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1 **A novel Cyphos IL 104-based polymer inclusion membrane (PIM) probe to mimic biofilm zinc**
2 **accumulation**

3
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8 **Abstract**

9 *The presence of Zn in surface waters from abandoned mining zones is a critical issue since*
10 *excess Zn concentrations may affect aquatic life and whole ecosystems. We present, for the*
11 *first time, a simple tool based on a polymer inclusion membrane (PIM) intended to monitor Zn*
12 *in river water by mimicking metal accumulation in the biofilm. The PIM-based probe contains a*
13 *polymeric membrane prepared using cellulose triacetate (CTA, 50% w/w) as the base polymer,*
14 *nitrophenyloctyl ether (NPOE) as the plasticizer (20% w/w), and the ionic liquid (IL) Cyphos 104*
15 *as the extractant (30% w/w). The accumulation of Zn in the acceptor phase (0.01 M HNO₃) was*
16 *evaluated for different free metal concentrations at 4 h accumulation time resulting in a good*
17 *correlation between the free metal concentration and the accumulated one. We also found*
18 *that the metal accumulated agrees with the free metal fraction upon addition of EDTA in the*
19 *donor solution. The results for Zn accumulation with the PIM-based probe were found to be*
20 *comparable to those obtained for a biofilm that was grown in a stream from an abandoned*
21 *mine area and subsequently translocated to the laboratory and put in contact with Zn polluted*
22 *stream water, so confirming the effectiveness of this new probe in mimicking Zn accumulation*
23 *in the biofilm.*

24

25

26 **Keywords** Zn · Polymer inclusion membrane · Cyphos IL 104 · River water · Metal accumulation

27 · Biofilm

28

29 1. Introduction

30 The presence of metals in the environmental is an issue of great concern both for the general
31 public and the scientific community. Metals not only affect water quality but can also
32 accumulate in living organisms, having an adverse effect on them. Zinc is a common
33 contaminant of surface freshwater, with no observed toxicity at concentrations below $10 \mu\text{g L}^{-1}$
34 ¹. However, it may be toxic at the concentrations found in many fluvial systems which vary
35 from μg to mg [1], where mining activity has been the main source of contamination[2–5]. For
36 instance, Gozzard et al. (2011) [4], reported that zinc is the most commonly encountered metal
37 in the surface waters of metal mining regions in the United Kingdom. High zinc concentrations
38 (above $1000 \mu\text{g L}^{-1}$), are common in stream water in the Dalarna region of Sweden, which is
39 known for metal exploitation. Zinc occurrence has motivated environmental regulation in
40 countries such as Portugal (APA, 2016), Canada (ATSDR, 2014), USA (CEPA, 1999) and Spain
41 (BOE, 2011), which set limits in the range of $30\text{--}59 \mu\text{g L}^{-1}$ and $210\text{--}500 \mu\text{g L}^{-1}$ (depending on the
42 water hardness). The toxicity of Zn is associated with an overproduction of reactive oxygen
43 species leading to oxidative stress resulting in lipid peroxidation, protein damage and DNA
44 alterations affecting most aquatic organisms [5]. It can become toxic for fish at increased
45 water levels as the main target is the gills, where the calcium uptake is disrupted, leading to
46 hypocalcaemia and eventually to death [6].

47 The term toxicity, applied to aquatic ecosystems, is closely related to the internalization
48 process of the pollutant and its specificity. It is broadly accepted that the bioavailability and
49 toxicity of metals cannot be predicted by total concentrations, but rather by the concentration
50 of various chemical species, in particular the free ion [7]. Therefore, the assessment of
51 bioavailability requires either the manipulation of biological entities, such as microorganisms,
52 algae, insects, plants or biofilm in aquatic ecosystems, or a chemical test can be performed if
53 the uptake of the contaminant by an organism can be mimicked by a chemical process [8].

54 Permeation liquid membranes (PLM) have been proposed as chemical probes to measure
55 bioavailability for inorganic species [9]. The separation of species with PLMs is based on
56 carrier-mediated mechanism. The extractant interacts with the species of interest initially
57 present in the donor, forming a complex at the interface donor solution-PIM. The complex
58 then diffuses to the interface PIM-acceptor solution where the species is back-extracted and
59 finally released into the acceptor. This chemical pumping allows the accumulation of the
60 analyte in the acceptor solution even against the concentration gradient. As a consequence,
61 the efficiency of the PLM is mainly dictated by the extractant used. PLMs incorporating
62 selective extractants have been used as analytical tools to study copper, lead, cadmium, zinc
63 and nickel speciation [10,11]. However, the use of PLM is often hampered due to the solubility
64 of the extractants in the adjacent water media affecting membrane stability and effectivity.

65 Polymer inclusion membranes (PIM) are advanced liquid extracting membranes that are
66 composed of a base polymer, providing mechanical strength, an extractant, and, in some
67 cases, a plasticizer, which provides elasticity to the membrane [12]. Since the extractant is
68 immobilized within the chains of the base polymer, PIMs are more stable than PLMs, and
69 highly selective when the appropriate extractant is used. As general purpose extractant for
70 cations, the compound di(2-ethylhexyl)phosphoric acid (D2EHPA) has been extensively used.
71 Moreover, D2EHPA has been incorporated in a passive sampler PIM-based device to determine
72 Zn in pond waters [13]. In our previous work, we developed a device incorporating a PIM
73 containing D2EHPA for the measurement of free Zn concentrations in hydroponic solution and
74 its performance has been compared with the fluxes measured in the roots of potato plants
75 [14].

76 Recently, more lipophilic, less-water soluble, and less toxic extractants have been investigated
77 and implemented in membrane systems, and among them, ionic liquids (IL) have received
78 particular attention. Ionic liquids (ILs) are liquid molten salts at room temperature formed by
79 an organic cation (dialkylimidazolium, tetralkylammonium, pyridinium, and phosphonium) and

80 an organic or inorganic anion, which exhibit certain remarkable characteristics, such as
81 negligible vapour pressure, great thermal and chemical stability, high ion conductivity, and low
82 volatility. They have been exploited in analytical sciences in different application fields (e.g.,
83 chromatography, electrochemistry or extraction). Moreover, they have been used as tailor-
84 made extractants exhibiting greater selectivity than other conventional extractant reagents
85 [15]. In particular, the IL trihexyl(tetradecyl)phosphonium bis-2,4,4-(trimethylpentyl)
86 phosphinate (Cyphos IL 104 or Cyphos 104) has been used for Cr(VI) transport using a
87 plasticized PIM [16], Au(III) transport from hydrochloric acid solutions [17], Cd(II) and Cu(II)
88 separation also from hydrochloric acid solutions [18], and ~~Zn(II) transport or for the~~ separation
89 of Zn(II) from Fe(III) from highly acidic solutions [19,20].

90 In the present work, a new PIM-based probe containing Cyphos IL 104 has been evaluated in
91 natural water for the first time to perform Zn accumulation measurements and to model metal
92 accumulation (Zn) in biofilm. The PIM-based probe was prepared and calibrated in the
93 laboratory using model solutions. The selectivity of the PIM-probe towards the free metal
94 species will also be investigated by the addition of an organic ligand
95 (ethylenediaminetetraacetate, EDTA). The assessment of the new probe in mimicking the Zn
96 accumulation in biological systems has been tested by comparison with fluvial biofilms that
97 were grown on submerged substrata in the Osor Stream, a river polluted with Zn due to past
98 mining activity, and then translocated to the laboratory.

