1 **A MINIATURIZED BISMUTH-BASED SENSOR TO EVALUATE THE MARINE**
2 **ORGANISM *STYELA PLICATA* BIOREMEDIATION CAPACITY TOWARD HEAVY**
3 **METAL POLLUTED SEAWATER**

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ABSTRACT

Cadmium and lead are highly toxic heavy metals which cause a severe worldwide pollution. In addition to the toxic effect produced by the direct exposure, they can be bioconcentrated and accumulated in living organisms, including humans. Herein, a miniaturized and disposable electrochemical sensor was improved for the simultaneous detection of cadmium and lead ions to study the bioremediation of polluted seawater in presence of the filter-feeding marine organism *Styela plicata*. A screen-printed electrode modified *in situ* with a bismuth film was selected using the anodic stripping analysis as detection technique. This sensor was coupled with a portable potentiostat and the detection of cadmium and lead ions was carried out by Square Wave Anodic Stripping Voltammetry, allowing the simultaneous detection of both heavy metals at ppb level (LOD = 0.3 ppb for lead, 1.5 ppb for cadmium).

This analytical tool was then applied to assess the bioremediation capacity of *S. plicata* through a bioremediation experiment, in which the organism has been exposed to seawater artificially polluted with 1000 ppb of Cd^{2+} and Pb^{2+} . The matrix effect of both seawater and acid digested biological samples was evaluated. A bioconcentration phenomenon was observed for both heavy metals through the analysis of *S. plicata* tissues. In details, Pb^{2+} resulted to be about 2.5 times more bioconcentrated than Cd^{2+} , giving an effective bioremediation level in seawater of 13% and 40% for Cd^{2+} and Pb^{2+} , respectively. Thus, our results demonstrate the capability of *S. plicata* to bioremediate Cd^{2+} and Pb^{2+} polluted seawater as well as the suitability of the electrochemical sensor for contaminated marine environment monitoring and bioremediation evaluation.

Keywords

Bioremediation, Lead and Cadmium pollution, Ascidians, Environmentally-friendly electrochemical sensor, *In situ* analysis

1. Introduction

Heavy metals are among the most dangerous pollutants because of their high toxicity for living organisms and for their low biodegradability, due to their persistence and accumulation in the environment (Glorennec, 2006; Valls and Lorenzo, 2002). Among heavy metals, cadmium and lead are non-essential elements with well-known toxic effects on humans (Godt et al., 2006; Gracia et al., 2007; Fewtrell et al., 2003; International Agency for Research on Cancer, 2006. Summaries & evaluation: Inorganic and organic compounds, Vol. 87. <http://www.inchem.org/documents/iarc/vol87/volume87.pdf>; Satarug et al., 2010). They are ubiquitous, according to their diffuse utilization in human activities, such as industrial processes, mining and metallurgic sectors, incinerators, batteries, pigments, antifouling and anticorrosive coatings (Glorennec et al, 2006; Kustin et al, 1983). Although some countries banned many products that have been important sources of these metals, they are still widely present in the world. Thus, cadmium and lead pollution is an important problem that needs to be faced by elaborating innovative strategies for the monitoring and the remediation of the contaminated environment. In this scenario, innovative approaches, which exploit different scientific disciplines, are designed to study and furnish sustainable solutions in bioremediation field.

91 Aquatic environments are worthy of note because they simultaneously represent accumulation
92 reservoirs and secondary sources of pollutants, such as heavy metals. The Marine Strategy
93 Framework Directive (MSFD, Directive 2008/56/EC of the European Parliament and of the
94 Council), which is the European regulation for assessment of the good environmental status of
95 marine system (European Commission, 2008), promotes an ecosystem-based approach, considered
96 as important requirement for sustainable management and environmental monitoring programs.
97 Thus, some recent studies are based on a combination of chemical contaminant measurements and
98 biological response (Hagger et al., 2008; Thain et al., 2008). There are many strategies to remediate
99 contaminated waters, based on mechanical, chemical, and biological techniques. The biological
100 approach involves the *bioremediator* organisms, which can naturally concentrate in their tissues
101 some kinds of pollutants (process known as *bioconcentration* phenomenon) and remove them from
102 the surrounding environment (Gifford et al., 2007). Furthermore, they may be able to give
103 recognizable responses about the contamination level (*bioindicators* organisms) or even degrade the
104 pollutants (Patron et al., 2013; Aresta et al., 2015). The use of bioremediators for the
105 decontamination of the environment is known as *bioremediation*: it is an ecosystemic approach as it
106 takes into consideration the role that each living and non-living component of an ecosystem plays in
107 it, giving information on the bioavailability of pollutants, on their direct effects on organisms and
108 on trophic relations.

