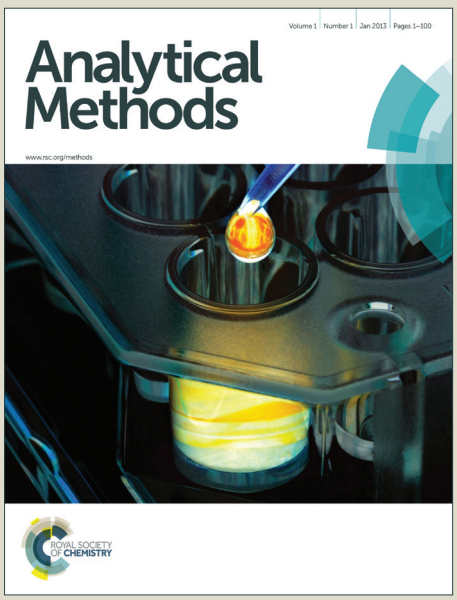
Analytical Methods

Accepted Manuscript



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Application of an ELISA-type amperometric assay to the detection of *Vibrio* species with screen-printed electrodes

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Abstract

We describe an electrochemical method for the rapid detection of *Vibrio* species. An avidin-biotin binding strategy was tested with 12 different *Vibrio* strains and applied onto gold screen-printed electrodes. *V. parahaemolyticus* cells were then amperometrically detected at a minimum abundance of 4×10^2 *V. parahaemolyticus* cells mL^{-1} within 60 min.

Main text

Vibrio bacteria species are ubiquitous in warm waters globally and show substantial spatiotemporal heterogeneity in their distribution[1]. Many strains are pathogenic to humans and marine animals and represent significant human health risks as well as a threat to the aquaculture industry[2-4]. Therefore, *Vibrio* outbreaks have been generating increasing interest amongst the scientific community and there is a growing need for establishing rapid, on-site detection techniques for pathogenic marine bacterial groups, including the *Vibrio* species.

Approaches used to examine these organisms rely on time-consuming procedures, including culturing or quantitative molecular biological approaches (e.g. quantitative PCR (QPCR)), often resulting in management decisions being made days after the collection of samples[5-7]. Tools incorporating biosensor technology allow for real-time quantitative assessment of *Vibrio* population in environmental samples offer considerable advantages

over well-established methods, including low analysis cost, relatively short time-to-result, high potential for miniaturisation, and the possibility of performing the measurements *in situ* without highly qualified personnel. Biosensing devices also allow for online monitoring of water systems enabling the development of ecosystem health and disease surveillance platforms in near real-time.

This study applies a robust, previously optimized protocol allowing the specific capture and detection of total *vibrios* in seawater samples. This protocol was used for the functionalisation of screen-printed electrodes[8] in order to develop an immunosensor that would allow on-site, near real time monitoring of vibrio outbreaks in aquatic systems.

The bacterial strains were obtained either from the American type culture collection (*Vibrio harveyi* 14126, *Vibrio coralliilyticus* BAA-450, *Vibrio shiloi* BAA-91, *Vibrio splendidus* 33125, *Vibrio ordalii* 33509, *Vibrio tubiashii* 19109 and *Serratia marcescens* BAA-632) or the University of Technology, Sydney (UTS) culture collection (*Vibrio rotiferianus* DAT722[9], *Vibrio alginolyticus* 12G1[10], *Vibrio natriegens* C5, *Vibrio campbellii* C7, *Vibrio parahaemolyticus* C8 and *Vibrio cholerae* S10[11]). All strains from the UTS culture collection had been typed using 16S rRNA or other housekeeping gene sequencing. Unless otherwise stated, all chemicals and reagents used in this study were obtained from Sigma-Aldrich (Sydney, Australia). The different strains were grown at 26 ºC in Marine Broth 2216 medium (Difco) overnight. For the surface immuno-functionalisation protocol, fresh cultures were aliquoted into 1.5 ml eppendorf tubes, and centrifuged for 10 min at 14,000 g. The resultant supernatants were discarded and the pellets were retrieved in PBS and stored at -20 ºC. Prior to freezing, total viable counts of colony forming units (CFU) were determined by serially diluting and plating onto Marine agar (2216 medium; Difco). Prior to experiments, frozen pellets were thawed to room temperature and reconstituted in

