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Amperometric biosensor based on a single antibody of dual function for rapid detection of *Streptococcus agalactiae*

Gersson Vásquez [a], Alba Rey [a], Camilo Rivera [a], Carlos Iregui [a], Jahir Orozco [b,*]

[a] Grupo de Patología Veterinaria, Universidad Nacional de Colombia, Carrera 30 No. 45-03, Bogotá 111321, Colombia
[b] Max Planck Tandem Group in Nanobioengineering, Universidad de Antioquia, Complejo Ruta N, Calle 67 No. 52-20, Medellín 050010, Colombia

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ABSTRACT

Pathogenic bacteria are responsible for several diseases in humans and in a variety of hosts. Detection of pathogenic bacteria is imperative to avoid and/or fight their potential harmful effects. This work reports on the first amperometric biosensor for the rapid detection of *Streptococcus agalactiae* (*S. agalactiae*). The biosensor relies on a single biotinylated antibody that immobilizes the bacteria on a screen-printed carbon electrode while is further linked to a streptavidin-conjugated HRP reporter. The biotinylated antibody provides selectivity to the biosensor whereas serves as an anchoring point to the reporter for further amplification of the electrochemical signal. The resultant immunosensor is simple, responds rapidly, and allows for the selective and highly sensitive quantification of *S. agalactiae* cells in a concentration range of 10^1–10^7 CFU ml^{-1}, with a detection limit of 10 CFU ml^{-1}. The approach not only enables a rapid detection and quantification of *S. agalactiae* in environmental samples but also opens up new opportunities for the simple fabrication of electrochemical immunosensors for different target pathogens.

1. Introduction

S. agalactiae is a Gram-positive pathogen that affects mainly human, cattle and fish whereas can cause sporadic diseases in many other hosts (Elliott et al., 1990; Hetzel et al., 2003; Johri et al., 2006; Yildirim et al., 2002). This bacterium is responsible for several pathologies, such as neonatal meningitis and sepsis in human beings, mastitis in cattle and meningoencephalitis, epicarditis, and choroiditis in fish (Evans et al., 2002; Hernández et al., 2009; Mian et al., 2009; Mitchell, 2003; Oliveira et al., 2005). However, the phylogenetic relationships among the *S. agalactiae* populations in the three hosts have not been clearly established (Delannoy et al., 2016; Finch and Martin, 1984; Pattison et al., 1955). *S. agalactiae* has shown to have a profound impact in both, public health and aquaculture. For example, as a primary commensal bacterium of the gastrointestinal and genitourinary tracts, *S. agalactiae* is commonly misdiagnosed because it coexists with multiple bacteria populations, affecting public healthcare systems especially in developing countries (Timoney, 2010). It is the major cause of morbidity and mortality in Tilapia, producing international economic losses up to $ 150 million for the aquacultural industry (Amal and Zamri-Saad, 2011). A precise identification of the bacterium and the source of its derived infections and determination of dissemination pathways and its maintenance in the different hosts and environments are prevailing questions that are currently under investigation. Therefore, the development of a fast, sensitive, and accurate analytical tool for detection of *S. agalactiae* in patients, in animals and in their natural habitats is of high priority.

Although significant efforts have been devoted to the development of diagnosis tools for pathogens, most technologies are still far from ideal, being either time consuming or complex (requiring specialized personnel and equipment); or unspecific and not sensitive, costly and not available in all laboratories (Fournier et al., 2013). Current ways for identifying *S. agalactiae* are based on bacteriological examinations (Keefe, 1997), serological methods (Skinner and Quesnel, 1978) and PCR, while histopathology is frequently implemented in fish pathology (Iregui et al., 2014). Diagnostic tests and devices based on biosensors are being increasingly tested as alternatives to standard laboratory instrumentation for clinical diagnosis (Elliott et al., 1990; Liébana et al., 2009; Sotillo et al., 2014) and environmental monitoring (Orozco and Medlin, 2011; Orozco et al., 2009). Among them, the high affinity of those based on antibodies has been extensively demonstrated (*e.g.* Mendoza et al., 2008). Biosensors are highly sensitive and respond rapidly. They are inexpensive, easy to operate and can be integrated into portable and automatic measurement systems. Only one biosensor for detection of *S. agalactiae*

* Corresponding author.
E-mail address: grupotandem@udea.edu.co (J. Orozco).