109 In this work, the bioremediation capacity of *Styela plicata* (Tunicata, Ascidiacea) for lead and
110 cadmium contaminated seawater was investigated. This organism belongs to the group of benthic
111 filter-feeding organisms, which live anchored to the hard bottom and retain nutrients from waters to
112 feed. *S. plicata* grows abundantly as a fouling organism on natural seabeds, as well as on
113 submerged man-made structures and on artificial substrates for shellfish and fish culture. These
114 suspension-feeding organisms are known to show a high bioconcentration capacity, due to their
115 well developed gill structure, the presence of vanadocytes and the absence of kidneys, and to have a
116 high filtering activity with respect to other ascidian organisms (Thiyagarajan and Qian, 2013). The
117 possibility of exploiting the combination of their abundant growth in polluted coastal areas and their
118 ability to remove contaminants from seawaters has generated considerable interest. *S. plicata* is
119 characterized by good properties as bioremediator and has been the subject of many studies for the
120 bioconcentration of cadmium, lead, arsenic, mercury and heavy metal compounds in natural
121 environments (Aydın-Önen, 2016; Bellante et al., 2016; Choi et al., 2014; Philp et al., 2003;
122 Radford et al., 2000;), as well as bacteria (Stabili et al., 2016) and for the biodegradability of some
123 organic chemicals (Cestone et al., 2008). It has been reported that they can bioconcentrate persistent
124 compounds present in the aquatic environment, resulting quite resistant to their toxic effects
125 (Petersen, 2007; Sumerel and Finelli, 2014; Abdul et al., 2015). However, to our knowledge, its
126 capacity to bioconcentrate Cd^{2+} and Pb^{2+} under a time-controlled exposure to seawater artificially
127 contaminated with known quantities of both the heavy metals has never been evaluated.

128 Herein, we present for the first time a time-controlled bioremediation experiment, in which *S.*
129 *plicata* specimens were exposed to known concentrations of Cd^{2+} and Pb^{2+} . In addition, the
130 bioremediation study was carried out using both reference methods and an innovative and
131 miniaturized electrochemical sensor. In details, a cost-effective, portable and easy-to-use device
132 based on a bismuth modified screen-printed electrode associated with a portable potentiostat was
133 used as an environmentally friendly analytical tool. This experimental setup was successfully
134 employed by our research group for the detection of cadmium and lead in fresh water and for
135 phytoremediation study using *Lemna Minor* aquatic plant (Neagu et al., 2014). The use of bismuth

permits the measurement of cadmium and lead without the removal of dissolved oxygen, necessary for the more traditional mercury-based electrodes, providing a wide negative potential window (until -1 V), suitable for the detection of these cations (Economou, 2005; Wang, 2005; Kokkinos and Economou, 2008; Svancara et al., 2010); moreover bismuth is considered much less toxic than mercury (Armenta et al., 2008; Economou, 2005; Wang, 2005; Arduini et al., 2010). Such electrodes can be obtained either by *in situ* or *ex situ* deposition of Bi^{3+} , or even by using bismuth-based materials, and many kinds of substrates can be employed (Arduini et al., 2010). In this work, screen-printed graphite electrodes modified with Bi^{3+} by *in situ* electrodeposition and a Nafion polymeric films have been chosen since characterized by high sensitivity (Calvo Quintana et al., 2011). Nafion/bismuth-modified sensor performances (Naf/Bi-SPE) were firstly investigated and then used to measure Cd^{2+} and Pb^{2+} both in seawater and in biological tissues of *S. plicata*, after the exposure of the organisms to artificially contaminated seawater. To evaluate the responses obtained by the sensor, benchmark methods have been used for comparison (ICP-MS and ICP-OES). In this way, the bioremediation ability of *S. plicata* with respect to Cd^{2+} and Pb^{2+} has been estimated within a time-controlled bioremediation experiment.

2. Experimental section

2.1. Reagents and apparatus

Nitric acid ($\text{HNO}_3 \geq 69\%$), sodium hydroxide ($\text{NaOH} \geq 30\%$), sodium chloride (NaCl) and sodium acetate anhydrous ($\text{CH}_3\text{CO}_2\text{Na}$) were obtained from Fluka (Trace Select). Bismuth (BiCl_3 1 mg/mL in 1% p/v HCl) and lead standard stock solutions ($\text{Pb}(\text{NO}_3)_2$ 1000 $\mu\text{g/mL}$ in 1% p/v HNO_3) were acquired from Carlo Erba, whereas cadmium standard stock solution ($\text{Cd}(\text{NO}_3)_2$ 1007 $\mu\text{g/mL}$ in 1.2% p/v HNO_3) was acquired from Sigma Aldrich. Commercial available Nafion[®] 117 (5% v/v in ethanol) was obtained from Aldrich. All the solutions were prepared using double-distilled water (by a Milli-Q system, Millipore). Teflon or polyethylene were chosen for containers (beakers, vessels, tubes, pipettes, round-bottomed flasks). All containers were soaked with HNO_3 3% v/v for at least 4 hours before utilization. PVDF filters (Minisart[®], Sartorius Stedim, Biotech) of 0.45 μm pore size were employed for filtering seawater and biological samples. Square-Wave Anodic Stripping Voltammetry was performed by means of a portable potentiostat, PalmSens[®] (Palm Instrument, The Netherlands), connected to a personal computer. Spectrometric analysis was carried out by means of Agilent 7500 Series ICP-MS instrument and Perkin Elmer Optima 8300 ICP-OES instrument. Microwave-assisted acid digestion was carried out with Anton Paar Multiwave 3000. *S. plicata* specimens and seawater samples were collected in natural areas of Central Tyrrhenian Sea along the Latium coast (Italy) and kept in the aquariums of the Aquaculture and Ecology laboratories (Biology Department of University of "Tor Vergata") until their involvement within the bioremediation experiments.

