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1 A novel DNA biosensor using a ferrocenyl intercalator applied to the potential
2 detection of human population biomarkers in wastewater

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13 **Abstract:** A new label-free electrochemical DNA (E-DNA) biosensor using a custom
14 synthesized ferrocenyl (Fc) double-stranded DNA (dsDNA) intercalator as a redox marker is
15 presented. Single-stranded DNA (ssDNA) was co-immobilized on gold electrodes with 6-
16 mercapto-hexanol (MCH) to control the surface density of the ssDNA probe, and hybridized
17 with complementary DNA. The binding of the Fc intercalator to dsDNA was measured by
18 differential pulse voltammetry (DPV). This new biosensor was optimized to allow the
19 detection of single base pair mismatched sequences and, able to detect as low as 10 pM target
20 ssDNA with a dynamic range from 10 pM to 100 nM. DNA extracted from wastewater was
21 analysed by quantitative polymerase chain reaction (qPCR) targeting human-specific
22 mitochondrial DNA (mtDNA). The aim of this approach is to enable the analysis of
23 population biomarkers in wastewater for the evaluation of public health using wastewater-
24 based epidemiology (WBE). The E-DNA biosensor was employed to detect human-specific
25 mtDNA from wastewater before and after PCR amplification. The results demonstrate the
26 feasibility of detecting DNA biomarkers in wastewater using the developed biosensor, which
27 may allow the further development of DNA population biomarkers for public health using
28 WBE.

29 **Keywords:** population biomarkers, wastewater-based epidemiology, DNA biosensor,
30 intercalator,

31 **1. Introduction**

32 Wastewater-based epidemiology (WBE) has shown to be a powerful tool for the
33 evaluation of community-wide drug¹⁻³, alcohol⁴ and tobacco use⁵, and has the potential to be
34 utilized for the evaluation of public health by assessing disease biomarkers⁶. The approach is
35 based on the analysis of specific urinary biomarkers such as drug residues and/or their
36 metabolites in wastewater following their excretion by humans, using urban wastewater
37 collected at a treatment plant selected for sampling. WBE is a cost-effective, fast and near
38 real-time tool for the evaluation of population health compared with traditional questionnaire
39 survey approaches⁷. Until now, the quantitative measurement of specific drug biomarkers in
40 wastewater has been performed to study the drug use habits of numerous communities^{1, 3}. As
41 an example, illicit drug use trends were recently evaluated in 21 countries (42 cities, total
42 population 24.74 million) across Europe³. Furthermore, some drugs and their metabolites
43 have also been proposed as a potential population biomarker for WBE such as creatinine⁸,
44 cholesterol and coprostanol⁹, as well as caffeine and nicotine metabolites¹⁰. Chen *et al*¹¹
45 outlined criteria for a candidate population biomarker: (1) must be quantifiable; (2) have little
46 affinity to particulate matter in wastewater or to filter paper; (3) be stable in wastewater; (4)
47 be constantly excreted and (5) the total excretion should correlate with census population,
48 meaning there should be no contribution other than human metabolism. The potential
49 population biomarkers reported in the literature were evaluated by Chen *et al*¹¹ and few of
50 them could strictly meet the proposed criteria. The major limitation of these chemical
51 biomarkers is that they rely on the stability and the contribution from non-human metabolism.

52 Completion of sequencing the human genome in 2003 has shown that up to 1% of the
53 human coding sequences are associated to cancer by mutation¹². This valuable information is
54 increasingly being utilized for diagnostic purposes by using known cancer associated
55 mutations as biomarkers¹³⁻¹⁶. DNA is a surprisingly resilient biomolecule capable of
56 persisting in the environment for many centuries, as shown by the complete genome
57 sequencing of a Neanderthal individual from a bone sample¹⁷. DNA is also naturally shed
58 into the environment by virtually all living organisms through urine, faeces, exudates or
59 tissue residues as demonstrated by the advent of environmental DNA studies¹⁸. These robust
60 characteristics have allowed the association of human specific mitochondrial DNA (mtDNA)
61 to human faecal contamination to assess water quality^{19, 20}. Since mtDNA also has mutations
62 known to be associated to cancer^{21, 22}, with a high copy number per cell²³, it has the potential
63 to be suitable for evaluating the feasibility of using novel analytical tools like biosensors to

64 detect human specific DNA from wastewater samples, with the perspective of further
65 developing the system to identify population biomarkers for WBE studies. Consequently,
66 mtDNA is clearly a potential population biomarker for WBE because it meets the outlined
67 criteria such as stability, being associated to cancer, presenting human specific sequences and
68 being quantifiable.

69 Electrochemical biosensors have great promise for the detection of disease biomarkers
70 in body fluids, in particular due to being cost-effective, offering fast response times and being
71 easily integrated with other devices²⁴. The advantages over conventional analytical
72 techniques are the possibilities of portability, miniaturisation and ability to measure complex
73 matrices with minimal sample preparation. Typically, E-DNA biosensors are designed with
74 the following elements: immobilization of the single-stranded DNA (ssDNA) probe,
75 hybridisation with complementary target sequence, introduction of an electroactive indicator,
76 and the electrochemical investigation of the surface²⁵. Differential pulse voltammetry (DPV)
77 is a convenient electrochemical tool for the detection of DNA, and the signal transducer may
78 consist of either labelled sequences with an active redox maker, or a double-stranded DNA
79 (dsDNA) intercalator such as metal ion complexes with iron, cobalt, osmium or ruthenium,
80 and organic compounds like methylene blue, daunomycin, Hoechst 33258, or anthraquinone
81 derivatives²⁶⁻²⁸. Amongst these compounds, threading intercalators that carry bulky substituents
82 on the periphery of the intercalating moiety, are reported to be very efficient and stable to
83 interact with the DNA duplex²⁸. Naphthalene diimide derivatives (like ferrocenyl derivatives)
84 have been proven to be active candidates as dsDNA intercalators and the constructed
85 biosensors demonstrate excellent analytical performance for the detection of DNA^{28, 29}.

86 Here we present an electrochemical biosensor using a custom synthesized ferrocenyl
87 (Fc) dsDNA intercalator derived from naphthalene diimide as a redox marker for
88 amperometric detection. This biosensor is able to detect as low as 10 pM complementary
89 DNA and is shown to be suitable for single mismatched base pair detection, potentially
90 enabling the detection of single-nucleotide polymorphism (SNP) cancer markers. Human-
91 specific mtDNA was successfully detected from wastewater using this E-DNA sensor with
92 and without prior qPCR amplification. To the best of our knowledge, this is the first time a E-
93 DNA biosensor has been reported for the detection of a mtDNA biomarker in wastewater.
94 We hope this technique may be further improved for the purpose of population DNA
95 biomarkers study to evaluate public health by means of a WBE approach.

