ADVANCING SAXITOXIN DETECTION IN AQUACULTURE: ESTABLISHING A PORTABLE BIOSENSOR DEVICE FOR ON-SITE APPLICATIONS

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ABSTRACT

Saxitoxin (STX) is a paralytic shellfish poison (PSP) produced by harmful algal blooms (HABs), particularly by Alexandrium minutum. Shellfish can easily become contaminated by these biotoxins in natural environments or during cultured farming processes. PSP toxins can cause organ numbness, muscle paralysis, breathing difficulties, and, in severe cases, fatalities. Therefore, routine and continuous monitoring is essential to prevent poisoning incidents. Traditional techniques like HPLC and LCMS are costly, time-consuming, and require trained personnel to operate instrumentation. In this context, a portable biosensor device offers a strategic solution for early detection of harmful algal blooms. Here, we describe a portable electrochemical immunosensor system for on-site detection of HABs and STX in the aquaculture industry. This handheld device is integrated with the Internet of Things (IoT) and a wireless sensor network. The system utilizes a direct assay immunosensor employing differential pulse voltammetry (DPV) for electrochemical detection of HABs or STX. We investigated the sensitivity of the developed immunosensor on a modified screen-printed electrode in three different matrices (phosphate buffer saline buffer, seawater, and shellfish). The results demonstrated a limit of detection (LOD) of the sensor in these matrices: 7.7 ppb, 6.38 ppb, and 2.09 ppb, respectively. Subsequently, the biosensor's performance was evaluated using real samples collected from Sg. Merbuk, Kedah and coastal area of Kuantan; and cockles from local market in Selangor.

Keywords: differential pulse voltammetry (DPV), harmful algal blooms (HABs), immunosensor, saxitoxin (STX)

INTRODUCTION

Harmful algal blooms (HABs) are natural phenomena characterized by an increase in the density of microalgal species in marine and freshwater environments. These occurrences raise concerns about human health, environmental preservation, and economic challenges. Massive blooms of microalgae can lead to fish kills in natural habitats or mariculture settings due to the release of bioactive compounds or hypoxia/anoxia in the surrounding environments. Factors such as industrialization, urbanization, and commercial agricultural practices contribute to nutrient runoff and enrichment in freshwater and marine coastal systems, which in turn promote algal blooms. Consumers and the fisheries industry are particularly concerned about marine biotoxins produced by certain HAB species. Filter-feeding shellfish, including mussels, oysters, clams, and crabs, can accumulate these biotoxins to dangerously high levels when they ingest harmful algae. The accumulation of algal-origin toxins in filter-feeding shellfish has led to human food poisoning, causing severe effects such as tingling or numbness in the mouth and extremities, dizziness, headache, fever, respiratory distress, and even death (McPartlin et al., 2016).

Over the past decade, several major harmful algal bloom (HAB) events have been reported in the country. The most recent cases were reported by the Ministry of Health of Malaysia on April 2, 2024, involving eight individuals who consumed mussels purchased from markets in Port Dickson, Negeri Sembilan, Malaysia. Seven people were hospitalized, and two of them were admitted to the Intensive Care Unit (ICU) due to severe symptoms, including paralysis (The Star, April 3, 2024). Earlier in 2001, an incident of shellfish intoxication occurred following a massive bloom of a marine dinoflagellate in a semi-enclosed lagoon in Tumpat, northeastern Peninsular Malaysia. The event gained widespread media attention, with six individuals hospitalized and one fatality due to severe intoxication. The organism responsible was identified months later as *Alexandrium minutum* based on cultures and wild specimens collected from the site. Toxin analyses conducted on both the organism and contaminated clams (known locally as 'lokan') from the area provided strong support for this identification (Usup et al., 2022). Following the incident, shellfish collection from the lagoon was banned by the Health Department for several months.

