www.elsevier.com/locate/talanta

Electrochemical RNA genosensors for toxic algal species: enhancing selectivity and sensitivity

Jahir Orozco, Elisa Villa, Carmem-Lara Manes, Linda K. Medlin, Delphine Guillebault

PII: S0039-9140(16)30658-0
DOI: http://dx.doi.org/10.1016/j.talanta.2016.08.073
Reference: TAL16837

To appear in: *Talanta*

Received date: 18 July 2016
Revised date: 18 August 2016
Accepted date: 28 August 2016

Cite this article as: Jahir Orozco, Elisa Villa, Carmem-Lara Manes, Linda K Medlin and Delphine Guillebault, Electrochemical RNA genosensors for toxi algal species: enhancing selectivity and sensitivity, *Talanta* http://dx.doi.org/10.1016/j.talanta.2016.08.073

Electrochemical RNA genosensors for toxic algal species: enhancing selectivity and sensitivity

Jahir Orozco*, Elisa Villa, Carmem-Lara Manes, Linda K. Medlin and Delphine Guillebault

[a] *Microbia Environnement. Observatoire Océanologique, F-66651 Banyuls/mer, France*
*Corresponding author. Tel.: +33-468-887-325. jahir.orozcoholguin@microbiaenvironnement.eu

Abstract
Harmful algal blooms (HABs) are becoming more frequent as climate changes, with tropical species moving northward. Monitoring programs detecting the presence of toxic algae before they bloom are of paramount importance to protect aquatic ecosystems, aquaculture, human health and local economies. Rapid and reliable species identification methods using molecular barcodes coupled to biosensor detection tools have received increasing attention over the past decade as an alternative to the impractical standard microscopic counting-based techniques. This work reports on a PCR amplification-free electrochemical genosensor for the enhanced selective and sensitive detection of RNA from multiple Mediterranean toxic algal species. For a sandwich hybridisation (SHA), we designed longer capture and signal probes for more specific target discrimination against a single base-pair mismatch from closely related species and for reproducible signals. We optimized experimental conditions, viz., minimal probe concentration in the SHA on a screen-printed gold electrode and selected the best electrochemical mediator. Probes from 13 Mediterranean dinoflagellate species were tested under optimized conditions and the format further tested for quantification of RNA from environmental samples. We not only enhanced the selectivity and sensitivity of the state-of-the-art toxic algal genosensors but also increased the repertoire of toxic algal biosensors in the Mediterranean, towards an integral and automatic monitoring system.

graphical abstract
To create your abstract, type over the instructions in the template box below. Fonts or abstract dimensions should not be changed or altered.

Keywords: RNA-biosensor, Sandwich hybridization, Toxic algae, Dinoflagellate, Environmental monitoring

1. Introduction

Climate change has been linked to increases in frequency, duration and geographical range of HABs [1]. HABs threat not only farmed animals, but also human health and local economies. Their biomass can also cause damage to ecosystems to alter habitats by oxygen depletion and species displacement [2]. Rapid and reliable species identification is being increasingly introduced in more regulations and monitoring programs searching for early warning systems to prevent devastating effects caused by HABs. However, there are no standard methods for monitoring HABs [3]. Standard identification methods are based on culturing and microscopic counting, which is tedious, time consuming, and requires specialized expertise [4]. Molecular-based methods can identify species faster and more accurately [5], but their integration into real time *in-situ* monitoring is extremely challenging. Electrochemical biosensors as molecular identification tools are robust, versatile, portable, mass produced and low-cost [6], which make them suitable candidates for novel automated networked systems for real-time *in-situ* monitoring of toxic algae. [1]

Specificity is crucial for the success of nucleic acid-based biosensors. In a SHA-based electrochemical biosensor either the capture or the signal probe must be specific. Here, a set of probes developed for a microarray assay [7] were translated into a SHA format. In environmental microbiology, a target sequence has to be identified from large and often unknown genetic materials [8]. The ability to differentiate a single nucleotide mismatch is a major challenge for genosensors development. Both selectivity and sensitivity of electrochemical biosensors are much improved today [9, 10]. For toxic algal detection, specificity has been predicted *in-silico* but tested separately for each probe in a dot-blot [11], fluorescent *in-situ* hybridization (FISH) [12], biosensors[13] and microarray assays [7]. The fabrication process has been widely studied for hybridization conditions [14], transducer platform geometry, electrochemical performance [13], and probe orientation [6]. Other achievements include a first prototype for a semi-automated-based rRNA toxic algal biosensor [15], flow injection analysis systems [16] and hand held devices [17]. However, algal biosensors still fall short of the ultimate goal: an *in-situ* integral monitoring system for all toxic species.

We report on a highly specific PCR amplification-free electrochemical genosensor for monitoring of several Mediterranean toxic algal species. We designed longer capture and signal probes than those reported [6, 13, 18, 19] and tested their specificity in a SHA format. We demonstrated highly reproducible discrimination of target from non-target sequences with a single base pair-mismatch. We optimized concentrations of the probes and of the antidigoxigenin (antiDIG)-HRP antibody, and tested different mediators and their working pHs. We interrogated 13 dinoflagellate species, under optimal conditions, and finally tested the feasibility of using the biosensor for toxic algal monitoring of contaminated seawater samples. The genosensor could discriminate RNA targets coming from samples containing 10^5 cells, thus demonstrating its great potential for toxic algal monitoring.

2. Materials and methods

2.1. Apparatus and materials

Voltammetric measurements were performed with an EmStat (Electrochemical Sensor Interface), using the PSTrace software from Palm Instruments BV (The Netherlands) and a connector from Dropsens (Spain). Measurements were carried out with a three electrode cell-containing screen printed gold electrodes (C223BT and DRP-220AT, Dropsens Spain), whose total volume is 50 µl. This cell consists of a DNA-modified gold working electrode of 1.6 and 4 mm diameter, respectively and an on-chip gold counter and silver pseudo-reference electrodes.