96 2. Experimental

97 2.1 Materials

98 Gold disc working electrodes with a radius of 1.0 mm were purchased from IJ
99 Cambria Scientific Ltd (Cambridge, UK). If not stated otherwise, all chemicals were
100 purchased from Sigma-Aldrich (UK) and used as received.

101 Single stranded DNA, used as reverse and forward primers for PCR amplification,
102 was synthesized by Eurofins MWG (Ebersberg, Germany). The oligonucleotides used as
103 DNA probes for the biosensors were modified with a thiol group at the 5' end to form HS-
104 (CH₂)₆-ssDNA to assemble on gold electrodes. The thiolated DNA sequences for elaboration
105 of biosensors were synthesized by Sigma-Aldrich (UK). The sequence 5'-HS-(CH₂)₆-CCA
106 CGT CGA GCG ATG-3' (15 bps) was used as capture probe, and one fully complementary
107 and three mismatched sequences (5'- CAT CGC TCG ACG TGG-3', 5'- CAT CGC TCA
108 ACG TGG-3', 5'- CAT CGC TAA ACG TGG-3' and 5'- CAT CGC AAA ACG TGG-3',
109 respectively) (Sigma-Aldrich) were employed to hybridize with the DNA probe. The human
110 specific mtDNA forward and reverse primers²⁰, respectively 5'-CAG CAG CCA TTC AAG
111 CAA TGC-3 and 5'- GGT GGA GAC CTA ATT GGG CTG ATT AG-3' were used for PCR
112 amplification targeting the human mitochondrial gene NADH dehydrogenase subunit 5, and
113 the modified forms with a HS-(CH₂)₆ group on the 5'-end of forward and reverse primers
114 were used for the elaboration of biosensors. Additionally, two thiolated random sequences,
115 which have the same number of base pairs with either the forward or reverse primer (5'-HS-
116 (CH₂)₆-AGA AGA AAC GGA GGA AGG GAA-3', 21 bps and 5'- HS-(CH₂)₆-AAG AAC
117 TGA AGG GCC AAA TGA GCC GA-3', 26 bps), were also synthesized for specificity
118 studies. The HPLC DNA powders were aliquoted in 10 mM Tris-HCl, 1mM
119 ethylenediaminetetraacetic acid (EDTA) upon receipt and stored in a freezer at -20 °C for
120 long-term usage.

121 The buffer for DNA probe immobilisation (IB) consisted of 0.8 M phosphate buffer
122 (PB) + 1.0 M NaCl + 5 mM MgCl₂ + 1 mM ethylenediamine tetraacetic acid (EDTA) pH 7.0.
123 The DNA hybridisation and measurement buffer was composed of 50 mM PB and 100 mM
124 K₂SO₄ (pH 7.4).

125 The influent wastewater was collected from the VEAS wastewater treatment plant
126 (WWTP; Oslo, Norway), and a sample of pooled urine was collected from portable public
127 toilets from Oslo during July, 2014. The collected samples were immediately frozen (-20 °C)
128 and stored frozen until analysis.

129 2.2. *Synthesis of Fc DNA intercalator*

130 The synthesis of dsDNA intercalators N,N' -((((((1,3,6,8-tetraoxo-1,3,6,8-
131 tetrahydrobenzo[*lmn*][3,8]phenanthroline-2,7-diyl)bis(ethane-2,1-diyl))bis(oxy)) bis (ethane-
132 2,1-diyl) bis(oxy)) bis(ethane-2,1-diyl)) diferrocenamide (**1**) was generated by N -(2-(2-(2-
133 aminoethoxy)ethoxy)ethyl)-2-ferrocenamide (**2**) and 1,4,5,8-Naphthalenetetracarboxylic
134 dianhydride (**3**). The synthesis procedure is briefly illustrated in Scheme 1, and the detailed
135 protocol of synthesis and characterization **2** and **1** is described in the supporting information.

136

137

138

Scheme 1

139

140 2.3. *Elaboration of biosensors*

141 Gold electrodes were cleaned following a procedure described elsewhere³⁰. In brief,
142 they were polished with 50 nm aluminium oxide particles (Buehler, USA) on a polishing pad
143 (Buehler) for 5 min, followed by sonication in ultrapure water, polishing on a blank polishing
144 pad, and sonication in ultrapure water to remove any particles. Gold electrodes were rinsed
145 with fresh piranha solution (H₂SO₄/H₂O₂, v/v 7/3) followed by rinsing thoroughly with
146 deionized water, and readily used for electrochemical cleaning. Electrodes were
147 electrochemically cleaned in a classical three-electrode cell by immersing them into H₂SO₄
148 (0.5 M) solution and the potential scanned between the oxidation and reduction potentials of
149 gold, 0 V and +1.5 V *versus* an Ag/AgCl reference electrode, with a scanning rate at 0.2 V/s
150 for 60 cycles until there was no further change in the voltammogram.

151 Gold electrodes then were rinsed with deionised water, dried in a stream of nitrogen
152 and incubated with mixed ssDNA/6-mercapto-1-hexanol (MCH) immobilization solution for
153 16 h in a humidity chamber at 4 °C. The molar ratio between ssDNA and MCH was 1:3, with
154 1 μM ssDNA in immobilization buffer. After immobilization, electrodes were rinsed in 50
155 mM PB + 100 mM K₂SO₄ + 10 mM EDTA (pH 7.0) to remove any remaining Mg²⁺. In order
156 to ensure complete thiol coverage of the gold surface and make favourable ssDNA
157 conformation for hybridization, the electrodes were backfilled with MCH (1 mM, H₂O) for 1
158 h, followed by rinsing with ultra-pure water and slightly drying with N₂ stream. The
159 electrodes was electrochemically characterized and then incubated with Fc intercalator at

160 desired concentration for 1 h. The electrodes were extensively rinsed with 0.1 % Tween-PB
161 buffer with vortex for 30 s, followed by rinsing with DI water and drying in N₂ stream.

162 *2.4. Isolation and characterization of DNA from wastewater*

163 The DNA in wastewater from Oslo (sample ‘WW1’) and in the pooled urine (sample
164 ‘WW2’) was isolated with a PowerWater® DNA Isolation Kit (14900-50-NF, (MO BIO
165 laboratories, Inc), without previous concentration according to manufacturer’s instructions to
166 obtain 100 µL DNA sample. Sterilized distilled water was included as a negative extraction
167 control. The concentration of the isolated DNA was determined with a Nanodrop®
168 spectrophotometer (ND-1000, Nanodrop Technologies, Wilmington, Delaware, USA).