99 **2. Experimental**

100 2.1 Reagents and solutions

101 A Zn stock solution (500 mg L^{-1}) was prepared from the solid $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (Panreac) and a
102 standard solution of 1000 mg L^{-1} Zn (Sigma Aldrich) was used to prepare the calibration
103 standards for ICP analysis. 2-(N-morpholino) ethanesulfonic acid (MES) (Fluka) was used as a

104 buffer. Other solids used were: Cu(NO₃)₂·6H₂O, Ni(NO₃)₂·6H₂O, Pb(NO₃)₂, Cd(NO₃)₂·4H₂O, all of
105 them from Panreac.

106
107 To prepare the membranes, cellulose triacetate (CTA) polymer (Acros Organics), the plasticizer
108 2-nitrophenyloctyl ether (NPOE) (Sigma-Aldrich), and chloroform (Panreac) were used.
109 Trihexyl(tetradecyl)phosphonium bis-2,4,4-(trimethylpentyl) phosphinate (Cyphos IL 104) was
110 kindly donated by Cytec (USA).

111 The organic ligands trans-1,2-diaminocyclohexan-N,N,N',N'-tetraacetic acid (CDTA) and
112 ethylenediamine N,N,N',N'-tetraacetic acid disodium salt (EDTA) were purchased from Sigma-
113 Aldrich. HNO₃ 69% (Panreac) and sodium sulphate (Merck) were also employed.

114 Ultrapure water with resistivity $\geq 18 \mu\text{S cm}^{-1}$ was taken from a MilliQ system (Millipore).

115

116 2.2 Instruments

117 A GLP-22 pH-meter (Crison) was used to measure the pH of the samples. Conductivity was
118 monitored with an Ecoscan portable conductometer from Entech Instruments. Total organic
119 carbon was measured with a TOC-V CSH from Shimadzu.

120 Zn concentrations were determined using an inductively-coupled plasma atomic emission
121 spectrometer, ICP-AES 5100 (Agilent Technologies).

122

123 2.3 Water samples

124 The appropriate volume of Zn stock solution was added to simulated natural water (SNW)
125 containing 2 mM NaHCO₃, 0.25 mM Na₂SO₄, 0.75 mM CaCl₂ and 2.5 mM MES solution (pH 6.3).

126 The preparation of SNW was adapted from [21] and the chemical parameters of Osor Stream
127 water (Table S1) were also taken into account.

128 Water samples from the Osor Stream (41°56'51"N, 2°33'25"E), a small tributary of the River
129 Ter (Girona, Spain), were collected during different time periods (July 2017, March-April 2018).

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130 This stream is influenced by continuous (effluent) and diffuse (run-off) metal inputs from a
131 disused mine [22].

132 Two sampling points were selected: a reference point (Osor-R), where Zn was not detected by
133 ICP-MS ($< 1.5 \mu\text{g L}^{-1}$), and a point downstream of the effluent from the mine area (Osor-M),
134 where $[\text{Zn}]_{\text{total}}$ varied from 200 to $450 \mu\text{g L}^{-1}$ (depending on the sampling period). The main
135 chemical parameters of these samples were pH 8.2-8.3, conductivity $400\text{-}500 \mu\text{S cm}^{-1}$, and TOC
136 $2\text{-}3 \text{ mg C L}^{-1}$ (see Table S1). Additionally, to compare the influence of the metal concentration
137 on both the PIM-probe and the biofilm, another water sample, Osor-d, was obtained by
138 diluting Osor-M with Osor-R (60:40, v/v) in order to ensure the same chemical characteristics.

139

140 2.4 Membrane preparation and PIM-probe experiments

141 A PIM containing 50% CTA: 30% Cyphos 104: 20% NPOE (% in w/w) [19] was prepared by a
142 casting method. First, the ability of the PIM to extract Zn was investigated by contacting a
143 piece of PIM (2 cm x 2cm) with $500 \mu\text{g L}^{-1}$ Zn in 15 mL of ultrapure water, SNW, or in solutions
144 separately containing 2 mM hydrogen carbonate, 0.25 mM sulphate, or 1.5 mM chloride (the
145 same concentration as in SNW). The contact time was 4 h under rotary agitation. The metal
146 remaining in the solution was determined and the zinc extracted in the PIM was calculated by
147 mass balance. The same procedure was followed to study the selectivity of the PIM using
148 solutions containing Cu(II), Pb(II), Ni(II) or Cd(II) at a concentration $500 \mu\text{g L}^{-1}$ each.

149 For the accumulation measurements, the PIM was mounted on a special device [14], which
150 was immersed 1 cm in a vertical position in the donor water sample containing Zn, under
151 agitation. The exposed area was 2.5 cm^2 and the acceptor solution (2.5 mL) was located inside
152 the device. The volume of donor phase was 250 mL and magnetic stirring was applied.
153 Different acceptor phase compositions, 0.01 M HNO_3 , 5×10^{-4} M CDTA and 0.5 M Na_2SO_4 , were
154 tested to recover the extracted zinc from the PIM. Calibration was performed, fixing 4 h as the
155 accumulation time, 0.01 M HNO_3 as acceptor solution under magnetic stirring. The same

156 experimental conditions as in the calibration were used when free zinc was measured in spiked
157 SNW and in spiked Osor-R (both at 0.50 mg L⁻¹ Zn), and also in Osor-M.

158 An additional accumulation experiment was performed using Osor-M in 10 L buckets under
159 agitation (teflon blades -Heidolph Hei-TORQUE Value 100) at 150 rpm.

160

161 2.5 Metal accumulation in biofilm

162 Biofilms were grown on submerged artificial substrata (7 cm x 7 cm sand-blasted glass
163 supports) at the colonization site (Osor-R) for 14 days, in April 2018, under optimum conditions
164 of light and water velocity. The substrata colonized with biofilm were then taken to the
165 laboratory and subjected to different treatments (see the experimental lay-out presented in
166 Figure S1): three biofilm samples were immediately separated for analysis; two biofilm
167 samples were exposed to Osor-M, and another two biofilm-colonized glasses were exposed to
168 Osor-d in 10 L buckets under agitation (teflon blades -Heidolph Hei-TORQUE Value 100) at 150
169 rpm. Experiments for the determination of Zn accumulated in the PIM-based probe were
170 simultaneously run in the 10 L buckets where biofilm was exposed.

171 After 4 h exposure time, the biofilm was recovered in a Petri dish, and dried in the oven at 50
172 °C for 15 h to determine the dry weight (DW). Around 200-400 mg DW biofilm was digested
173 with 4 mL of concentrated HNO₃ (65% suprapure) and 1 mL H₂O₂ (31% suprapure) in a
174 microwave ETHOS SEL High Performance Extraction System (Milestone). The Zn concentration
175 in the acid extract of the biofilm as well as the metal accumulated in the acceptor phase of the
176 PIM-probe were determined by ICP-AES after filtration through a 0.45 µm cellulose acetate
177 filter.

