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A simple *Streptomyces* spore-based impedimetric biosensor to detect lindane pesticide

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ABSTRACT

Lindane is a pesticide potentially harmful to environment and human health and in general, its detection involves mostly tedious, expensive and time-consuming techniques. A new strategy to detect lindane can be focused on the activity of *Streptomyces* strain M7 (SM7), which is capable to grow in the presence of organochlorine pesticides (OCPs) as carbon source. On this regard, the aim of this work is to present an optimized design of a microbial biosensor prototype based on a SM7 spore platform to detect and quantify lindane by electrochemical impedance spectroscopy (EIS) as the transduction method. By means of this technique, the metabolic bacterial activity was evaluated by analyzing a set of electrical parameters calculated by fitting the measured impedance spectra through an equivalent electrical circuit. Activity studies were performed by incubating the SM7 spore platform with (optimized incubation conditions) and without (to be used in a practical biosensor) shaking in the presence of lindane. In all cases, bacterial activity was measured by the impedance changes produced in the cells due to lindane removal from the culture medium. This proposed label-free impedimetric biosensor is simple, cost-effective and feasible to detect changes in the lindane concentration as low as 120 $\mu g\,L^{-1}$ in less than 2 days without sample pretreatment. In addition, the surface of electrodes can be reused for several different tests. This sensor prototype represents a valuable alternative for OCPs detection.

1. Introduction

Lindane (γ-hexachlorocyclohexane) is one of the organochlorine pesticides (OCPs) recently classified as a persistent organic pollutant in accordance with the Stockholm Convention [1,2]. OCPs are potentially harmful to environment and human health since they are lipophilic compounds with high resistance to degradation and half-life from a few to more than 10 years [3]. These persistent chemicals can be transferred and magnified to higher trophic levels through the food chain due to their relative stability and bioaccumulation properties [4,5], causing several adverse and toxic effects [2]. Lindane has been used for crop protection and prevention of vector-borne diseases for many decades. Negative impacts of lindane on the environment and human health have been reported worldwide [6]. It is neurotoxic, an endocrine disrupter in humans and it is classified as a possible carcinogen and teratogen by the United States Environment Protection Agency and the World Health Organization [7,8].

Despite the fact the use of lindane has been banned, some countries are still using it for economic reasons [9,10]. Residues

Abbreviations: SM7, *Streptomyces* strain M7; MM, modified liquid medium; MMLin, modified liquid medium supplemented with lindane; SCA, solid culture medium of starch-casein agar.

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of lindane have been determined in numerous diverse matrices [11,12] even in geographic zones where this pesticide has never been used [13]. Therefore, numerous methods have been applied for the detection and quantification of lindane, including: (a) colorimetric measurement of chloride ions based on phenol red [14], or using zinc in acetic acid [15,16], (b) complete mineralization to $^{14}CO_2$ [15], (c) electronic noses [17] and (d) liquid and gas (GC) chromatographies [18,19], coupled with selective detectors, such as electron-capture detection (ECD), nitrogen-phosphorus detection and mass spectrometry. Even though some of these methods are sensitive [20,21], in general, they include two key previous steps: lindane extraction from the bulk of the matrix and clean-up from the matrix coextractives [22]. These procedures are mostly tedious, expensive and time-consuming techniques [23]; hence, they are not suitable for on-site monitoring [24]. From the methods listed above, and given lindane nature (i.e. volatile and thermally stable), GC-ECD is the most commonly used detection method with appropriate detection limits [21,25]. Therefore, a simple method for sensitive and selective detection of lindane contamination in environmental matrices is highly desirable.

Biosensors present a promising alternative to conventional techniques because they offer a rapid, simple, specific, and sensitive way for the detection of environmental pollutants, including OCPs without the need of sophisticated instruments [25]. A major limitation of the development of lindane biosensor is the availability of biorecognition elements, which can specifically detect it [26]. Different organisms have been found to be capable of using lindane as a growth substrate, such as fungi [27–31], cyanobacteria [32–34], aerobic [35–40] and anaerobic bacteria [41–43]. *Streptomyces* strain M7 (SM7) is capable to grow in the presence of lindane as carbon source [35,37,44]. This SM7 metabolic activity can be valuable used in a biosensor to detect and, eventually, quantify this pesticide.