169 *2.5. qPCR analysis of human-specific DNA in wastewater*

170 Quantitative real-time PCR (qPCR) was performed using a Bio-Rad CFX96
171 instrument (Bio-Rad Laboratories, Hercules, CA, USA). The human specific mtDNA ND5
172 primers were used for PCR amplification from wastewater DNA samples²⁰. The final PCR
173 reaction volume was 15 µL, consisting of 7.5 µL SsoFast mastermix (Bio-Rad) 0.6 µL
174 reverse and forward primers (0.4 µM final concentration), 2 µL DNA sample and completed
175 with 4.3 µL ddH₂O. The amplification conditions were modified as following: 2min hot start
176 step at 98 °C, followed by 35 cycles of 98 °C for 5 s and 60 °C for 5 s. The amplification was
177 followed by a melt curve analysis with a temperature incrementation of 0.2 °C from 65 °C to
178 95 °C. A positive control consisting of human blood DNA sample and two negative controls
179 (one from sterilized water during DNA isolation control and one is non-nucleotide water)
180 were added in each run of amplification. The amplification was considered to be valid if only
181 one unique product peak was identified by melting curve analysis.

182 Human mitochondrial gene ND5 nucleotide sequences were retrieved from
183 GeneBank (<http://www3.ncbi.nlm.nih.gov>), and the PCR amplicon as part of AY972053 was:
184 5’-CAGCAGCCATTCAAGCAATCCTATAACAACCGTATCGGCGATATCGGTTTCATC
185 CTCGCCTTAGCATGATTTATCCTACACTCCAACCTCATGAGACCCACAACAAATAG
186 CCCTTCTAAACGCTAATCCAAGCCTCACCCCACTACTAGGCCTCCTCCTAGCAGC
187 AGCAGGCAAATCAGCCCAATTAGGTCTCCACC (195 bps)-3’. An aliquot (3 µL) of
188 each amplification reaction was analysed on 1% (w/v) agarose gel in TAE 1x buffer (pH 8).
189 The image was recorded by Bio-Rad reader (Image Lab 4.1 software). In addition, an 182bp
190 non-complementary PCR product amplified from the sequence of fibroblast growth factor

191 receptor 3 FGFR3 S249C) as described previously³¹, was randomly used to evaluate the
192 selectivity of the biosensors.

193 2.6. Electrochemical characterization of biosensors

194 All electrochemical measurements were performed on a three-electrode cell, with an
195 Ag/AgCl reference electrode (Radiometer Analytical, Lyon, France) against which all
196 potentials are quoted, and a Pt counter electrode (BASi, USA). Before measurement,
197 electrodes were placed into measurement buffer for 1 h to stabilize the electrodes. PCR
198 products were heated at 95 °C for 10 min and then cooled down to room temperature before
199 the incubation with ssDNA probes.

200 Differential pulse voltammetry (DPV) measurements were performed by placing
201 electrodes in 100 mM PB with Ag/AgCl as reference electrode. DPV scans run between -0.2
202 V and 0.7 V vs Ag/AgCl (scan rate 0.05 V/s, step potential 0.005 V, modulation amplitude
203 0.05 V, modulation time 0.05 s, interval time 0.1 s). The schematic illustration of elaboration
204 of DNA sensors with Fc intercalator as redox marker is presented in Scheme 2.

205

206

Scheme 2

207 3. Results and discussion

208 3.1 Development of biosensors based on Fc intercalators

209 A crucial control parameter for electrochemical DNA biosensors, is the surface
210 density of the immobilized ssDNA probe onto the electrode surface in order to effectively
211 hybridize with complementary DNA. Many studies have shown that a low density of ssDNA
212 ($1\text{-}3 \times 10^{12}$ molecules/cm²) on the surface of the electrodes could achieve high hybridization
213 efficiency for DNA biosensors^{30, 32, 33}. In our early study³⁰, we optimised the surface density
214 to maintain high hybridization efficiency by controlling the molar fraction of ssDNA with
215 MCH on the electrode surface. Here, we used the optimal molar ratio between ssDNA and
216 MCH (1:3) (ssDNA surface density of 1.6×10^{12} molecules/cm²) in order to allow
217 complementary DNA to effectively hybridize with the DNA probe. The concentration of Fc
218 intercalators was optimized to 1 μ M for binding with dsDNA. As shown in Figure 1A, the
219 current peak at around 0.49 V *versus* Ag/AgCl was associated with the oxidation of ferrocene
220 on the electrodes. The peak current increases proportionally to complementary DNA
221 hybridization to the ssDNA probe, enabling target complementary DNA concentration

222 measurement. The synthesized dsDNA intercalator can specifically bind with matched bases
223 in-between the double-helix of DNA; the detailed synthesis and characterization is described
224 in the supporting information. The number of paired bases on the electrode surface increases
225 with increasing concentration of complementary DNA, leading to more intercalators to bind
226 with dsDNA and a current value increase. The signal response to the complementary DNA
227 hybridization shows a good dynamic range spanning from 10 pM to 100 nM ($R^2=0.98$), and
228 the limit of detection was determined to be 10 pM (3σ). These results suggest that our custom
229 synthesized intercalator displays effective and specific binding with the dsDNA.

230 Several groups have developed voltammetric biosensors to detect DNA using
231 different surface modification to improve sensitivity and analytical performances as presented
232 in Table S1 on the Supplementary Information³⁴⁻³⁸. As an example, Bo *et al*³⁵ reported a
233 voltammetric biosensor based on the chemically modified graphene paste with a limit of
234 detection of 0.02 nM and a dynamic range of 0.02 - 21.2 nM. Liu *et al*³⁶ developed a sensitive
235 DNA biosensor with a limit of detection at 0.001 nM using a hollow gold nanosphere to
236 enhance the immobilized DNA probe amount. However, the elaboration of biosensors
237 required complicated surface coating procedures and allowed non-specific adsorption of the
238 analyte onto the surface. Compared to those biosensors, the sensor described here could
239 effectively control the surface density by the molar ratio between the MCH and thiolated
240 ssDNA. The elaboration procedure was robust, easy-to-operate, time-saving whilst still
241 sensitive enough for complementary DNA detection at pM range. The stability of the DNA
242 biosensors was also investigated by storage of the immobilized DNA probes at 4 °C. The
243 results show that the peak current value from hybridization with the 1 nM complementary
244 DNA only reduced to 88%, 79.3 % and 68 % following storage for 1, 3 and 7 days,
245 respectively (See Figure 1). This is due to the robust co-immobilized SAMs layer on the gold
246 electrodes.