2.2. Screen-printed electrode preparation

Screen-printed electrodes were home-produced with a 245 DEK (Weymouth, UK) machine. Working and counter-electrodes were printed using graphite-based ink (Electrodag 421) and pseudo-reference electrode was printed using silver-based ink (Electrodag 477 SS), both from Acheson (Milan, Italy). Electrodes were printed on a flexible polyester substrate (Autostat HT5)

from Autotype Italia (Milan, Italy), with thickness of 0.175 mm. An insulator (Argon Carbonflex 25.101S) was used to define a working surface area, which results in a diameter of 3 mm. The printing procedure used has already been described in a previous paper (Arduini et al., 2012).

2.2.1. Amperometric pre-treatment

Screen-printed electrodes were pre-treated through an amperometric process in which an oxidative potential of 1.6 V is applied for 300 s to the sensor, covered by a drop of acetate buffer 50 mM + 50 mM NaCl at pH 4.6, utilizing a portable potentiostat PalmSens® (Palm Instruments, The Netherlands). Subsequently, the sensor was rinsed with double-distilled water and dried at room temperature.

2.2.2. Nafion drop-casting

The dried sensors were then modified with 5 µL of Nafion solution (0.5% v/v) by drop-casting and immediately placed in a heater at 37 °C for 30 min to form a nanometric polymeric film (Paul et al, 2013; Li et al., 2011) through this thermal treatment. Hence, the sensors were stored at room temperature until their use.

2.3. Heavy metal detection by stripping analysis

All the voltammetric measurements were performed in 5 mL of acetate buffer 50 mM + NaCl 50 mM at pH 4.6 in beakers made of Teflon. Bi-SPEs were obtained by *in situ* electrodeposition of bismuth into 5 mL of acetate buffer solution containing 0.5 ppm of Bi³⁺ (selected concentration, as described later). A first film of metallic bismuth was created during the blank recording at an applied potential of -1.0 V, then various quantities of Cd²⁺ and Pb²⁺ from standard solutions were simultaneously added and measured. During each measurement, a new Bi-layer was overlaid on the electrode surface, favoring the accumulation of cadmium and lead. Anodic stripping voltammetry technique with square wave mode (SW-ASV) was chosen for the electrochemical experiments. Parameters and conditions of the three conventional phases of this technique (accumulation, rest period and stripping) were investigated. The definitive SW-ASV parameters are: E_{begin/deposition} = - 1 V; E_{end/condition} = - 0.4 V; t_{deposition} = 300 s; t_{equilibrium} = 10 s; E_{step} = 0.005 V; E_{amplitude} = 0.02 V; frequency = 50 Hz. The system was coupled with a switch box (Palm Instruments, The Netherlands), which automatically controlled the magnetic stirring during the voltammetric phases, turning on the stirring only during the conditioning and the deposition periods. The experimental setup was reported in Figure 1.

Calibration curves were obtained in order to extract linear relations for both cadmium and lead and the analytical features of the sensor were determined.

Fig. 1. Experimental setup composed of portable potentiostat (PalmSens®) associated with personal computer. The electrochemical sensor, which is connected to the system by an electrical contact, is also shown in the inset. During the measure, the sensor is immersed into a beaker, where the solution is stirred by means of a magnetic plate and the stirring is controlled through a suitable device (switchbox).

2.4. Heavy metal detection by ICP-OES

For ICP-OES measurements, all seawater samples were filtered with 0.45 μm pore size PVDF filters, diluted 1:20 (v/v) with double-distilled water and acidified with 1% (v/v) of HNO_3 . The experiments were carried out with standard parameters: RF power 1400 W; plasma gas (Ar) flow rate 15 L/min; auxiliary gas (air) flow rate 0.2 L/min; nebulizer gas (Ar) flow rate 0.55 L/min. A Meinhard nebulizer with cyclonic spray chamber was employed to introduce the sample into the plasma. The emission wavelengths considered were: 214.440 and 226.502 nm for Cd; 220.353 nm for Pb.

2.5. Heavy metal detection by ICP-MS

For ICP-MS measurements, all samples from *S. plicata* were filtered with 0.45 μm pore size PVDF filters, diluted 1:50 (v/v) with double-distilled water. The experiments were carried out with standard parameters: RF power 1500 W; plasma gas (Ar) flow rate 15 L/min; make-up gas (He) flow rate 10 L/min; nebulizer gas (Ar) flow rate 1 L/min. A Meinhard nebulizer with cyclonic spray chamber was employed to introduce the sample into the plasma. The analytical masses (amu) chosen were: ^{159}Tb as internal standard for ^{111}Cd ; ^{175}Lu as internal standard for ^{206}Pb .