As biorecognition element determines the degree of selectivity and specificity of the biosensor, the transducer method has a great influence on the sensitivity, speed, efficiency and simplicity of the device. Electrochemical impedance is considered an effective, cheap, and non-destructive transducer technique for sensing the biorecognition event. The metabolic activity of microorganisms can be evaluated by measuring the impedance change caused by modifications in the medium resistance and in the interface reactance [45–49]. In fact, the biorecognition event can be detected either on the surface of the electrode, by probing the electrode–electrolyte interfacial properties when the adhered bacterial cells interact with an analyte [50–52], or in the electrolyte solution, by monitoring the metabolites produced by bacterial cells as a result of growth in the presence of an analyte [53–55]. Among the different types of label free methods, impedimetric biosensors are of particular interest due to their reduced matrix interferences, simple electrical measurements and possibility of automation [56].

In previous works, we designed a biofunctional surface to be integrated in a prototype of a biosensor to detect OCPs employing a SM7 spore-based platform as biorecognition element, optimized the composition of the liquid medium to determine the SM7 activity by impedimetric detection, and evaluated impedance at two fixed frequencies as the transduction method (measured with the commercially available Quantibac equipment, TecnoVinc SRL, Argentine) [57,58]. The results of our studies showed that SM7 adhered to SCA (spore-based platform) immersed in MM represented the optimum culture medium to determine the presence of a carbon source with this impedimetric method. In addition, we confirmed that adhered spores germinated to fully active cells responding faster to analytes than suspended bacteria. Therefore, in these works we combined the spore-based platform with the Quantibac equipment as a first approximation to detect carbon source with an electronic prototype based on a SM7 [57,58]. However, we could not detect SM7 metabolic activity in the presence of lindane as a sole carbon source with this simple impedimetric determination performed at just two fixed frequencies. As the following step toward the development of the impedimetric biosensor, this work presents an optimized design of a sensor prototype based on a SM7 spore platform to detect lindane by electrochemical impedance spectroscopy (EIS).

2. Materials and methods

2.1. Reagents

Lindane (99% pure) was purchased from Accustandard Inc. (New Haven, USA), hexane (≥99% pure, for pesticide residue analysis) and $(NH_4)_2SO_4$ were purchased from Sigma–Aldrich (St. Louis, USA), $FeSO_4$ and K_2HPO_4 from J.T. Baker (Center Valley, USA), starch and $MgSO_4$ from Cicarelli (San Lorenzo, Argentine), casein from Calbiochem (Darmstadt, Germany) and agar–agar from Britania (Buenos Aires, Argentine). These chemicals were reagent grade and used without further purification. All solutions were prepared with ultrapure water (ρ = 18 MΩ cm) from a Millipore-MilliQ system.

2.2. Bacteria strain and culture media

The strain *Streptomyces* sp. M7 [59,60] was adhered by surface dissemination on a solid culture medium (starch-casein agar, SCA [61]) and cultured at 30.0 °C without shaking during 7 days. SM7 adhered to SCA (spore-based platform) was immersed in a modified liquid medium (MM) [58] which was supplemented with 1.50×10^{-3} g L^{-1} lindane (MMLin) as sole carbon source. All culture media were adjusted to pH 7.00 and sterilized by autoclaving for 20 min at 120 °C. Lindane stock solutions were sterilized by filtration (0.22 μm pore size Millipore filter) and then added aseptically to the autoclaved media.

2.3. Cells, electrodes and equipments

EIS measurements were performed with a Solartron 12,508 W impedance analyzer composed by a Solartron 1287 electrochemical interface and a Solartron 1250 frequency response analyzer, commanded by the corresponding software provided by the manufacturer (ZPlot® and ZView®, Scribner Associates Incorporated).

EIS measurements were performed in 100 mL culture flasks containing the SM7 spore-based platform in contact with MM or MMLin as shown in Fig. 1 (panel a). Stainless steel electrodes (57 mm length, 1 mm diameter) with a 2 mm separation between them, were inserted in a commercial gypsum material (Poximix®) maintaining this gap fixed as presented in Fig. 1 (panel b). After that, electrodes were sterilized in 75% ethanol for 1 h, and rinsed thoroughly with deionized water followed by a sonication step in deionized water for 20 min. Finally, they were mounted on a suitable porous plug at the top of the flasks.

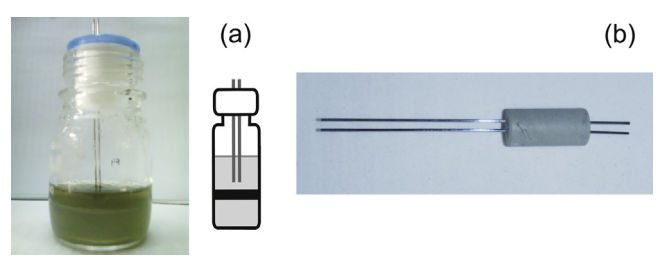

Fig. 1. 100 mL culture flasks containing the SM7 spore-based platform in contact with MMLin used as the EIS cell, sketch of the final montage of the culture cell (a) and the stainless steel electrodes (57 mm length, 1 mm diameter) with a 2 mm separation between them inserted in a commercial gypsum material used for impedance determinations (b).