247 **Figure 1**

248
249 In order to further investigate the specific interaction of the Fc intercalator, fully and
250 partially complementary DNA containing 1, 2 and 3 mismatched base pairs were employed to
251 hybridize with the ssDNA probe on the electrode. As shown in Figure 1B, the peak current
252 decreased with the increasing number of mismatched base pairs, suggesting that our Fc
253 intercalator is able to distinguish a single mismatched base pair, and therefore potentially be
254 employed for the detection of single-nucleotide polymorphisms (SNPs) disease biomarkers.

255 The naphthalene diimide moiety in the Fc intercalator is able to insert or intercalate between
256 adjacent base pairs of dsDNA. The intercalator carries bulky ferrocene on the periphery of
257 intercalating moiety, and it becomes placed in the major and minor grooves simultaneously
258 when intercalated to the DNA duplex³⁹. This kind of intercalator has a strong affinity with
259 dsDNA because of this peculiar binding mode, and enables to easily discriminate between
260 double- and single-stranded DNA with a large margin. More importantly, due to its highly
261 specific affinity to well matched base pairs it can even discriminate a single mismatched base
262 pair. SNPs are the most common type of genetic variation among people, and each SNP
263 represents a difference in a single DNA nucleotide. In this study, we investigate feasibility for
264 SNPs detection in wastewater samples for the purpose of public health epidemiology. Pänke
265 *et al*²⁵ electrochemically detected single base-pair mismatch using a competitive binding
266 assay between un-labelled and labelled DNA with an electroactive redox-marker methylene
267 blue, and methylene blue was also demonstrated to be useful for the detection of point
268 mutation in DNA voltammetric biosensor⁴⁰. In our study, we simply use the affinity between
269 Fc intercalator and fully/partially matched base pairs to transduce the signal, which is label-
270 free and rapid for single base-pair mismatch detection⁴¹. Besides, this intercalator also shows
271 an enhancement of impedimetric signal not only for DNA hybridization (see Figure S9) but
272 also for aptamer sensors for the detection of protein⁴¹. Furthermore, to evaluate the feasibility
273 of E-DNA biosensor for the detection of complex matrix, 1 μ M fully complementary DNA
274 (5'- CAT CGC TCG ACG TGG-3', 15 bps) was spiked into wastewater and deionized water
275 for assay, respectively, the peak values from all the blanks are negligible (0.02 μ A in buffer,
276 0.04 μ A in wastewater and 0.03 I deionized water), indicating that our sensors are specific for
277 complex matrices detection. The peak value (3.95 μ A) from DNA spiked in wastewater is
278 only slightly higher than that (3.72 μ A) in buffer, and the current value (3.61 μ A) from DNA
279 spiked in deionized water is a bit lower than that in buffer. However, in general the responses
280 of the spiked DNA samples in three matrixes are not significant.

281 *3.2 qPCR analysis of human-specific mitochondrial DNA from wastewater*

282 In addition to mtDNA mutations associated to cancer^{21, 22}, mutations in the ND5
283 subunit of complex I of the mitochondrial DNA are a frequent cause of oxidative
284 phosphorylation disease⁴². Therefore, the ND5 sequence could potentially be used as a
285 population biomarker candidate. DNA was extracted from the Oslo wastewater sample WW1)
286 and the pooled urine sample WW2; human-specific mtDNA was amplified by qPCR and the
287 product specificity was checked by melting curves analysis and gel electrophoresis. The

288 qPCR protocol²⁰ was modified for the optimal use of the qPCR instrument and the reagents
289 used in this study. A human DNA sample was included as a positive control (PC), and two
290 negative controls were amplified (one from sterilized water during DNA isolation control
291 NC1, another from non-nucleoid water NC2). As shown in Figure 2A, the PC sample yielded
292 the highest signal, followed by WW2 and then WW1 samples. This correlated well with the
293 fact that the mtDNA template content in WW1 was less than that in WW2 due to around 1-10
294 dilution factor for WW1 collected from the wastewater treatment plant. Figure 2B presents
295 the melting curve of the PCR amplicons. A single well defined melting peak at around 83 °C
296 was observed for both the samples studied and the human positive control sample confirming
297 the specificity of the PCR amplification. Furthermore, specificity was also confirmed by gel
298 electrophoresis showing a product size corresponding to the expected 195 bp (see Fig 2C).
299 The two negative controls show no or negligible amplification, confirming that the tested
300 positive samples are true positives. We can therefore conclude that the DNA sample
301 preparation and qPCR assay produced specific and quantitative results from urine and
302 wastewater samples.

303 **Figure 2**

304

305 *3.3. Detection of human specific mtDNA from wastewater with developed biosensors*

306 The thiolated forward and reverse primers were separately immobilized onto
307 electrodes for the detection of mtDNA qPCR products from the three tested samples: PC,
308 WW1 and WW2. The measurements were performed using the same procedure as applied to
309 the standard samples and the developed biosensor. As shown in Figure 3, three PCR samples
310 (PC, WW1 and WW2) diluted 1 in 50, were hybridized with two different probes on the
311 sensor and the current peak at around 0.49 V *versus* Ag/AgCl, attributed to the oxidation
312 peak of the Fc intercalator, was obtained from both probes each binding with each of the
313 three samples. The peak current value, proportional to the concentration of mtDNA PCR
314 products, is the highest for PC and it shifts to lower values in the urine-pool WW2 and
315 wastewater WW1 due to the decreased concentration of mtDNA amplicons. The results are in
316 good agreement with the qPCR results (Figure 2). However, the peak current value (0.17 μ A)
317 from the forward primer probe (21 bp) is lower than that (0.20 μ A) from the reverse primer
318 probe (26 bp) when detecting the same WW2 sample. This is due to the fact that the reverse
319 primer contains 5 more bases than the forward one, therefore resulting in more Fc

320 intercalators interacting with dsDNA on the electrodes. The highest peak current was
321 achieved using the reverse primer as probe and therefore this probe was selected for further
322 detection.

