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Optimization of Bioluminescence of *Vibrio fischeri* and Assessment of Hg++, Cd++, As++, Zn++, Ag+, Cu++ and Ni++ Ions

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Authors' contributions

This work was carried out in collaboration between both authors. Author MB designed the study, performed the statistical analysis and wrote the protocol. Author AAAH wrote the first draft of the manuscript, managed the analyses of the study and managed the literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

Various forms of metallic elements are ubiquitous in the environment. The presence of heavy metals in the environment results from natural causes and human activities. They are necessary and even essential for growth and well-being of living organisms. Meanwhile, at high concentration they pose a particular problem by exhibiting more or less strong toxicity. Unlike other toxicants, these elements are not biodegradable. Therefore, they accumulate by marine organisms throughout food chain, which represent a potential danger for flora and fauna. Their toxicity on organisms and their impact on the environment were very different. One of the most important part to evaluate environmental pollution is assessment of heavy metals. Therefore, it is important to use a simple and cost effective method to evaluate toxicants. In this regard, a bioluminescence

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inhibition method has been optimized. This method consisted to optimize the intensity and stability of luminescence emitted by bacteria *Vibrio fischeri* (*V. fischeri*) strain NRRL-B-11177. Toxic effects of Cd^{++}, Zn^{++}, As^{++}, Ag^{+}, Ni^{++}, Cu^{++} and Hg^{++} ions have been determined using the method. Results showed aeration (900 ml/min) and agitation (250 rpm) were essential to *V. fischeri* to emit light. Hg^{++} ion was the most toxic compared to other tested ions with EC_{50} value of 0.008 ± 0.001 mg/l. Thus, this method is simple, rapid, sensitive and cost effective, could be used to assess a considerable amount of samples.

Keywords: Vibrio fischeri; bioluminescence; methodology; heavy metals; environment.

1. INTRODUCTION

Various forms of metallic elements are ubiquitous in the environment. However, their concentrations were generally very low, which explains why they were called "trace metals" or "metallic trace elements" [1]. The presence of heavy metals in the environment results from natural causes and human activities. They are naturally redistributed in the environment by geological processes, biological cycles, technological and industrial activities. Therefore, they form new metal compounds and introduce them into the atmosphere through transport, incineration of waste, petrochemical activities and use of fossil fuels [1-12]. Heavy metals are necessary and even essential for the growth and well-being of living organisms [13]. Otherwise, at high concentration, they pose a particular problem by exhibiting more or less strong toxicity [14,15]. Unlike other toxicants, these elements are not biodegradable. Therefore, they accumulate by marine organisms throughout food chain, which represent a potential danger for flora and fauna [16,17]. Heavy metals present different risks because of their impact on the environment in addition to their toxicity on organisms, i. e., chemical, physicochemical and biological properties [18]. One of the most important part to evaluate environmental pollution is assessment of heavy metals. In order to assess their toxicities, many different approaches have been used to study the metal toxicity on living organisms, such as algae, crustaceans, fish and on bacteria either as single isolates or mixed cultures in solution, or in communities [19-26]. Generally, the aim of these approaches was to determine the inhibition of an organism [27]. In case of bacteria several types of tests could be considered on isolated bacterial populations. Toxic effects were studied from bacterial growth, bacterial enzymatic activity, adenosine triphosphate content or inhibition of bioluminescence [28-31]. Therefore, to evaluate the pollution, it was preferable to use a simple, rapid, sensitive and cost effective such as bioluminescence inhibition method could indicate specific information on the toxicity of heavy metals [32]. The objective of this study was to optimize the luminescence of *Vibrio fischeri* (*V. fischeri*) in order to assess several heavy metals.

2. MATERIALS AND METHODS

This study carried out between March, 2017 and Febrary, 2018 at Laboratory of Microbiology, Pharmacology, Biotechnology and Environment, Faculty of Sciences Aïn Chock, Hassan II University.

2.1 Bacterial Strains

Vibrio fischeri (*V. fischeri*) strain NRRL-B-11177.