The recent event has raised concerns among relevant authorities regarding the need for improved monitoring of harmful algal blooms (HABs) in the country. Current HABs studies are deemed insufficient to safeguard public health and ensure seafood safety. This incident has underscored the shortcomings in the existing monitoring programs by relevant authorities. Despite potential costs, ensuring the safety of our seafood products is crucial, not only for export purposes but also for local consumption. To address this issue, there is a critical need for a rapid, simple, and sensitive device capable of real-time and in-situ analysis for detecting saxitoxin-producing dinoflagellates. Biosensor devices present an attractive alternative technology for contaminant detection due to their rapid, sensitive, and user-friendly nature, enabling real-time and on-site analysis. Real-time detection of biotoxin contaminants is essential as it provides immediate interactive information about the tested sample, allowing authorities and seafood industries to take corrective measures before products are released for consumption. Biosensor technology has been widely applied in diverse fields, including pollution control, detection of toxic gases, environmental monitoring, quality control, drug development, and agriculture. We present here the development of an antibody-based sensor for saxitoxin detection and its application in a portable device designed for rapid and routine monitoring.

MATERIALS AND METHODS

Chemicals and Materials

Polyclonal antibodies against *Alexandrium minutum* were developed in-house at the Animal Complex, MARDI Serdang, using locally isolated cultures. The screen-printed carbon electrodes (SPCEs) used in the biosensor study were sourced from Biogenes Technologies Sdn. Bhd., Malaysia. All chemicals were obtained from Sigma-Aldrich, USA, unless otherwise stated. Phosphate-buffered saline (PBS) solution was prepared by dissolving one PBS tablet in 200 mL of deionized (DI) water, yielding a 0.01 M phosphate buffer with a pH of 7.4 at 25 °C. Blotto non-fat dry milk was purchased from Santa Cruz Biotechnology, USA. Seawater samples were collected from Kuantan coastal area and Sungai Merbok, Kedah, Malaysia; while shellfish were obtained from breeding cages along Sg. Merbok, Kedah, Malaysia. All electrochemical biosensor analyses were conducted using a portable electrochemical biosensor reader with the MARDIsense app. An ABRAXIS® Saxitoxins (PSP), 96-test, Enzyme Linked Immunosorbent Assay (ELISA) kit (Gold Standard Diagnostics Horsham Inc., USA) was used for validation study.

Calibration Plot Determination

The calibration plot for Saxitoxin detection was determined using a three-electrode system with a screen-printed electrode (SPCE) consisting of working, reference, and counter electrodes made of carbon, utilized as the sensing medium in electrochemical measurements. The carbon working electrode was modified with polypyrrole and gold nanoparticles (AuNP) using a drop-casting technique. Saxitoxin calibration plot was determined by applying direct assay protocol on the modified SPCE. Briefly, a 10 µL solution of 0.7 mg/mL anti-saxitoxin antibody (anti-STX Ab) was immobilized on the modified SPCE and incubated for 1 hour. The SPCE was washed three times with phosphate-buffered saline (PBS) and then rinsed with distilled water. The remaining active sites on the SPCE were blocked by adding 20 µL of 0.05% dry milk in 0.01 M PBS and incubating for 15 minutes. After washing the SPCE again, 10 µL of each saxitoxin standard solution, ranging from 0 to 15 ppb in both buffer and matrix systems (i.e. seawater, and shellfish) was immobilized on the working electrode and incubated for 15 minutes. The SPCE was washed again, and prior to analysis, 100 µL of 5 mM ferricyanide/ferrocyanide in 0.1 M KCl (redox solution) was added to the SPCE. Differential pulse voltammetry (DPV) analysis was conducted over a potential range of -400 mV to +400 mV using a portable biosensor reader. All steps were performed at room temperature.

Sample Extraction Method

Muscle tissues were removed from the shellfish and rinsed with distilled water to remove contamination. Next, the muscle tissues were homogenized to a soupy texture, and 5 grams of the homogenized sample was added to 10 mL of 0.01 M PBS buffer (pH 7.4). The sample was vortexed at maximum speed and then centrifuged at 4,000 rpm for 10 minutes. The supernatant was diluted to an optimized dilution factor. For standard development in shellfish matrix, the diluted supernatant was added with different standard concentrations of saxitoxin. For sample analysis, the diluted supernatant was used directly for sample application.