Lindane determinations by GC-ECD were made with a Hewlett–Packard Model HP 6890 GC equipped with a ^{63}Ni ECD and a HP5 capillary column (Agilent J&W, cross-linked methylpolysiloxane; length, 30.0 m; internal diameter, 0.25 mm; film thickness, 0.25 μm). The chromatographic conditions were: carrier gas (helium > 99% pure) flow rate, 1.5 mL min^{-1}; injector temperature, 200.0 °C; detector temperature, 280.0 °C; and injection volume 1.0 μL (splitless mode). At a first stage, column was purged at 300.0 °C to remove any attached impurities. The oven temperature program began at 80.0 °C and increased to 150.0 °C at 10.0 °C min^{-1} for 8 min, then increased to 200.0 °C at 7.0 °C min^{-1} for 5 min, after that increased to 250.0 °C at 10.0 °C min^{-1} for 5 min and finally increased to 280.0 °C at 20.0 °C min^{-1} for 1.86 min. Under these conditions, the lindane retention time was 42 min. The detection limit was 10 μg L^{-1} of lindane and the average percentage recovery of lindane was 85.8 ± 5.2%. Data analysis was performed with HP 3398A GC Chemstation A.01.01 (Hewlett–Packard 1998) software.

2.4. Experimental

EIS determinations of bacterial activity were made in the culture flasks employing a bipolar electrode configuration (Fig. 1b). 20 mL MMLin were added over previously prepared SM7 spore-based platform (1 × 10^7 U.F.C. mL^{-1} initial concentration inoculums) and cultured at 30.0 °C with and without shaking for 48 h. Electrodes were submerged 10 mm in the liquid medium, close (without touching) the spore-based platform. Two types of reference controls were also prepared: (a) non-inoculated SCA in contact with MMLin (SCA + MMLin) and (b) inoculated SCA in MM without additional carbon source (SM7 + MM). Considering that chloride ions were expected to be released to the culture medium during SM7 activity in the presence of lindane, impedance spectra were also obtained through successive additions of a concentrated chloride solution (10 mg L^{-1}) to MMLin to reach a range between 0 and 750 μg L^{-1}. Each experiment was carried out by duplicate and the results are informed as the arithmetic means.

Bacterial activity was evaluated by changes in the impedance spectra measured in the 1 Hz to 65 kHz AC frequency range with a small amplitude sine wave voltage (20 mV) [62]. Impedance spectra were recorded for each cell at 0, 20 and 40 h incubation time at 30.0 °C. For each measurement, eight cycles were averaged registering seven points for decade at 25 °C.

In addition, daily supernatant samples of centrifuged cultures (9.900 × *g*, 10 min) were used to determine residual lindane concentration during 48 h of cultivation. Lindane determination was carried out by GC-ECD, the recommended standard method by American Public Health Association [63]. The pesticide was extracted from the culture supernatants by liquid–liquid extraction method with hexane. These samples were kept at −18.0 °C and defrosted before chromatography measurements at 25.0 °C. Quantitative analysis of samples was performed by using appropriate lindane calibration standards prepared in MM, comparing the residual lindane concentration to SCA + MMLin, and also considering the lindane sorption percentage in SCA. All the manipulations were made under sterile conditions.

3. Results and discussion

EIS measurements were used in the sensor prototype, in order to overcome the drawbacks encountered when only two fixed frequencies were used to detect SM7 metabolic activity in the presence of lindane [58].

When a whole lindane molecule (γ-hexachlorocyclohexane) is degraded (dechlorinated), six chloride ions are released to the medium, which mainly modifies the conductivity of the solution.

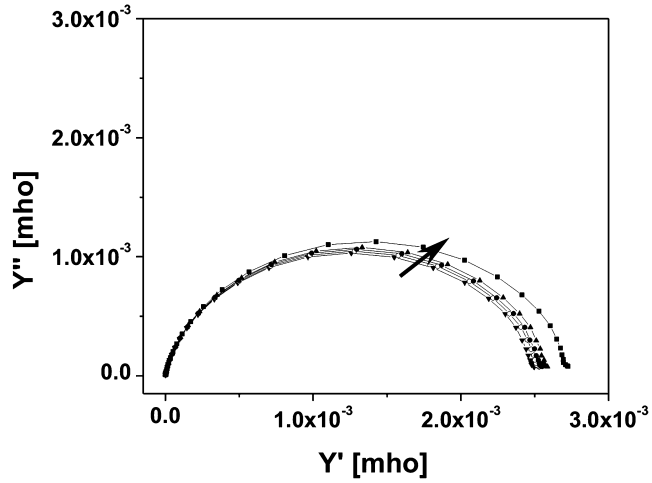

Fig. 2. Argand diagrams of admittance for successive additions of a concentrated chloride ions solution (10 mg L^{-1}) to MMLin. The arrow points out the increase in chloride ions concentration from 0 to 750 μg L^{-1}. EIS measurements were performed with stainless steel electrodes (57 mm length, 1 mm diameter) with a 2 mm separation between them at 25 °C.