323 **Figure 3**

324
325 In order to evaluate the specificity of the binding between the ssDNA probes and the
326 PCR products, two thiolated ssDNA that contain the same number of bases as either the
327 forward or the reverse primers and are non-complementary to the mtDNA PCR product
328 sequence, were immobilized and named probe F(non) and probe R(non). Figure 4A presents
329 the peak current value obtained from the 4 different probes with WW1 PCR products diluted
330 1 in 50. The peak currents from non-complementary probes are low compared to those from
331 complementary ones, indicating that the intercalator could specifically bind with matched
332 base pairs. The same experiment was performed in the analysis of the WW2 sample, which
333 shows similar results (Figure 4B). Though there are small peaks from the non-complementary
334 probes, it is likely that the long-sequence mtDNA may foul the electrode surface introducing
335 spurious effects. To further determine the non-specific adsorption of probes, a non-
336 complementary PCR product (182 bps) diluted 1 in 50 was introduced to hybridize on a
337 probe R electrode under the same conditions. As shown in Figure 4C, the current signal from
338 non-complementary sample gives a negligible signal. These results demonstrate that the
339 biosensors can specifically detect human mtDNA PCR products extracted from wastewater
340 using the custom synthesized ferrocenyl intercalator.

342 **Figure 4**

341
342
343
344 The optimized biosensor was used to detect mtDNA of PCR products from WW1 and
345 WW2 with various dilution factors. As shown in Figure 5, the peak current signal decreased
346 with the increasing dilution-factor for WW1 and WW2, displaying a good dynamic range
347 spanning from dilution-fraction 1/5 to 1/100, with a LOD determined to be 7.1 nM from
348 WW1 and 4.4 nM from WW2 (1/100 and 1/200 dilution respectively; the concentration was
349 measured with a spectrophotometer). The threshold value was determined from the average
350 signal of blanks (“PCR non-com”) plus 3 times standard deviation. However, to obtain a

351 more reliable threshold value, a cohort of non-complementary PCR products may need to be
352 evaluated. On the basis of these promising results, we then attempted to directly detect target
353 DNA from the WW1 (12.2 $\mu\text{g}/\text{mL}$) and WW2 (33.7 $\mu\text{g}/\text{mL}$) samples (extracted from 100 mL
354 wastewater) without PCR amplification. Although the peak current values obtained were
355 quite close to that from non-complementary PCR products (Figure 5C), WW2 was above the
356 threshold while WW1 scored negative. This difference is quite understandable since WW2 is
357 undiluted urine, while WW1 is approximately 1% urine. Wastewaters are complex matrices
358 with a high bacterial content along with diverse cell residues from plants and animals aside of
359 human shed material. Such a dilution of the target human DNA, along with the interference
360 of non-target DNA is expected to be challenging for direct detection as signal may also be
361 associated to non-specific adsorption from self-fouling of long DNA sequences. However,
362 the concentrated DNA extracted from pooled urine (WW2) produced a stronger signal than
363 that obtained with WW1, as expected as it is likely to contain proportionally more target
364 human DNA, as already shown by qPCR. To our knowledge this is the first time that an E-
365 DNA biosensor is used for direct detection of target DNA from a wastewater sample. The

366

367

Figure 5

368

369 Although direct detection and quantification of human mtDNA from extracted
370 wastewater samples remains challenging, we believe that this proof-of-concept study will
371 accelerate the development of ultrasensitive biosensing technology for the analysis of
372 biomarkers in wastewater for epidemiology studies. For example, this system may be further
373 improved by using nanomaterials for signal enhancement⁴³ for a more sensitive DNA
374 biomarker analysis in wastewater without PCR amplification.

375 In summary, a sensitive E-DNA biosensor using a custom synthesized Fc intercalator
376 as the electrochemical signal transducer was developed. The optimized biosensor could detect
377 complementary DNA at concentrations as low as 10 pM with a dynamic range spanning from
378 10 pM to 100 nM, also enabling the detection of single nucleotide mismatches. To further
379 evaluate the feasibility of using this technology for analysing human population DNA
380 biomarkers from wastewaters, primers designed to amplify human-specific mtDNA were
381 used for qPCR from wastewater and pooled urine samples. Serial dilutions of the human
382 mtDNA PCR products were specifically detected by the customized biosensors down to a

383 200-fraction dilution (4.4 nM). Furthermore, E-DNA biosensors appear well suited for
384 implementation in portable PCR microdevices directed at the rapid detection of DNA
385 biomarkers⁴⁴, including wastewater for the purpose of WBE. Finally, our study demonstrates
386 the feasibility of analysing human specific DNA biomarkers directly in wastewater by using
387 an E-DNA biosensor. This shows that E-DNA biosensors have a strong development
388 potential for the monitoring of DNA-disease biomarkers in wastewater, such as SNPs, for the
389 evaluation of public health by WBE.

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399 pooled urine samples.

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Captions:

Scheme 1 Synthesis of Fc dsDNA intercalator

Scheme 2 Illustration of detection of human mitochondrial DNA from wastewater with electrochemical biosensor based on Fc intercalator.

Figure 1 DPV curve of 1 mM ferrocenyl intercalator for the detection of various concentrations of complementary DNA and the current as the function of the complementary DNA concentration (insert) (A), and of DPV curve from effects of the number of mismatched base pairs and the current value as the function of the number of mismatched base pairs (insert) (B).

Figure 2 Amplified cycles (A) and melting curve (B) of qPCR analysis of mtDNA in wastewater sample using prime expected products 195 bps, and image (C) of electrophoresis gel analysis of PCR products with double replicates from each sample. PC: human positive control, WW1: wastewater treatment plant, WW2: urine pool, NC1: extraction negative control, NC2: qPCR mastermix negative control.

Figure 3 DPV curve of detection of human sample (PC), WW1 and WW2 samples amplified with PCR using thiolated forward (A) and reverse (B) primers as probes

Figure 4 Detection of PCR products (1/50 dilutions) of WW1 (A) and WW2 (B) with different thiolated probes (probe F: thiolated forward primer; probe R: thiolated reverse primer probe; F/R (non): 21/26 bps non-complementary to the mtDNA PCR product), and (C) current signal response to the complementary and non-complementary PCR products hybridisation to probe R.

Figure 5 Detection of WW1 (A) and WW2 (B) PCR product serial dilution, and (C) the current response from the detection of mtDNA extracted from wastewater with/without PCR amplification, reference PCR products of WW1 and WW2 were diluted 1 in 50.