2.6. Seawater sample treatment

Seawater samples were collected from the aquarium or from the containers used during the bioconcentration experiments and stored at 4 °C until analysis. Various dilutions (v/v) were performed in order to eliminate any seawater matrix effect: 1:2, 1:5, 1:10, 1:20, 1:50 (v/v) in acetate buffer for SW-ASV. For ICP-OES analysis, seawater samples were filtered before 1:20 dilution (v/v) in double-distilled water and then acidified with 1% HNO₃. The content of cadmium and lead in seawater samples was calculated through the corresponding calibration curves and then corrected with the dilution factor.

2.7. Biological sample treatment

In order to analyze heavy metals bioconcentrated into *S. plicata* specimens, biological samples were taken from the organisms tissue and subjected to microwave-assisted acid-digestion. Animals were selected from the aquarium before and after the bioremediation experiments and then frozen until analysis. Subsequently, they were defrosted and weighed (wet weight, ww), then 0.3 g of tissue samples were cut and digested in 10 mL of ultrapure HNO₃. A vessel with only ultrapure HNO₃ was used as a control. The digestion was carried out with a microwave oven (Anton Paar Multiwave 3000) using the following parameters: pressure rate 0.3 bar/s; IR 210 °C; power 1220 W. After the digestion, samples were filtered with 0.45 µm PVDF and filled up to 15 mL with double-distilled water. Digested biological samples were stored at 4 °C until their use. Samples were analyzed through SW-ASV after dilution 1:50 (v/v) in acetate buffer, and through ICP-MS after filtration and dilution 1:50 (v/v) in ultrapure water. The content of cadmium and lead in *S. plicata* samples was calculated through the corresponding calibration curves and then corrected with the dilution factor.

2.8. Bioremediation experimental setup

Three specimens of *S. plicata*, having similar sizes (about 10 g, ww) and belonging to the same sampling from their natural environment, were chosen from the storage aquarium, in the same condition, and employed for bioremediation experiments. Hence, 4 polyethylene beakers, previously cleaned with HNO₃ 3% (v/v), were filled with 1,5 L of seawater and spiked with 1 ppm of Cd²⁺ and Pb²⁺. One specimen of *S. plicata* was carefully placed at the bottom of each beaker, except one that was used as the blank, taking care of their stability. A tube system was designed and used to insufflate air in each beaker, in order to oxygenate the internal environment. Beakers were sealed with additional beakers and polyethylene film, in order to avoid the seawater evaporation and the loss of heavy metals due to the blown air. The time-zero of the bioconcentration experiment was taken as the organisms appeared to be acclimatized (when they showed well-opened siphons). Then, seawater samples at 0 h, 24 h, 168 h were collected and immediately analyzed. Seawater samples to be subsequently analyzed with ICP-OES were filtered and stored at 4 °C. Organisms were all collected at 168 h, when they still showed opened siphons, rinsed with double-distilled water and frozen. A picture of the bioremediation experiment setup is reported in Fig. 2. The quantities of Cd²⁺ and Pb²⁺ accumulated inside *S. plicata* tissues were calculated using the following equation:

$$[HM_{\text{conc.sol.}} (\mu\text{g/L}) * 0.015 \text{ L}] / m_{\text{tissue}} (\text{g}) = HM_{\text{conc.tissue}} (\mu\text{g/g})$$

where the concentrations of cadmium and lead in tissue were obtained from the concentration measured in acid digested solutions, corrected for the dilution to 15 mL and normalized for the wet weight of the sampled tissue.

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Fig. 2. Picture of the bioremediation experiment setup. 1, 2, and 3 represents three replicates of the microsystem containing one specimen of *S. plicata*, while 4 represents the blank.

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2.9. Statistical analysis

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Statistical comparison of Cd^{2+} and Pb^{2+} concentrations obtained with the electrochemical sensor and the spectroscopic reference methods (ICP-OES and ICP-MS) was carried out using the non-parametric rank test Wilcoxon for each sample of seawater and tissues. The null hypothesis assumed is that there is no significant difference between data found by the electrochemical sensor and those by reference methods. The statistical analyses were performed using the program PAST (Paleontological Statistics 3.12).

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3. Results and discussion

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3.1. Study of experimental variables

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In order to develop a sensitive sensor to be applied in seawater matrix, Naf/Bi-SPE performances were improved starting from our previous work, where the sensor was applied for phytoremediation studies in fresh water (Neagu et al., 2014).

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3.1.1. Amperometric pre-treatment

Before their use, bare sensors were treated by applying an oxidative potential to eliminate heavy metals in inks and to activate the electrode surface (Calvo Quintana et al., 2011). Different applied potentials and oxidative times were investigated. The oxidative potentials were studied in the range 1.4 - 1.8 V (Fig. S1, A), showing an increase in sensitivity at the increment of the applied potential, while at 1.8 V no response was observed; then the applied potential of 1.6 V was selected. Several treatment times were evaluated, observing a typical bell-shaped behavior; 300 sec was selected as a compromise between sensitivity and repeatability (Fig. S1, B).