2.2 Bacterial Growth Media

V. fischeri strain was grown at 25°C in a modified Luria-Bertani Salt (LBS) medium (10 g nutrient broth, 5 g yeast extract, 20 g NaCl, 50 ml 1 M Tris buffer, pH 7.5±0.02, in 1 l of distilled water). All products were purchased from Biokar Diagnostics.

2.3 Chemicals and Samples

Hg^{++} ($HgCl_2$) 1 mM, Cd^{++} ($CdCl_2$) 30 mM, As^{++} (K^{+} Arsenic) 30 mM, Zn^{++} ($ZnSO4•7H_20$) 30 mM, Ag^{+} ($AgNO_3$) 1 mM, Cu^{++} ($CuSO_4$) 30 mM, Ni^{++} ($NiSO_4$) 30 mM suspensions were prepared. Thereafter, dilutions of the mineral salts were directly prepared. 2% NaCl (pH7.5±0.02) served as a control and diluent solution for all concentrations except Ag^{+} ion prepared and diluted with distilled water. Concentrations of tested mineral salts were presented in Table 1. All chemicals were purchased from Biokar Diagnostics.

2.4 Luminometer

Luminescence of marine bacterium *V. fischeri* was performed using a 96-well microplate

Luminoskan Ascent Luminometer (LAL) Thermo, manipulated by Ascent software version 2.6 ThermoLab systems. The bacteria growth was determined using spectrophotometer (UV-Visible) at the optical density of 600 nm (OD_{600} nm). The inhibition of luminescence intensity of *V. fischeri* caused by toxicants was determined as described by A. A. Abdel-hamid et al. [33].

2.5 Statistical Analysis

All data were analyzed using GraphPad Prism (GraphPad Software version 6.0), values are means ± standard errors. All experiments were done in triplicate.

2.6 Intensity and Stability of Luminescence Emission of Marine Bacterium *V. fischeri*

Freeze-dried luminescent bacteria *V. fischeri* were stabilized first at 4°C for 30 min, then inoculated in 5 ml of a modified LBS medium and incubated at 25°C for 24 h. Cultures were harvested when they grew and 0.05 ml were inoculated into a fresh modified LBS medium (5 ml) therefore incubated at 25°C for 24 h. Thereafter, aliquots of bacterial suspension (2 ml, OD = 0.25) were transferred into a fresh modified LBS medium (100 ml), incubated at 25° C, aerated 900 ml/min and stirred (250 rpm). A series of samples were taken during incubations and immediately measured. The bioluminescence was measured by LAL and the bacterial growth by spectrophotometer at OD_{600} nm. For bioluminescence measurements, 0.1 ml of 2% NaCl (pH 7.5±0.02) was added into well in duplicate and acclimated at 25°C for 30 min and then supplemented with 0.1 ml of bacterial suspension manually and measured. Prior each measurement, samples were automatically shaken for 10 sec in LAL.

2.7 Assessment of Hg^{++}, Cd^{++}, As^{++}, Zn^{++}, Ag^{+}, Cu^{++} and Ni^{++} Ions

Suspensions of Hg^{++}, Cd^{++}, As^{++}, Zn^{++}, Ag^{+}, Cu^{++} and Ni^{++} ions were prepared. 2% NaCl (pH7.5±0.02) served as a diluent solution for all suspensions except Ag^{+} ion diluted with distilled water. Prepared dilutions were presented in Table 1. Diluents were placed at 25°C for 30 min. Prior toxicity testing, luminescent bacteria *V. fischeri* were reconstituted in a modified LBS medium, aliquots of bacterial suspension (2 ml) were transferred into a fresh modified LBS medium (100 ml), incubated at 25°C, stirred (250 rpm) and aerated (900 ml/min) for 22 h. 0.1 ml of each concentration was added into different wells in duplicate and was supplemented with 0.1 ml of bacterial suspension by automatic dispensing in the LAL testing chamber, followed by incubation at 25°C for 15 min. The bioluminescence was recorded after microplate was automatically shacked. The signal of the light was measured after 15 min in duplicate.