178 All experiments were run at least in duplicate at 22 ± 2 °C.

179

180 **3. Results and discussion**

181 3.1 Performance of the PIM-based probe

182 The compositions of the PIM, as well as the chemical characteristics of the donor and the
183 acceptor phases, are key factors in the design of an effective transport system. Baczynska et al.
184 studied the effect of the plasticizer on the extraction of anionic zinc chlorocomplexes [19],
185 finding that a PIM composition of 50% CTA: 30% Cyphos 104: 20% NPOE resulted in a higher Zn
186 transport rate. Therefore, we fixed this composition for the PIM-based probe and we focussed
187 in evaluating, for the first time, its effectiveness for the transport of Zn in natural waters. For
188 this, a $500 \mu\text{g L}^{-1}$ Zn solution in SNW at pH = 6.3 was used as a donor phase, where Zn exists
189 mainly as a free cation (94.5%, according to visual MINTEQ calculations), and it was found that
190 the metal was successfully transported to a 0.01M HNO_3 solution used as the acceptor phase.
191 Some exploratory experiments were performed to better understand the transport
192 mechanism, since, to the best of our knowledge, all published work dealing with extraction of
193 metals with Cyphos 104 has been performed with anionic species present at high hydrochloric
194 acid concentrations [16–20]. The experiments were conducted using $500 \mu\text{g L}^{-1}$ Zn in both
195 ultrapure water or in solutions separately containing carbonate, sulphate, or chloride (Figure
196 S2). Similar percentages of Zn extraction were found in all cases except for ultrapure water,
197 showing out that Cyphos 104 can extract positive charged Zn species through an ion-pair
198 mechanism that also involves the extraction of an anion. The extraction in SNW was higher
199 (Figure S2), around 85%, probably due to the cumulative effect of the anions that were
200 simultaneously present. Selectivity was also studied. To this end, pieces of the PIM were
201 contacted with Cu(II), Pb(II), Ni(II) or Cd(II) contained in SNW solution at pH 6.3. Only around
202 20% extraction for Cu(II) was found, and less than 5% in the other cases.

203 The accumulation of Zn in the PIM-based probe was then studied. Acceptor phases of different
204 composition such as 0.01 M HNO_3 , as was used in previous works [11,13], a complexing agent,
205 CDTA (5×10^{-4} M) and a 0.5 M Na_2SO_4 solution [19] were tested. The results are shown in Figure
206 1 (a) as the time dependence accumulation of Zn. As it can be observed, a 0.01 M HNO_3
207 solution provided a more effective transport of metal, resulting in a linear relationship

208 between the Zn accumulated and the accumulation time until 17 h of exposure. The Zn
209 accumulation in the acceptor phase is in agreement with a steady-state regime, and the
210 average flux calculated is $15.5 \pm 1.8 \text{ nmol cm}^{-2} \text{ h}^{-1}$ [23]. However, experiments over longer
211 periods of time showed a decrease of Zn in the acceptor phase due to the deployment of the
212 metal in the donor solution. For this reason, and fixing 0.01 M HNO_3 for further studies, the
213 same type of experiment was carried out using 10 L of Osor-M, which naturally contained 314
214 $\mu\text{g L}^{-1}$ Zn, as a donor phase (see Figure 1 (b)). The trend observed is similar to that in Figure 1(a)
215 for 0.01 M HNO_3 , with a linear accumulation at shorter times and a maximum value at 17 h.
216 The higher volume of the donor solution ensured that no depletion of the metal occurred,
217 reaching an equilibrium state.

218

219 **Figure 1**

220 Membrane stability in SNW was checked by measuring the weight difference of the PIM at
221 different accumulation times *versus* the initial weight. After 6 h contact time, the mass loss of
222 the Cyphos 104-based PIM was 3%, being 13-15% for a PIM prepared with D2EHPA (50% CTA:
223 30% D2EHPA: 20% NPOE (w/w)). Moreover, the reuse of the PIM was also checked and we
224 obtained similar fluxes when a new membrane was compared with a PIM which has previously
225 been in contact for 7 hours with the Osor-R, being 12.2 ± 0.2 and $9.8 \pm 0.5 \text{ nmol cm}^{-2} \text{ h}^{-1}$,
226 respectively (fluxes obtained at 28 h accumulation time, n=2). The stability and the long-term
227 performance are clear advantages for the application of the probe for extended time periods.

228

229 3.2 Calibration and speciation measurements

230 A calibration curve was obtained for an exposure time of 4 h affording an intermediate
231 accumulation of Zn within steady state conditions and without depletion of the metal even

232 when 250 mL of donor solution was used. The results obtained are depicted in Figure 2,
233 together with the equation that relates the accumulated Zn to the free concentration of metal
234 in the donor solution. The good correlation between the two variables is particularly
235 noteworthy.

236

237 **Figure 2**

238

239 To test whether the PIM-probe can be applied to measure free Zn, different experiments were
240 performed using SNW as a donor solution, with and without the EDTA ligand, and Osor-R and
241 Osor-M, which contain natural ligands. The total Zn concentration (measured with ICP-AES)
242 was between 0.48-0.51 mg L⁻¹, in agreement with the amount added. The accumulated Zn in
243 the acceptor phase and the free Zn measured with the PIM-probe in the different water
244 samples are presented in Table 1, and compared with Zn free values calculated by MINTEQ.

245 **Table 1**

246 The addition of EDTA in SNW diminishes the value of free Zn to 31.7% as calculated by
247 MINTEQ, which is in good agreement, within the experimental error, with the 29% obtained
248 with the PIM-based probe. Therefore, the addition of EDTA results in the formation of ~~non-~~
249 ~~labile~~ complexes with Zn that cannot be transported through the PIM (non-reactive species).
250 For both Osor-R (with added Zn) and Osor-M water samples, around 72-87% of the total metal
251 is measured, meaning that Zn is either in the free form, ~~or~~ forming labile complexes (i.e.
252 complexes that dissociate at the membrane interface), or forming neutral lipophilic complexes
253 soluble in the PIM phase. The remaining metal forms stable complexes, probably with organic
254 ligands, or complexes that are kinetically inert ~~from the kinetic point of view~~. The results
255 obtained highlight the good ability of the developed PIM-based probe for Zn speciation

256 measurements in natural waters to determine the PIM concentration of Zn (Zn_{PIM} in analogy to
257 the C_{DGT} defined for the diffusion gradients in thin film technique or DGT [23]).

258 3.3 Comparison with Zn accumulated in biofilm

259 The developed PIM-based probe was investigated as a tool to mimic metal accumulation in
260 living organisms. We selected biofilm communities, formed by benthic microbial communities
261 in a structure of highly complex extracellular polymeric substances, which were previously
262 tested for metal accumulation [22]. It was found that biofilm accumulated 150–225 $\mu\text{g Zn g}$
263 DW^{-1} in microcosms, and 200–500 $\mu\text{g Zn g DW}^{-1}$ in the Osor-M Stream (acute toxicity, 6-24h)
264 and that the metal in biofilm continued to accumulate until the end of the experiment (5
265 weeks). This exposure was found to affect the structure and function of the biofilm
266 communities [22,24].

267 We have compared Zn accumulation in the PIM-based probe with that in the biofilm
268 translocated to the laboratory (see experimental section for details and Figure S1). Laboratory
269 conditions were selected in order to exercise a tight control over the experiments. The results
270 obtained are shown in Figure 3. The background Zn concentration in the biofilm was
271 determined to be 110 $\mu\text{g Zn g DW}^{-1}$.

272

273 **Figure 3**

274

275 The high capacity of the biofilm to accumulate Zn is observed in Figure 3: the values found are
276 in good agreement with the concentration values reported by Corcoll et al. [22]. Moreover, the
277 Zn accumulation in both the biofilm and the PIM-probe respond in concordance with the
278 amount of metal present in the water samples. The trend that is observed constitutes a
279 promising result for the application of the PIM-based probe for Zn bioavailability
280 measurements and to predict metal accumulation in biofilm. Thus, the use of this probe can

281 avoid the long and tedious biofilm manipulation steps (collection, transportation to the
282 laboratory, biofilm drying and acid digestion) needed to investigate metal contamination.
283 Moreover, the PIM-probe can be easily prepared at a low cost as the estimated costs for a PIM
284 is less than 1 euro. It is worth mentioning that this is the first study to present a direct
285 comparison between the accumulation of a PIM-based probe and biofilm grown under natural
286 conditions.