3.1.2. Nafion and bismuth modification

After the amperometric treatment, sensors were modified with Nafion[®] solution, since the Nafion film improves mechanical resistance of the working electrode as well as reduces the fouling of the electrode by organic components (Hoyer et al., 1987; Torma et al., 2008). Successively, the sensor was modified with bismuth film by *in situ* electroplating. This approach was selected since it allows to obtain more sensitive detection of Cd²⁺ and Pb²⁺ than the *ex situ* electroplating (Rico et al., 2008; Calvo Quintana et al., 2011), in which Bi-films are created out of the target solution. In the following section the study of crucial parameters such as Bi³⁺ concentration, deposition time, pulse frequency, equilibrium time, and step potential was carried out to achieve an accurate measurement of Cd²⁺ and Pb²⁺. For the time of conditioning, pulse potential amplitude, and speed of stirring, values were selected from our previous study and reported in Table 1 (Neagu et al., 2014).

3.1.3. Bi film study and SW-ASV parameters

Bi³⁺ concentration determines the thickness of the Bi-film which is formed during the deposition phase of the ASV. The Bi-film thickness affects the sensitivity of the sensor, because a too thick film disfavors the electron transfer at the working electrode surface, while a too thin film may result in an insufficient covering of the electrode surface. Usually, Bi³⁺ concentration must be about one order of magnitude higher than the analyte concentration (Svancara et al., 2003). Repeatability can also be affected by Bi³⁺ concentration, so it must be improved both in terms of sensitivity and repeatability. The study of the experimental values was performed in acetate buffer, which is known to be the ideal medium for Bi-sensors (Hocevar et al., 2005; Arduini et al. 2010). As shown in the Fig. 3A, the best compromise between sensitivity and repeatability for both heavy metals tested was observed at 0.5 ppm of Bi³⁺. This concentration is in agreement with the values (400 to 1000 ppb) reported in literature (Kefala et al., 2004), and it is sufficiently in excess with respect to the expected analyte concentrations.

Effect of the deposition time was then investigated. This parameter plays an important role in stripping voltammetry, because analytes are concentrated from the solution onto the working electrode surface during this period, allowing a higher sensitivity than the other voltammetric techniques. For longer deposition times, lower detection limits can be achieved, since a larger quantity of analytes is reduced at the working electrode surface. As can be seen in the Fig. 3B, the maximum current intensities were obtained for a deposition time of 10 min, but 5 min was chosen as a compromise between sensitivity and a short time of analysis. Moreover, for a longer deposition time, a broadening of peaks and hence a loss of resolution were observed.

Pulse frequency of the square wave function and potential step increment were also studied, since they can contribute to the resulting sensitivity and the resolution of peaks. By increasing the pulse frequency, an increment of peak height was observed but also a decrease of repeatability and peaks resolution. Thus, the value of 50 Hz was selected, as a compromise between sensitivity and

359 repeatability (Fig. 3C). For the step potential, which is the increment between each potential pulse,
 360 the best compromise among sensitivity, repeatability, and well-resolved peaks were observed for 5
 361 mV, thus this value was selected (Fig. 3D).
 362 Finally, the effect of the equilibrium time was also investigated. This is the time between the
 363 deposition and the stripping period, when potential as well the stirring were not applied, favoring
 364 the homogenization of the metallic film formed on the working electrode surface. The time of 10
 365 sec was chosen (data not shown).
 366 The definitive SW-ASV parameters resulting from this study for cadmium and lead ion detection
 367 are summarized in Table 1.
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369 **Fig. 3.** Study of the fundamental parameters of the SW-ASV analysis: Bi³⁺ concentration in the
 370 electro-plating solution (A), deposition time (B), frequency (C) and step potential (D) of the SW
 371 pulses. Currents have been recorded in duplicate in acetate buffer 50 mM with NaCl 50 mM, pH
 372 4.6, containing 30 ppb of Cd²⁺ (black bars) and Pb²⁺ (grey bars), by SW-ASV. Initial parameters:
 373 E_{cond}= -0.4 V, t_{cond}= 30 s, E_{dep}= -1.0 V, t_{dep}= 300 s, t_{eq}= 20 s, E_{begin}= -1.0 V, E_{end}= -0.4 V, E_{pulse}=
 374 0.02 V, E_{step}= 0.01 V, Frequency= 100 Hz. Resulting parameters: E_{cond}= -0.4 V, t_{cond}= 30 s, E_{dep}= -

376 1.0 V, $t_{\text{dep}} = 300$ s, $t_{\text{eq}} = 10$ s $E_{\text{begin}} = -1.0$ V, $E_{\text{end}} = -0.4$ V, $E_{\text{pulse}} = 0.02$ V, $E_{\text{step}} = 0.005$ V, Frequency=
377 50 Hz).

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379 **Table 1.** SW-ASV parameters used in this work for the detection of Cd^{2+} and Pb^{2+} in acetate buffer
380 50 mM with NaCl 50 mM, pH 4.6, and containing 500 ppb of Bi^{3+} .