RESULTS AND DISCUSSION

Portable IoT-Immunosensor Determination

A portable biosensor device integrated with Internet-of-Things (IoT-Biosensor) was tested for its sensitivity in detecting saxitoxin (STX) in both buffer and matrix systems. Prior to application, modification of the sensor platform is essential to enhance biosensor sensitivity. In this study, polypyrrole (Ppy) and gold nanoparticles (AuNP) were introduced onto the sensor surface to amplify the sensor signal. Polypyrrole (Ppy)-based polymer was selected for sensor platform modification due to its intrinsic chemical and electrical properties (Sheikhzadeh et al., 2016), which are valuable for biosensor fabrication and functional surfaces. Incorporating Ppy with AuNP aimed to improve both the sensitivity and selectivity of the biosensor. The unique properties of AuNP, including high surface area, electrical and thermal conductivity, and electrochemical stability, were significant factors in choosing this nanomaterial for sensor surface modification (Auwal et al., 2023; Truong et al., 2019; Zhang et al., 2016; Xiao et al., 2020; Yildirim et al., 2012). Ppy and AuNP were drop-casted onto the working electrode (WE) for 30 minutes to adhere to the carbon surface of the screen-printed carbon electrode (SPCE). The direct assay format was conducted on the modified SPCE, as illustrated in Figure 1, and the sensor's sensitivity was assessed using differential pulse voltammetry (DPV), with a 5 mM ferricyanide/ferrocyanide solution in 0.1 M KCl utilized as the redox solution.

Figure 1: Schematic diagram of direct assay format for saxitoxin (STX) sensor detection

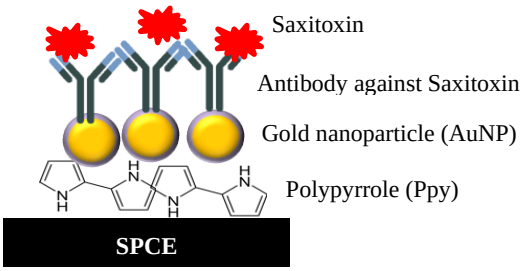
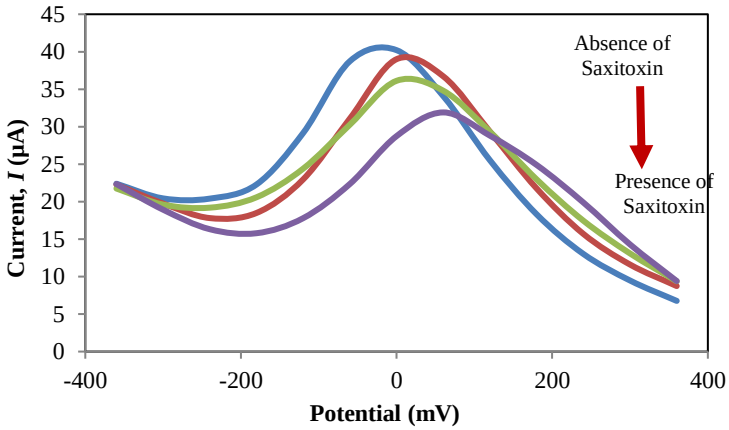