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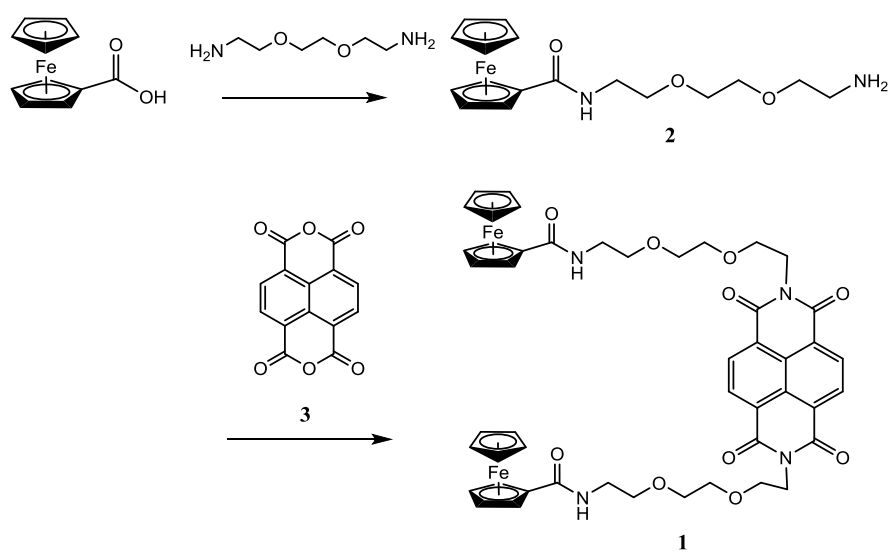
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581 **Schemes and Figures**

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Scheme 1 Synthesis of Fc dsDNA intercalator

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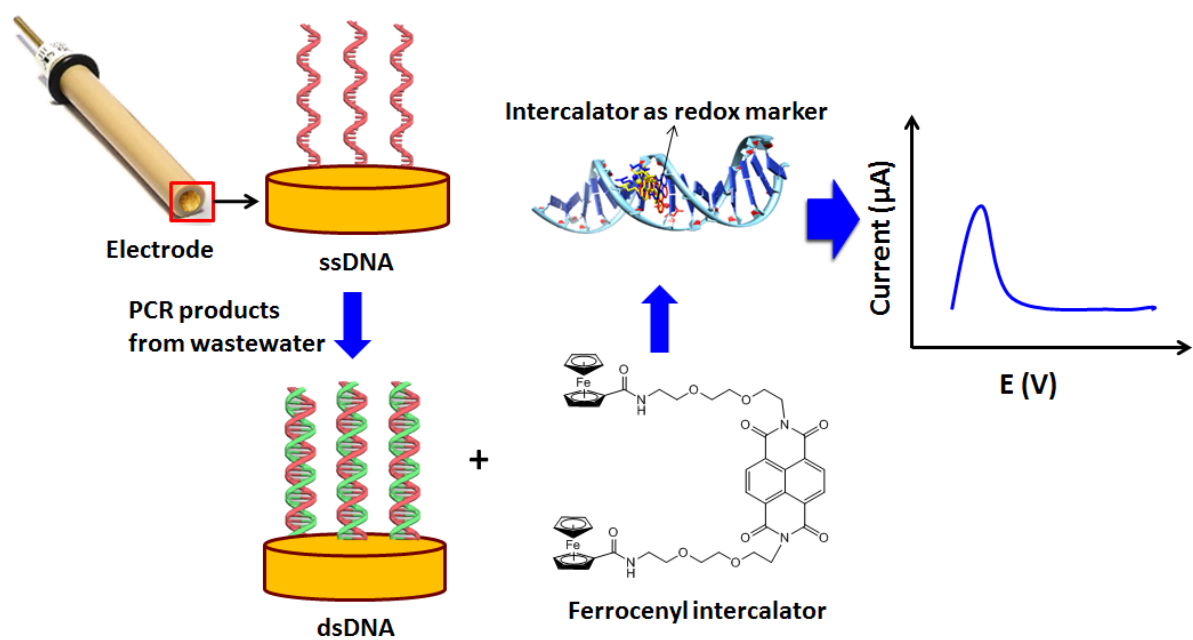
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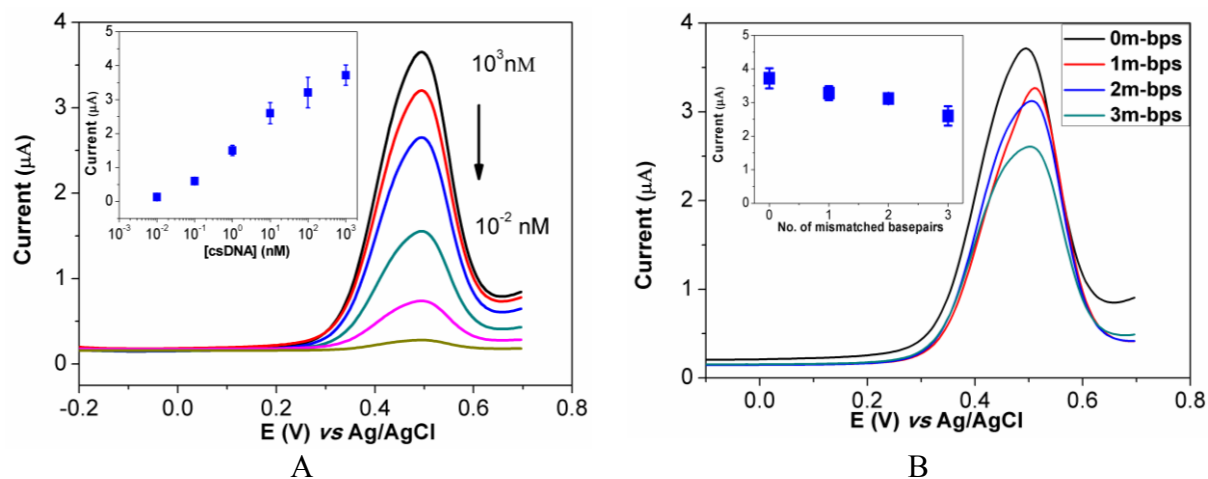
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Scheme 2 Illustration of detection of human mitochondrial DNA from wastewater with electrochemical biosensor based on Fc intercalator.

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619 Figure 1 DPV curve of 1 mM ferrocenyl intercalator for the detection of various concentrations of
620 complementary DNA and the current as the function of the complementary DNA concentration
621 (insert) (A), and of DPV curve from effects of the number of mismatched base pairs and the current
622 value as the function of the number of mismatched base pairs (insert) (B).