The concentration of the released ions to the culture medium, measured as conductivity variation, may be implemented as a cost-effective method to quantify the pesticide from EIS measurements. With such a purpose, impedance spectra for successive additions of a concentrated chloride ions solution to MMLin were previously registered. Fig. 2 shows the Argand diagrams of admittance performed in the 0–750 μg L^{-1} chloride concentration range. The maximum value was chosen considering the complete degradation of 1 mg L^{-1} lindane. As pointed out by the arrow in Fig. 2, the admittance slightly increases with an increment in the ionic concentration at low frequencies. Results in Fig. 2 clearly indicate that a chloride concentration as low as 150 μg L^{-1} (the lowest added concentration) produces a change in the Argand diagram. Hence, even though MMLin is a culture medium with a high concentration of different ions, small changes in the chloride concentration can be detected by EIS. Considering the stoichiometry of the total degradation of lindane, 150 μg L^{-1} of chloride ions corresponds to the total degradation of 215 μg L^{-1} of the pesticide for this particular set up (cell and electrodes). Fig. 3 shows the Argand diagrams of admittance obtained for the SCA + MMLin

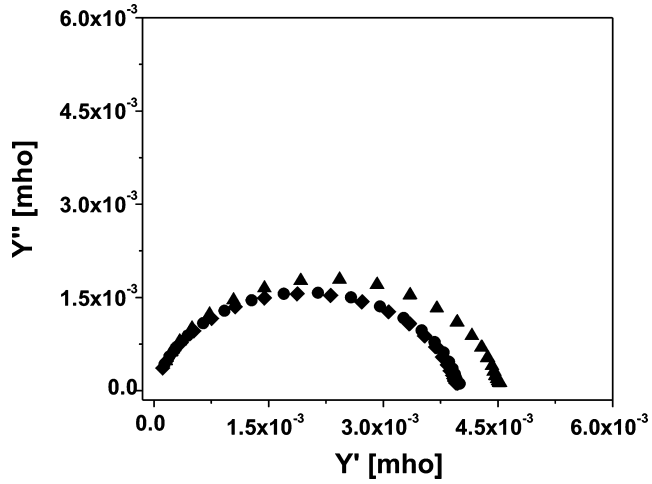

Fig. 3. Argand diagrams of admittance for reference cell of non-inoculated SCA in the presence of MMLin (SCA + MMLin) cultured without shaking at 0 ♦, 20 ● and 40 ▲h incubation time. EIS measurements were performed with stainless steel electrodes (57 mm length, 1 mm diameter) with a 2 mm separation between them at 25 °C.

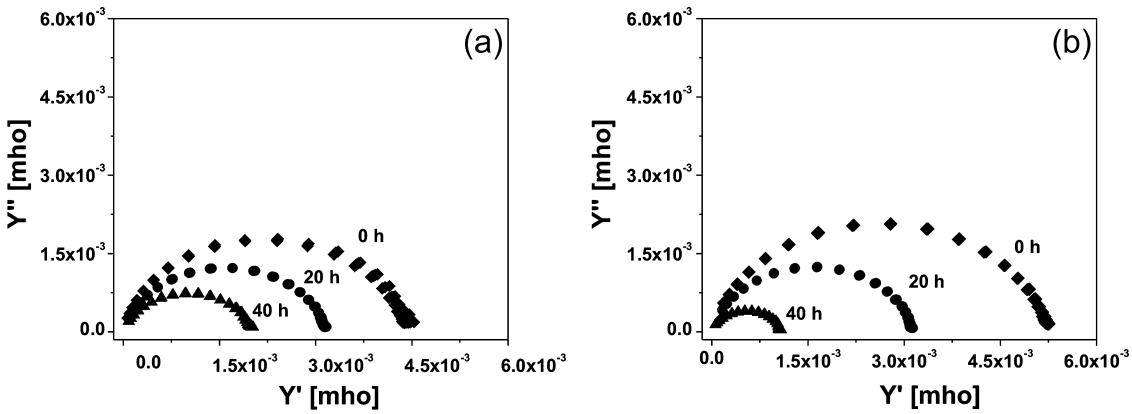

Fig. 4. Argand diagrams of admittance for SM7 spore-based platforms immersed in MMLin at 0 ♦, 20 ● and 40 ▲h incubation time, cultured with (a) and without (b) shaking. EIS measurements were performed with stainless steel electrodes (57 mm length, 1 mm diameter) with a 2 mm separation between them at 25 °C.

cultured without shaking at different incubation times (0, 20 and 40 h), as an example of the reference cell response. The admittance slightly increases as a function of the incubation time. This general behavior was also observed with the other reference cells, suggesting a similar trend as adding ions to MMLin.