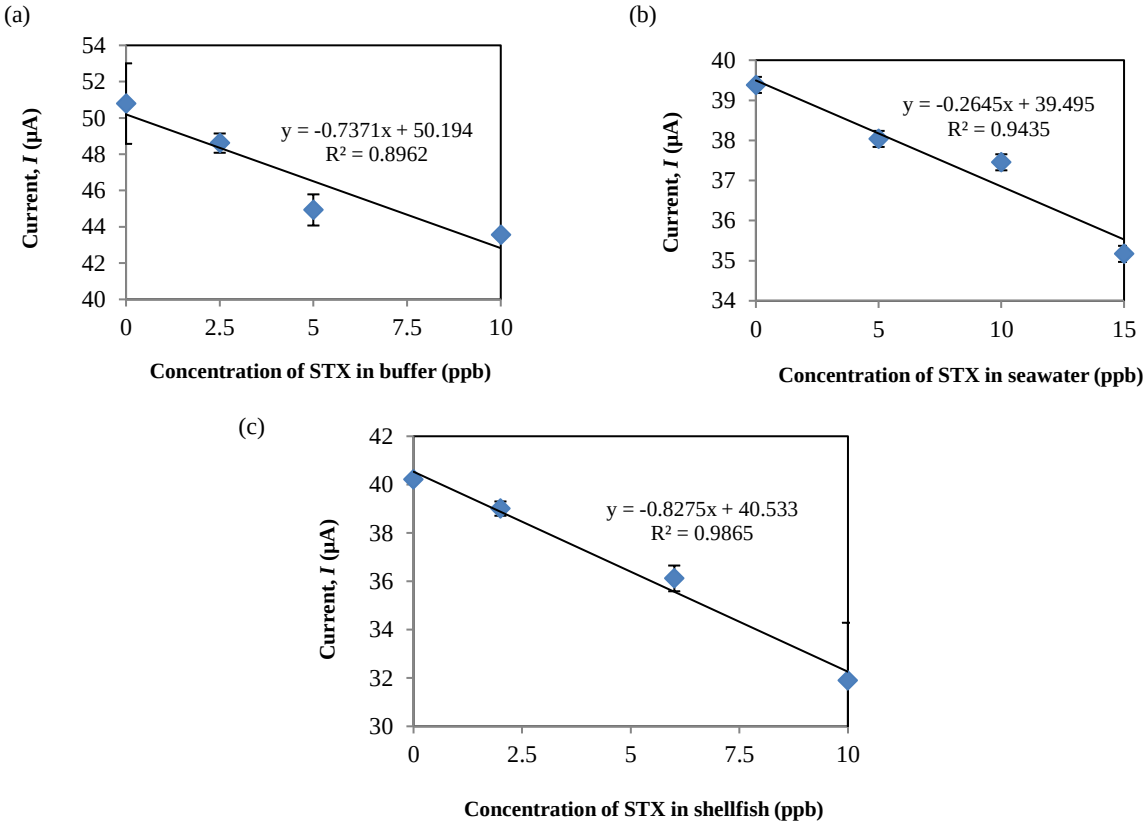
Figure 2 depicts the DPV response of the modified electrode with the addition of saxitoxin (STX) at different concentrations. A distinguishable anodic peak current was observed, which decreased with increasing antigen concentration. The binding of antigen-antibody complexes can increase steric hindrance and electrostatic influences within the biorecognition layer, leading to a decrease in electron-transfer rate and mediator diffusion (Police Patil et al., 2023). Following the affinity reaction, the peak current decreased with increasing concentrations of the nonconductive antigen. DPV is often utilized for label-free detection in biosensor system, showing an inverse correlation between the peak current and STX concentration.

Figure 2: Differential Pulse Voltammetry (DPV) of ppy/AuNP/STX-ab at different concentration of standard Saxitoxin in 5mm redox solution



Under optimal conditions, the sensitivity of the developed portable sensor for saxitoxin (STX) was investigated at different concentrations in three diverse matrix systems namely buffer solution, seawater matrix, and shellfish matrix (Figure 3). The direct assay was conducted on the Ppy/AuNP-modified electrode using various standard concentrations of STX ranging from 0 to 10 ppb in the buffer and shellfish matrix, and from 0 to 15 ppb in the seawater matrix. An antibody concentration of 0.7 mg/mL, which exhibited the most optimal electrochemical response towards the target analyte, was immobilized on the carbon working electrode (WE). A 0.01 M pH 7.4 phosphate-buffered saline (PBS) solution was used as a blank or for the 0 ppb standard. The developed biosensor demonstrated high sensitivity and specificity; however, the components of biological fluids or sample matrices can influence the reliability of quantitative results by amplifying or attenuating the analyte signal. This phenomenon, known as the matrix effect, is critical for evaluating bioanalytical method validation. In this study, a fifty-fold dilution factor of the shellfish matrix was optimized to minimize the matrix effect on sensor development. Consequently, the STX standard was added at a fifty-fold dilution of the extracted shellfish sample for calibration curve generation in the shellfish matrix, while no dilution factor was applied to the seawater samples. All measurements were recorded using DPV analysis to obtain current readings.

