of interest (enzymatic activity, ATP content, organic matter concentration or biomass). As shown, the enzymatic activity is the parameter that needs more time (13 min), and hence, more cumulated electric charge, to be assessed. At least 3 min of reconnection would be needed for the organic matter concentration bioindication. Nevertheless, biomass concentration and ATP content could be determined ($R^2 >$ 0.9, *p*-value <0.04) even within the first minute of the biosensor reconnection (1 min of the discharge). According to these results, the authors recommend using the electric current cumulated within the first 5 min after the reconnection of the M3C to assess the metabolic activity of the microbial population (as ATP), biomass content, and organic matter concentration with a good estimation ($R^2 > 0.95$). This will significantly reduce the time that the potentiostat needs to work, and therefore, the data processing needs and the energy consumption of the biosensor will be also reduced. Detailed results of the regression analyses can be found in the Supplementary material Table S1.

A key aspect in biosensing technologies is the charge transfer between the electron's donor/acceptor (electroactive microorganisms and their surroundings, in this case) and the electrodes themselves. Indeed, there is a lot of discussion around BES electrodes' influence area (Müller et al., 2010; Thapa et al., 2022; Zhang et al., 2015) and the strategies to improve the charge transfer. The use of modified organisms and conducting polymers are among these strategies. Also, conductive polymers demonstrated to be biocompatible, which could be very beneficial for a better biofilm development onto the electrodes (Andriukonis et al., 2022; Kisieliute et al., 2019; Ramanavicius and Ramanavicius, 2020).

Furthermore, the time that the bioelectrochemical system can remain under open circuit conditions should be assessed in order to guarantee the survival of the electroactive bacteria and, therefore, the viability of the electroactive metabolism pathway. Since the present works aimed at a proof of concept of the biosensing potential of biocapacitance, the effect of different disconnection times on the biosensing accuracy was not surveyed. Although in the present study the open circuit conditions were maintained for very few minutes (2–5 min), some reports of longer disconnected periods exist. For example, Sim et al. (2018) demonstrated good correlation results for BOD assessment with a discontinuous operation of a BES-biosensor up to 40 min of charging (open circuit) time. Similarly, in Chung et al. (2020) charging–discharging times of up to 120 min were evaluated for naphthenic acid concentrations biosensing. However, nature-based solutions such as vertical flow treatment wetlands, green walls, or infiltration basins, are fed discontinuously, with typical feeding cadencies of four to twelve times per day and, therefore, the BES-biosensor would remain disconnected for several hours. In a study performed by Ruiz et al. (2015), the performance of an open circuit MFC under starvation conditions for several days was addressed. They found that the MFC lost activity after

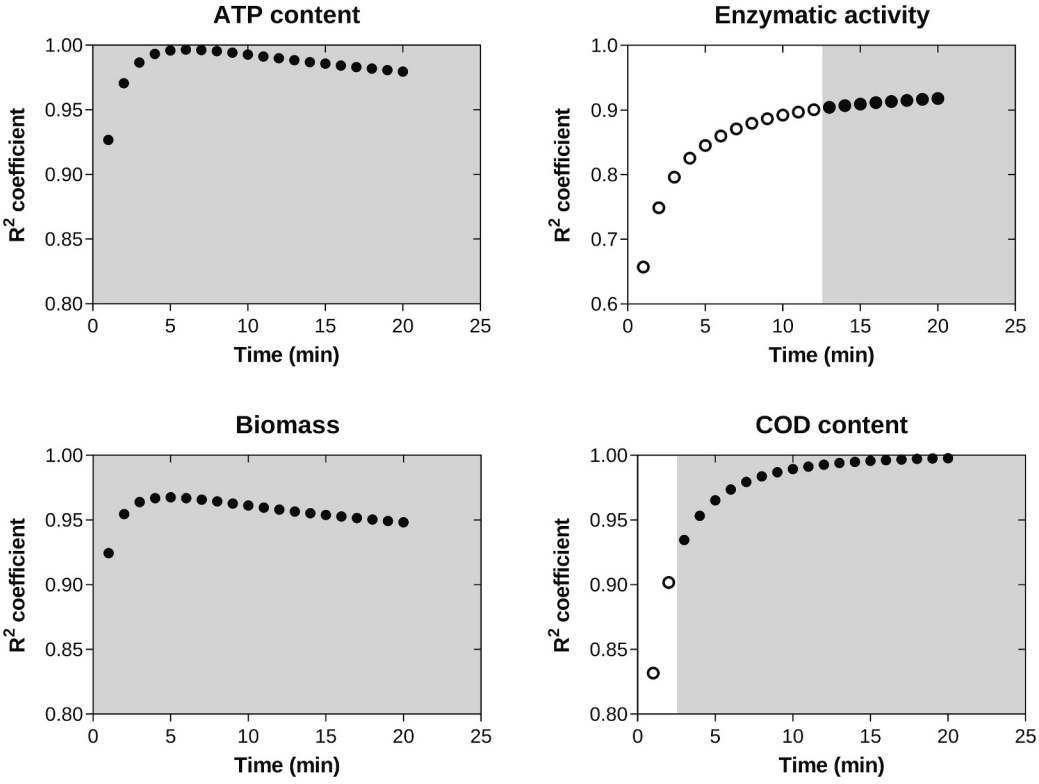

Fig. 4. Results of R^2 coefficients of the linear regression tests performed between the cumulated electric charge at different discharge times and the different parameters assessed. Statistically significant regressions (p-value <0.05) are displayed with solid dots and a shaded background.

7 days of starvation, although this process was reversible. However, after 21 days, the MFC was irreversibly damaged. Albeit the biocapacitance of the microbial electrochemical system was not specifically addressed, the study demonstrates that, even in very hostile conditions (starvation and circuit disconnected for several days), the electroactive pathway could still withstand.

In order to assess the real application of M3C-based biosensors, some uncertainties should be addressed in further works. Among them, we want to highlight: a) the effect of different charging times on the sensitivity and reproducibility of the biosensor; b) the range in which the biosensor is accurate should be determined for each parameter, as well as the stability over time of the biosensor itself and the reference electrode; c) assess the viability and technical feasibility of sustainable and independent power sources (such as solar panels) implementation to run the potentiostat; and d) the effect of different charge transfer improvement strategies.

4. Conclusions

In conclusion, the present study demonstrates the feasibility of using the biocapacitance phenomenon of an M3C system to estimate the microbial activity, biomass and COD concentration of wastewater. More specifically, significant regression analysis with $R^2 > 0.9$ were obtained for all parameters assessed, being the COD concentration and ATP content the best represented ($R^2 = 0.99$ and $R^2 = 0.95$, respectively). Moreover, it has been determined a reconnection time of at least 5 min to obtain reliable results.

Even though the proof-of-concept nature of the present study and the future work recommended previously, the use of biocapacitance for biosensing boosts the possibility of developing a new accurate, low-cost, online, remote, self-maintained, and quasi-real-time biosensor, widening its application to environments working under water content fluctuations and reducing its energy demand.

CRediT authorship contribution statement

Marta Fernandez-Gatell: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft, Visualization. **Xavier Sanchez-Vila:** Formal analysis, Writing – review & editing. **Jaume Puigagut:** Conceptualization, Methodology, Supervision, Writing – review & editing.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.166510.

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