3. RESULTS AND DISCUSSION

The aim of this study was to optimize the intensity and stability of luminescence emitted by bacteria *V. fischeri* cultivated in a modified LBS medium prior to assess Hg^{++}, Cd^{++}, As^{++}, Zn^{++}, Ag^{+}, Cu^{++} and Ni^{++} ions.

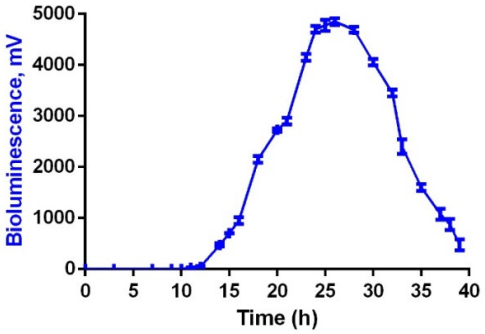

Fig. 1. Luminescence intensity of *V. fischeri* in a modified LBS medium incubated in a water bath at 25° C, aerated (900 ml/min) and stirred (250 rpm) for 39 h

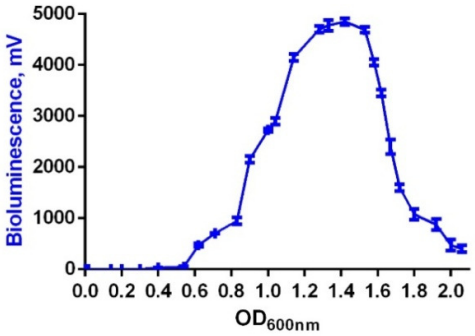

Fig. 2. Growth and luminescence intensity of *V. fischeri* in a modified LBS medium incubated in a water bath at 25° C, aerated (900 ml/min) and stirred (250 rpm)

Table 1. Concentrations of tested Cd^{++}, Zn^{++}, As^{++}, Ag^{+}, Ni^{++}, Cu^{++} and Hg^{++} ions. 2% NaCl (pH7.5 ± 0.02) served as a diluent solution for all concentrations except Ag^{+} ion diluted with distilled water

Cd^{++}	Zn^{++}	As^{++}	Ag^{+}	Ni^{++}	Cu^{++}	Hg^{++}
6.57	8.62	3.42	0.17	8.42	7.49	0,033
5.25	6.90	2.72	0.14	4.21	3.74	0,02
4.38	5.75	2.28	0.11	2.80	2.49	0,017
3.75	4.92	1.95	0.09	2.10	1.87	0,01
3.28	4.31	1.71	0.08	1.68	1.49	0,008
2.62	3.45	1.36	0.06	1.40	1.24	0,004
2.19	2.87	1.14	0.05	1.20	1.07	0,002
1.87	2.46	0.97	0.048	1.05	0.93	
1.64	2.15	0.85	0.042	0.93	0.83	
1.31	1.72	0.68	0.034	0.84	0.74	
1.09	1.43	0.57	0.028			
0.93	1.23	0.48	0.024			
0.82	1.07	0.42	0.021			
0.65	0.86	0.34	0.017			
0.54	0.71	0.28	0.014			
0.46	0.61	0.24	0.012			
0.41	0.53	0.21	0.010			

Table 2. EC_{50} values of tested Cd^{++}, Zn^{++}, As^{++}, Ag^{+}, Ni^{++}, Cu^{++} and Hg^{++} ions

Tested ions	EC_{50} (mg/l)
Cd^{++} (tested as $CdCl_2$)	1.7 ± 0.2
Zn^{++} (tested as $ZnSO_4 \cdot 7H_20$)	1.8 ± 0.2
As^{++} (tested as K^{+} Arsenic)	1.0 ± 0.07
Ag^{+} (tested as $AgNO_3$)	0.02 ± 0.01
Ni^{++} (tested as $NiSO_4$)	1.5 ± 0.1
Cu^{++} (tested as $CuSO_4$)	1.5 ± 0.08
Hg^{++} (tested as $HgCl_2$)	0.008 ± 0.001