287

288 **Conclusions**

289 A novel ionic liquid-based PIM probe has been designed for Zn bioavailability monitoring in
290 surface waters. The accumulation of Zn from both simulated natural water or water samples
291 from a polluted stream was possible using a membrane containing 50% CTA: 30% Cyphos 104:
292 20% NPOE (w/w) and a 0.01 M HNO₃ solution as the acceptor phase. The calibration of the
293 PIM-based probe was successfully performed and by the addition of EDTA to the donor
294 solution, it has been demonstrated that the PIM-probe can be used for speciation studies as
295 only ~~a fraction of the total the free metal and the labile complexes are is~~ measured by the PIM.

296 Finally, the comparison of the metal measured by the PIM-probe and the metal accumulated
297 by biofilm, showed the ability of the developed tool to perform metal bioavailability studies.

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- 412

413 **Figure captions**

414

415 **Figure 1.** Zn accumulated at different exposure times. (a) Donor solution (250 mL): $500 \mu\text{g L}^{-1}$
416 Zn in SNW; (b) Donor solution: Osor-M ($314 \mu\text{g L}^{-1}$ Zn), 10 L. Acceptor phase 0.01 M HNO_3 .

417 **Figure 2.** Relationship between Zn accumulated in the acceptor phase and free Zn in SNW (250
418 mL). Acceptor phase 0.01 M HNO_3 . Exposure time: 4 h.

419 **Figure 3.** Comparison of Zn accumulated in PIM-based probe (acceptor phase 0.01 M HNO_3) vs.
420 Zn in the biofilm. Donor solution: Osor-M and Osor-d, exposure time: 4 h.

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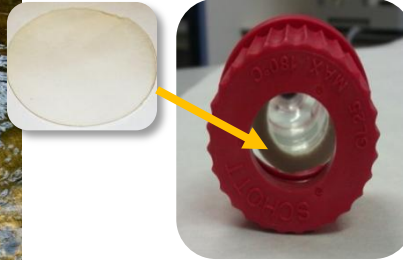
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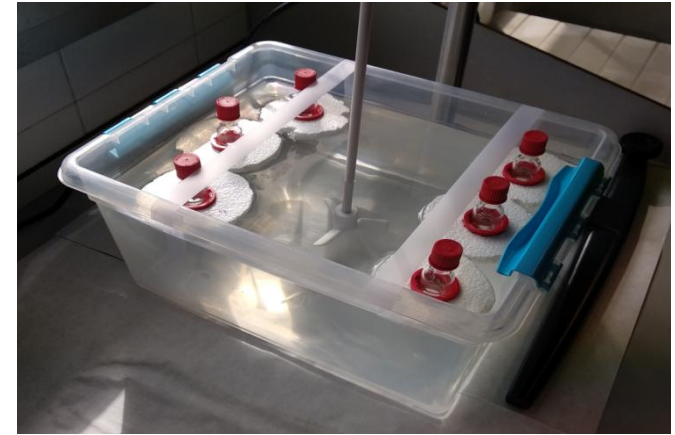
Osor Steam



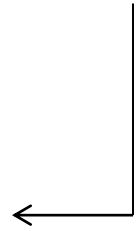
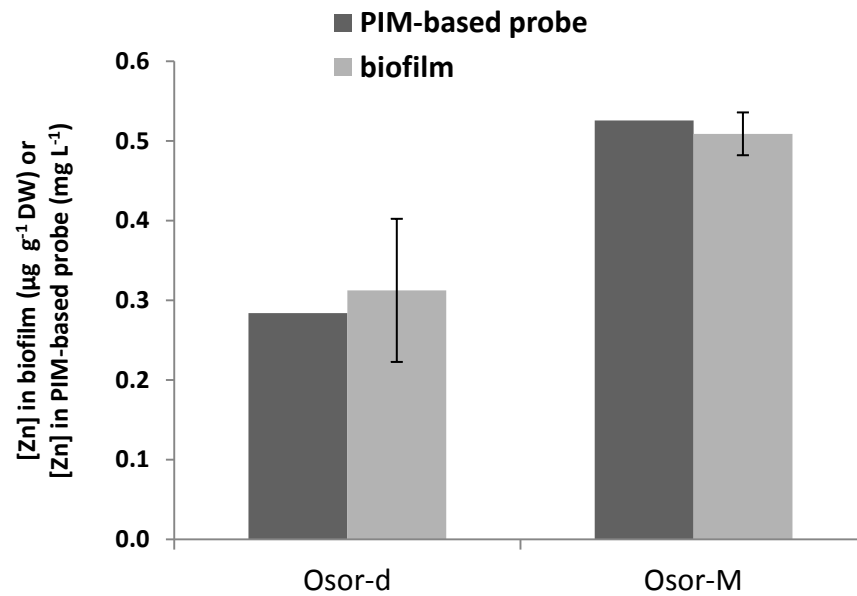
PIM



Laboratory



Biofilm grown on glasses placed in a non polluted site



Highlights

- Design of a new probe containing a polymer inclusion membrane for Zn measurements
- PIM prepared with the green ionic liquid Cyphos 104 with improved stability
- Only 3% mass loss after 6 h contact time for the Cyphos 104-based PIM
- The probe measures free Zn in simulated water and water from an abandoned mine site
- The new probe is effective in mimicking Zn accumulation in biofilm

1 **A novel Cyphos IL 104-based polymer inclusion membrane (PIM) probe to mimic biofilm zinc**
2 **accumulation**

3

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8 **Abstract**

9 *The presence of Zn in surface waters from abandoned mining zones is a critical issue since*
10 *excess Zn concentrations may affect aquatic life and whole ecosystems. We present, for the*
11 *first time, a simple tool based on a polymer inclusion membrane (PIM) intended to monitor Zn*
12 *in river water by mimicking metal accumulation in the biofilm. The PIM-based probe contains a*
13 *polymeric membrane prepared using cellulose triacetate (CTA, 50% w/w) as the base polymer,*
14 *nitrophenyloctyl ether (NPOE) as the plasticizer (20% w/w), and the ionic liquid (IL) Cyphos 104*
15 *as the extractant (30% w/w). The accumulation of Zn in the acceptor phase (0.01 M HNO₃) was*
16 *evaluated for different free metal concentrations at 4 h accumulation time resulting in a good*
17 *correlation between the free metal concentration and the accumulated one. We also found*
18 *that the metal accumulated agrees with the free metal fraction upon addition of EDTA in the*
19 *donor solution. The results for Zn accumulation with the PIM-based probe were found to be*
20 *comparable to those obtained for a biofilm that was grown in a stream from an abandoned*
21 *mine area and subsequently translocated to the laboratory and put in contact with Zn polluted*
22 *stream water, so confirming the effectiveness of this new probe in mimicking Zn accumulation*
23 *in the biofilm.*

24

25 **Keywords** Zn · Polymer inclusion membrane · Cyphos IL 104 · River water · Metal accumulation
26 · Biofilm