Figure 3: Calibration curve in three different matrix systems (a) PBS buffer system; (b) Seawater matrix system; and (c) Shellfish matrix



The calibration plots in the three different systems demonstrated a decrease in current signal that was inversely proportional to the concentration of saxitoxin (STX), showing a strong correlation (high R^2 value) and a linear relationship over a wide working range up to 15 ppb of saxitoxin. The developed immunosensor exhibited excellent sensitivity, as evidenced by the low limits of detection (LOD) and quantification (LOQ) shown in Table 1.

Table 1: Coefficient of determination (R^2), Limit of Detection (LOD) and Limit of Quantification (LOQ) of STX immunosensor in different matrix system

Matrix system	R^2	LOD (ppb)	LOQ (ppb)
PBS buffer	0.896	7.70	23.30
Seawater	0.943	6.38	19.33
Shellfish	0.986	2.09	6.35

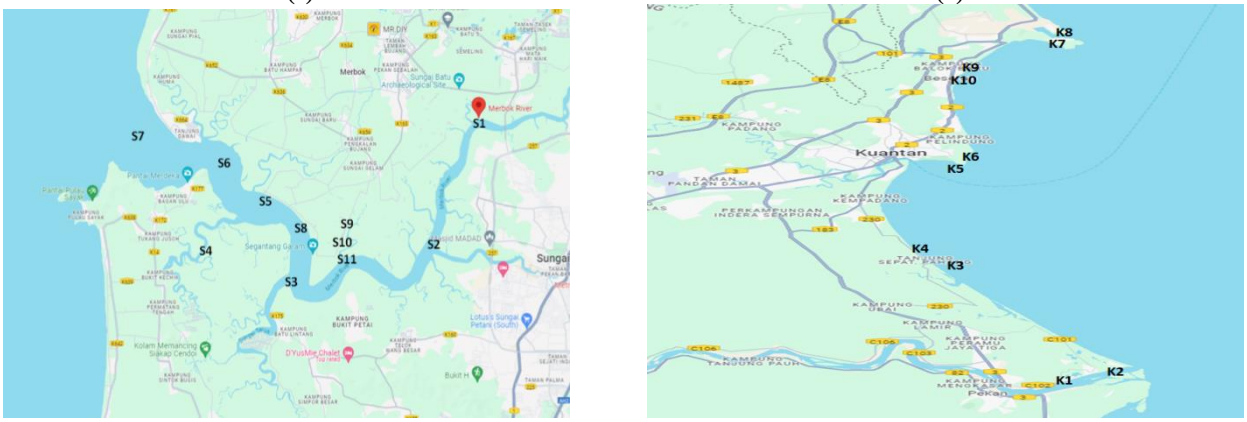
The detection in the shellfish matrix demonstrated the lowest limit of detection (LOD), capable of detecting as low as 2.09 ppb, compared to 7.70 ppb and 6.38 ppb for the buffer and seawater matrix systems, respectively. Similarly, the limit of quantification (LOQ) was also lowest in the shellfish matrix system, at 6.35 ppb, compared to 23.30 ppb and 19.33 ppb in the buffer and seawater matrix systems, respectively. These results confirm that the developed portable immunosensor is highly sensitive for detecting saxitoxin even at very low concentrations.