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in clinical samples has been published so far, using impedance spectroscopy as the detection system (Chiriacò et al., 2016). However, to the best of our knowledge, this work is the first amperometric biosensor for screening the bacterium in environmental samples.

Amplification is a crucial step in the fabrication of biosensors. Different strategies for amplification of the analytical signal have been explored, including incorporation of nanomaterials, biomaterials or a combination of them into nanobiocomposites (Crespilho et al., 2009). Amplification of biotinylated sites can intensify histochemical reactions to increase the sensitivity of staining procedures up to 100-fold employing horseradish peroxidase (HRP) and/or biotin in tissues (Adams et al., 1996). Such histopathologic affinity reactions have inspired the development of the biosensor format presented here. Biotin can be linked to different molecules, and then attached with high affinity and specificity to avidin (neutravidin or streptavidin), which, in turn, can be coupled to enzymes, nanoparticles, or fluorochromes etc., for a versatile range of application in (bio)sensing (Bobrow et al., 1989).

Herein, we describe the development of an amperometric immunosensor for the detection of *S. agalactiae* isolated from Tilapia fish. The biosensor relies on a single biotinylated antibody of dual function that selectively immobilizes the bacterium on a screen-printed carbon electrode (SPCE) surface while is further linked to a streptavidin-conjugated HRP reporter for amplification of the electrochemical signal. The resultant immunosensor is fast, simple, selective and highly sensitive. The protocol led to an efficient signal amplification with reduced assay steps and incubation times. The approach permitted the rapid screening of *S. agalactiae* in environmental samples of interest to the Tilapia fish industry. However, it could be implemented in human *S. agalactiae* clinical monitoring, opening up new opportunities for the fabrication of simple electrochemical immunosensors for different target pathogens.

2. Materials and methods

2.1. Equipment and reagents

Electrochemical measurements were performed using a potentiostat-galvanostat PalmSens and SPCEs with a standard three-electrode configuration: platinum, silver, and carbon as the counter, reference and working electrodes, respectively, DRP-150 (Dropsens, Oviedo-Spain). NeutrAvidin (Molecular Probes); biotinylated polyclonal anti-*Streptococcus* group B antibody (ab 19983) and 3,3′,5,5′-Tetramethylbenzidine (TMB) containing hydrogen peroxide (abcam); Streptavidin Horseradish Peroxidase (HRP) SA-5004 (Vector Laboratories); were used as received. Phosphate buffered saline (PBS) 1X pH 7.2 and 7.4; PBS pH 7.2, containing 0,05% Tween-20; 0,05 M $NaHCO_3$ buffer pH 9.6 and 0,01 M acetate buffer pH 5.0, were prepared with deionized sterilized water.

2.2. Bacteria cultivation

S. agalactiae reference strain (SaTiBe0818) was cultivated in brain heart infusion (BHI) agar plates for 24 h at 37 °C and the cultures resuspended in sterile PBS. Optical density (OD) of bacterial culture was measured to determine the bacterial growth stationary phase. Cultures of *S. agalactiae* strain were grown to late log phase (OD600=0.4). The number of viable cells was determined by the spread-plate technique.

2.3. Fabrication of the immunosensor

The SPCEs were incubated overnight in 7 μl of 100 μg/ml neutravidin dissolved in 0,05 M $NaHCO_3$ solution, at 4 °C covering all electrodes when placed in a wet chamber. After neutravidin adsorption, the electrodes were rinsed with PBS-tween buffer (3 times) and 3% bovine serum albumin (BSA) was dropped onto the chips for 1 h for blocking of unspecific sites. Bacteria were pre-incubated in a 1–100 μg/ml solution of anti-*S. agalactiae* antibodies, under agitation for 30–90 min. The resultant bio-conjugated was dropped onto the chips and incubated for 15–60 min with further rinsing with PBS-Tween buffer (3 times). Finally, the SPCEs were incubated in a 1–10 μg/ml streptavidin-HRP conjugated for 30 min and washed 3 times with PBS-Tween buffer. The amperometric signal was recorded by placing 45 μl 0,01 M acetate buffer pH 5.0 on the SPCE surface. Current was recorded at −200 mV/s for 60 s, after which 5 μl TMB were added to the electrochemical cell and the current further registered for additional 140 s (Salam and Tothill, 2009). HRP molecules catalyze the enzymatic oxidation of TMB in the presence of H_2O_2. The oxidized TMB is reduced back at the surface of the SPCE, thus producing a signal that is proportional to the number of cells. We optimized the biosensor fabrication process in terms of sensitivity, reproducibility and time of analysis. The parameters were sequentially changed at a time while keeping others constant. At optimal conditions, we studied the biosensor response towards different *S. agalactiae* bacterial concentrations and results were processed using the PStrace 4.2 software.