2.2. Reagents and solutions

~25 and ~50-mer oligonucleotides probes were synthesized from Thermo Scientific (Germany) and diluted to 100 µM in 18 MΩcm DNA- and RNA-free ultrapure deionized water. The capture and signal probes for the different species were designed with similar lengths and 52.3 % average GC content, for average annealing temperatures of 59.3ºC (Table 1).

Buffer solution were prepared as follow: 0.5 M $NaH_2PO_4.H_2O$ containing 1.54 M NaCl (10X PBS, pH 7.4), 0.1 M Tris containing 0.3 M NaCl (Bead buffer, pH 7.6), 0.08 M Tris containing 0.3 M NaCl and 0.04% sodium dodecyl sulphate (SDS) (4X hybridization buffer, pH 8.0), 0.5 M $NaH_2PO_4.H_2O$ containing 1.0 M NaCl (10X POP buffer, pH 6.45), 1.0 M PBS containing 0.1 % bovine serum albumina (BSA) and 0.05 % Tween 20 (PBS-T-BSA, pH 7.4). Herring sperm DNA (3.48 $\mu g \mu l^{-1}$) was used as blocking reagent. All reagents (analytical grade, Sigma-Aldrich, France) were prepared in 18 MΩcm deionized water and stored at 4ºC until needed. Sulphuric acid, potasium hexacianoferrate, mercaptohexanol, methylene blue (MB), and H_2O_2 were supplied by Sigma (France). N-Phenyl-p-phenylenediamine monohydrochloride 99% (ADPA) was purchased by Acros Organics (Belgim), 3,3′,5,5′-tetramethylbenzidine (TMB) from Interchim (France) and Anti-DIG-HR0.05 fragments were fabricated by Roche (France).

2.3. Characterization of the transducer platform and development of the biosensor device

Gold screen-printed electrodes were cleaned, pre-treated and electrochemically characterized as described in [20], showing excellent performance and high reproducibility. We report some results expressed as current density to better compare signals coming from electrodes with different sizes. Herein current density was estimated by normalizing the obtained current intensity with respect to the corresponding geometric area of the working electrode. The target is captured in a sandwich hybridization-type format between a 3' thiolated capture probe and a 5' DIG labelled signal probe. The capture probe is chemisorbed at the surface of a screen printed working electrode by incubation overnight of 4 µl of a 1-10 µM thiolated capture probe dissolved in 1 M KCl, in a wet chamber. Post-treatment of the electrodes was achieved by incubation of the chips in 3 µl of 1 mM 6-mercapto-1-hexanol, for 50 min. After washing with 1X PBS, target and signal probe oligonucleotides were then assembled over the capture probe-modified chips in a unique incubation step in a 3 µl hybridization mixture, containing 7.5 µl milliQ water, 3.5 µl 4 X hybridization buffer, 1 µl herring sperm DNA, 1 µl 0.1% BSA (PBS-T-BSA), 1 µl DIG-marked DNA probe (0.4 µM to 1.4 µM), and either 1 µl target DNA (0.1 to 1.4 µM) for the positive control or 1 µl non target DNA (0.7 µM) for the negative control; and incubated at 57 ºC during 30 min in a closed wet chamber, after which the electrochemical detection takes place. The hybridized products react with the antiDIG-HRP labelled antibody, which, in the presence of H_2O_2, oxidizes a mediator that is then reduced back at the surface of the electrode by applying a fix potential of -200 mV. The registered current-time profile is used for tracking the concentration of DNA (RNA) target. A calibration curve must be determined for each target species, with further estimation of the RNA concentration per cell; and cells number in field samples.

2.4. Culture conditions and extraction of genetic material

Alexandrium minutum was cultivated in enriched seawater medium (F/2) (Guillard 1983) at 19°C and 100 µE $m^{-2}s^{-1}$ light intensity under a 12:12 light:dark cycle, harvested in exponential growth and counted with a FACSCanto II flow cytometer (BD Biosciences, USA).

A known number of *A. minutum* cells was transferred to 15 ml tubes and centrifuged at 5000 g for 8 min. The supernatant was removed leaving around 2 ml of sample. Tubes were centrifuged a second time at 10000 g for 1 min, and the remaining supernatant completely removed without disturbing the pellet. 1 ml of TRI-Reagent (Sigma, France) or 1 ml of Quick-RNA™ MiniPrep lysis buffer (Zymo Research, USA) was immediately added to each pellet and homogenized. Bashing-bead lysis tubes (0,5 mm, Zymo Research, USA) were added to the cell lysate to break and open the cells with a Tissue Lyser (Qiagen, USA) for 2 minutes at maximum speed. Total RNA was isolated using Quick-RNA™ MiniPrep kit or TRIReagent. RNA concentration was measured with a Nanodrop Spectrophotometer (Peqlab, Erlangen, Germany). Samples were stored at -80 °C until further processing.

2.5. Environmental samples

Seawater samples were collected at the SOLA coastal monitoring station from the Bay of Banyuls sur Mer, France (42°29′18″N, 3°8′42″E). 10^2-10^5 cells from laboratory-cultured *A. minutum* were resuspended in 1 ml of seawater. One l of seawater was spiked in triplicate with various *A. minutum* cell concentrations. Negative controls were seawater with no added cells. Samples were filtered onto a 3 µm polycarbonate filter (Whatman® Nuclepore Track-Etched Membranes), and immediately immersed in Quick-RNA™ MiniPrep lysis buffer (Zymo research, USA) for RNA extraction as described above.