3.1 Intensity and Stability of Luminescence Emission of Marine Bacterium *V. fischeri*

The best conditions for intensity and stability of luminescence emission and growth of *V. fischeri* were determined in a modified LBS medium prior to assess mineral salts toxicities. During the incubation of the bacteria in a water bath at 25°C, aerated (900 ml/min) and stirred (250 rpm). It was found that the emission of luminescence started just after 11 h of aeration (Fig. 1). The intensity of luminescence rose with cell growth until it reached a peak of 4850 mV after 26 h, the corresponding cell growth was OD_{600} = 1.4. Then the luminescence dropped as cell went into stationary phase (Fig. 2). In this study, *V. fischeri* were cultivated in a modified LBS medium, containing 10 g nutrient broth, 5 g yeast extract, 2% NaCl, 50 m/l M Tris buffer, pH 7.5±0.02, temperated at 25°C, aerated (900 m/min) and stirred (250 rpm), was in accordance with previous studies, which the best conditions of growth obtained when *V. fischeri* were grown in a media at pH (7.2-7.8), best temperatures between (20-25°C), strong aeration and stirring between (300-400 rpm) [34-38]. Therefore, in these best conditions, the intensity of luminescence increased with cell growth. Furthermore, the intensity of bioluminescence obtained was comparable with previously published work by A. A. Abdel-hamid et al. [33]. In present work, we added aliquots of bacterial suspension (2 ml, OD = 0.25) into a fresh modified LBS medium (100 ml) and aerated 900 ml/min, in state of bacterial suspension (0.1 ml) added into 10 ml of modified LBS medium and aerated 600 ml/min in previously published work. Besides, when using 100 ml of modified LBS medium, the best time to evaluate samples was between 18-23 h. Therefore, it was between 16-22 h in the case of 10 ml. Thus, using 100 ml in state of 10 ml of modified LBS medium, had allowed us to assess a considerable amount of mineral salts.