Fig. 4 displays the Argand diagrams of admittance of the spore-based platform in the presence of MMLin at 0, 20 and 40 h incubation times, cultured with (A) and without (B) shaking. Although shaking is the optimized culture condition of these microorganisms, it is not practical for the purpose of a biosensor; so it is necessary to evaluate if the culture conditions are compatible with a good activity of the cells. The results showed that in both cases, the admittance strongly diminishes at low frequencies as the incubation time increases, thus shaking can be avoided in this case. Impedance values are highly sensitive to lindane consumption by SM7. However, the trend observed is not ruled by the increment in the concentration of ionic species in the liquid medium, as observed by the addition of chloride ions to MMLin (Fig. 2). Instead, it seems that the metabolic activity produces stronger changes in the electrode-solution interface as indicated by Felice et al. [52,64]. Fig. 5 displays the Bode diagrams corresponding to the impedance measurements shown in Fig. 4, giving the modulus on the left (primary axis) and the phase angle on the right (secondary axis) of the figures. Both the impedance modulus and the angle phase change as a function of the incubation time. The impedance decreases, while the phase trends to a more capacitive value in the low frequencies range, (from 1 to 10^3 Hz). This effect is more evident on the Argand diagrams, where the scale is linear. In this frequency range, the impedance value is dominated by the double layer capacitance [46]. These impedance values decrease logarithmically when increasing the frequency up to 10^3 Hz. At higher frequencies (from 10 kHz to 1 MHz, the resistance region, also evidenced by a phase angle close to 0°) no significant difference is observed, indicating that the detection of SM7 activity in the presence of lindane is only possible below 10 kHz. Some authors have assigned the impedance changes at low frequencies with bacterial adhesion to the electrodes [65,66] or with the growth of microorganisms [64,67,68]. These conditions do not apply to our case because SM7 spores are adhered during the impedimetric measurement; hence, the observed impedance change can only be attributed to the modification of the interface impedance due to metabolic activity of the bacteria.

To better understand the physical meaning of the observed changes, the impedance spectra (Fig. 5) were analyzed using a suitable equivalent electric circuit, the Randles model [69]. Impedance changes were evaluated with the model shown in the inset of Fig. 5 [55,70], commonly used for non-faradaic impedance biosensors [70] as an appropriate equivalent electric circuit when no electroactive species are involved in the biorecognition event [51]. In this equivalent electric circuit, R_s represents the electrolyte solution resistance, R_{ct} represents the charge transfer resistance across the electrode–electrolyte interface, and CPE represents a constant phase element. CPE is used instead of the ideal capacitance; it has no physical meaning, and it takes into account the frequency dispersion of the capacitance value [85]. It was further assumed that the two pairs of electrodes were identical; thus, the equivalent circuit represents both electrodes, which is beneficial for obtaining