Phase	Potential (V)	Time (s)	Square Wave	
Conditioning	-0.4	30	Pulse Frequency (Hz)	50
Deposition	-1	300	Pulse Amplitude (V)	0.02
Equilibrium	/	10	Step Potential (V)	0.005
Stripping	-1 to -0.4	3		

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383 *3.2. Analytical performances in standard solution*

384 The analytical features of the sensor were characterized using the selected experimental conditions
385 (Table 1). Calibration curves were obtained in acetate buffer with 0.5 ppm of Bi^{3+} and by additions
386 of Cd^{2+} and Pb^{2+} from standard solutions. Typical voltammograms, obtained during a calibration
387 study, are shown in Fig. 4A. The linear relationship, calculated as the average of three sensors, is
388 reported in Fig. 4B. LOD ($S/N = 3$) and LOQ ($= 3.3\text{LOD}$) are calculated as the minimum
389 concentration detectable and quantifiable respectively. The overall analytical features are reported
390 in Table 2.

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394 **Fig. 4.** Voltammograms and corresponding calibration curves obtained by SW-ASV for
395 consecutive additions of Cd^{2+} and Pb^{2+} in acetate buffer 50 mM with NaCl 50 mM, pH 4.6, and
396 containing 500 ppb of Bi^{3+} : 5 ppb (red line), 10 ppb (blue line), 15 ppb (green line), 20 ppb (orange
397 line), 30 ppb (dark red line), 40 ppb (light blue line), 50 ppb (brown line), 60 ppb (violet line).
398 Currents have been recorded in triplicate by SW-ASV ($E_{\text{cond}} = -0.4$ V, $t_{\text{cond}} = 30$ s, $E_{\text{dep}} = -1.0$ V, $t_{\text{dep}} =$
399 300 s, $t_{\text{eq}} = 10$ s, $E_{\text{begin}} = -1.0$ V, $E_{\text{end}} = -0.4$ V, $E_{\text{pulse}} = 0.02$ V, $E_{\text{step}} = -0.005$ V, Frequency= 50 Hz).

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Table 2. Analytical performances of the Naf/Bi-sensor obtained in the different working solutions: acetate buffer (50 mM with NaCl 50 mM, pH 4.6), seawater diluted 1:20 (v/v) with acetate buffer, digested tissue diluted 1:50 (v/v) with acetate buffer. The measurements were carried out in triplicate.

	Working solution	Linear range (ppb)	Equation $y = (m \pm \sigma_m)x + (y_0 \pm \sigma_{y0})$	r^2	LOD (ppb)	LOQ (ppb)
Cd²⁺	Acetate buffer	5-50	$y = (0.53 \pm 0.01)x - (3.1 \pm 0.3)$	0.991		
	Seawater (1:20)	5-60	$y = (0.454 \pm 0.008)x - (2.2 \pm 0.3)$	0.991	1.5	5
	Digested tissue (1:50)	10-40	$y = (0.28 \pm 0.04)x - (1 \pm 1)$	0.938		
Pb²⁺	Acetate buffer	5-60	$y = (0.60 \pm 0.01)x - (2.4 \pm 0.4)$	0.991		
	Seawater (1:20)	3-60	$y = (0.51 \pm 0.01)x - (2.8 \pm 0.4)$	0.986	0.3	1
	Digested tissue (1:50)	10-40	$y = (0.37 \pm 0.05)x - (1 \pm 1)$	0.927		

3.3. Study of matrix effect

In the perspective to apply the developed sensor in seawater and in *S. plicata* tissues for evaluating the bioconcentration capacity of the marine organism, the effect of both matrices on the electrochemical measurements was investigated.

3.3.1. Seawater

The organic components of seawater samples, including organic debris and microorganisms, can cause the fouling of the working electrode surface, with the consequent decrease of the voltammetric signals. Moreover, the high salinity of seawater, that usually in Tyrrhenian Sea is about 36-37 g/L, can affect the electrochemical measure. In order to evaluate the overall importance of seawater matrix effect, several dilutions of seawater samples in acetate buffer (from 1:2 to 1:50 v/v with the acetate buffer) were analyzed, highlighting a significant matrix effect at low dilution factors. The dilution of 1:20 (v/v) was chosen since the matrix effect was overcome, obtaining calibration curves for cadmium and lead described in Table 2. Comparing the slope of the linear regression obtained in seawater and the slope of the linear regression obtained in standard solution, a ratio of 0.86 and 0.84 was obtained for Cd²⁺ and Pb²⁺, respectively, indicating a low matrix effect. This means a slight decrease of the sensor sensitivity in seawater matrix at the dilution of 1:20 (v/v), which can be attributed mostly to the organic content in seawater rather than the high salinity. In fact, it is known that this sensor can be used in conditions with high ionic strength; moreover it has been shown that salinity does not cause a significant matrix effect using this kind of sensors (Güell et al., 2008). Hence, the sensor resulted to be suitable for the detection of seawater contaminated at level equal or greater than 100 and 60 ppb for Cd²⁺ and Pb²⁺, respectively, which may corresponds to polluted coastal environments at ppm levels.