27

28 1. Introduction

29 The presence of metals in the environmental is an issue of great concern both for the general
30 public and the scientific community. Metals not only affect water quality but can also
31 accumulate in living organisms, having an adverse effect on them. Zinc is a common
32 contaminant of surface freshwater, with no observed toxicity at concentrations below $10 \mu\text{g L}^{-1}$
33 ¹. However, it may be toxic at the concentrations found in many fluvial systems which vary
34 from μg to mg [1], where mining activity has been the main source of contamination[2–5]. For
35 instance, Gozzard et al. (2011) [4], reported that zinc is the most commonly encountered metal
36 in the surface waters of metal mining regions in the United Kingdom. High zinc concentrations
37 (above $1000 \mu\text{g L}^{-1}$), are common in stream water in the Dalarna region of Sweden, which is
38 known for metal exploitation. Zinc occurrence has motivated environmental regulation in
39 countries such as Portugal (APA, 2016), Canada (ATSDR, 2014), USA (CEPA, 1999) and Spain
40 (BOE, 2011), which set limits in the range of $30\text{-}59 \mu\text{g L}^{-1}$ and $210\text{-}500 \mu\text{g L}^{-1}$ (depending on the
41 water hardness). The toxicity of Zn is associated with an overproduction of reactive oxygen
42 species leading to oxidative stress resulting in lipid peroxidation, protein damage and DNA
43 alterations affecting most aquatic organisms [5]. It can become toxic for fish at increased
44 water levels as the main target is the gills, where the calcium uptake is disrupted, leading to
45 hypocalcaemia and eventually to death [6].

46 The term toxicity, applied to aquatic ecosystems, is closely related to the internalization
47 process of the pollutant and its specificity. It is broadly accepted that the bioavailability and
48 toxicity of metals cannot be predicted by total concentrations, but rather by the concentration
49 of various chemical species, in particular the free ion [7]. Therefore, the assessment of
50 bioavailability requires either the manipulation of biological entities, such as microorganisms,
51 algae, insects, plants or biofilm in aquatic ecosystems, or a chemical test can be performed if
52 the uptake of the contaminant by an organism can be mimicked by a chemical process [8].

53 Permeation liquid membranes (PLM) have been proposed as chemical probes to measure
54 bioavailability for inorganic species [9]. The separation of species with PLMs is based on
55 carrier-mediated mechanism. The extractant interacts with the species of interest initially
56 present in the donor, forming a complex at the interface donor solution-PIM. The complex
57 then diffuses to the interface PIM-acceptor solution where the species is back-extracted and
58 finally released into the acceptor. This chemical pumping allows the accumulation of the
59 analyte in the acceptor solution even against the concentration gradient. As a consequence,
60 the efficiency of the PLM is mainly dictated by the extractant used. PLMs incorporating
61 selective extractants have been used as analytical tools to study copper, lead, cadmium, zinc
62 and nickel speciation [10,11]. However, the use of PLM is often hampered due to the solubility
63 of the extractants in the adjacent water media affecting membrane stability and effectivity.

64 Polymer inclusion membranes (PIM) are advanced liquid extracting membranes that are
65 composed of a base polymer, providing mechanical strength, an extractant, and, in some
66 cases, a plasticizer, which provides elasticity to the membrane [12]. Since the extractant is
67 immobilized within the chains of the base polymer, PIMs are more stable than PLMs, and
68 highly selective when the appropriate extractant is used. As general purpose extractant for
69 cations, the compound di(2-ethylhexyl)phosphoric acid (D2EHPA) has been extensively used.
70 Moreover, D2EHPA has been incorporated in a passive sampler PIM-based device to determine
71 Zn in pond waters [13]. In our previous work, we developed a device incorporating a PIM
72 containing D2EHPA for the measurement of free Zn concentrations in hydroponic solution and
73 its performance has been compared with the fluxes measured in the roots of potato plants
74 [14].

75 Recently, more lipophilic, less-water soluble, and less toxic extractants have been investigated
76 and implemented in membrane systems, and among them, ionic liquids (IL) have received
77 particular attention. Ionic liquids (ILs) are liquid molten salts at room temperature formed by
78 an organic cation (dialkylimidazolium, tetralkylammonium, pyridinium, and phosphonium) and

79 an organic or inorganic anion, which exhibit certain remarkable characteristics, such as
80 negligible vapour pressure, great thermal and chemical stability, high ion conductivity, and low
81 volatility. They have been exploited in analytical sciences in different application fields (e.g.,
82 chromatography, electrochemistry or extraction). Moreover, they have been used as tailor-
83 made extractants exhibiting greater selectivity than other conventional extractant reagents
84 [15]. In particular, the IL trihexyl(tetradecyl)phosphonium bis-2,4,4-(trimethylpentyl)
85 phosphinate (Cyphos IL 104 or Cyphos 104) has been used for Cr(VI) transport using a
86 plasticized PIM [16], Au(III) transport from hydrochloric acid solutions [17], Cd(II) and Cu(II)
87 separation also from hydrochloric acid solutions [18], and separation of Zn(II) from Fe(III) from
88 highly acidic solutions [19,20].

89 In the present work, a new PIM-based probe containing Cyphos IL 104 has been evaluated in
90 natural water for the first time to perform Zn accumulation measurements and to model metal
91 accumulation (Zn) in biofilm. The PIM-based probe was prepared and calibrated in the
92 laboratory using model solutions. The selectivity of the PIM-probe towards the free metal
93 species will also be investigated by the addition of an organic ligand
94 (ethylenediaminetetraacetate, EDTA). The assessment of the new probe in mimicking the Zn
95 accumulation in biological systems has been tested by comparison with fluvial biofilms that
96 were grown on submerged substrata in the Osor Stream, a river polluted with Zn due to past
97 mining activity, and then translocated to the laboratory.

98 **2. Experimental**

99 2.1 Reagents and solutions

100 A Zn stock solution (500 mg L^{-1}) was prepared from the solid $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (Panreac) and a
101 standard solution of 1000 mg L^{-1} Zn (Sigma Aldrich) was used to prepare the calibration
102 standards for ICP analysis. 2-(N-morpholino) ethanesulfonic acid (MES) (Fluka) was used as a

103 buffer. Other solids used were: $\text{Cu}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$, $\text{Ni}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$, $\text{Pb}(\text{NO}_3)_2$, $\text{Cd}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, all of
104 them from Panreac.

105 To prepare the membranes, cellulose triacetate (CTA) polymer (Acros Organics), the plasticizer
106 2-nitrophenyloctyl ether (NPOE) (Sigma-Aldrich), and chloroform (Panreac) were used.
107 Trihexyl(tetradecyl)phosphonium bis-2,4,4-(trimethylpentyl) phosphinate (Cyphos IL 104) was
108 kindly donated by Cytec (USA).

109 The organic ligands trans-1,2-diaminocyclohexan-N,N',N'-tetraacetic acid (CDTA) and
110 ethylenediamine N,N',N'-tetraacetic acid disodium salt (EDTA) were purchased from Sigma-
111 Aldrich. HNO_3 69% (Panreac) and sodium sulphate (Merck) were also employed.

112 Ultrapure water with resistivity $\geq 18 \mu\text{S cm}^{-1}$ was taken from a MilliQ system (Millipore).

113

114 2.2 Instruments

115 A GLP-22 pH-meter (Crison) was used to measure the pH of the samples. Conductivity was
116 monitored with an Ecoscan portable conductometer from Entech Instruments. Total organic
117 carbon was measured with a TOC-V CSH from Shimadzu.

118 Zn concentrations were determined using an inductively-coupled plasma atomic emission
119 spectrometer, ICP-AES 5100 (Agilent Technologies).

120

121 2.3 Water samples

122 The appropriate volume of Zn stock solution was added to simulated natural water (SNW)
123 containing 2 mM NaHCO_3 , 0.25 mM Na_2SO_4 , 0.75 mM CaCl_2 and 2.5 mM MES solution (pH 6.3).