phosphate buffered saline (PBS) solution, to the desired cell concentration. Initially, a sandwich immunoassay format involving a biotinylated anti-*Vibrio* polyclonal capture antibody (Bt-α*Vib* Pab) and a horseradish peroxidase (HRP)-conjugated anti-*Vibrio* polyclonal signal antibody (HRP-α*Vib* Pab) (Kirkegaard & Perry Laboratories Inc; Washington, D.C, US) for *Vibrio* species was developed based on ELISA and colorimetric detection of HRP. A neutravidin layer (100 µL of 20 µg mL^{-1} in 10 mM of pH 7.4 phosphate buffered saline (PBS)) was immobilised on the surface of maxisorb Nunc 96-well plates by direct adsorption. After 3 washes with 100 µL of PBS (10 mM pH 7.4) containing 0.05% v/v Tween 20 (PBST buffer), the well surfaces were blocked with 200 µL bovine serum albumin (BSA 2% w/v) at 37 ºC for 2 h. Then, 100 µL of 20 µg mL^{-1} Bt-α*Vib* Pab was added into each well and incubated at 37ºC for 1 h. After washing the wells, increasing concentrations of *vibrio* cells (ranging from 0 to $1x10^9$ cells mL^{-1}) were added and left in contact with the well surface for 30 min at room temperature (23±2ºC). This was followed by addition of 100 µL of a 1/500 dilution of the HRP-α*Vib* Pab prepared in PBST buffer. After a 30-min incubation at room temperature and another similar washing step, 100 µL of the HRP substrate, 3,3′,5,5′-tetramethylbenzidine (TMB), was introduced in each plate well and left to react for 10 min. The reaction was quenched using 50 µL of a 1 M H_2SO_4 solution. A yellow colour arising from the formation of a 3,3′,5,5′-tetramethylbenzidine diimine derivative then developed and the absorbance of the solution was recorded at 450 nm using a (Fluostar optima, BMG Labtech Pty. Ltd.). For each concentration of *Vibrio* cells, the standard deviation was calculated as the mean of 3 absorbance replicates.

Figure 1 shows the absorbance results from increasing vibrio cells concentration. Here, a relatively low absorbance was observed until a concentration of 1×10^3 cells mL^{-1}

was reached, where the absorbance then steadily increased to a plateau at 1 x 10^9 cells mL^{-1} resulting from a saturation of the antibody binding sites.