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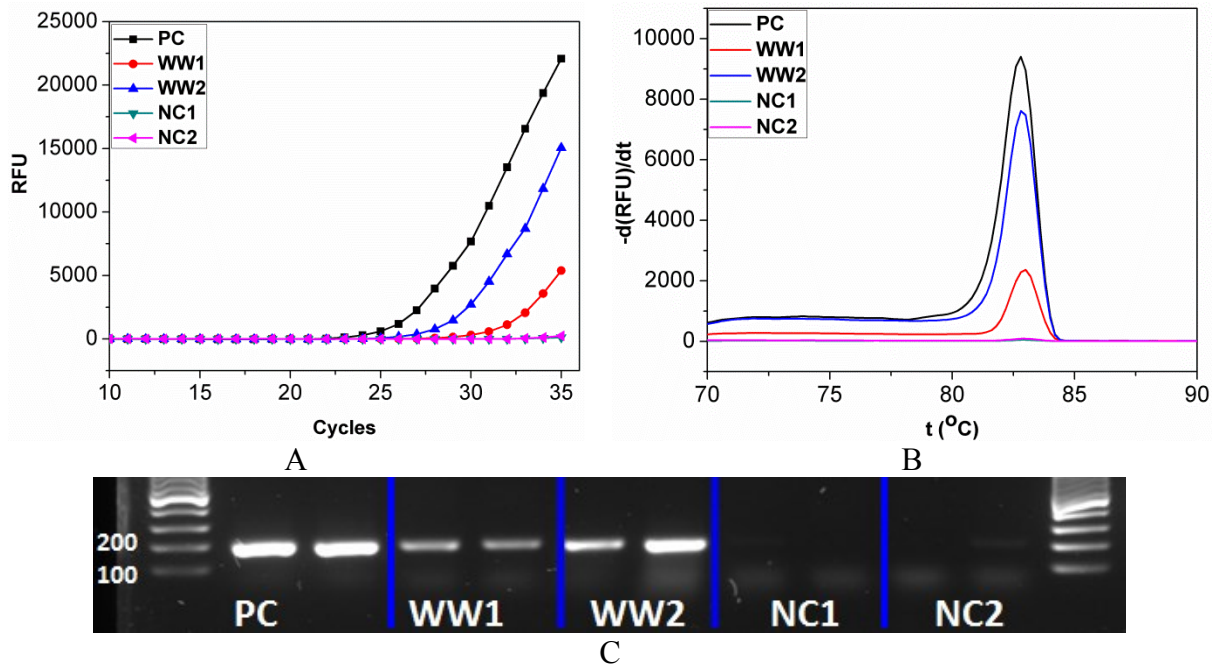
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641 Figure 2. Amplified cycles (A) and melting curve (B) of qPCR analysis of mtDNA in wastewater
642 sample using primer expected products 195 bps, and image (C) of electrophoresis gel analysis of PCR
643 products with double replicates from each sample. PC: human positive control, WW1: wastewater
644 treatment plant, WW2: urine pool, NC1: extraction negative control, NC2: qPCR mastermix negative
645 control.