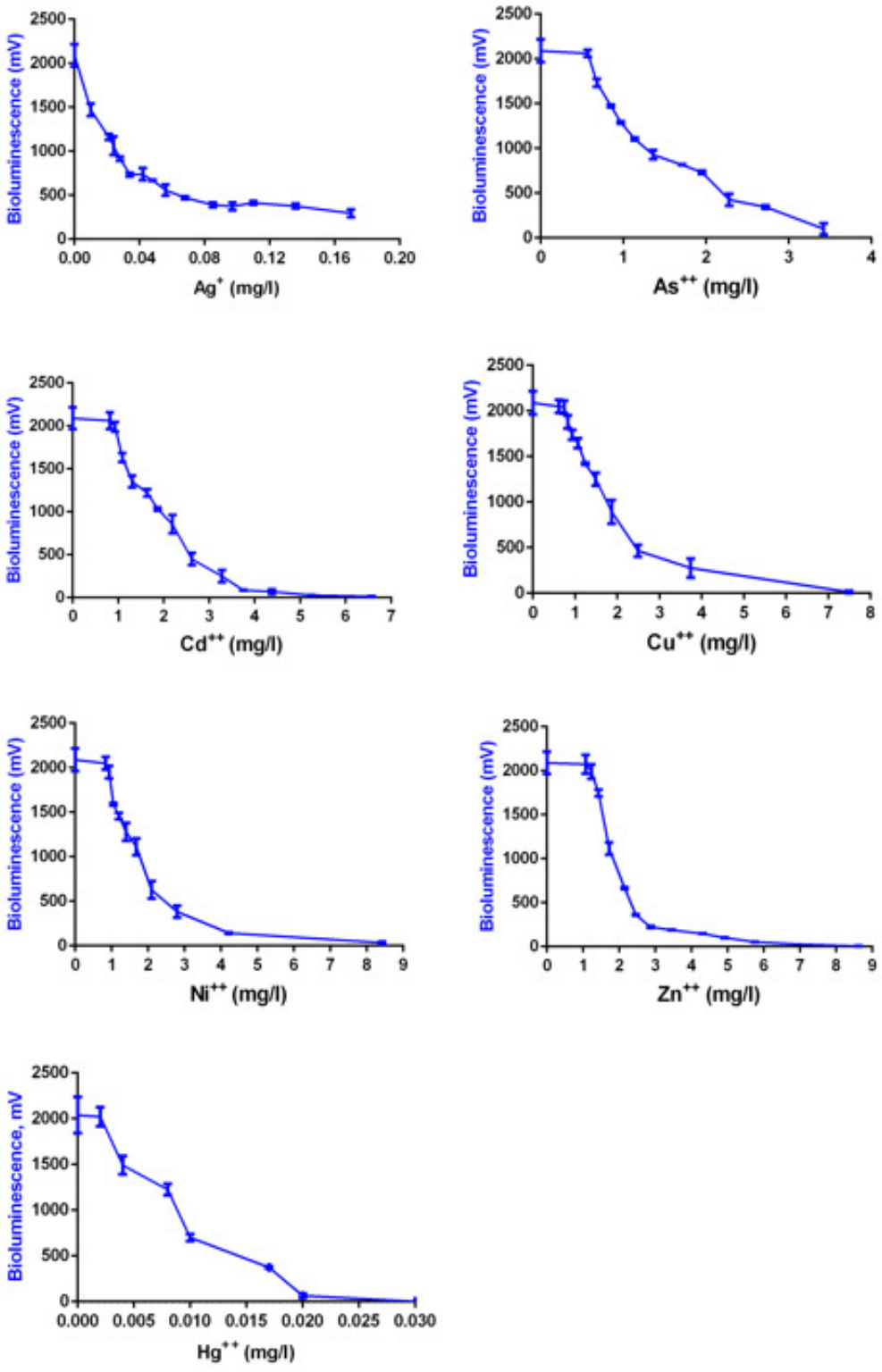

Fig. 3. Inhibition of luminescence of *V. fischeri* exposed to Cd^{++}, Zn^{++}, As^{++}, Ag^{+}, Ni^{++}, Cu^{++} and Hg^{++} ions, measured in a 96-well microplate LAL instrument. 2% NaCl served as control

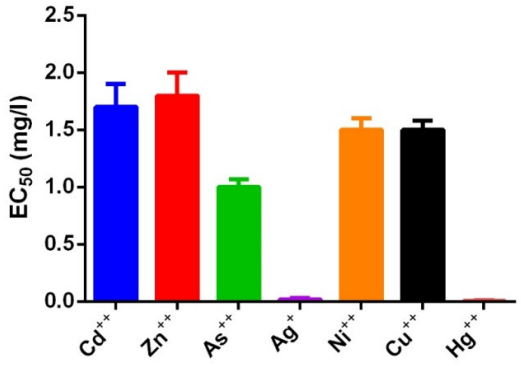

Fig. 4. Comparison of EC_{50} tested Cd^{++}, Zn^{++}, As^{++}, Ag^{+}, Ni^{++}, Cu^{++} and Hg^{++} ions

