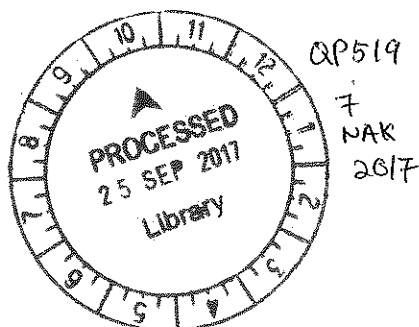i

Haematococcus pluvialis WHOLE CELL BIOSENSOR FOR HEAVY METAL DETECTION

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ABSTRACT

Tonnes of heavy metals are deposited onto the environment every year causing high water contamination which has become a major concern for health regulatory bodies world-wide. So there is a need to develop screening methods for rapid and accurate detection of heavy metals. Hence a novel *Haematococcs pluvialis* whole cell biosensor was developed utilizing astaxanthin as a bio-reporter for detection of presence of heavy metals Cd, Pb and Ni. *Haematococcus pluvialis* cells were immobilized with agarose and exposed to heavy metals cadmium, lead and nickel. The astaxanthin response was detected spectrophotometrically at wavelength 482 nm. The biosensor produced the highest absorbance response with cell immobilized at day 3 with a density of 5×10^5 cells/ cuvette. pH was found to affect the response of biosensor, with the highest response produced at pH 5. The biosensor was able to detect presence of both Cd and Pb with in a linear range of 0.001 to 1.00 mg/L for both Cd and Pb while the linear detection range for Ni was found to be with 0.001-5.00 mg/L range. The results showed that astaxanthin is naturally present in *Haematococcus pluvialis* at low concentration but can be induced due to oxidative stress from increased reactive oxygen species induced by the heavy metals in the cells which triggers increased biosynthesis of astaxanthin.Astaxanthin is an antioxidant and acts a defense mechanism to protect the cell against oxidative damage. Additionally, *Haematococcus pluvialis* biosensor produced a high and rapid response within 15 minutes on exposure to heavy metal hence the designed biosensor, showed good potential for detection of heavy metals for environmental toxicity monitoring.

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LIST OF ABBREVIATIONS

HPLC	High performance liquid chromatography
HMs	Heavy metals
ROS	Reactive oxygen species
$^1O_2$	Singlet oxygen
^3Chl	Triplet chlorophyll
^1Chl	Singlet chlorophyll

Pb	Lead
Ni	Nickel
Cd	Cadmium
OD	Absorbance
Ppm	Parts per million
^0C	Degree Celsius
nm	Nanometer
mL	Milliliter
L	Liter
ATP	Adenosine triphosphate
λ	Wavelength
As	Nickel

1.0 INTRODUCTION

With the rapid development in urbanization, industrial and agricultural processes, tonnes of heavy of metal (HMs) laden effluents are released into the environment every year (Wong, Lee, & Surif, (2013) and (Turdean, 2011). The increasing levels of HMs contamination in aquatic systems has become a major concern for environmental regulatory bodies (Jarup, (2003) and (Tchounwou, Yedjou, Patlolla, & Sutton, 2012). Hence there is a need to develop screening methods for HMs detection in water.

Currently they are powerful analytical techniques used for HMs detection like atomic absorption and emission spectrophotometry, chromatographic techniques like gas chromatography and high performance liquid chromatography Han, Zhu, Yuan, & Li, (2001) and (Thompson, Maliwal, Feliccia, Fierke, & McCall, 1998).

These methods are highly reliable and accurate but they require highly expensive equipments, skilled personnel to operate them and they have difficulty in analyzing water samples with very low HMs concentrations hence they usually require some form of sample preparation and purifications before analysis hence there time consuming Han, Zhu, Yuan, & Li, (2001); Thompson et al., (1998) and Turdean, (2011). Furthermore they do not reflect the cumulative effects of the pollutants on biota and habitat alterations in the ecosystem James & Ellen, (1999) and (Emily & Scott, 2011).

Consequently, there is a vast interest in the use of simple, inexpensive, sensitive, portable and easy to use biosensors to monitor water pollutants Thompson et al., (1998) and (Rogers, 1995). Biosensors are analytical devices that integrate bio-receptors coupled to a physical transducer that generates a measurable signal proportional to the concentration of the analytes Durrieu, Guedri, Berezhetskyy & Chovelon, (2007) and Turdean, 2011).

To date, several bio-reporter molecules have been utilized in development of biosensors for HMs detection like activities of enzymes like glucose oxidase coupled to electro-active materials Ghica, Carvalho, Amine & Brett, (2013) and Soldatkin et al., (2012) and immobilized phytochelatins coupled to capacitive signal transducer to measure conformational changes (Bontidean et al., 1998).

However the use of immobilized enzyme(s) and proteins as bio-reporters is more tedious and costly due to production and purification procedures, additionally optimizations of protein stability is difficult Berezhetskyy et al., (2007) and (Durrieu et al., 2007). Therefore whole cell biosensors provide a more attractive option because whole cells contain several-enzymes and cofactors and can reflect the real physiological effects like bioavailability, toxicity, genotoxicty and morphological alterations of HMs on the cells. Additionally enzymes are within already optimized conditions inside the cells so more stable and protected from interfering substances stables Turdean, 2011; Belkin, (2003) and (Renella & Giagnoni, 2016)

Whole cells can be utilized for *in-situ* and continuous HMs detection (Aisyah, Chai & Wong, 2015). Several different whole cells have been investigated for use in biosensors for HMs detection like use of Cyanobacteria Wong & Surif, (2013) and several micro-algae species have been investigated.

Microalgae species are preferred for use as bio-reporters because they are relatively easy and cost effective to culture, show rapid increase in biomass and high sensitivity to HMs (Bellinger & Sigee, 2015). Additionally micro-algae show several indicator responses like production of reactive oxygen species (ROS) Ernani et al., (2003), reduction in photosynthetic oxygen release in *A. torulosa* Chia et al., (2005), heavy metal absorption Adriana et al., (2015) and production of phyto-plankton-photo pigments like chlorophyll in *C. vulgaris* (Wong, Lee, Koh, Ong & Choong, 2016).

Other pigments like anti-oxidant beta-carotenes have been studied but to a lower extent, a few carotenoid based biosensors have been reported for HMs detection (Wong & Chong, 2014). However the developed bio-sensors so far often utilized transgenic organisms and require longer exposure time for HM detection which has limited there practicality (Wong et al., 2015).

In this paper, responses of *invivo* astaxanthin in immobilized *H. pluvialis* cells on exposure to HMs Cd, Pb and Ni was reported. Among algae species, currently *H. pluvialis* have been shown to accumulate the highest quantity of astaxanthin of up to 1-5% total cell dry weight, under conditions of stress (Wan et al, 2014).

Astaxanthin is a strong antioxidant and protects the cell membranes against oxidative damage through inhibiting lipo-peroxidation and through rigidifying the membranes, preventing penetration of ROS Barros, Pinto, Colepicolo, & Pedersén, (2001) and (Wisniewska & Subczynski, 1998) and quenching of excitation energy of Singlet oxygen ($^1O_2$) Bing et al., (2003) and (Koboyahsi et al., 1997).

ROS are produced when disruption/ blockage of electron chain flow during photosynthesis occurs, which causes leakage of electrons that leads to formation of ROS (Volland et al, 2014), additionally, blocking electron flow inhibits transfer of single chlorophyll ^1ChI energy to reaction centers of photosystem II, so the singlet chlorophyll is transformed into triplet chlorophyll that reacts with O_2 to form highly reactive singlet oxygen.

ROS have been shown to indirectly induce astaxanthin biosynthesis via several mechanisms Collén, Pinto, Pedersen, & Colepicolo, (2003) and (Sharma et al., 2012). Due to high astaxanthin production in *H. pluvialis*, a strong biochemical response was predicted with shorter exposure time. The produced red astaxanthin can absorbs light at 482 nm wavelength, so an optical transducer was used in construction of the biosensor. The aims of this study were:

- To investigate the response of immobilized *Haematococcus pluvialis* to heavy metals Cd, Pb and Ni spectrophotometrically.
- To determine the linear detection range for the heavy metals Cd, Pb and Ni.

2.0 LITERATURE REVIEW

2.1 *Hematococcus pluvialis*

Haematococcus pluvialis is fresh water, unicellular biflagellate green microalgae that belongs to phylum *Chlorophyta*, order *volvocales* and family *Haematococcaseae* Shah et al., (2016) and (Herrero et al., 2012). *Hematococcus pluvialis* is widely distributed across various habitats and environments, it can be found in temporal water bodies, ponds, and dried fountains, on rocks with brackish water and on the sea shores Shah et al., (2016). *H. pluvialis* is well suited to survive under conditions of extreme light, salinity, nutrient deprivation and high temperature due to its ability to form cysts (Boussiba & Vonshak, 1991).

Hence *H. pluvialis* has potential to be used for long term monitoring of effects of pollutants as a bio-indicator. The life cycle of *H. pluvialis* consists of mainly two stages; the 'green' flagellated stage and the 'red' immotile stage. In between those two stages, *H. pluvialis* undergoes four morphological cellular changes Koboyashi et al., (1997); Sha et al., (2016) and (Sun et al., 2016).

The cellular types include macrozooids which are green vegetative flagellated cells while microzooids are green non-flagellated cells that both majorly dominate the culture when conditions are optimal Sha et al., (2016) and (Lorenz, 1999). When conditions become unfavorable then plamella and thick walled alpanospores cells with accumulated astaxanthin dominate the culture Koboyashi et al., (1997) and Lorenz, (1999), when conditions return to normal, the red alpanospores germinate into macrozooids and a new cell cycle begins.

Due to the ubiquitous nature of *H. pluvialis*, the non-motile alpanospores are microscopic and have size ranging between <10-50μm Wayama et al., (2013) and (Shah et al., 2016). The small size of the cells makes them potential candidates for immobilization in whole cell biosensors. Similar to *C. vulgaris* another type of algae species is commonly utilized in whole cell biosensor (Nguyen-Ngoc & Tran-Minh, 2007).

Additionally, *H. pluvialis* can easily be cultured in a range media heterotrophic, mixotrophic and phototrophic cultures Shah et al., (2016); Herrero et al., (2012) which makes it cost effective to use in whole cell biosensors. Due the thick cell wall of *H. pluvialis* when it forms alpanospores

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accumulated with astaxanthin, the cell is ideal for immobilization on agarose without morphological damage, hence viable and functional.

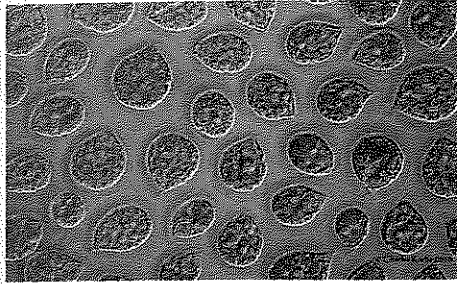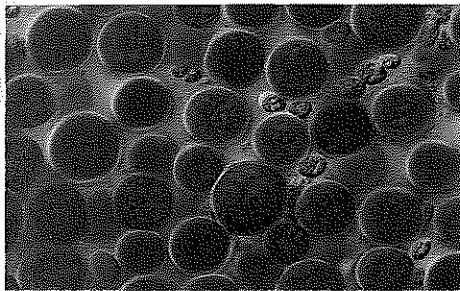Figure 2.1.a Green motile *Hamatococcus pluvialis* under normal environmental conditions (encyclopedia, 2012).

Figure 2.1.b Red immotile alpanospores of *Haematococcus pluvialis* under conditions of stress (AlageMart, 2014).

2.1.1 ASTAXANTHIN

Hematococcus pluvialis is considered the richest source of natural astaxanthin (3,3'- dihydroxy-ß-carotene-4,4'-dione) a C_{40} tetraterpene and is a strong antioxidant Sha et al., (2016) and Sun et al., (2016). As observed in figure 2.1.1, astaxanthin is a secondary keto-carotenoid that is bio-synthesized via the carotenoid pathway from pyruvate and Glyceraldehyde-3-phosphate (G3P).

Both pyruvate and G3P are glycolysis products that later enter the non-Mevelonate pathway (MEP) to produce Isopentyl-diphosphate (IPP) an intermediate for astaxanthin biosynthesis and other carotenoid synthesis Lorenz, (1999) and (Koboyashi et al., 1997).

The pathway is initiated under conditions of stress in *H. pluvialis* like high temperature, nutrient deprivation, intense irradiation and others Sun et al., (2016); Koboyashi et al., (1997) and (Wang & Zarka, 2003).

Apart from the photo-protective role, astaxanthin has also been shown to increase the spectrum for absorption of light by the microalgae into the red region hence the algae can absorb light energy even in reduction of chlorophyll content in the alpanospores Koboyashi et al., (1997). Additionally astaxanthin is produced as protective antioxidant to quench reactive oxygen species Aflalo, Meshulam, Zarka & Boussiba, (2007) and to protect the cell from oxidative damage Shah et al., (2016) and (Minhas et al., 2016).

Astaxanthin acts as a photo-protective agent through dissipation of excessive light energy hence shielding the photosynthetic apparatus from damage, in addition astaxanthin acts as physico-chemical barrier around the nucleus that protects DNA from reactive oxygen species (Yong & Lee, (1991) and (Koboyashi et al., 1997).

During the red stage, lipid deposition increase by 40% in the cytoplasm of the cell and astaxanthin deposition occurs in the lipid droplets and exists as esters in the alpanospores (Wang & Zarka, (2003) and (Aflo et al., 2007). In the green macrozooid cells, other pigments like chlorophyll a and b, lutein 75-80%, β-carotene and other primary carotenes exists, however in the red stage there is a decrease in all other pigments and an increase astaxanthin up to 89-99% of total carotenoids Shah et al., (2016) and (Wang & Zarka, 2003).

The ability of *Haematococcuc pluvialis* to accumulate up to 1-5% astaxanthin of total cell dry weight under conditions of stress Park & Lee, (2001) and Wan et al., (2015) which enables it absorb more energy to repair cellular damage (Koboyashi et al., 1997). Astaxanthin acts as an accessory light harvesting pigment that utilizes light over a wide spectral range including the blue region (Wang & Zarka, 2003). That gives the algae an essential characteristic as a bio-reporter as it can absorb light at specific wavelength Kobayashi et al., (1997) and Lorenz, (1999), so if coupled to an optical transducer, could be used in a biosensor.

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