Natural phytoplankton without target species (checked by microscopic observation) were collected by several horizontal net hauls using a 50 µm mesh plankton net. 10^4 and 10^5 cells from an *A. minutum* culture were added to two aliquots of 50 ml from a net plankton sample and immediately fixed in Quick-RNA™ MiniPrep lysis buffer (Zymo research, USA). After extraction, RNA concentration was determined using a NanoVue spectrophotometer (GE Healthcare) and Agilent Bioanalyzer 2100 (Agilent Biotechnologies). Natural phytoplankton from the Bay of Banyuls with no toxic species (checked by microscopic observation), were used as a negative control.

3. Results and Discussion.

3.1. Optimization of the genosensor fabrication process.

Table 1. DNA probe sets (25 nts) for each target species, their melting temperature and % GC content. A DNA strand spanning the entire capture and signal probe (50 nts) regions was synthesized as a target.

Target specie	5'SH-Capture probe[a]		Signal probe-DIG3[b]	
	Tm (ºC)	GC (%)	Tm (ºC)	GC (%)
Alexandrium minutum	59.3	52	59.1	54
Alexandrium tamarense	55.7	48	57.6	48
Alexandrium ostenfeldii	59.3	52	59.7	48
Gymnodinium catenatum	62.5	63	61.0	56
Karenia brevis	59.3	52	57.4	50
Karenia mikimotoi	59.3	52	61.3	52
Dinophysis acuminata	60.6	61	61.1	54
Dinophysis acuta	57.7	48	57.5	37
Dinophysis norvegica	57.4	50	61..3	52
Heterosigma akashiwo	62.6	60	61.3	52
Chattonella subsalsa	59.3	52	58.9	40
Lingulodinium polyedrum	62.6	60	59.3	52
Protoceratium reticulatum	57	39	59.9	46

[a]Capture probe sequences are reported in the patent PCT/GB2013/051938

[b]Signal probes are patent pending.

Concentration of capture and signal probes, hybridization time and concentration of antiDIG-HRP antibody were systematically optimized while keeping other variables constant. Capture probe involves a back-and-forth of forces from the short- range chemical interaction of the gold/thiol chemical attachment and from the long-range electrostatic repulsion among DNA strands. Optimizing the capture probe concentration is important to ensure high yield hybridization efficiency and rapid hybridization kinetics. It has been reported that 100% of target sequences can be hybridized at the lowest probe density regimes and that the binding follows a Langmuir-like kinetics. At high probe density, efficiency can drop up by 10% with slower kinetics [21]. Long-term surface coverage of adsorbed thiol-ssDNA oligonucleotides shorter than 24 bases has shown to be largely independent of oligonucleotide length as they tend to organize in end-tethered, highly extended configurations. Strands longer than 24 bases arrange less ordered and the surface coverage start to dramatically decrease with probe length[22]. Herein, a probe length of 25 bases was chosen such that surface coverage is not affected while taking advantage of the longer probes in terms of selectivity and sensitivity. Surface coverage was then very reproducible and any variability was most likely caused by chip-to-chip variation in roughness of the gold surface where higher probe density was reached. We used the 1.6 mm diameter

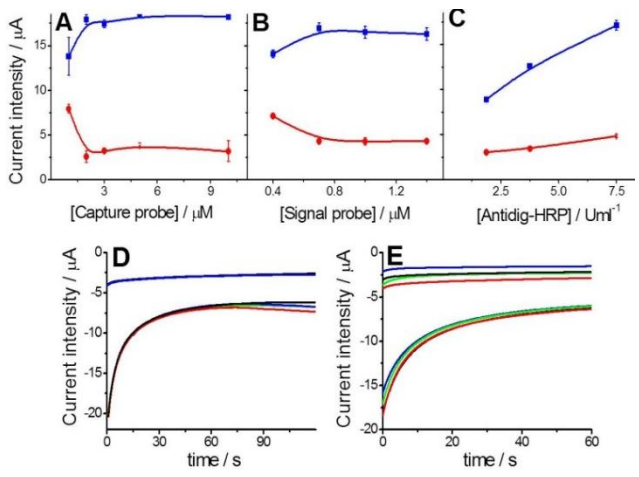


Figure 1. Optimization of experimental conditions. Resultant current obtained with increasing concentrations of capture probe (A), signal probe (B) and antiDIG-HRP antibody (C). In A) synthetic target DNA concentration of 0.7 µM (blue squares) was compared to a non-target DNA sequence of identical length as negative control (red circles). Error bars indicate the standard deviation of three different biosensors. D and E show the chronoamperometry of 4 different genosensors at a hybridization time of 60 and 30 min, respectively, with optimal conditions, with respect to negative controls.

C223BT Dropsens chips with a smaller diameter and rougher surface as compared to the 4 mm diameter DRP-220AT Dropsens previously used [6, 13]. Capture probe concentration can be dropped to 2 µM without compromising the optimal intensity previously achieved with 10 µM of shorter probes (Figure 1A). Attempts to reduce the capture probe incubation time from overnight to 90 min, where the kinetics of the chemisorption process has been reported to be faster and gold surface coverage optimal [21], did not lead to better target hybridization intensities. This fact indicates that the kinetic limiting step may be the slower reorganization of the monolayer of thiolated capture probes at the electrode surface, respect to their fast thiol chemisorption, which is in agreement with reported studies [22]. Ionic strength of the capture probe solution has a strong effect on kinetics. The higher ionic strength, the higher probe adsorption because of lower electrostatic repulsion among probe strands [21]. Stability of the anchored captured probe was improved with higher ionic strength solutions (1 M KCl) for capture probe dilution rather than water as done in [6, 13, 23]. Such stability likely improves reproducibility (Figures 1 D and E). With lower capture probe concentrations, the signal probe concentrations must be concomitantly reduced. Figure 1B shows the signal probe concentration reduced to 0.7 µM. Lowering antiDIG-HRP antibody concentration is also required to minimize the unspecific adsorption of this protein complex, which enhances the signal/noise signal. Figure 1C shows how all attempts to reduce the concentration of antiDIG-HRP antibody used in [13], starting from 7.5 Uml^{-1} were fruitful. Background was minimized by diminishing the volume of the antibody to 1.5 µl at the same concentration.