2.4. Selectivity and environmental samples testing

Selectivity was evaluated by incubating the immunosensor in 10^5 CFU of *S. agalactiae* isolated from Tilapia fish suffering from streptococcosis and comparing its response to that from incubation in the same number of CFU of two species of bacteria that commonly coexist with *S. agalactiae* in Tilapia (i.e,. *Aeromonas hydrophila* and *Edwardsiella tarda*), under the same experimental conditions. The response of the immunosensor with all the reagents but in the absence of bacteria and only in buffer were also included as negative controls. Finally, we evaluated the immunosensor response in samples of interest from the fish industry. Current intensities of the immunosensor were tested in samples from different farm water sources named Source 1, Pond 1 and Sludge 1 (4°15′42″N, 73°33′51″W) and Source 2, Pond 2, Sludge 2 (3°41′49″N, 73°41′55″W) from the Colombian departments of Tolima and Meta, respectively. Samples from Lake Betania located at Huila, Colombia (2°41′6″N, 75°26′24″W), in absence of the bacteria or inoculated with 10^1, 10^4 and 10^7 *S. agalactiae* cells were tested as negative and positive controls, respectively.

3. Results and discussion

3.1. Immunosensor optimization

The underlying immunosensor principle relies on the immobilization of *S. agalactiae* bacteria on the surface of a neutravidin-coated SPCE and further amplification of the amperometric signal with streptavidin-linked HRP enzyme. SPCEs were selected taking into account their outstanding electrochemical properties and having in mind a further application of the resultant biosensor in field settings. The first parameter to be optimized was the antibody concentration and its incubation time. In preliminary experiments, biotinylated antibodies solutions of different concentrations were first anchored on the surface of a neutravidin coated SPCE, as explained above, and the bacteria linked (and further labeled) as described. However, the resultant amperometric signals were very poor independent of the antibody concentrations and incubation times (data not shown).

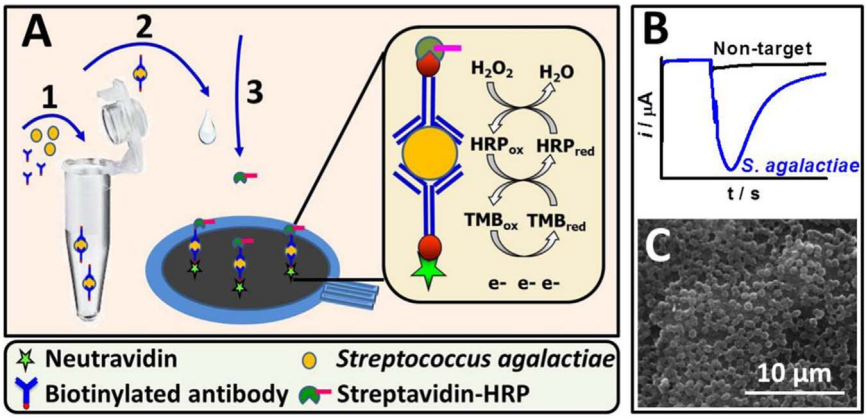

Fig. 1. Immunosensor-based *S. agalactiae* detection strategy. A) Different steps in the immunosensor development. *S. agalactiae* cells incubated with a biotinylated anti *S. agalactiae* antibody under stirring conditions (1). The solution then placed at a neutravidin-coated SPCE surface (2). The conjugated further labeled with a biotinylated HRP enzyme (3), for subsequent electrochemical detection. Such a reaction followed by means of TMB as the mediator in the presence of H_2O_2. B) The resultant amperometric signal recorded in the presence (blue line) and in the absence (black line) of target cells. C) SEM of the SPCE incubated with 107 CFU *S. agalactiae* antibody-coated cells. Note the typical coccoid appearance of the cell aggregates. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