124 The preparation of SNW was adapted from [21] and the chemical parameters of Osor Stream
125 water (Table S1) were also taken into account.

126 Water samples from the Osor Stream (41°56'51"N, 2°33'25"E), a small tributary of the River
127 Ter (Girona, Spain), were collected during different time periods (July 2017, March-April 2018).

128 This stream is influenced by continuous (effluent) and diffuse (run-off) metal inputs from a
129 disused mine [22].

130 Two sampling points were selected: a reference point (Osor-R), where Zn was not detected by
131 ICP-MS ($< 1.5 \mu\text{g L}^{-1}$), and a point downstream of the effluent from the mine area (Osor-M),
132 where $[\text{Zn}]_{\text{total}}$ varied from 200 to $450 \mu\text{g L}^{-1}$ (depending on the sampling period). The main
133 chemical parameters of these samples were pH 8.2-8.3, conductivity $400\text{-}500 \mu\text{S cm}^{-1}$, and TOC
134 $2\text{-}3 \text{ mg C L}^{-1}$ (see Table S1). Additionally, to compare the influence of the metal concentration
135 on both the PIM-probe and the biofilm, another water sample, Osor-d, was obtained by
136 diluting Osor-M with Osor-R (60:40, v/v) in order to ensure the same chemical characteristics.

137

138 2.4 Membrane preparation and PIM-probe experiments

139 A PIM containing 50% CTA: 30% Cyphos 104: 20% NPOE (% in w/w) [19] was prepared by a
140 casting method. First, the ability of the PIM to extract Zn was investigated by contacting a
141 piece of PIM (2 cm x 2cm) with $500 \mu\text{g L}^{-1}$ Zn in 15 mL of ultrapure water, SNW, or in solutions
142 separately containing 2 mM hydrogen carbonate, 0.25 mM sulphate, or 1.5 mM chloride (the
143 same concentration as in SNW). The contact time was 4 h under rotary agitation. The metal
144 remaining in the solution was determined and the zinc extracted in the PIM was calculated by
145 mass balance. The same procedure was followed to study the selectivity of the PIM using
146 solutions containing Cu(II), Pb(II), Ni(II) or Cd(II) at a concentration $500 \mu\text{g L}^{-1}$ each.

147 For the accumulation measurements, the PIM was mounted on a special device [14], which
148 was immersed 1 cm in a vertical position in the donor water sample containing Zn, under
149 agitation. The exposed area was 2.5 cm^2 and the acceptor solution (2.5 mL) was located inside
150 the device. The volume of donor phase was 250 mL and magnetic stirring was applied.
151 Different acceptor phase compositions, 0.01 M HNO_3 , 5×10^{-4} M CDTA and 0.5 M Na_2SO_4 , were
152 tested to recover the extracted zinc from the PIM. Calibration was performed, fixing 4 h as the
153 accumulation time, 0.01 M HNO_3 as acceptor solution under magnetic stirring. The same

154 experimental conditions as in the calibration were used when free zinc was measured in spiked
155 SNW and in spiked Osor-R (both at $0.50 \text{ mg L}^{-1} \text{ Zn}$), and also in Osor-M.

156 An additional accumulation experiment was performed using Osor-M in 10 L buckets under
157 agitation (teflon blades -Heidolph Hei-TORQUE Value 100) at 150 rpm.

158

159 2.5 Metal accumulation in biofilm

160 Biofilms were grown on submerged artificial substrata (7 cm x 7 cm sand-blasted glass
161 supports) at the colonization site (Osor-R) for 14 days, in April 2018, under optimum conditions
162 of light and water velocity. The substrata colonized with biofilm were then taken to the
163 laboratory and subjected to different treatments (see the experimental lay-out presented in
164 Figure S1): three biofilm samples were immediately separated for analysis; two biofilm
165 samples were exposed to Osor-M, and another two biofilm-colonized glasses were exposed to
166 Osor-d in 10 L buckets under agitation (teflon blades -Heidolph Hei-TORQUE Value 100) at 150
167 rpm. Experiments for the determination of Zn accumulated in the PIM-based probe were
168 simultaneously run in the 10 L buckets where biofilm was exposed.

169 After 4 h exposure time, the biofilm was recovered in a Petri dish, and dried in the oven at 50
170 °C for 15 h to determine the dry weight (DW). Around 200-400 mg DW biofilm was digested
171 with 4 mL of concentrated HNO_3 (65% suprapure) and 1 mL H_2O_2 (31% suprapure) in a
172 microwave ETHOS SEL High Performance Extraction System (Milestone). The Zn concentration
173 in the acid extract of the biofilm as well as the metal accumulated in the acceptor phase of the
174 PIM-probe were determined by ICP-AES after filtration through a $0.45 \mu\text{m}$ cellulose acetate
175 filter.

176 All experiments were run at least in duplicate at $22 \pm 2 \text{ }^\circ\text{C}$.

177

178 3. Results and discussion

179 3.1 Performance of the PIM-based probe

180 The compositions of the PIM, as well as the chemical characteristics of the donor and the
181 acceptor phases, are key factors in the design of an effective transport system. Baczynska et al.
182 studied the effect of the plasticizer on the extraction of anionic zinc chlorocomplexes [19],
183 finding that a PIM composition of 50% CTA: 30% Cyphos 104: 20% NPOE resulted in a higher Zn
184 transport rate. Therefore, we fixed this composition for the PIM-based probe and we focussed
185 in evaluating, for the first time, its effectiveness for the transport of Zn in natural waters. For
186 this, a $500 \mu\text{g L}^{-1}$ Zn solution in SNW at $\text{pH} = 6.3$ was used as a donor phase, where Zn exists
187 mainly as a free cation (94.5%, according to visual MINTEQ calculations), and it was found that
188 the metal was successfully transported to a 0.01M HNO_3 solution used as the acceptor phase.
189 Some exploratory experiments were performed to better understand the transport
190 mechanism, since, to the best of our knowledge, all published work dealing with extraction of
191 metals with Cyphos 104 has been performed with anionic species present at high hydrochloric
192 acid concentrations [16–20]. The experiments were conducted using $500 \mu\text{g L}^{-1}$ Zn in both
193 ultrapure water or in solutions separately containing carbonate, sulphate, or chloride (Figure
194 S2). Similar percentages of Zn extraction were found in all cases except for ultrapure water,
195 showing out that Cyphos 104 can extract positive charged Zn species through an ion-pair
196 mechanism that also involves the extraction of an anion. The extraction in SNW was higher
197 (Figure S2), around 85%, probably due to the cumulative effect of the anions that were
198 simultaneously present. Selectivity was also studied. To this end, pieces of the PIM were
199 contacted with Cu(II), Pb(II), Ni(II) or Cd(II) contained in SNW solution at $\text{pH} 6.3$. Only around
200 20% extraction for Cu(II) was found, and less than 5% in the other cases.