Sample Application Using Portable Device and IoT Biosensor

The monitoring of harmful algal bloom (HAB) species and saxitoxin (STX) currently relies heavily on instrumentation methods such as light microscopy, HPLC, and LCMS (Wang et al., 2024). However, these methods are limited by factors such as being highly time-consuming, labor-intensive, and requiring trained personnel. Therefore, there is a pressing need for easy-to-use, rapid, and sensitive techniques that can facilitate toxin monitoring outside of laboratory settings or for on-site monitoring. The developed portable biosensor has the potential to address this need. In this study, the application of the developed portable IoT biosensor for on-site monitoring was tested in two locations: Sg. Merbok, Kedah in the northern part of Peninsular Malaysia (Figure 4a), and the coastal area of Kuantan, Pahang in the eastern part of Peninsular Malaysia (Figure 4b). Seawater samples were collected at eleven stations along Sg. Merbok and ten sampling points along the Kuantan coastal area and were tested using the developed biosensor. The samples were tested directly on-site and were validated using the ELISA technique.

Figure 4: Sampling location (a) Sg. Merbok, Kedah located North of Peninsular Malaysia and (b) Kuantan coastal area, Pahang located East of Peninsular Malaysia

(a) (b)



The correlation between the analyzed data using the developed portable IoT biosensor and the ELISA technique is tabulated in Table 2. Seawater samples showed an 80% and 50% correlation between the IoT biosensor and ELISA analysis for samples from Sg. Merbok, Kedah, and the Kuantan coastal area, Pahang, respectively. The STX concentrations in seawater samples S1 and K8 were detected as 5.565 ppb and 3.146 ppb, respectively, using the IoT biosensor, while they were not detected by ELISA due to the higher limit of detection (LOD) of 10 ppb for the ELISA technique. In contrast, the STX concentrations in samples S7, K2, K4, K5, and K10 were not detected by the IoT biosensor. However, ELISA data showed readings at very low concentrations below the LOD for ELISA.

Table 2: Detection of STX in seawater sample from Sg. Merbok, Kedah and Kuantan coastal area, Pahang using portable IoT reader and its validation study using ELISA technique

Sampling Point	STX concentration (ppb)	
	IoT-Biosensor	ELISA
Sg. Merbok, Kedah		
S1	5.565	**ND*
S2	*ND*	*ND*
S3	*ND*	*ND*
S4	*ND*	*ND*
S5	*ND*	*ND*
S6	*ND*	*ND*
S7	*ND*	0.001
S8	*ND*	*ND*
S9	*ND*	*ND*
S10	*ND*	*ND*
S11	*ND*	*ND*
Coastal Area Kuantan, Pahang		
K1	*ND*	*ND*
K2	*ND*	0.007
K3	*ND*	*ND*
K4	*ND*	0.005
K5	*ND*	0.008
K6	*ND*	*ND*
K7	*ND*	*ND*
K8	3.146	*ND*
K9	0.410	0.004
K10	*ND*	0.08

**ND* = Not Detect

The viability of the developed portable IoT biosensor for STX detection in shellfish samples was also investigated. Shellfish samples, such as cockles from markets in Selangor (Figure 5b), and oysters and mussels from Sg. Merbok, Kedah (5c and 5d), were tested using the portable biosensor. The shellfish samples were extracted following the extraction method mentioned earlier. The supernatant of the extracted samples was diluted fifty times, which was the optimized dilution factor for shellfish samples to minimize matrix effects. The results obtained from both techniques were recorded in Table 3, showing a 100% correlation for the shellfish samples analyzed.

Figure 5: (a) Oyster and mussels farming area at Sg. Merbok, Kedah; (b) Cockles sample from market in Selangor; (c) Oysters sample; and (d) Mussels sample from Sg. Merbok, Kedah

(a) (b) (c) (d)

Table 3: Detection of STX in shellfish sample from local market and Sg. Merbok, Kedah using portable IoT reader and its validation study using ELISA technique

Shellfish Sample	STX concentration (ppb)	
	IoT-Biosensor	ELISA
Cockles (Market)	*ND	ND
Oyster S1 (Sg. Merbok)	ND	ND
Oyster S3 (Sg. Merbok)	ND	ND
Oyster S4 (Sg. Merbok)	ND	ND
Mussels S3 (Sg. Merbok)	ND	ND

*ND = Not Detected

All the analyzed results on the portable biosensor will be stored on the central cloud server with their data log details and real-time location. The website can be accessed at https://mardisense.com. The details for Mardi*sense* Apps and website will describe in other publication.