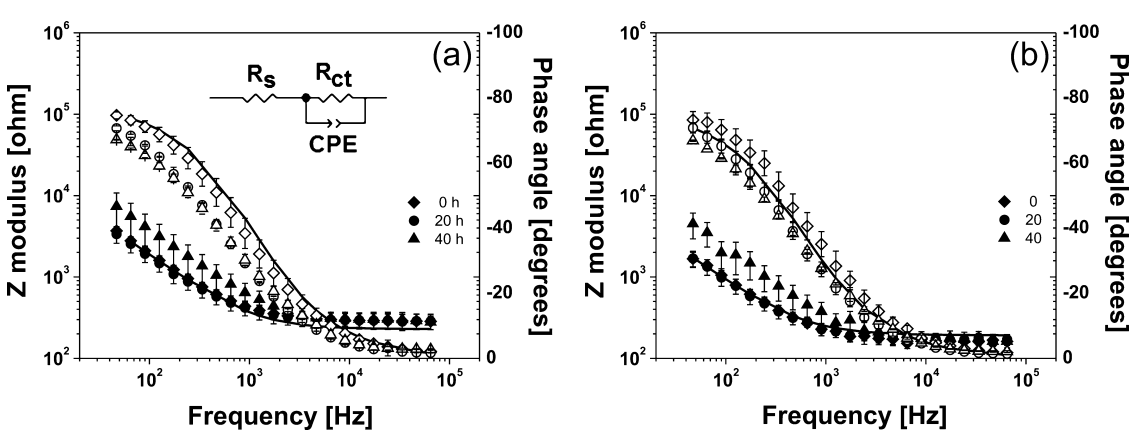

Fig. 5. Bode diagrams for SM7 spore-based platforms immersed in MMLin at 0 ♦, 20 ● and 40 ▲h incubation time, cultured with (a) and without (b) shaking. Open symbols: impedance (*Z*) modulus. Solid symbols: angle phase. Lines correspond to the fitting obtained with the equivalent electrical circuit displayed in the inset. EIS measurements were performed with stainless steel electrodes (57 mm length, 1 mm diameter) with a 2 mm separation between them at 25 °C.

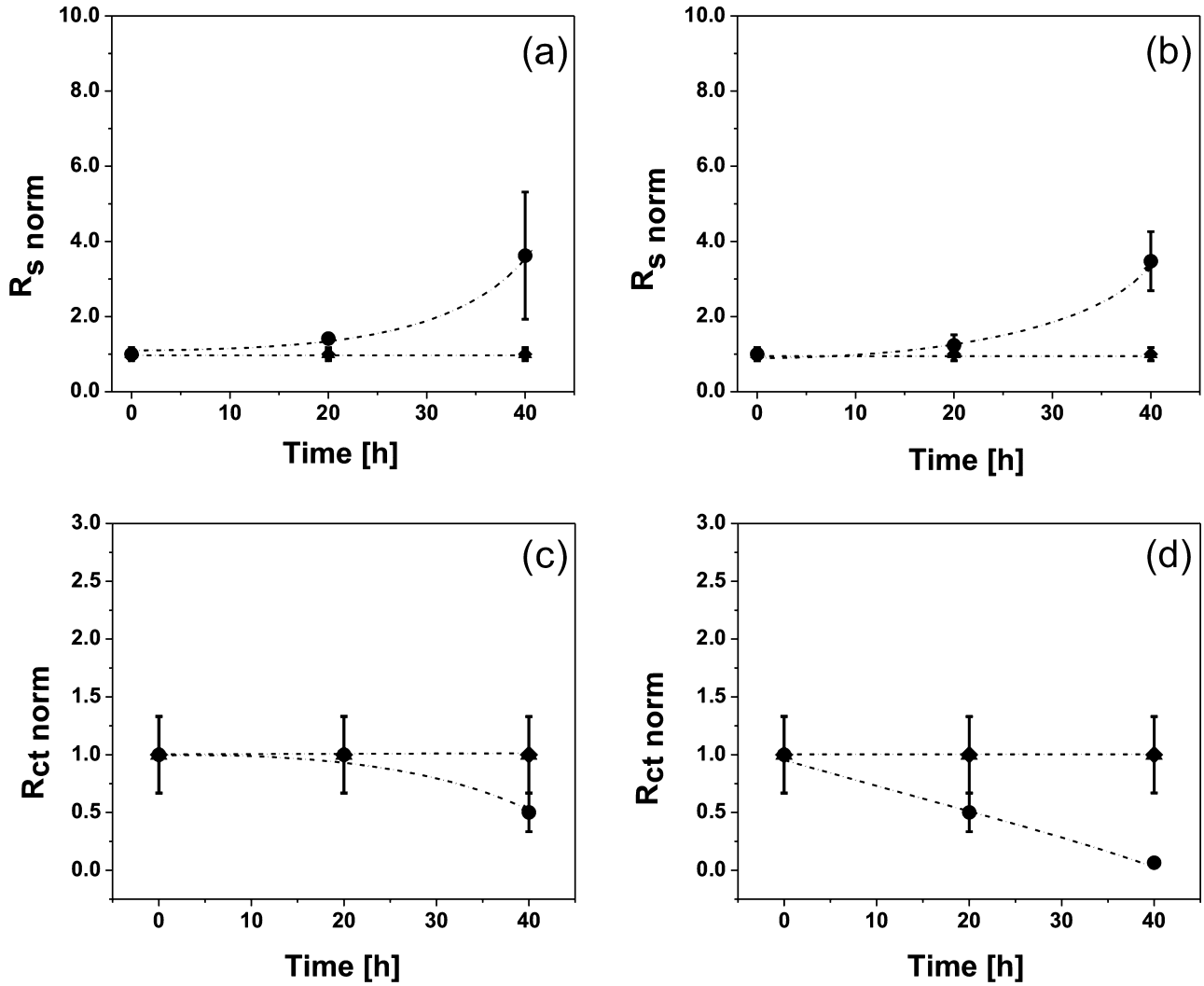

Fig. 6. Normalized R_s (a, b) y R_{ct} (c, d) determined from the Randles circuit for SM7 spore-based platforms immersed in MMLin (a, c) with and (b, d) without shaking, as a function of incubation time. (♦) SCA + MMLin (control 1), (▲) SM7 spores adhered to SCA + MM (control 2).