3.2 Assessment of Hg^{++}, Cd^{++}, As^{++}, Zn^{++}, Ag^{+}, Cu^{++} and Ni^{++} Ions

V. fischeri were cultivated in a modified LBS medium and were successfully applied to assess the toxicity of Cd^{++}, Zn^{++}, As^{++}, Ag^{+}, Ni^{++}, Cu^{++} and Hg^{++} ions. Tested concentrations of the mineral salts were shown in Table 1. 2% NaCl (pH7.5±0.02) served as a control and diluent solution for all concentrations, except Ag^{+} ion diluted with distilled water. EC_{50} values of tested suspensions were shown in Table 2. In the case of Cd^{++} ion, the control and two concentrations (0.93, 0.98 mg/l) were clearly similar which showed these concentrations were not sufficient to inhibit the light emitted by *V. fischeri* consequently they were not toxic to this strain. Therefore, four concentrations (6.57, 5.25, 4.38, 3.75 mg/l) were extremely toxic, similarly capable to inhibit totally the luminescence. The concentration 1.87 mg/l presented moderate toxicity (Fig. 3). In the case of Zn^{++} ion, two concentrations (1.07 and 1.23 mg/l) did not show toxicity. Whereas, from the concentration 2.87 to 8.62 mg/l showed great inhibition of light. Therefore, the concentration 1.72 mg/l presented moderate toxicity (Fig. 3). In the case of As^{++} ion, the concentration 0.57 mg/l was at the same level of the control. Therefore, concentrations from 0.85 mg/l to 1.95 mg/l presented moderate toxicity. Thereafter, the concentration 3.42 mg/l inhibited the light of the bacteria totally (Fig. 3). In the case of Ag^{+} ion, the concentration 0.01 mg/l was moderately toxic to the bacteria strain. Meanwhile concentrations form 0.056 mg/l to 0.17 mg/l presented the same toxicity (Fig. 3). In the case of Ni^{++} ion, the concentration 8.42 mg/l inhibited totally the light of the bacteria. Therefore, the concentration 1.68 mg/l presented moderate toxicity. Thereafter, at the concentration 0.84 mg/l there was no toxicity (Fig. 3). In the case of Cu^{++} ion, two concentrations (0.62 and 0.74 mg/l) did not show toxicity. Whereas, the concentration 7.49 mg/l inhibited totally the light of the bacteria. Therefore, concentrations 1.24, 1.49 and 1.87 mg/l showed moderate toxicities (Fig. 3). In the case of Hg^{++} ion, the control and the concentration 0.002 mg/l were clearly similar which showed this concentration was not sufficient to inhibit the light emitted by *V. fischeri* consequently it was not toxic to this strain. Therefore, two concentrations (0.02 and 0.03 mg/l) were extremely toxic, similarly capable to inhibit the luminescence totally. Thereafter, the concentration 0.008 mg/l showed moderate toxicity (Fig. 3). Ni^{++} and Cu^{++} ions presented the same toxicity their EC_{50} values were 1.5 ± 0.1 and 1.5 ± 0.08 mg/l respectively (Table 2 and Fig. 4). Whereas, the value of Cu^{++} ion obtained was comparable with that obtained by Mortimer et al. [31]. Thus, the difference was 0.5 mg/l. Otherwise, Cd^{++} and Zn^{++} ions presented approximately the same toxicity their EC_{50} values were 1.7 ± 0.2 and 1.8 ± 0.2 mg/l respectively (Table 2 and Fig. 4) consequently they were less toxic than other tested ions. The value of Zn^{++} ion was different from that in published work [31]. Meanwhile, As^{++} ion showed moderate toxicity compared to other tested ions. Therefore, Ag^{+} ion was very toxic compared to other tested ions, with EC_{50} value of 0.02 ± 0.01 mg/l. (Table 2 and Fig. 4). Thereafter, Hg^{++} ion was the most toxic with EC_{50} value of 0.008 ± 0.001. Eventually, inhibition of bioluminescence was closely related to concentrations of tested ions. Greater concentration induce greater inhibition of luminescence.

4. CONCLUSION

According to the different results obtained during this study, the best conditions of intensity and stability of bioluminescence obtained, when *V. fischeri* cultured in a modified LBS medium, aerated (900 ml/min) and stirred (250 rpm). The intensity of luminescence rose with cell growth until it reached a peak of 4850 mV after 26 h, the corresponding cell growth was OD_{600} = 1.4. Then the luminescence dropped as the cell went into stationary phase. Thus, the best time of sample analysis by bioluminescence was between 18-23 h. It was found that, $Ni^{++}$and Cu^{++} ions presented the same toxicity with EC_{50} values of 1.5 ± 0.1 and 1.5 ± 0.08 mg/l respectively. Otherwise, Cd^{++} and Zn^{++} ions presented also approximately the

same toxicity, consequently they were less toxic than other tested ions. Meanwhile, As^{++} ion showed moderate toxicity compared to other tested ions. On the other hand, Hg^{++} ion was the most toxic with EC_{50} value of 0.008 ± 0.001. It was concluded that luminescence of *V. fischeri* which cultivated in a modified LBS medium is simple, rapid, sensitive and cost effective, could be used to analyse a considerable amount of samples.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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