CONCLUSION

In this study, we have successfully demonstrated a highly effective smart portable immunosensor for saxitoxin detection. This biosensor system showed excellent sensitivity across diverse matrix systems, including buffer, seawater, and shellfish matrices. Notably, the immunosensor exhibited an impressively low limit of detection (LOD) in all three matrices, with the shellfish matrix showing the lowest LOD at 2.09 ppb. To assess its practical utility, the portable biosensor was tested with real samples and used in on-site applications. The correlation and validation study with the ELISA method demonstrated good agreement, underscoring the reliability of the sensor system. These findings emphasize the potential of this immunosensor for on-site sampling in monitoring harmful algal bloom intoxication and detecting saxitoxin presence, offering a promising tool for environmental and food safety applications.

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REFERENCES

Auwal, M. M., Janice, K., Richard, L.& Kevin, C. H. (2023). An Electrochemical Screen-Printed Sensor Based on Gold-Nanoparticle-Decorated Reduced Graphene Oxide–Carbon Nanotubes Composites for the Determination of 17-β Estradiol. *Biosensors* 13, 491. https://doi.org/10.3390/bios13040491

McPartlin, D. A., Lochhead, M. J., Connell, L. B., Doucette, G. J. & O'Kennedy, R. J. (2016). Use of Biosensors for the Detection of Marine Toxins. *Essays in Biochemistry* 60:49-58. https://doi.org/10.1042/EBC20150006

Police Patil, A. V., Chuang, Y. S. & Wu, C. C. (2023). Recent advances in electrochemical immunosensors with nanomaterial assistance for signal amplification. *Biosensors* 13:125. https://doi.org/10.3390/bios13010125

Sheikhzadeh, E.,Chamsaz, M., Turner, A. P. F., Jager, E. W. H. & Beni, V. (2016). Label-Free Impedimetric Biosensor for Salmonella Typhimurium Detection Based on Poly [pyrrole-co-3-carboxyl-pyrrole] Copolymer Supported Aptamer. *Biosensors and Bioelectronics* 80: 194-200

The Star, 3 April 2024. page 5. https://www.moh.gov.my/moh/resources/Keratan%20Akhbar/2024/APRIL/Keratan_Akhbar_03.04_.2024

Truong, T.N.L., Van Toan, P., Hao, N. Q. (2019). Using AuNPs-modified screen-printed electrode in the development of molecularly imprinted polymer for artificial bioreceptor fabrication to improve biosensor sensitivity for 17 β -estradiol detection. *Adv. Nat. Sci. Nanosci. Nanotechnol. 10*, 015015

Usup, G., Pin, L. C., Ahmad, A., & Teen, L. P. (2002). *Alexandrium* (Dinophyceae) species in Malaysian waters. *Harmful Algae*, 1(3), 265-275

Wang, Y., Javeed, A., Jian, C., Zeng, Q. & Han, B. (2024). Precautions for Seafood Consumers: An Updated Review of Toxicity, Bioaccumulation, and Rapid Detection Methods of Marine Biotoxins. *Ecological and Environmental Safety* 274:116201

Xiao, T.; Huang, J.; Wang, D.; Meng, T.; Yang, X. (2020). Au and Au-Based nanomaterials: Synthesis and recent progress in electrochemical sensor applications. *Talanta* , *206*, 120210

Yildirim, N.; Long, F.; Gao, C.; He, M.; Shi, H.-C.; Gu, A.Z. (2012). Aptamer-Based Optical Biosensor for Rapid and Sensitive Detection of 17β-Estradiol In Water Samples. *Environ. Sci. Technol.*, *46*, 3288–3294

Zhang, D.; Zhang, W.; Ye, J.; Zhan, S.; Xia, B.; Lv, J.; Xu, H.; Du, G.; Wang, L. (2016). A Label-Free Colorimetric Biosensor for 17β-Estradiol Detection Using Nanoparticles Assembled by Aptamer and Cationic Polymer. *Aust. J. Chem.*, *69*, 12–19.