quantitative parameters during the fitting procedure [70]. As a matter of fact, lines in Fig. 5 show the good agreement between the measured spectra and the fitting curves. This equivalent circuit was used for all the measured spectra, including chloride addition and reference cells. The electrical parameters were calculated with relative errors lower than 1% in the measured frequency range, indicating that the model was suitable and meaningful for this electrochemical system.

Chloride addition to MMLin caused small but noticeable changes in R_s (from 400 to 370 Ω) and in CPE (from 1.47 to 1.72 μF). However, the presence of either SCA or SM7 produced larger differences among reference cells (resulting from small variations in the electrodes, cell set-up or SM7 spore-based platform) than the observed changes when adding ions to MMLin. In fact, the calculated electrical parameters for the impedance spectra recorded with the reference cells (see supplementary information), showed approximately 20% difference among cells, a rather large value compared to the relative errors provided by fitting the spectra. Within experimental error, R_s, R_{ct} and CPE of the reference cells did not change by increasing the incubation time. On the other hand, Fig. 6 shows R_s and R_{ct} normalized against the first measured value as proposed by Farrow et al. [71]. A noticeable change, as function of the incubation time in both culture conditions (with and without shaking), is observed during SM7 metabolic activity in the presence of lindane. The CPE-P value is a parameter linked with the phase angle which oscillates from 1, for planar surfaces, to 0.5 to very rough ones. It is related to the angle of rotation of a purely capacitive line on the complex plane plot. That means, when the CPE-P is 1.0 the CPE can be considered as a pure capacitor [85]. In the present case, CPE-P values were higher than 0.8 indicating a closely capacitive behavior of interface in all the studied conditions, while CPE-T values were constant between 1 and 3 μF. As expected from the Argand diagrams, R_s values increased about three to four times during the 48 h of lindane consumption, whereas R_{ct} drastically dropped orders of magnitude (from 1×10^{13} to 1×10^{6} Ω). Therefore, the processes taking place at the electrode–electrolyte interface [72] controlled the impedance change caused by the spore-based SM7 metabolic activity in the presence of lindane. Diverse mechanisms have been proposed to explain the electrochemical processes that change the impedance measurements due to bacterial activity: (a) the production of electroactive secondary metabolites that facilitate a charge transfer at the electrode–electrolyte interface [73–75], (b) the deposition of biofilm material on the electrode surface that affects capacitance and/or charge transfer, (c) the direct attachment of microbial through pili, flagella and outer membrane proteins that facilitate charge transfer reactions [65,76,77], (d) the presence of microbial cells in close proximity to the electrode surface, (e) the breakdown of nutrients within the electrolyte [58,64,73,78,79], and