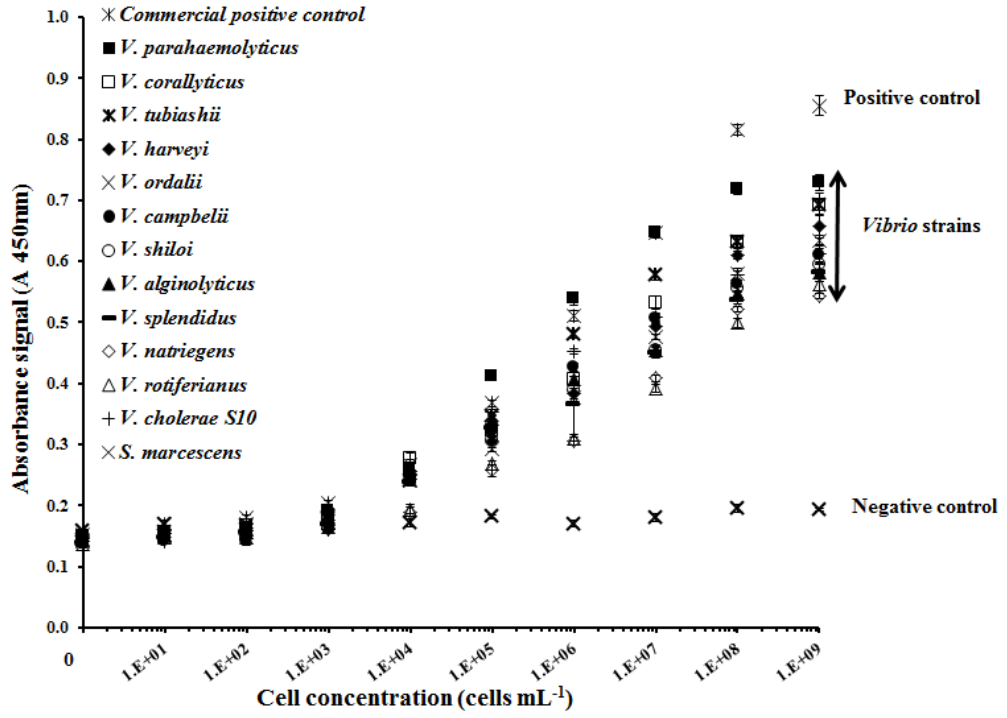

Figure 1. Affinity of the different *Vibrio* strains and commercial positive control to the functionalised well plate surface using optimised conditions**.** Absorbance signals obtained after a 30 min cell capture step on the functionalised surface and a 30 min detection step using a 1/1000 dilution of horseradish peroxidase anti-*Vibrio* antibody (HRP-α*Vib* Pab). For each condition, the standard deviation was calculated as the mean of 3 replicates.

There was substantial variation in signal amplitudes, demonstrating differences in the affinity of the antibody for the different *Vibrio* strains. However, the limit of detection (LOD) (as concentration for the mean of 10 blanks plus 3 times the standard deviation) remained similar for all strains tested, ranging from 7 x 10^3 to 3 x 10^4 cells mL^{-1}. *V. parahaemolyticus* showed a LOD slightly lower at 7 x 10^3 cells mL^{-1} and was thus chosen as the target for subsequent electrochemical detection. No cross-reactivity or cross-binding was observed for the non-target control bacterium, *Serratia marcescens*.

Having optimised the antibody mediated capture and detection strategy using the colorimetric method, the next step was to adapt the detection strategy to an amperometric detection system. For this purpose, screen-printed gold electrodes were purchased from Dropsens, S.L., Spain. Screen-printed electrodes have been used increasingly in the past decades as a cost effective alternative to photolithografically fabricated electrodes and common redox probes [12, 13]. Each electrode consists of a gold high temperature screen-printed working electrode (220AT, 4 mm diameter, Au/HTSPEs), a gold counter electrode and an Ag/AgCl reference electrode. In order to carry out the electrochemical measurements, we used a CHI1232B hand-held bipotentiostat device (CHInstruments, Inc, USA). These electrodes have been characterised and used in previous studies [14, 15, 16], however, a characterisation of the electrodes was carried out using a 10^{-3} M solution of potassioum hexocyanoferrate (III) in 0.1 M H_2SO_4. The results showed a linear relation of the peak current versus the square root of the scan rate (0.999) and the relative standard deviation (%RSD) obtained from 12 of the screen-printed electrodes tested was 3.1%. In this work, the working electrode was modified using the protocol described above for the ELISA format by incubating it in the neutravidin and Bt-*αVib* Pab solutions, respectively. The functionalised electrodes were then successively incubated in both *V. parahaemolyticus* and *S. marcescens* cells solutions of increasing concentration (0 to 1 x 10^9 cells mL^{-1}) for 30 min and in the 1/500 dilution of HRP-*αVib* Pab for 30 min before being introduced in the HRP substrate/mediator solution containing 1mM hydrogen peroxide (H_2O_2) and 1mM hydroquinone (HQ) in deoxygenated PBS (pH 7). In this system, HRP catalyses the reduction of H_2O_2 coupled to the oxidation of HQ into benzoquinone (BQ). BQ is then electrochemically reduced back into HQ at the electrode

surface, generating a reduction wave around −0.35V vs. Ag/AgCl[17]. In the presence of HRP, production of BQ is directly related to the amount of enzyme (Figure 2).