3.3.2. Acid digested samples of *S. plicata*

A first specimen of *S. plicata* was selected from the storage aquarium for a preliminary analysis of its matrix, so a sample of tissue (about 0.3 g, ww) was acid digested and analyzed, by applying several dilutions from 1:5 to 1:100 v/v with the acetate buffer. In this case, a dilution of 1:50 (v/v) was chosen because it represented a good compromise between the reduction of matrix effect and the dilution factor. Corresponding calibration curves obtained for Cd^{2+} and Pb^{2+} are described in Table 2. The ratio between the slope of the linear regression obtained in *S. plicata* samples and the slope of the linear regression obtained in standard solution resulted to be equal to 0.53 and 0.61 for Cd^{2+} and Pb^{2+} , respectively, indicating that a relevant matrix effect occurs. This higher decrease of the sensor sensitivity in the acid digested biological samples can be associated to the complex matrix of *S. plicata* organism. The preliminary analysis of a *S. plicata* specimen, operated with both the electrochemical sensor and ICP-MS, revealed that no traces of cadmium and lead were naturally present in the organism tissue. In this way, it was possible also to prove that neither the storage aquarium conditions nor the microwave-assisted acid-digestion procedure contributed to any lead or cadmium traces found in the digested samples.

3.4. Bioremediation experiment

Since *S. plicata* sample analyzed showed no presence of cadmium and lead at the detection limit of the analytical system used, we supposed that all these organisms belonging to the same sampling have not been subjected to pollution in nature. Thus, any amount of heavy metals found inside tissues of specimens used for the bioremediation experiments may be attributed to the quantity of heavy metals artificially added in each container at the beginning of the experiment (1000 ppb of Cd^{2+} and Pb^{2+}). A picture of the bioremediation experiment setup is reported in Fig. 2.

The Wilcoxon test revealed no significant differences ($p \geq 0.25$) between the results from SW-ASV and spectroscopic reference methods in all samples examined: in the seawater microsystems, where *S. plicata* has been exposed for 0, 24 and 168 h to 1000 ppb of Cd^{2+} and Pb^{2+} , in the control microsystem and in the ascidian tissues.

3.4.1. Cd^{2+} and Pb^{2+} content in seawater

The monitoring of the cadmium and lead concentration in the seawater during the bioremediation experiment was performed through SW-ASV and ICP-OES analysis. ICP-OES was used as reference method, suitable for the simultaneous heavy metal detection with a wide linear range (from 5 ppb to 100 ppb). Seawater sampled at several times (0 h, 24 h and 168 h) of the bioremediation experiments were immediately analyzed by SW-ASV, in order to avoid changes in the metal content due to the biotic component, using the previously chosen dilution 1:20 (v/v). For the spectrometric analysis, seawater samples have to be filtered in order to reduce the high content of organic compounds, which can adversely affect the measurement. The content of Cd^{2+} and Pb^{2+} calculated using SW-ASV and ICP-OES is reported for one microsystem in the Fig. 5, in which a comparison is made between results concerning the seawater in presence of *S. plicata* (A, B) and without *S. plicata* (C, D). One microsystem (corresponding to microsystem 2, Fig. 2) was chosen to show the resulting concentration of Cd^{2+} and Pb^{2+} found in the seawater after the exposition of 168 h to the ascidian. Analogous results were found for the other two microsystems in which *S. plicata* was present (data not shown). At the zero-time of the experiment, around 1000 ppb of Cd^{2+} and Pb^{2+} were detected for all samples, but an overestimation is observed for SW-ASV analysis,

479 particularly for Cd^{2+} . This might be explained considering the high variability of the seawater
 480 matrix, which can be very different from the samples used for obtaining the calibration curves, and
 481 consequently affect the results. This matrix effect can be attributed particularly to the organic
 482 component of seawater samples rather than the high salinity, as discussed before (paragraph 3.3.1).
 483 As the exposure time increases, a decreasing trend of Cd^{2+} and Pb^{2+} concentrations is clearly
 484 evident in seawater in presence of *S. plicata*, for both methods (Fig.5 A and B). In details, it can be
 485 noted that the time-dependent decrease of Pb^{2+} is greater with respect to Cd^{2+} . Differently, no
 486 appreciable trend can be observed for seawaters without the marine organism.
 487

488
 489 **Fig. 5.** Comparison between the content of Cd^{2+} (A, C) and Pb^{2+} (B, D) obtained by SW-ASV
 490 (black bars) and ICP-OES (grey bars) analysis of seawater (diluted 1:20 v/v) sampled at several
 491 times during the bioremediation experiment, from solution in presence (A, B) and in absence of *S.*
 492 *plicata* (C, D). Concentrations are reported as medium value of two replicates for SW-ASV and
 493 three replicates for ICP-OES, and related to the beaker 2 (Fig. 2).
 494