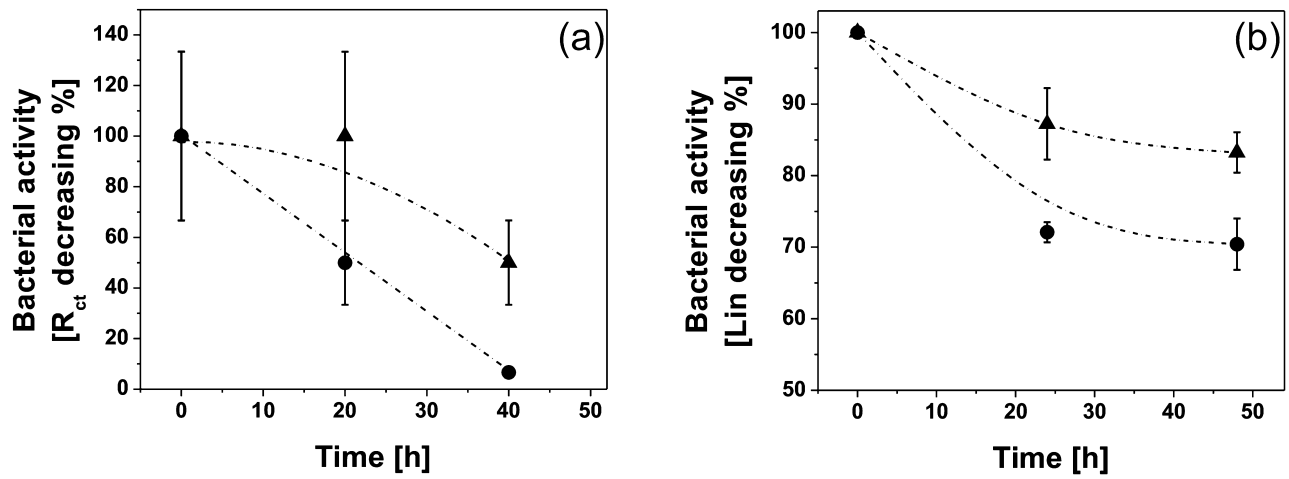

Fig. 7. Bacterial activity as a function of the incubation time for SM7 spore-based platforms immersed in MMLin, cultured with (▲) and without (●) shaking, determined from the percentage of R_{ct} (a) and lindane residual concentration as measured by GC-ECD (b). Lines only point out data trend.

(f) the adsorption of metabolites (proteins, macromolecules, etc.) on the electrode surface [80–82]. Taking into account the particular features of the proposed impedimetric set up, some of these mechanisms (like bacterial adhesion to the electrode or its presence in MM) do not operate with the spore-based platform. On the other hand, either the production of electroactive metabolites and/or the breakdown of nutrients during lindane consumption by the SM7 spore-based platforms may drastically reduce R_{ct}.

Fig. 7 presents the bacterial activity of the SM7 spore-based platform in the presence of MMLin (cultured with and without shaking) as a function of the incubation time, stated as the percentage of (A) R_{ct} (calculated using 1×10^{13} Ω as 100%) and (B) the residual lindane concentration (determined by GC-ECD). Lindane concentration decreased about 30% from the initial value in 48 h. Our previous studies showed that SM7 consumed 10.0 g L^{-1} of glucose in the same period of time [58]. Hence, lindane degradation is slower than glucose consumption as sole carbon source. This is expected since pesticide degrading mechanism needs to be activated in the absence of glucose before the metabolic process begins [83]. Table 1 compares the percentage of R_{ct} to the lindane concentration consumed by the SM7 spore-based platform cultured without shaking at different incubation times. Within 2 days, R_{ct} suffered a sharply drop nearly 90% from the initial value. However, as can be noticed in the table this fall does not mean a total consumption of the pesticide from the culture medium. As a matter of fact, the table shows that a 50% reduction of R_{ct} is due to the consumption of 120 μg L^{-1} lindane by the SM7 spore-based platform. Therefore, EIS is a sensitive method to evaluate SM7 metabolic activity allowing perceiving lindane concentrations close to environmentally found values [84] in less than 2 days. EIS coupled to culture flasks operating as cells with a pair of stainless steel electrodes can be successfully used to monitor the activity of adhered SM7 spores to SCA in the presence of a liquid culture medium, such as MM supplemented with lindane as a sole carbon source without shaking.

Table 1
The percentage of R_{ct} compared to the lindane concentration consumed by the SM7 spore-based platform cultured without shaking as a function of the incubation time.

Time [h]	%R_{ct}	Lindane concentration [μg L^{-1}]
0	$(1.0 \pm 0.2) \times 10^2$	$(4.6 \pm 0.1) \times 10^2$
20	$(5 \pm 2) \times 10^1$	$(3.4 \pm 0.1) \times 10^2$
40	$(7 \pm 1) \times 10^0$	$(3.2 \pm 0.1) \times 10^2$

4. Conclusions

In this work, we presented results regarding evaluation of a previously designed and prepared biofunctional surface based on a platform of adhered SM7 spores to be used in a sensor prototype to detect and quantify OCPs. By means of impedance spectroscopy, the metabolic bacterial activity was evaluated by analyzing a set of electrical parameters calculated by fitting the measured impedance records through an equivalent electrical circuit. Activity studies were performed by incubating the SM7 spore platform with and without shaking in the presence of lindane as an analyte of OCPs. In all cases bacterial activity was found by the impedance changes produced in the cells due to lindane removal from the culture medium. Our proposed label-free impedimetric biosensor is simple, cost-effective and feasible to detect changes in the concentration of only 120 lindane μg L^{-1} in less than 2 days without sample pretreatment.

The released of ionic species to the liquid culture medium set up, changes the impedance in the electrochemical system that can be registered by impedance spectroscopy. These impedance modifications allow perceive and quantify lindane in the culture medium easily and economically even in small concentrations. The surface of electrodes can be reused for several different tests as there is no biorecognition element used on the surface of electrodes. In addition, our proposed design does not require a sample pretreatment for pesticide extraction of the supernatants regularly used in chromatographic methods of analysis.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.snb.2014.10.030.

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