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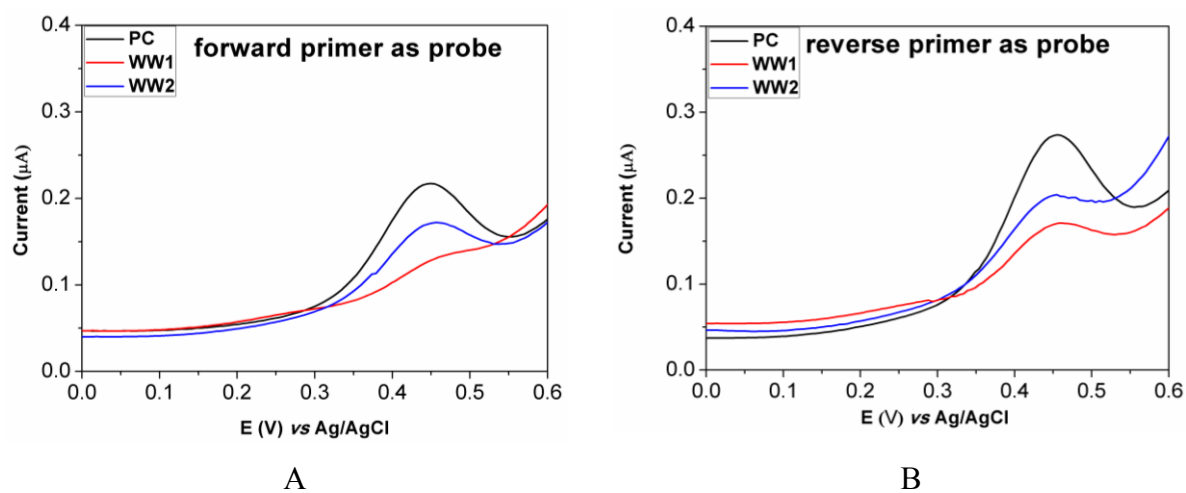
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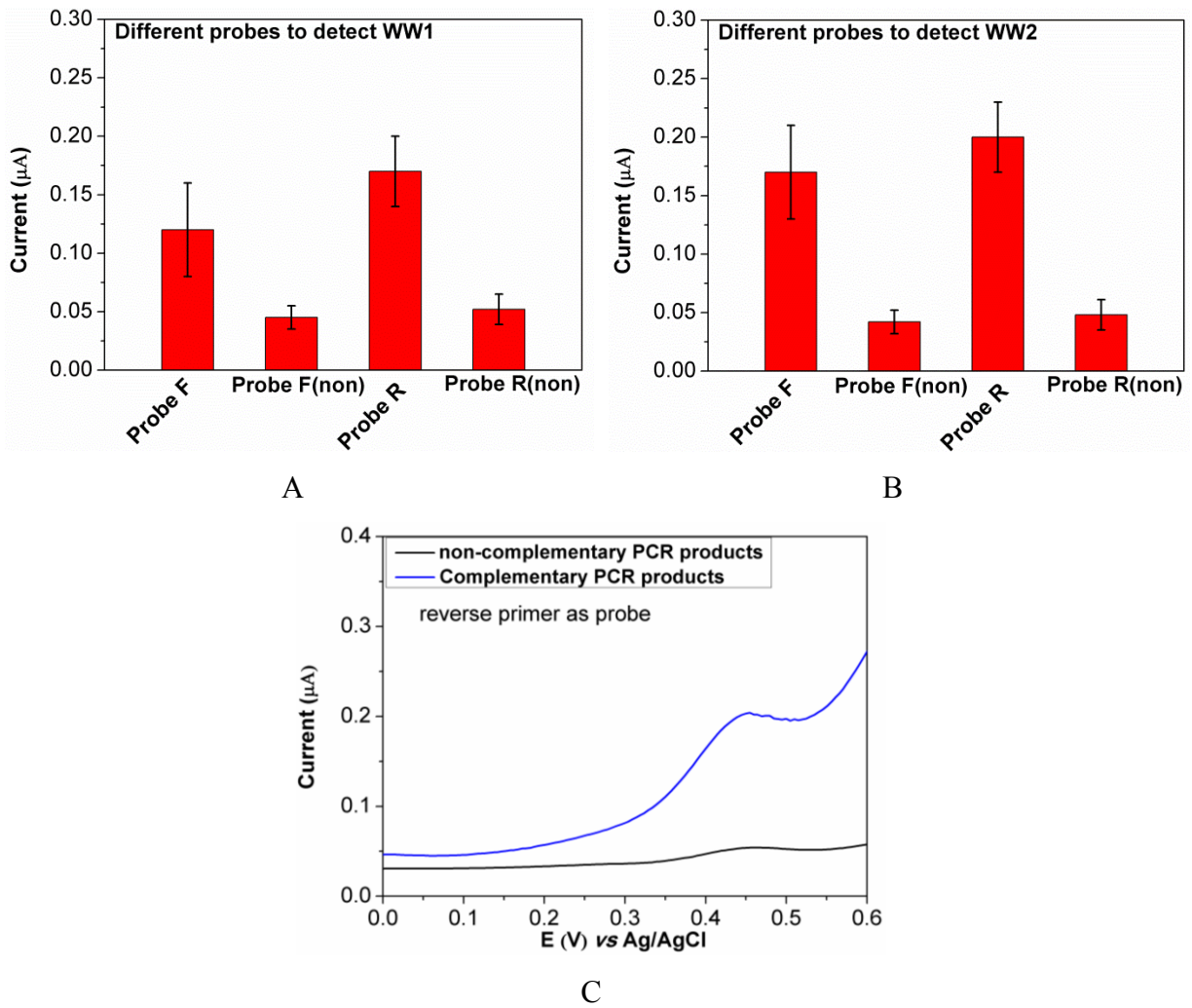
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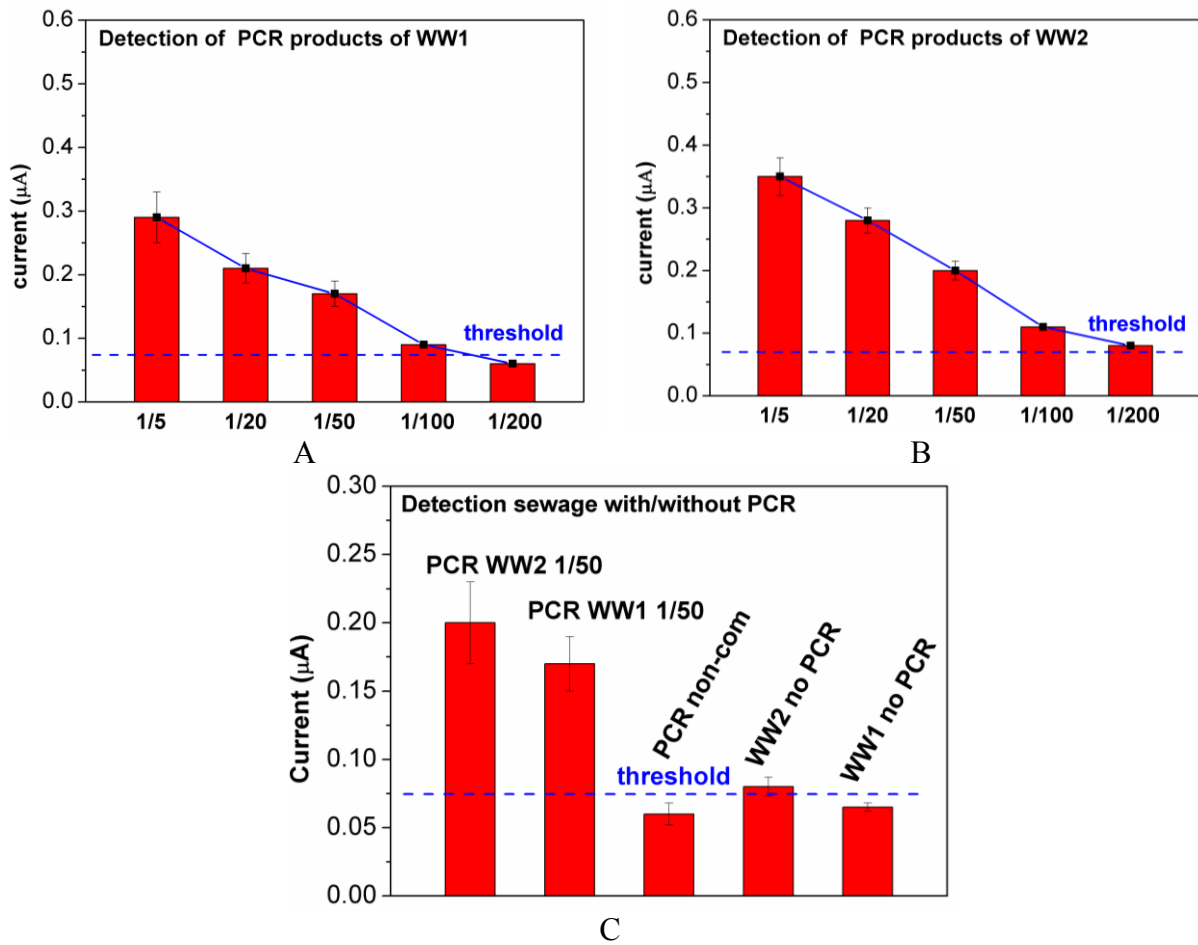
662 Figure 3 DPV curve of detection of human sample (PC), WW1 and WW2 samples amplified with
663 PCR using thiolated forward (A) and reverse (B) primers as probes

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677 Figure 4 Detection of PCR products (1/50 dilutions) of WW1 (A) and WW2 (B) with different
 678 thiolated probes (probe F: thiolated forward primer; probe R: thiolated reverse primer probe; F/R
 679 (non): 21/26 bps non-complementary to the mtDNA PCR product), and (C) current signal response to
 680 the complementary and non-complementary PCR products hybridisation to probe R.

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690 Figure 5 Detection of WW1 (A) and WW2 (B) PCR product serial dilution, and (C) the current
 691 response from the detection of mtDNA extracted from wastewater with/without PCR amplification,
 692 reference PCR products of WW1 and WW2 were diluted 1 in 50.

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