495 **3.4.2. Cd^{2+} and Pb^{2+} content in *S. plicata* tissue**
 496

497 Samples of the three specimens of *S. plicata* involved in the bioremediation experiment were
 498 analyzed in order to evaluate its bioconcentration capacity and to test the ability of the sensor to
 499 detect Cd^{2+} and Pb^{2+} . ICP-MS was preferred as reference method because of its capacity to detect
 500 simultaneously heavy metals until very low concentration (below 1 ppb), which results more
 501 suitable taking into consideration the high dilution factor used for these samples. The content of
 502 Cd^{2+} and Pb^{2+} ($\mu\text{g/g}$) was calculated as described in paragraph 2.8 and is reported in Fig. 6.
 503 Considerable quantities of Cd^{2+} and Pb^{2+} were revealed with both techniques, confirming that Cd^{2+}
 504 and Pb^{2+} have been bioconcentrated into the organism tissues. The concentrations calculated with
 505 the electrochemical sensor resulted underestimated with respect the concentrations from ICP-MS
 506 measurements, but a similar trend is observed, particularly for Pb^{2+} . It is worthy of note that *S.*
 507 *plicata* showed a different bioconcentration capacity between Cd^{2+} and Pb^{2+} , since the content of
 508 Pb^{2+} found in *S. plicata* tissues was greater than the content of Cd^{2+} for each specimen. In details,
 509 average values obtained by SW-ASV were $12 \pm 5 \mu\text{g/g}$ for Cd^{2+} and $32 \pm 10 \mu\text{g/g}$ for Pb^{2+} , and by
 510 ICP-MS were $19 \pm 2 \mu\text{g/g}$ for Cd^{2+} and $43 \pm 8 \mu\text{g/g}$ for Pb^{2+} . Hence, Pb^{2+} resulted to be about 2.5
 511 times more bioconcentrated than Cd^{2+} , giving an effective bioremediation level in seawater of 13%
 512 and 40% for Cd^{2+} and Pb^{2+} , respectively. The greater bioconcentration capacity observed for Pb^{2+} in
 513 our study is consistent with data reported in literature concerning the analysis of the natural content
 514 of heavy metals in tissues of *S. plicata* (Aydın-Önen, S., 2016; Bellante et al., 2016; Philp et al.,
 515 2003). However, Cd^{2+} and Pb^{2+} content in *S. plicata* specimens involved in our bioremediation
 516 experiment are significantly greater than the content found in specimens collected from natural
 517 coastal areas.
 518

519 **Fig. 6.** Comparison between the content of Cd^{2+} (A) and Pb^{2+} (B) obtained by SW-ASV (black
 520 bars) and ICP-MS (grey bars) analysis of acid digested tissues of *S. plicata* (diluted 1:50 v/v with
 521 acetate buffer), sampled from the three microsystems used for the bioremediation experiment ($t =$
 522 168 h). Measurements in triplicates.
 523
 524
 525

526 4. Conclusions

527 In this work, the suitability of a miniaturized, disposable and cost-effective sensor for simultaneous
 528 detection of Cd^{2+} and Pb^{2+} in both seawater and *S. plicata* tissues was demonstrated. The
 529 bioconcentration capacity of *S. plicata* in cadmium and lead polluted seawater was confirmed

through bioremediation experiments in controlled conditions. Specifically, we observed a greater bioconcentration for Pb^{2+} than for Cd^{2+} , according with previous results reported in literature. Our study gives further evidence of the possibility to use this ascidian in bioremediation strategies, as well as a bioindicator of metal contaminants in seawaters, and so as a recommendable tool for marine pollution monitoring plans.

The matrix effect of both samples has been investigated, revealing a significant effect on the electrochemical measurements for both samples, requiring the dilution of the matrix. This is due to the high, complex and variable content of organic compounds of these samples. It has to be noted that these samples are not easy to be analyzed also with reference methods, because several specific pre-treatments are required. However, the high salinity that characterized the samples, in particular seawater, did not adversely affect the voltammetric measurement, whereas drastically affected the sample preparation for the spectrometric analysis. The possibility to avoid the sample desalinization represents an interesting advantage of the electrochemical sensor.

The Naf/Bi-SPE resulted to be a useful tool for the environmental screening analysis of natural areas contaminated with cadmium and lead. No significant differences between results obtained with the electrochemical sensor and with spectroscopic reference methods (ICP-OES and ICP-MS) resulted from statistical analysis (Wilcoxon test). Moreover, this sensor can be applied for several kinds of samples, including highly saline solutions and highly acidic and complex mineralized biological tissues of animals. Taking into consideration our previous work, in which an aquatic plant and its growth medium were studied, these results amplify the field of the sensor applications. The versatility of this device, combined with the simplicity of use, the portability, the cost-effectiveness and the reduced impact on the environment make it a competitive alternative to the classical analytical methods, as well as a novel approach for water quality assessment.

Acknowledgments

F.A. thanks the project "Electrochemistry and Electrochemical cost-effective sensors for remediation and detection of heavy metals in polluted water and soils" in the frame of scientific and technological protocol between Italy and Algeria 2016-2018, research activity supported by Italian Ministry of Foreign Affairs in the frame of International Cooperation; D.M. and F.A. thanks Ocean 2013 Project-SMS FP7-OCEAN-2013 (grant number 613844) for financial support.

The authors would like to thank Marcello Giorgi, University of Rome "Tor Vergata", for technical support in field and laboratory procedures with biological samples.

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