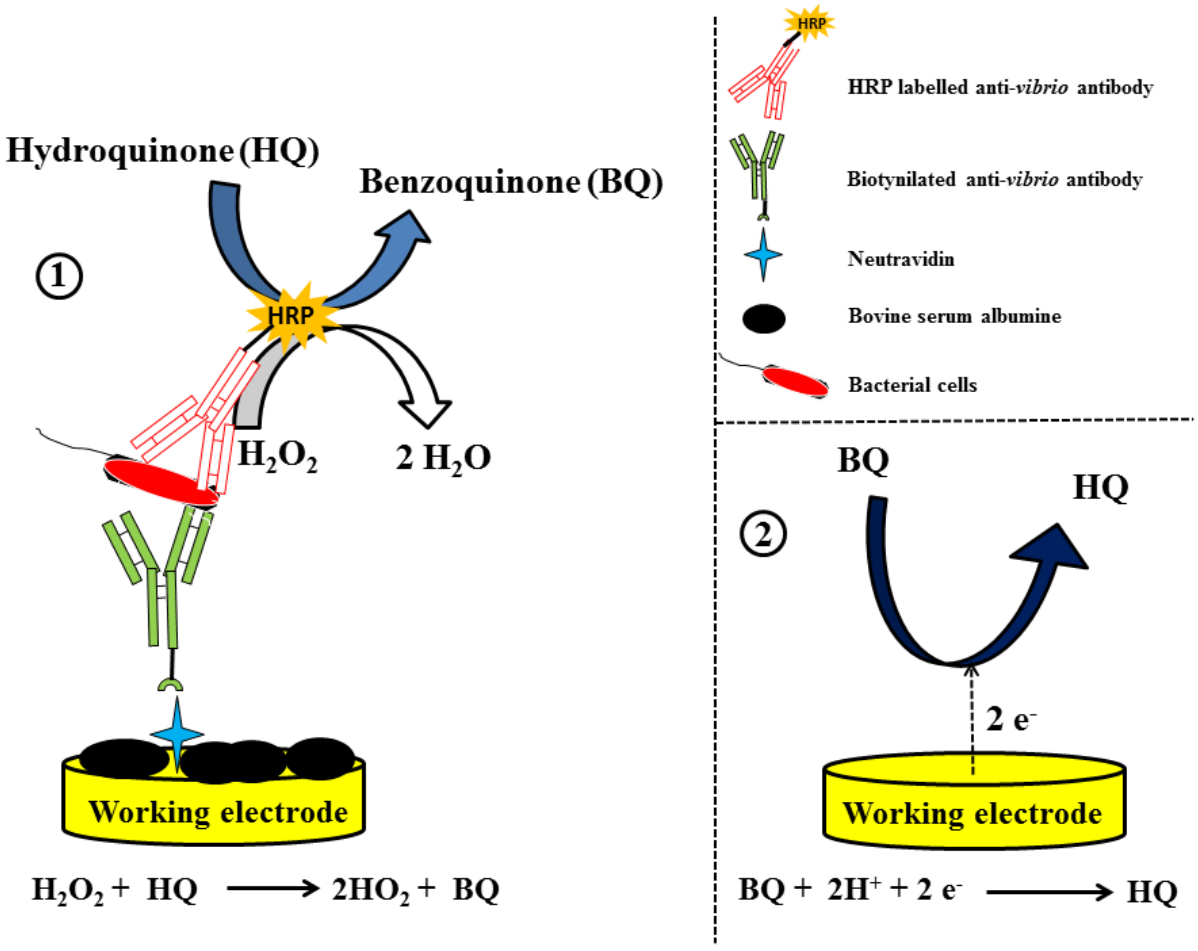

Figure 2. The principle of the amperometric immunosensor based on: 1) the reduction of hydrogen peroxide (H_2O_2) coupled with the oxidation of hydroquinone (HQ) into benzoquinone (BQ) catalysed by the horse radish peroxidase label of the anti-*vibrio* antibodies and 2) the reduction of the benzoquinone produced back into hydroquinone at the electrode surface at a potential of -0.35V versus Ag/AgCl.

The intensity generated by the reduction of BQ was recorded for 90 seconds and the slope of the curve generated calculated, representing the rate of current increase.