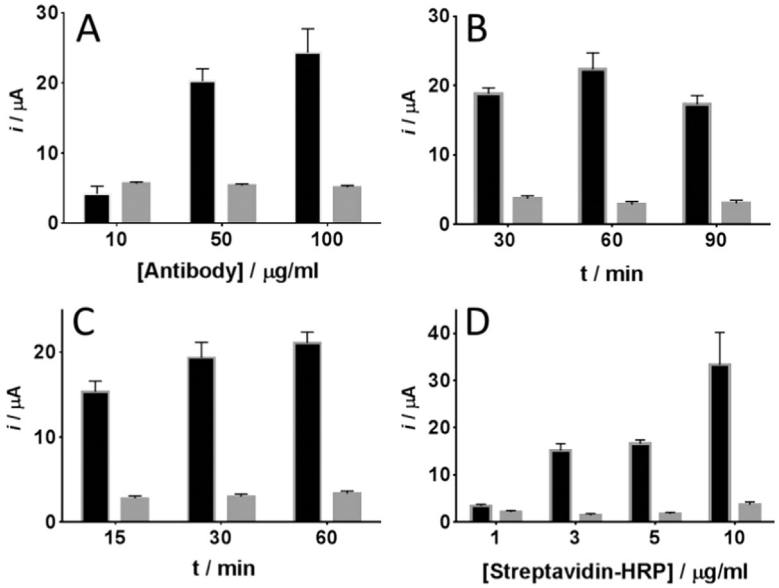

Fig. 2. Determination of optimal working conditions. Amperometric reduction current obtained with different concentrations of biotinylated anti *S. agalactiae* antibody (A), pre-incubation time (B), bacterium-antibody conjugated incubation time (C), and further incubation with different concentrations of streptavidin-HRP enzyme (D). Data are the mean and standard deviation of absolute values of 3 measurements. Black and gray bars are the currents registered for 10^7 *S. agalactiae* bacteria and the negative controls (without bacteria), respectively.

Alternatively, the antibody showed selectively to interact with the bacterial cell wall-expressed antigens when stirred (Fig. 1A-1). The resultant conjugated was then linked to the surface of the SPCE through neutravidin-biotin interactions (Fig. 1A-2), and further decorated with the HRP-labeled streptavidin through biotin-streptavidin interactions (Fig. 1A-3). The electrochemical signal was finally recorded with TMB as a mediator (Fig. 1B). Fig. 1C shows a scanning electron microscopy (SEM) image of 10^7 CFU of *S. agalactiae* cells. The bacteria, of about 0.9 µm size, are forming clusters and/or rows as typically arranged *S. agalactiae* (de Aguiar et al., 2016), thereby confirming their immobilization at the surface of the SPCE. We tested concentrations of antibodies ranging from 10 to 100 µg/ml, with a fix population of bacteria set at 10^7 CFU, for 30–90 min incubation time. Fig. 2A (black bars) shows that when the concentration of biotinylated antibody was raised to 100 µg/ml, the current intensity reached its highest value. The number of exposed biotin molecules coating the bacteria cells is speculated to be high, some of which working to immobilize the bacteria on the SPCE and others, free-exposed, serving as anchoring points for the HRP reporter. The resultant amplified signal was high enough to be discriminated *per se* from the concomitant signal counterparts (with no target bacteria) used as negative controls (Fig. 2A, gray bars). Such simplified detection strategy eliminates the need for a secondary antibody in the biosensor format, of especial utility when secondary antibodies are not available, thereby not only reducing the steps and time of the bioassay but also the cost associated with antibodies production. Yet, such high amplification also results in a significant decrease of the reproducibility as inferred from the high standard deviation values. 50 µg/ml gave a still high-intensity signal but with lower variability, so that we selected this concentration for further interrogations. The amplification permits much shorter incubations in primary antibodies than that with conventional techniques (Adams, 1992). Herein, the shorter incubation time-tested led to a higher current intensity with a lower variability (Fig. 2B). Henceforth, we pre-incubated bacteria-antibody for 30 min.