We studied the effect of hybridization time on the current intensity. Results showed slightly higher signals when the target/non-target DNA was hybridized for 1h (20.36/4.13 µA, n=4, Figure 1D) with respect to 30 min (18.25/2.94, n=4). Yet, due to the faster fabrication process and still high reproducibility (Figure 1D), 30 min was selected as hybridization time for further tests (Figure 1E). Signals of 4 biosensors tested during the same day showed an almost absolute 100% concordance recording for 60 s. However, signals decayed and readings diverged when the recording was done up to 120 s. This behavior may be explained by diffusion limitations of the mediator as time passes. 60 s was the ideal time for readings, when the signal was shown to be stable. The highly reproducible signals before 60 s allows us to interrogate their magnitude at the very beginning of the electrochemical reaction to take advantage of the wider working range generated. This result is in agreement with semi-infinite linear diffusion conditions that satisfies the Cottrell equation [24]. Further results are thus reported with currents read at such an initial point.

3.2. Effect of mediators on the genosensor sensitivity.

Digoxigenin specific antibody coupled to the enzyme-linked HRP immunomarker generates the electroanalytical signal. O-phenylenediamine (ODP) and ABTS, are two common mediators reported to track the catalysis of H_2O_2 to H_2O [14, 15]. However, they are mutagenic in the Ames test [25]. TMB, neither mutagenic nor carcinogenic, is more sensitive than ODP and ABTS in immunosorbent essays [26]. The TMB as mediator and its influence on the amperometric signal were tested with the ~25 mer newly designed probes and compared to that from (ADPA, and MB) from [6, 13]. Figure 2 shows the highest current density of the ADPA (1.39 μAmm^{-2}) as compared with TMB (0.53 μAmm^{-2}) and MB (0.24 μAmm^{-2}) under identical conditions (0.2 mM mediator and 5 mM H_2O_2, pH 7.6). However, there are several reports of the HRP-catalyzed oxidation of TMB at lower pHs [26]. A higher solubility and stability (up to 1.5 mM TMB in a

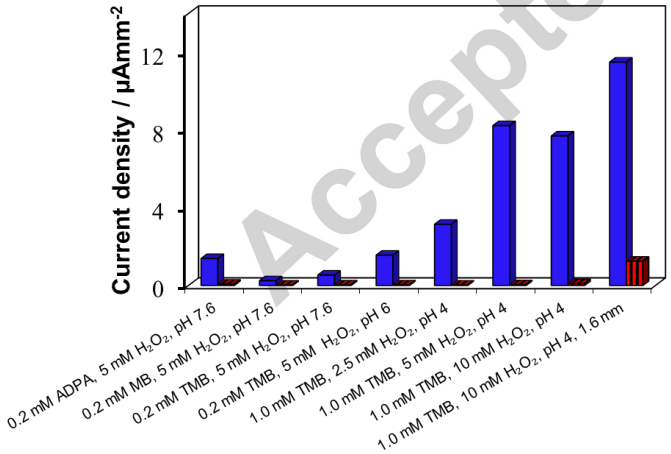


Figure 2. Comparative study of the effect of mediators on the genosenor analytical signal. Responses obtained by chronoamperomety with POP (experiments at pH 7.6) and citrate buffer (experiments at pH 4 and 6), containing ADPA, MB or TMB mediators and optimal H_2O_2 concentrations as indicated. Other conditions as reported in Figure 2. r.s.d lower than 6.7 % each case (n=3). Signal expressed as current density to compare 4 and 1.6 mm diameter electrodes.

potassium citrate buffer, for more than 1 h) has been reported at pH 4.0 without compromising the enzyme performance in immunoassay applications [27]. The biosensor performance was tested using TMB at pHs 7.6, 6.0 and 4.0. A 3-fold improved signal (1.56 μAmm^{-2}) was obtained with TMB at pH 6.0 as compared to pH 7.6. By lowering the pH to 4.0, it was possible to increase the

amount of TMB dissolved in the buffer up to 1.0 mM with a concomitant increase in the generated signal (8.23 μAmm^{-2}), at 5 mM H_2O_2 concentration, which is more than 15-fold and 6-fold as compared to the MB and ADPA systems, respectively, at pH 7.6. The signal magnitude is expressed as current density to compare genosensors developed with different sized electrodes. The magnitude of the non-target DNA signals were highly comparable, except for the last one performed with the 1.6 mm (signal at the very right in Figure 2) recorded by using the C223BT Dropsens chip. This corroborates the higher signals (11.5 and 1.25 μAmm^{-2} both specific and unspecific, respectively) coming from a rougher electrode surface and the great influence of electrode material and geometry reported [13]. Based on the results above, pH 4 citrate buffer, containing 1.0 mM of TMB and 5 mM H_2O_2 were chosen as the best conditions for further experiments. The resultant signals were in agreement with those recorded by a commercial TMB solution (results not shown).