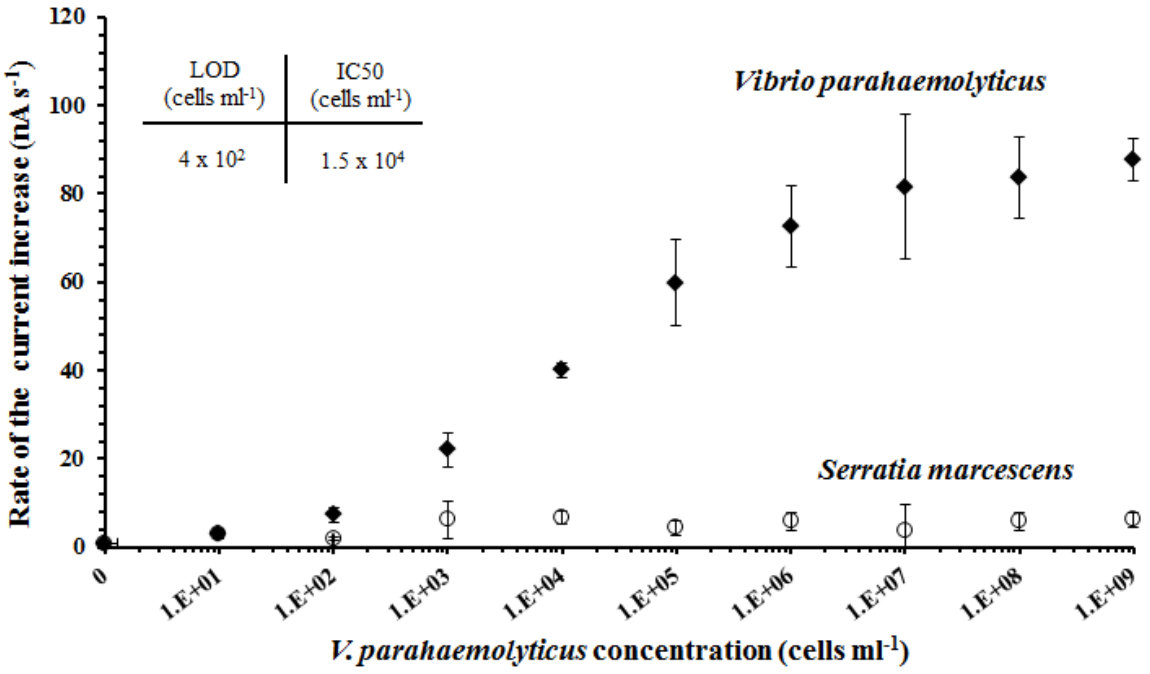

Figure 3. Amperometric detection of *Vibrio parahaemolyticus*. Rate at which the intensity increases at a potential of 0.30V Versus Ag/AgCl during the first 90 seconds of contact with the substrate solution. The electrodes were previously left in contact with increasing cell concentrations. For each condition, the standard deviation was calculated as the mean of 3 replicates.

Figure 3 shows the relationship between the rate of current increase and increasing bacterial cell concentrations previously put in contact with the electrode for both *S. parahaemolyticus* and the negative control *S. marcescens*. These results confirmed the lack of cross reaction with the negative control. A LOD of 4 x 10^2 cells mL^{-1} for *V. parahaemolyticus* was obtained which represents a ten-fold improvement compared to that obtained using the colorimetric method in Figure 1. A half maximal inhibitory concentration (the concentration that generates 50% of the maximum signal) was evaluated using the equation: [maximum rate – (maximum rate – minimum rate)/2]) of 1.5 x 10^4 cells mL^{-1}.

Conclusions

Amperometry was successfully applied as a detection technique for *Vibrios* species in an immunoassay using screen-printed electrodes. The limit of detection was 100 to 1000 fold lower than previously reported using similar methodologies [18, 19], and the assay took only 60 min. This improvement can be explained by the effectiveness of the capture format employed compared to the strategies used in these previous studies. The cost-effectiveness of the technology used in this study, its simplicity and robustness, along with its miniaturisation potential, would allow the development of an on-site, real-time detection system for the monitoring of vibrio outbreaks in water systems. Future work is aimed at including a pre-concentration step using functionalised magnetic particles[17] which might provide a significant improvement to the amperometric sensor in enhancing the detection sensitivity[20]. Additionally, this technique will be tested on more *Vibrio* species in the future and antibodies directed to specific vibrio strains of interest will be tested.

Acknowledgments

Olivier Laczka is supported by the UTS Chancellor's postdoctoral fellowship program and acknowledges the Transfield Foundation for its financial contribution to this project. The authors also acknowledge Dr David Bourne from the Australian Institute of Marine Science and Dr Justin Seymour from the University of Technology, Sydney for providing some of the bacterial strains used in this study.

Notes and references

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