Neutravidin is an avidin analogous protein with attractive features for the development of biosensors of entire cells and commonly used in surface bio-functionalization. Neutravidin-biotin interaction has been shown to be strong and rapid (Nobs et al., 2004). The fact that neutravidin lacks carbohydrate moieties implies that it reduces the capability of binding lectins from the cells surface while retaining its biotin-binding affinity (Linman et al., 2008). Furthermore, neutravidin isoelectric point is near-neutrality. Neutravidin dissolved in a higher pH than its isoelectric point, will be negatively charged, which is expected to minimize non-specific interactions with the negatively charged bacteria cell surface. We next varied incubation time from 15 to 60 min (Fig. 2C). Although current intensities resulted only slightly increasing with incubation time, 30 min seems to be enough for the biotin-surrounded bacteria to robustly set and saturate the neutravidin-coated SPCE (see Fig. 1C). Consequently, as no dramatic improvement of the amperometric signal was achieved by longer times 30 min was selected as the optimal, which will additionally reduce significantly the total assay time while giving high enough reproducible signals as shown below. Finally, we optimized the concentration of streptavidin-HRP complex used as the reporter of the electrochemical reaction. Fig. 2D shows the impact that concentration of the reporter has on the amperometric signal intensity and demonstrates that amplification is crucial for the development of biosensor-based analytical tools to get discriminable signals while minimizing variability and non-specific signals. We interrogated concentrations of the streptavidin-HRP complex ranging from 1 to 10 μg/ml. Whereas biosensors tested with 1 μg/ml gave signals for the positive and negative formats with differences no statistically significant ($p < 0.05$), 10 μg/ml gave statistically significant differences ($p < 0.05$), but with the highest variability. Although both 3 and 5 μg/ml concentrations were high enough to produce a distinguishable positive/negative signal and lower variability (lower standard deviation values), 3 μg/ml was selected as the optimal concentration of reporter complex. Table 1 summarizes the parameter tested and optimal conditions selected.

3.2. Analytical performance

We used the optimized conditions (see Table 1) to study the relationship between the current intensity and increasing changes of bacteria concentration in a buffered media. Fig. 3A shows the corresponding analytical signals recorded after following the optimized protocol detailed above. Unlike the typically limit current expected from amperometry, we observed a transient-like current-time profile. Communication (electron transfer) between the active center of the enzyme and the electrode surface through the mediator was not sustained. The big size and increasingly high population of bacteria speculate to inhibit the continuous diffusion of the mediator towards the electrode surface. Simple improvements have been suggested for sustained electronic communication (Campuzano et al., 2010; Zhong et al., 2010; Bange et al., 2005) and for counteracting the limited mediator diffusion (Orozco and Medlin, 2011). However, the biosensor was indeed responding to increasing concentrations of *S. agalactiae*, while the response in the absence of the target bacteria was considerably lower. This fact

Table 1
Analytical parameters tested and optimal conditions selected.

Parameter	Tested	Selected
Antibody concentration, μg/ml	10–100	50
Antibody-*S. agalactiae* preincubation time, min	30–90	30
Antibody-*S. agalactiae* incubation time, min	15–60	30
Streptavidin-HRP, μg/ml	1–10	3

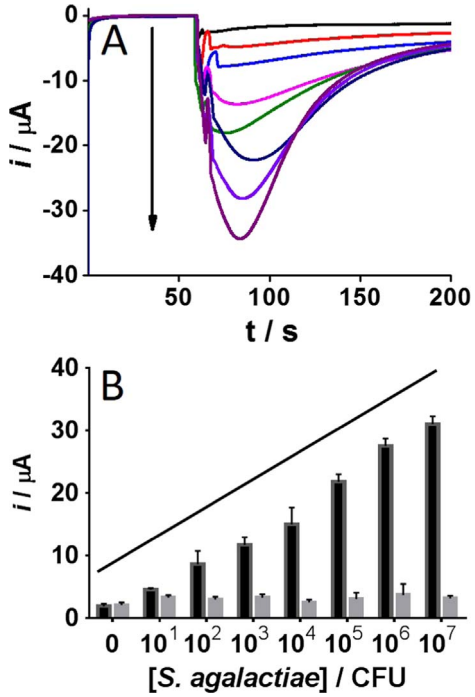

Fig. 3. Immunosensor calibration curve under optimized conditions. Chronoamperometric signals (A) and resultant current intensity (B), generated with increasing concentration of *S. agalactiae* cells from 0 (blank) to 10^7CFU/ml. Black and gray bars are the currents registered for the different concentrations of *S. agalactiae* and the negative controls (without no bacteria), respectively. Other conditions as were detailed in Fig. 2.