3.3. Selective performance of the optimized genosensors for multiple target species

We next assayed if the new set of 13 probes could specifically detect their respective target toxic alga. Figure 3 shows a variety of signals with currents ranging from 8.19 to 18.4 µA for the target DNA and from 0.99 to 3.83 for the non-target DNA, with relatively low device-to-device standard deviation, ranging from ± 0.03 to ± 1.3 µA. These results indicate the proper design of the probes and their potential for monitoring toxic algae in environmental samples. Specificity of the new probe sets was tested in the electrochemical genosensor format. Longer probes require higher hybridization temperatures because of the increased GC content of the sequences, which was ca. 50% of the total probe. These probes required 57 ºC for hybridization, which is higher than the 46 ºC

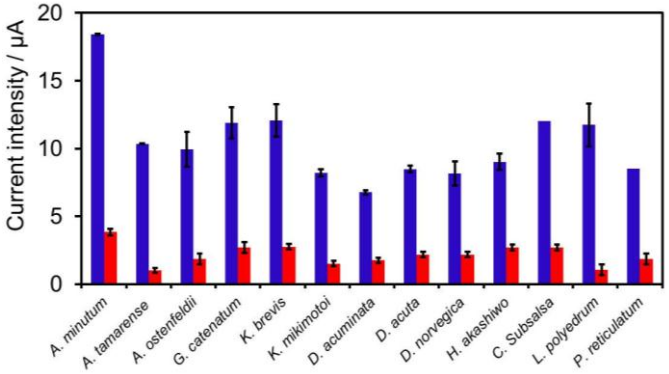


Figure 3. Current intensity of 13 toxic algal species tested at optimal conditions. The current intensities correspond to positive target (synthetic DNA) and negative controls (non-target DNA), respectively. Error bars represent the standard deviation of three genosensors.

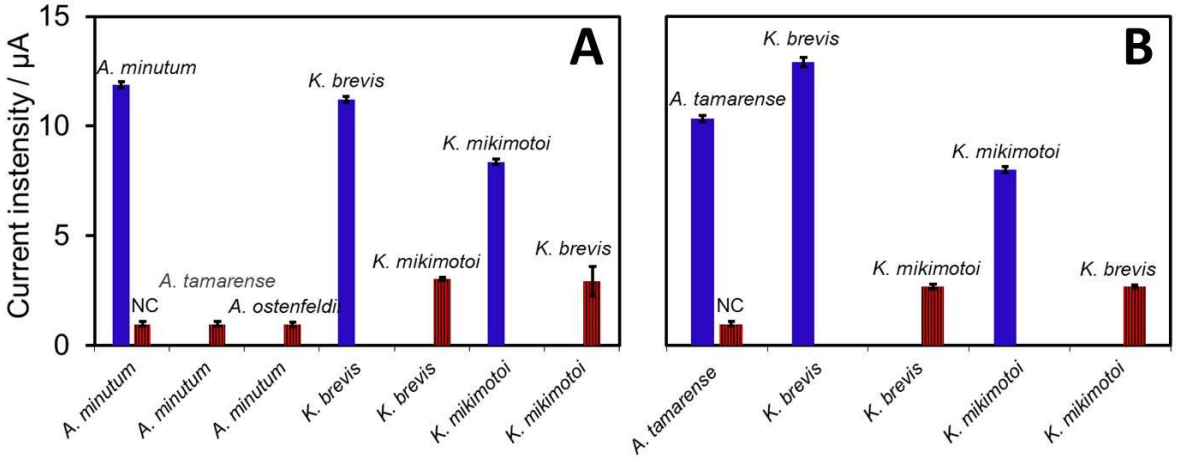


Figure 4. Selectivity of the genosensors with a super-optimal 60 ºC (A) and sub-optimal 57 ºC (B) hybridization temperature. A) DNA capture and signal probes belonging to algae of the genus *Alexandrium* and *A. minutum* target sequence interrogated against *A. tamarense* and *A. ostendeldii* (>2 mismatches to target). DNA capture and signal probes belonging to *Karenia brevis* and tested against *K. brevis* target sequence and *K. mikimotoi* (single-mismatch); and DNA capture and signal probes of *Karenia mikimotoi* interrogated against *K. mikimotoi* target and *K. brevis* single-mismatch non-target, respectively. B) Similar comparison with *A. tamarense* respect to a non-complementary DNA sequence and the cross-checking with *K. brevis* and *K. mikimotoi*, at 57 ºC. Capture and signal probes are denoted on the X axis and target in the upper part of the bars, respectively.

needed for shorter probes [6, 13, 23], which will impact positively on probe specificity. More G≡C nucleic base pairs, which are more stable than their A=T homologues, require more energy to form the higher number of hydrogen bonds between them and thus are more stable. The higher temperature applied for hybridization relaxes the secondary structures of DNA or RNA single strands, such that with more unfolded genetic material, the target accessibility by the capture and signal probes is favored. To evaluate the capture probes specificity at the species level, the electrochemical genosensor was tested with non-target DNAs of different species (*A. minutum* capture probe tested against *A. minutum*, *A. tamarense* and *A. ostendeldii* target sequences), and with DNA with one mismatch between target and non-target DNA (*K. brevis* and *K. mikimotoi*).