201 The accumulation of Zn in the PIM-based probe was then studied. Acceptor phases of different
202 composition such as 0.01 M HNO_3 , as was used in previous works [11,13], a complexing agent,
203 CDTA (5×10^{-4} M) and a 0.5 M Na_2SO_4 solution [19] were tested. The results are shown in Figure
204 1 (a) as the time dependence accumulation of Zn. As it can be observed, a 0.01 M HNO_3
205 solution provided a more effective transport of metal, resulting in a linear relationship

206 between the Zn accumulated and the accumulation time until 17 h of exposure. The Zn
207 accumulation in the acceptor phase is in agreement with a steady-state regime, and the
208 average flux calculated is $15.5 \pm 1.8 \text{ nmol cm}^{-2} \text{ h}^{-1}$ [23]. However, experiments over longer
209 periods of time showed a decrease of Zn in the acceptor phase due to the deployment of the
210 metal in the donor solution. For this reason, and fixing 0.01 M HNO_3 for further studies, the
211 same type of experiment was carried out using 10 L of Osor-M, which naturally contained 314
212 $\mu\text{g L}^{-1}$ Zn, as a donor phase (see Figure 1 (b)). The trend observed is similar to that in Figure 1(a)
213 for 0.01 M HNO_3 , with a linear accumulation at shorter times and a maximum value at 17 h.
214 The higher volume of the donor solution ensured that no depletion of the metal occurred,
215 reaching an equilibrium state.

216

217 **Figure 1**

218 Membrane stability in SNW was checked by measuring the weight difference of the PIM at
219 different accumulation times *versus* the initial weight. After 6 h contact time, the mass loss of
220 the Cyphos 104-based PIM was 3%, being 13-15% for a PIM prepared with D2EHPA (50% CTA:
221 30% D2EHPA: 20% NPOE (w/w)). Moreover, the reuse of the PIM was also checked and we
222 obtained similar fluxes when a new membrane was compared with a PIM which has previously
223 been in contact for 7 hours with the Osor-R, being 12.2 ± 0.2 and $9.8 \pm 0.5 \text{ nmol cm}^{-2} \text{ h}^{-1}$,
224 respectively (fluxes obtained at 28 h accumulation time, n=2). The stability and the long-term
225 performance are clear advantages for the application of the probe for extended time periods.

226

227 3.2 Calibration and speciation measurements

228 A calibration curve was obtained for an exposure time of 4 h affording an intermediate
229 accumulation of Zn within steady state conditions and without depletion of the metal even

230 when 250 mL of donor solution was used. The results obtained are depicted in Figure 2,
231 together with the equation that relates the accumulated Zn to the free concentration of metal
232 in the donor solution. The good correlation between the two variables is particularly
233 noteworthy.

234

235 **Figure 2**

236

237 To test whether the PIM-probe can be applied to measure free Zn, different experiments were
238 performed using SNW as a donor solution, with and without the EDTA ligand, and Osor-R and
239 Osor-M, which contain natural ligands. The total Zn concentration (measured with ICP-AES)
240 was between 0.48-0.51 mg L⁻¹, in agreement with the amount added. The accumulated Zn in
241 the acceptor phase and the free Zn measured with the PIM-probe in the different water
242 samples are presented in Table 1, and compared with Zn free values calculated by MINTEQ.

243 **Table 1**

244 The addition of EDTA in SNW diminishes the value of free Zn to 31.7% as calculated by
245 MINTEQ, which is in good agreement, within the experimental error, with the 29% obtained
246 with the PIM-based probe. Therefore, the addition of EDTA results in the formation of
247 complexes with Zn that cannot be transported through the PIM (non-reactive species). For
248 both Osor-R (with added Zn) and Osor-M water samples, around 72-87% of the total metal is
249 measured, meaning that Zn is either in the free form, forming labile complexes (i.e. complexes
250 that dissociate at the membrane interface), or forming neutral lipophilic complexes soluble in
251 the PIM phase. The remaining metal forms stable complexes, probably with organic ligands, or
252 complexes that are kinetically inert. The results obtained highlight the good ability of the
253 developed PIM-based probe for Zn speciation measurements in natural waters to determine

254 the PIM concentration of Zn (Zn_{PIM} in analogy to the C_{DGT} defined for the diffusion gradients in
255 thin film technique or DGT [23]).

256 3.3 Comparison with Zn accumulated in biofilm

257 The developed PIM-based probe was investigated as a tool to mimic metal accumulation in
258 living organisms. We selected biofilm communities, formed by benthic microbial communities
259 in a structure of highly complex extracellular polymeric substances, which were previously
260 tested for metal accumulation [22]. It was found that biofilm accumulated 150–225 $\mu\text{g Zn g}$
261 DW^{-1} in microcosms, and 200–500 $\mu\text{g Zn g DW}^{-1}$ in the Osor-M Stream (acute toxicity, 6-24h)
262 and that the metal in biofilm continued to accumulate until the end of the experiment (5
263 weeks). This exposure was found to affect the structure and function of the biofilm
264 communities [22,24].

265 We have compared Zn accumulation in the PIM-based probe with that in the biofilm
266 translocated to the laboratory (see experimental section for details and Figure S1). Laboratory
267 conditions were selected in order to exercise a tight control over the experiments. The results
268 obtained are shown in Figure 3. The background Zn concentration in the biofilm was
269 determined to be 110 $\mu\text{g Zn g DW}^{-1}$.

270

271 **Figure 3**

272

273 The high capacity of the biofilm to accumulate Zn is observed in Figure 3: the values found are
274 in good agreement with the concentration values reported by Corcoll et al. [22]. Moreover, the
275 Zn accumulation in both the biofilm and the PIM-probe respond in concordance with the
276 amount of metal present in the water samples. The trend that is observed constitutes a
277 promising result for the application of the PIM-based probe for Zn bioavailability
278 measurements and to predict metal accumulation in biofilm. Thus, the use of this probe can

279 avoid the long and tedious biofilm manipulation steps (collection, transportation to the
280 laboratory, biofilm drying and acid digestion) needed to investigate metal contamination.
281 Moreover, the PIM-probe can be easily prepared at a low cost as the estimated costs for a PIM
282 is less than 1 euro. It is worth mentioning that this is the first study to present a direct
283 comparison between the accumulation of a PIM-based probe and biofilm grown under natural
284 conditions.

285

286 **Conclusions**

287 A novel ionic liquid-based PIM probe has been designed for Zn bioavailability monitoring in
288 surface waters. The accumulation of Zn from both simulated natural water or water samples
289 from a polluted stream was possible using a membrane containing 50% CTA: 30% Cyphos 104:
290 20% NPOE (w/w) and a 0.01 M HNO₃ solution as the acceptor phase. The calibration of the
291 PIM-based probe was successfully performed and by the addition of EDTA to the donor
292 solution, it has been demonstrated that the PIM-probe can be used for speciation studies as
293 only a fraction of the total metal is measured by the PIM. Finally, the comparison of the metal
294 measured by the PIM-probe and the metal accumulated by biofilm, showed the ability of the
295 developed tool to perform metal bioavailability studies.

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300

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410 **Figure captions**

411

412 **Figure 1.** Zn accumulated at different exposure times. (a) Donor solution (250 mL): $500 \mu\text{g L}^{-1}$
413 Zn in SNW; (b) Donor solution: Osor-M ($314 \mu\text{g L}^{-1}$ Zn), 10 L. Acceptor phase 0.01 M HNO_3 .

414 **Figure 2.** Relationship between Zn accumulated in the acceptor phase and free Zn in SNW (250
415 mL). Acceptor phase 0.01 M HNO_3 . Exposure time: 4 h.

416 **Figure 3.** Comparison of Zn accumulated in PIM-based probe (acceptor phase 0.01 M HNO_3) vs.
417 Zn in the biofilm. Donor solution: Osor-M and Osor-d, exposure time: 4 h.