indicates that it is possible not only to discriminate the bacteria in a certain contaminated/uncontaminated sample but also to get sensitive analytic information that leads to quantification of such pathogens in an environmental sample. In a stirred solution, the local hydrodynamics expects such that the flux of TMB mediator is constant, thus attaining a steady-state current. Herein, as the experimental set up does not allow stirring, and the diffusion of the mediator was greatly limited, the estimated maximum current intensities at 80s were related to the logarithmic increasing concentrations of bacteria from 10^1 CFU and 10^7 CFU. At this time, the current values were maximum and with less variability, while the mediator diffusion process was still not limited. Fig. 3B shows such dependence with a current intensity (μA)=1.907 log [*S. agalactiae*]+0.0017 (μA), and a regression coefficient, $r^2=0.9817$. r^2 indicates a good correlation between the two variables tested. However, this calibration curve was from buffered solutions and thus it is not intrinsically considering the potential effect of the matrix from a certain sample as discussed in the next section. The experimental detection limit of the immunosensor was estimated to be 10^1CFU/ml, based on the signal threshold of at least 3 times the standard deviation of the blank signal.

3.3. Selectivity test and detection of S. agalactiae in environmental samples

The antibodies that have an affinity reaction with the bacterial cell wall-expressed antigens provided selectivity to the biosensor. Only the bio-conjugated biotinylated antibodies/*S. agalactiae* cells were then anchored at the SPCE. The response of the biosensor was tested against two species of bacteria that can eventually coexist with *S. agalactiae* in a Tilapia population in a natural water source, pond or sludge, *i.e. A. hydrophila* and *E. tarda* (Iregui et al., 2014). Fig. 4 shows that the biosensor response to *S. agalactiae* target bacteria is 25.21 ± 3.09μA, which is more than 6-fold higher

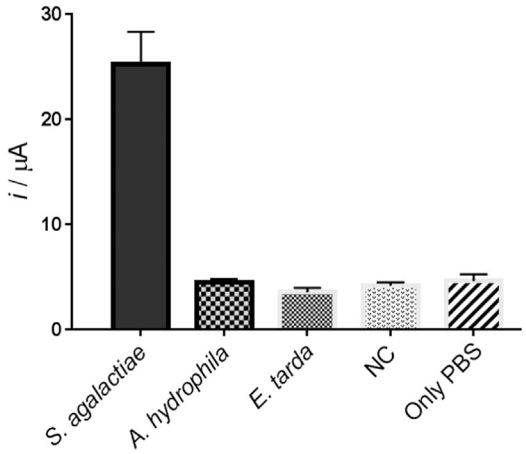

Fig. 4. Immunosensor selectivity. The current intensity that the immunosensor generates by incubation with 10^5 CFU of *S. agalactiae* isolated from Tilapia suffering from streptococcosis and comparison with the signal generated by the same CFU of *A. hydrophila* and *E. tarda* bacteria, respectively that can eventually co-exist with *S. agalactiae* in Tilapia and its natural environment. The response of the immunosensor with all the reagents but in the absence of bacteria (NC) and only in buffer (only PBS) were included as negative controls. Other conditions as were detailed in Fig. 2.

than the concomitant *A. hydrophila* and *E. tarda* bacteria counterpart responses. These results confirm that only the bio-conjugated that was formed in the pre-incubation step was then anchored to the neutravidin-coated SPCE. Antibodies lack affinity for the other tested species and consequently, lonely, were further linked to the SPCE. A negligible reaction with the streptavidin-HRP complex then occurred, producing comparatively very low signals. The resultant current intensities of 4.48 ± 0.31 and $3.52 \pm 0.42\mu A$ for *A. hydrophila* and *E. tarda* bacteria are comparable to those obtained in absence of bacteria ($4.58 \pm 0.33\mu A$) and only buffer ($4.58 \pm 0.65\mu A$), respectively, thereby demonstrating the selectivity of the as-prepared biosensor.

The next experiments were conducted to demonstrate the feasibility of using the as-prepared amperometric immunosensor for detection of *S. agalactiae* in samples of interest to the fish industry. For this purpose, a lake water sample spiked with 10^1, 10^4 and 10^7 *S. agalactiae* cells led to signals of 3.78 ± 0.2, 11.42 ± 3.88 and $12.99 \pm 3.7\mu A$, respectively (Fig. 5, squares filled bars). Such intensity values were perfectly distinguishable from the corresponding signal with no bacteria ($2.68 \pm 0.41\mu A$) used as negative control (NC, very left), with differences statistically significant ($p < 0.05$), thus demonstrating the feasibility of the biosensor for *S. agalactiae* detection in natural samples. However, the current

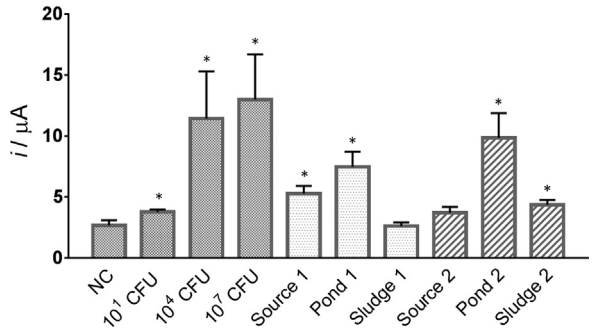

Fig. 5. Immunosensor response in environmental samples. The current intensity of the immunosensor tested in samples of two different Tilapia farm water sources (source, pond, sludge), respectively. Lake samples, in absence of the bacteria (NC) and spiked with 10^1, 10^4 and 10^7 *S. agalactiae* cells used as negative and positive controls, respectively. Other conditions as were detailed in Fig. 2. *Significantly different respect to the negative control ($p < 0.05$).

intensities were 83%, 76% and 42% lower compared to the same concentration of target bacteria at buffered solutions, respectively (Fig. 3). These results suggest that matrix effects are playing a key role in the resultant current intensity differences respect to those in buffered solutions, and the higher the target bacteria concentration, the stronger the matrix effect. The presence of particulate matter and/or sediments, or higher population of coexisting bacteria may account for these differences (Song et al., 2010). When we interrogated samples coming from the source, pond and sludge farm Tilapia sources, 1 (dots filled bars) and 2 (lines filled bars), respectively, we observed that current intensities of source 2 and sludge 1 were comparable with those from controls without bacteria in lake water (NC). No statistically significant differences ($p < 0.05$) indicate that they may be free of *S. agalactiae*. In contrast, samples from source 1, pond 1 and 2 and sludge 2 appear to be contaminated as current intensities were higher than that without bacteria and even higher than that with 10^1 *S. agalactiae* cells in samples of lake water, respectively, with statistically significant differences ($p < 0.05$). Contrastingly, none of the tested samples was contaminated based on standard microbiological tests, or molecular biology techniques. However, *S. agalactiae* has been indeed the cause of fish mortality in similar samples, diagnosed by histopathologic observation, even when they were not detected by molecular biology or microbiological techniques. Our results are in agreement with such histopathologic observations, which have been suggested as better diagnosis method for *S. agalactiae* (Hernández et al., 2009). The discrepancy in these results corroborates the difficulties to diagnose these particular bacteria in environmental samples and highlights the potential of the as-developed biosensor for this purpose, even though a better evaluation of the matrix effects on the analytical signal is needed. The overall results suggest that our immunosensor holds promise as a screening tool of *S. agalactiae* in samples of interest in fish industry and encourage us to broaden further the scope for samples of interest in human patients.

4. Conclusions

We developed an amperometric immunosensor for detection of *S. agalactiae*, isolated from Tilapia suffering from streptococcosis, in only 90 min. The strategy relies on a unique antibody to anchor bacteria at a SPCE and further generates a signal through a streptavidin-HRP reporter complex. The as-prepared biosensor showed to be selective and highly sensitive for the quantification of *S. agalactiae*. This simple approach led to an efficient signal amplification, greatly reduced the assay steps and incubation times, and showed great potential to be implemented by untrained personnel and with minimal requirements for instruments and reagents. Further efforts will be devoted not only to get a better electrochemical performance but also to minimize the observed matrix effects when testing environmental water samples. Overall, we demonstrated a *S. agalactiae* rapid screening method in samples of fish industry interest. We believe the biosensors could be readily implemented in human clinical monitoring to broaden the range of possibilities for detection of target pathogens in both environmental and clinical samples.

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