Hybridization duplexes depend on annealing temperatures that influence not only the yield but also the duplex purity. At both sub- and super-optimal annealing temperature values, both non-specific and reduced yield duplexes may be formed. Specificity was tested at 60 and 57 ºC (Figure 4A and 4B, respectively). Studies at 60 ºC (Figure 4A) show that for species belonging to the same genus but with a completely random target sequence, the target-non-target signal ratio is 12 (the non-specific hybridization minimally contributes to the signal with respect to that from the target sequence). However with a single-mismatch of the target to non-target, this signal ratio is only 3.7 and 2.8 (*K. brevis-K. mikimoto* and *K. mikimoto-K. brevis* cross hybridization as negative control, respectively). Although these ratios are considerable lower, they are sufficient to discriminate target from non-target closely related species with a single-mismatch. When specificity was investigated at 57 ºC, 2 º lower than the melting temperature (Figure 4B), results were very similar, with slightly higher target-non-target signal ratio of 4.8 and 3.0, respectively. These results indicate that the capture probes are highly specific to the target and that both sub- and super-optimal annealing temperature discriminates target from non-target even with a single-mismatch at both temperatures interrogated.

3.4. Performance of the toxic algal biosensor in environmental samples

With optimized conditions, the reduction current intensity was first interrogated with different concentrations of synthetic DNA from *A. minutum* (Figure 5A). Current intensity increased with increasing concentrations of synthetic target DNA from 0 to 0.42 µM (A). Figure 5B shows the resultant calibration curve (B), including a well-fit curve with a linear relationship between current intensity and target concentration in the inset, which follows the equation: current intensity (µA) = 392.94 [DNA *A. minutum*] (µM) + 1.69 (µA), with a regression coefficient, r^2 = 0.980, indicating a good correlation between the two variables tested. The limit of detection (LOD) was estimated by using the 3σ/m IUPAC criteria. LOD was 1.8 nM (27.28 ng/ml) of synthetic *A. minutum* DNA, which is half (58 ng/ml) of that reported by us for *Prymnesium parvum* [13] with 4 mm DRP-220AT, a smoother gold electrode surface. To determine the biosensors LOD with target RNA, we tested RNA extracted from harvested *A. minutum* in buffered solutions. Figure 6A shows current intensity increasing with concentrations of the target ranging from 0 to 35 ng/µl of extracted *A. minutum* RNA, respectively. The original chronoamperograms registered for each concentration are in the inset. Both variables were linearly correlated with the equation: current intensity (µA) = 93.05 [RNA *A. minutum*] (ng/µl) + 2.31 (µA), with an excellent regression coefficient, r^2 = 0.999. LOD was estimated to be 3.98 ng/µl or about 143 cells assuming 0.028 ng RNA/cell of *A. minutum* [28]. The different biosensor sensitivity towards synthetic DNA strands respect to RNA material is related to the fact that the concentration of synthetic DNA corresponds to only the highly abundant target specific strands, whereas the concentration of RNA extracted from cultivated target corresponds to the 'total' RNA that includes complementary (much less abundant) and non-complementary RNA strands from the target. Although the signal for the negative control, *i.e.* seawater samples containing natural algal population (with no RNA target) seems to be high; it was possible to discriminate 9 ng/µl of RNA target (the smallest concentration tested). Reduction of the background signal could be achieved, for example, with the use of ternary monolayers of thiols that block the unspecific adsorption of genetic material [29]. To demonstrate its practical utility, the biosensor was tested in seawater samples containing either only natural plankton or concentrated plankton (to increase the sample complexity in terms of abundance and diversity of planktonic species). In both cases, the samples were spiked with 104 and 105 laboratory cultured *A. minutum* (see details in the experimental section), and after RNA extraction, the concentration of RNA was determined by the biosensor. The 10^5–spiked seawater sample, containing diverse and abundant species from the concentrated phytoplankton, produced a detectable signal of 0.54 ± 0.114 µA (Figure 6B, blue signal and bar). Although the registered current is lower than that registered with the negative control in the RNA calibration curve in seawater containing algal population (2.28 ± 0.12 µA) by using a non-complementary RNA strand (Figure 6A, black signal and bar), this value was higher than that from the negative control run with only phytoplankton (0.13 ± 0.18 µA), (Figure 6B, black bar), containing no toxic species and used as a negative control in the environmental samples. Statistical analysis of these results showed relevant differences in the signal intensities ($p < 0.05$) and thus the presence of *A. minutum* was determined. It demonstrates the potential of this genosensor to detect *A. minutum* in a sample of at least 1 x 10^5 cells from a phytoplankton mixture. The seawater sample spiked with 17 ng/ml of RNA extracted from the cultured species (ca. 600 cells), did produced a current of 3.95 ± 0.12 µA (Figure 6B, cyan bar and signal), used as positive control for the experiment. It is also possible that unknown RNA in the environmental sample could be captured by the capture probe but not recognised by the signal probe, thus reducing that available for the target, in turn reducing the signal in environmental samples relative to that found with pure cultures.

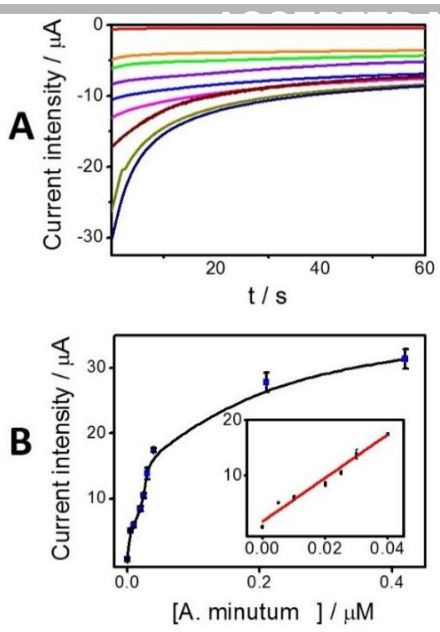


Figure 5. Chronoamperomety recorded with synthetic DNA of *A. minutum*. Increase of reduction current intensity with increasing concentrations of synthetic DNA target (0, 0.005, 0.01, 0.02, 0.03, 0.035, 0.04, 0.07, 0.21 and 0.42 μM), respectively (A) and the corresponding calibration curve (B). Inset, a well-fitted curve showing the linear relationship between current intensity and target concentration. Error bars represent the standard deviation of three devices. Other conditions as were detailed in Figure 2.

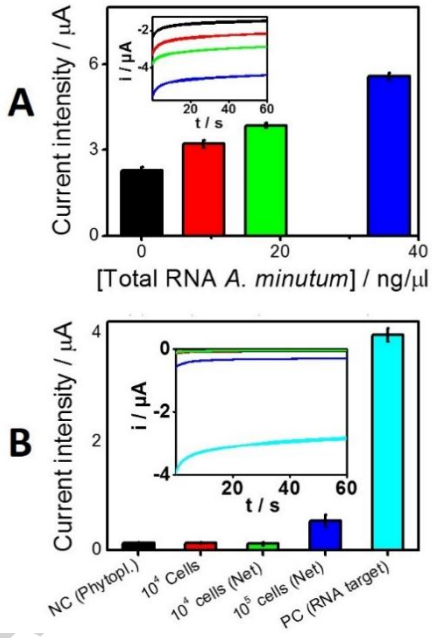


Figure 6. Chronoamperomety recorded with total RNA from *A. minutum* target and tested in environmental samples. A) Increase of reduction current intensity with increasing concentrations of RNA target, 0 (Negative control) 9, 17 and 35 ng/μl, respectively. B) Interrogation of the biosensor in environmental samples. Bars from left to right are the signals of seawater samples containing natural phytoplankton (as a negative control); and natural phytoplankton spiked with 1 x 10^4 cells, seawater samples containing concentrated phytoplankton and spiked with 10^4 and 10^5 cells; and seawater sample spiked with 17 ng/μl of RNA extracted from the cultured species (as a positive control). Original choronoamperograms are in the insets (A and B, respectively). Error bars represent the standard deviation of three devices. Other conditions as were detailed in Figure 2.

4. Conclusions.

This work reports on a PCR amplification-free electrochemical genosensor for the highly selective and sensitive detection of RNA from multiple Mediterranean toxic algal species. A new set of longer capture and signal probes was tested for the highly sensitive detection of toxic algal species in a SHA format. Conditions were optimized for minimal capture and signal probe concentrations, hybridization time and temperature, enzymatic label concentration; and optimal electrochemical mediator. The optimized genosensor

could discriminate the target sequence from its corresponding one-mismatch non-target sequences with reproducible signals. The biosensor was tested with synthetic DNA from a variety of species present in the Mediterranean area. Calibration curves with synthetic DNA, extracted target RNA and seawater samples spiked with *A. minutum* target cells were presented. Limits of detection could be as low as 1.8 nM (27.28 ng/ml) of synthetic DNA, 3.98 ng/µl of extracted RNA in buffered solutions (or ca. 143 cells) and 10^5 cells in spiked seawater samples. Overall, the work enhanced the selectivity and sensitivity of the electrochemical genosensors and increased the repertoire of toxic algal biosensors in the Mediterranean area. We believe that the enhanced biosensors are now at their optimal conditions to be integrated in any automated system, a step forward towards the integral monitoring of these species in the Mediterranean.

Acknowledgements

This work was funded by the EU FP7-OCEAN-2013.1 SMS project.

References.

[1] M. Edwards, D.G. Johns, S.C. Leterme, E. Svendsen, A.J. Richardson, Regional climate change and harmful algal blooms in the northeast Atlantic, Limnology and Oceanography 51(2) (2006) 820-829.
[2] K.L. Bruce, S.C. Leterme, A.V. Ellis, C.E. Lenehan, Approaches for the detection of harmful algal blooms using oligonucleotide interactions, Analytical and Bioanalytical Chemistry 407(1) (2015) 95-116.
[3] C.M. Scanlan, J. Foden, E. Wells, M.A. Best, The monitoring of opportunistic macroalgal blooms for the water framework directive, Marine Pollution Bulletin 55(1-6) (2007) 162-171.
[4] D. Roszak, R. Colwell, Survival strategies of bacteria in the natural environment, Microbiological reviews 51(3) (1987) 365.
[5] S.M. Dittami, V. Hostyeva, E.S. Egge, J.U. Kegel, W. Eikrem, B. Edvardsen, Seasonal dynamics of harmful algae in outer Oslofjorden monitored by microarray, qPCR, and microscopy, Environmental Science and Pollution Research 20(10) (2013) 6719-6732.
[6] J. Orozco, J. Baudart, L.K. Medlin, Evaluation of probe orientation and effect of the digoxigenin-enzymatic label in a sandwich hybridization format to develop toxic algae biosensors, Harmful Algae 10(5) (2011) 489-494.
[7] J.U. Kegel, Y. Del Amo, L.K. Medlin, Introduction to project MIDTAL: its methods and samples from Arcachon Bay, France, Environmental Science and Pollution Research 20(10) (2013) 6690-6704.
[8] J. Peplies, F.O. Glockner, R. Amann, Optimization strategies for DNA microarray-based detection of bacteria with 16S rRNA-targeting oligonucleotide probes, Applied and Environmental Microbiology 69(3) (2003) 1397-1407.
[9] M. Castoldi, V. Benes, M.W. Hentze, M.U. Muckenthaler, miChip: a microarray platform for expression profiling of microRNAs based on locked nucleic acid (LNA) oligonucleotide capture probes, Methods 43(2) (2007) 146-152.
[10] J. Wang, DNA biosensors based on peptide nucleic acid (PNA) recognition layers. A review, Biosensors & Bioelectronics 13(7-8) (1998) 757-762.
[11] D.I. Greenfield, R. Marin, S. Jensen, E. Massion, B. Roman, J. Feldman, C.A. Scholin, Application of environmental sample processor (ESP) methodology for quantifying Pseudo-nitzschia australis using ribosomal RNA-targeted probes in sandwich and fluorescent in situ hybridization formats, Limnology and Oceanography-Methods 4 (2006) 426-435.
[12] D.M. Anderson, D.M. Kulis, B.A. Keafer, K.E. Gribble, R. Marin, C.A. Scholin, Identification and enumeration of Alexandrium spp. from the Gulf of Maine using molecular probes, Deep-Sea Research Part Ii-Topical Studies in Oceanography 52(19-21) (2005) 2467-2490.
[13] J. Orozco, L.K. Medlin, Electrochemical performance of a DNA-based sensor device for detecting toxic algae, Sensors and Actuators B-Chemical 153(1) (2011) 71-77.
[14] K. Metfies, S. Huljic, M. Lange, L.K. Medlin, Electrochemical detection of the toxic dinoflagellate Alexandrium ostenfeldii with a DNA-biosensor, Biosensors & Bioelectronics 20(7) (2005) 1349-1357.
[15] S. Diercks-Horn, K. Metfies, S. Jaeckel, L.K. Medlin, The ALGADEC device: A semi-automated rRNA biosensor for the detection of toxic algae, Harmful Algae 10(4) (2011) 395-401.
[16] I. Shitanda, S. Takamatsu, K. Watanabe, M. Itagaki, Amperometric screen-printed algal biosensor with flow injection analysis system for detection of environmental toxic compounds, Electrochimica Acta 54(21) (2009) 4933-4936.
[17] M.J. LaGier, J.W. Fell, K.D. Goodwin, Electrochemical detection of harmful algae and other microbial contaminants in coastal waters using hand-held biosensors, Marine pollution bulletin 54(6) (2007) 757-770.
[18] S. Diercks, K. Metfies, L.K. Medlin, Development and adaptation of a multiprobe biosensor for the use in a semi-automated device for the detection of toxic algae, Biosensors & Bioelectronics 23(10) (2008) 1527-1533.
[19] C. Gescher, K. Metfies, L.K. Medlin, The ALEX CHIP - Development of a DNA chip for identification and monitoring of Alexandrium, Harmful Algae 7(4) (2008) 485-494.
[20] R. Olive-Monllau, J. Orozco, C. Fernandez-Sanchez, M. Baeza, J. Bartroli, C. Jimenez-Jorquera, F. Cespedes, Flow injection analysis system based on amperometric thin-film transducers for free chlorine detection in swimming pool waters, Talanta 77(5) (2009) 1739-1744.
[21] A.W. Peterson, R.J. Heaton, R.M. Georgiadis, The effect of surface probe density on DNA hybridization, Nucleic Acids Research 29(24) (2001) 5163-5168.
[22] A.B. Steel, R.L. Levicky, T.M. Herne, M.J. Tarlov, Immobilization of Nucleic Acids at Solid Surfaces: Effect of Oligonucleotide Length on Layer Assembly, Biophysical Journal 79(2) (2000) 975-981.
[23] J. Orozco, L.K. Medlin, Review: advances in electrochemical genosensors-based methods for monitoring blooms of toxic algae, Environmental Science and Pollution Research 20(10) (2013) 6838-6850.
[24] A.J.F. Bard, L. R. , Electrochemical Methods. Fundamentals and Applications, 2nd ed., New York. 2001.

[25] C.E. Voogd, J.J. Vanderstel, J. Jacobs, On the mutagenic action of some enzyme-immunoassay substrates, Journal of Immunological Methods 36(1) (1980) 55-61.

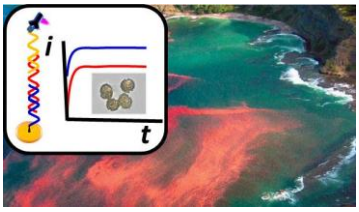
[26] E.S. Bos, van der Doelen, A.A., van Rooy, N. and Schuurs, A.H.W.M., 3,3', 5'5-Tetramethylbenzidine as an Ames test negative chromogen for horse-radish peroxidase in enzyme-immunoassay, Journal of Immunoassay 2(3-4) (1981) 187-204.
[27] A. Frey, B. Meckelein, D. Externest, M.A. Schmidt, A stable and highly sensitive 3,3 ',5,5 '-tetramethylbenzidine-based substrate reagent for enzyme-linked immunosorbent assays, Journal of Immunological Methods 233(1-2) (2000) 47-56.
[28] S. Diercks, L.K. Medlin, K. Metfies, Colorimetric detection of the toxic dinoflagellate Alexandrium minutum using sandwich hybridization in a microtiter plate assay, Harmful Algae 7(2) (2008) 137-145.
[29] F. Kuralay, S. Campuzano, D.A. Haake, J. Wang, Highly sensitive disposable nucleic acid biosensors for direct bioelectronic detection in raw biological samples, Talanta 85(3) (2011) 1330-1337.

Electrochemical RNA genosensors for toxic algal species: enhancing selectivity and sensitivity

Leave this area blank for abstract info.

Jahir Orozco*, Elisa Villa, Carmem-Lara Manes, Linda K. Medlin and Delphine Guillebault
Microbia Environnement. Observatoire Océanologique, F-66651 Banyuls/mer, France

Click here and insert/paste graph



Highlights

- A PCR amplification-free genosensor is proposed.
- Longer capture and signal probes were tested.
- Highly sensitivity and selectivity were achieved.
- A single base pair-mismatch was recognized.
- 10^5 cells were detected in spiked seawater samples.