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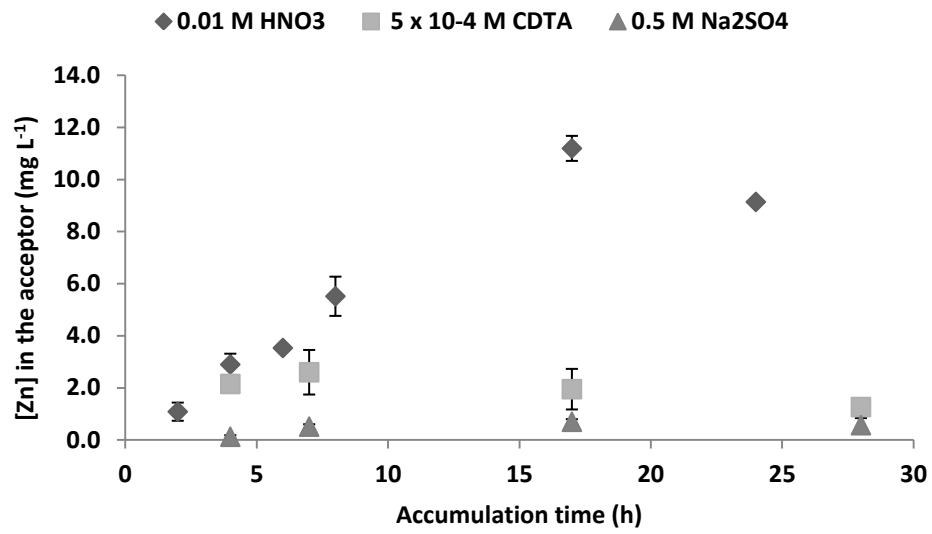
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Table[Click here to download Table: Table 1.docx](#)**Table 1.** Zn measured with the PIM-based probe (n = 2)) and calculated with visual MINTEQ. The donor volume was 250 mL. The exposure time was 4 h and the acceptor phase was 0.01 M HNO₃. Standard deviation is shown in between parenthesis.

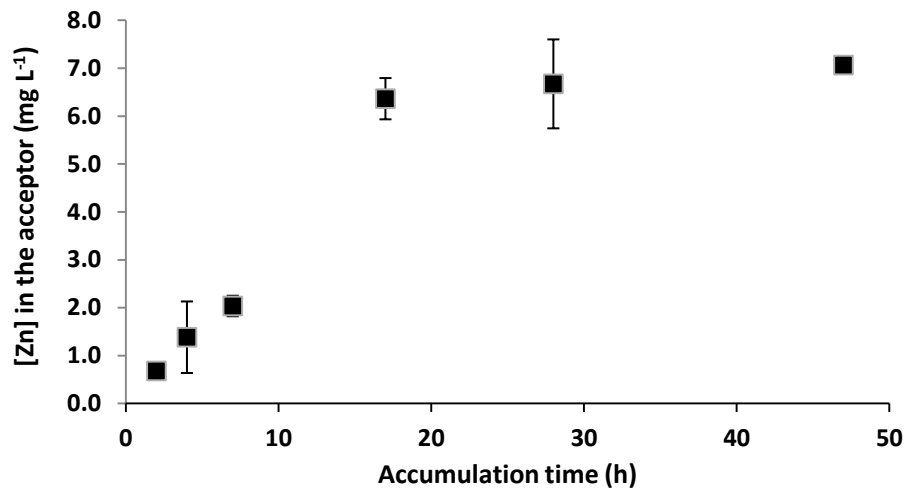
Sample	Added	Zn total content in sample	Total Zn in PIM acceptor	Zn free measured (PIM)	Zn free (PIM)	Zn free
		(mg L ⁻¹) ^a	(mg L ⁻¹)	(mg L ⁻¹) ^c	(%) ^d	(MINTEQ) (%)
SNW	Zn	0.50	2.9(0.4) ^b	0.59(0.09)	119	94.5
	Zn +EDTA	0.50	0.7 (0.2)	0.15(0.04)	29	31.7
Osor-R	-	<LOQ	0.07 (0.07)	<LOQ	n.d.	-
	Zn	0.50	1.74 (0.00)	0.36(0.00)	72	-
Osor-M 2017	-	0.45	1.9 (0.2)	0.39(0.04)	87	-
Osor-M 2018	-	0.31	1.3 (0.1)	0.27(0.02)	86	-

^ameasured with ICP-AES; ^bn = 5; ^ccalculated taking into account the measured free Zn and the total present in the donor sample, ^d Using the calibration curve shown in Fig. 2

(a)



(b)



Figure

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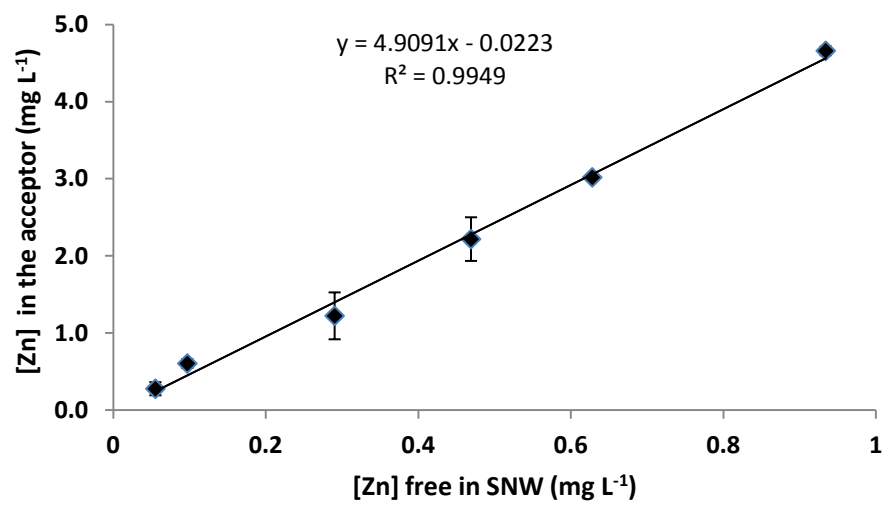
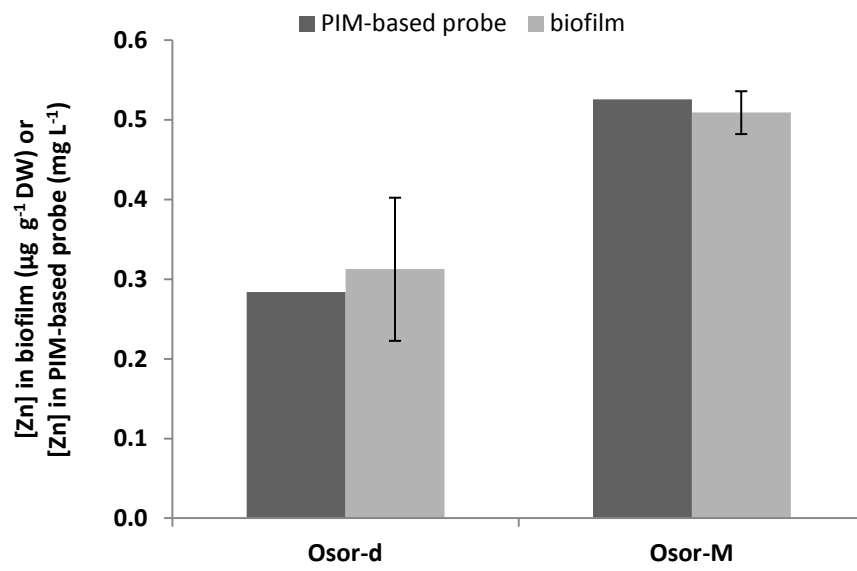


Figure
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Declaration of interests

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.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: