Zostera muelleri as a bioindicator of heavy metal pollution in marine ecosystems

by

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Abstract

Seagrass has been shown by previous researchers, over four decades, to be a useful bioindicator of heavy metal pollution in marine environments. Despite the large data base resulting from these studies to date there has been no method for exploiting this data in its entirety for the monitoring of sites in general and for identifying pollution events. This project has devised and tested a simple, standard method to identify heavy metal pollution in different marine environments. The assay is based on the benchmarking of any given set of analytical data for heavy metal levels in whole-plant seagrass tissue against heavy metal concentration magnitude ranges, derived from the totality of the available international data. Notably, this subsumes the broad range of geographical locations, climatic conditions, different seagrass species and the different plant organs analysed, that are inherent in the available data sets.

Thus, magnitude criteria have been established for “normal” (background) versus “elevated” and “polluted” levels for the “anthropogenic” metals, As, Cd, Co, Cr, Cu, Mn, Ni, Pb and Zn. Concurrently, an experimental case study was devised and carried out to implement and test the devised assay. Employing the regional seagrass species *Zostera muelleri* (Eelgrass), samples were collected from locations in Port Phillip Bay (PPB), Western Port (WP) and Corner Inlet (CI) over a five-year period, from 2015 to 2020, across six seasons, and were processed and analysed for their heavy metal content by Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES).

According to the derived assay, the metal levels found in the case studies carried out for the three bays, were compared to the totality of the international database. Notably, of the six seasonal periods, Winter 2018 data (except for Cu and Mn) showed evidence of a gross heavy metal contamination event, affecting all bays. Therefore, the data for the other seasons have been compared separately from that of the “aberrant” Winter 2018 season. This study has found that for all seasons and across all bays, As was very highly elevated and Cd and Pb significantly elevated. For As and Pb, PPB and WP were comparable, with CI being less affected for both metals. For Cd, the pollution levels were comparable across all three bays. For all seasons, Co was also generally elevated across the bays, except for (all bays) Summer 2015/16 and PPB Autumn

2019, with CI being less affected overall. Generally, over all seasons and bays, Cr levels were not highly elevated. However, a slight elevation was observed for WP and CI Summer 2019/18, PPB and WP Autumn 2019 and WP Winter 2019. PPB and WP appear more affected than CI. Cu levels were within background for all seasons, except for Summer 2015/2016, where a slight elevation was observed for PPB and CI. Generally, the Cu levels for PPB > WP > CI. For all seasons, Mn levels were well within background across all the bays and tended to be lower in WP than in PPB or CI. Across all seasons, Ni levels were well within background across all the bays, with the levels for PPB and WP being comparable and the levels for CI being consistently lower. For all seasons, Zn levels across all the bays were essentially within background, with evidence of a slight elevation in PPB Summer 2015/16. There was a progressive increase in Zn levels for WP from Spring 2018 to Winter 2019, with a slight elevation being evident for the latter season.

Declaration

“I, Yao-Han Lee, declare that the PhD thesis entitled *Zostera muelleri* as a bioindicator of heavy metal pollution in marine ecosystems is no more than 80,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work”.

“I have conducted my research in alignment with the Australian Code for the Responsible Conduct of Research and Victoria University’s Higher Degree by Research Policy and Procedures.

Signature

Date: 11th August 2023

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Presentations relevant to this thesis

International conferences (copies provided in the Appendix)

1. Lee, Y-H., Orbell, J. D. and Robinson, R. W. 2018. *Zostera muelleri* as a bioindicator for oceanic heavy metal levels in south-eastern Australia. ECSA 57 Changing estuaries, coasts and shelf systems – diverse threats and opportunities, September 3 – 6, 2018, Perth, Australia. (Poster Presentation) – Appendix 1

2. Lee, Y-H., Dear, E.J., Finger, A., Robinson, R. W. and Orbell, J. D. 2019. Magnitude ranges of heavy metals in seagrass throughout the world – evidence for heavy metal pollution I the bays of south-eastern Australia. CHEERS – Global changes in estuarine and coastal systems functioning innovative approaches and assessment tools, November 4 – 8, 2019, Bordeaux, France. (Platform Presentation) Appendix - 2

Local presentations (copies provided in Appendix)

1. Lee, Y-H. 2019. Bioindicators useful for human and environmental health. 3-minute thesis competition. Appendix 3

2. Lee, Y-H., Orbell, J.D. and Robinson, R. W. 2020. Seagrass as a bio-monitor of heavy metal pollution in the (ostensibly pristine) Victorian marine environment. The 2020 ISILC (Institute for Sustainable Industries and Liveable Cities) HDR Student Conference 2020. Appendix 4

3. Lee, Y-H., Orbell, J.D. and Robinson, R. W. 2022. A novel assay for the identification of heavy metal pollution in marine ecosystems. The 2022 ISILC (Institute for Sustainable Industries and Liveable Cities) HDR Student Symposium 2022. Appendix 5 – **Placed 3rd in the Engineering & Science Stream for Best Presenter for Themed Presentation won $50**

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List of Abbreviations

$(CH_3CH_2)_4Pb$: Tetraethyl lead
AAS: Atomic absorption spectroscopy
AGAL-6: Cabbage leaves
AGAL-10: Hawkesbury River Sediment:
AMSA: Australian Marine Sciences Association
AQA 16-12 S2: Cabbage leaves
BCFs: Bioconcentration factors
BCR-679: Cabbage leaves
BTEX: Benzene, Toluene, and Xylene isomers
CI: Corner Inlet
$CaNa_2$ EDTA: Calcium disodium ethylenediaminetetraacetate
CASS-4: Nearshore seawater Reference Material
CO_2: Carbon Dioxide
CRMs: Certified reference materials
DDT: Dichlorodiphenyltrichloroethane

DO: Dissolved oxygen

Dw: Dry weight

EPA: Environmental Protection Agency

FIMS: Flow Injection Mercury System

ICP-AES: Inductively Coupled Plasma Atomic Emission Spectroscopy

ICP-MS: Inductively Coupled Plasma Mass Spectrometry

ICP-OES: Inductively Coupled Plasma Optical Emission Spectrometry

IQR: Interquartile range

HCL: Hollow Cathode Lamp

HCl: Hydrochloric acid

HNO_3: Nitric acid

K: Kelvin

KMO: Kaiser-Meyer-Olkin

MFB: Metropolitan Fire Brigade

MLLW: Mean lower low water

NMI: National Measurement Institute

NO_2: Nitrogen dioxide

NTU: Nephelometric Turbidity Units

PAHs: Polycyclic aromatic hydrocarbons

PCA: Principal Component Analysis

PCB: Polychlorinated biphenyl

PFAS: Per- and polyfluoroalkyl substances

PINP: Phillip Island Nature Parks

ppb: Parts per billion

PPB: Port Phillip Bay

PPM: Parts per million

PPT: Parts per thousand

POPs: Persistent organic pollutants

PVC: Polyvinyl chloride

Q_1: 1st quartile

Q_3: 3rd quartile

RSD: Relative standard deviation

SRM: Standard Reference Material

SO_2: Sulphur dioxide

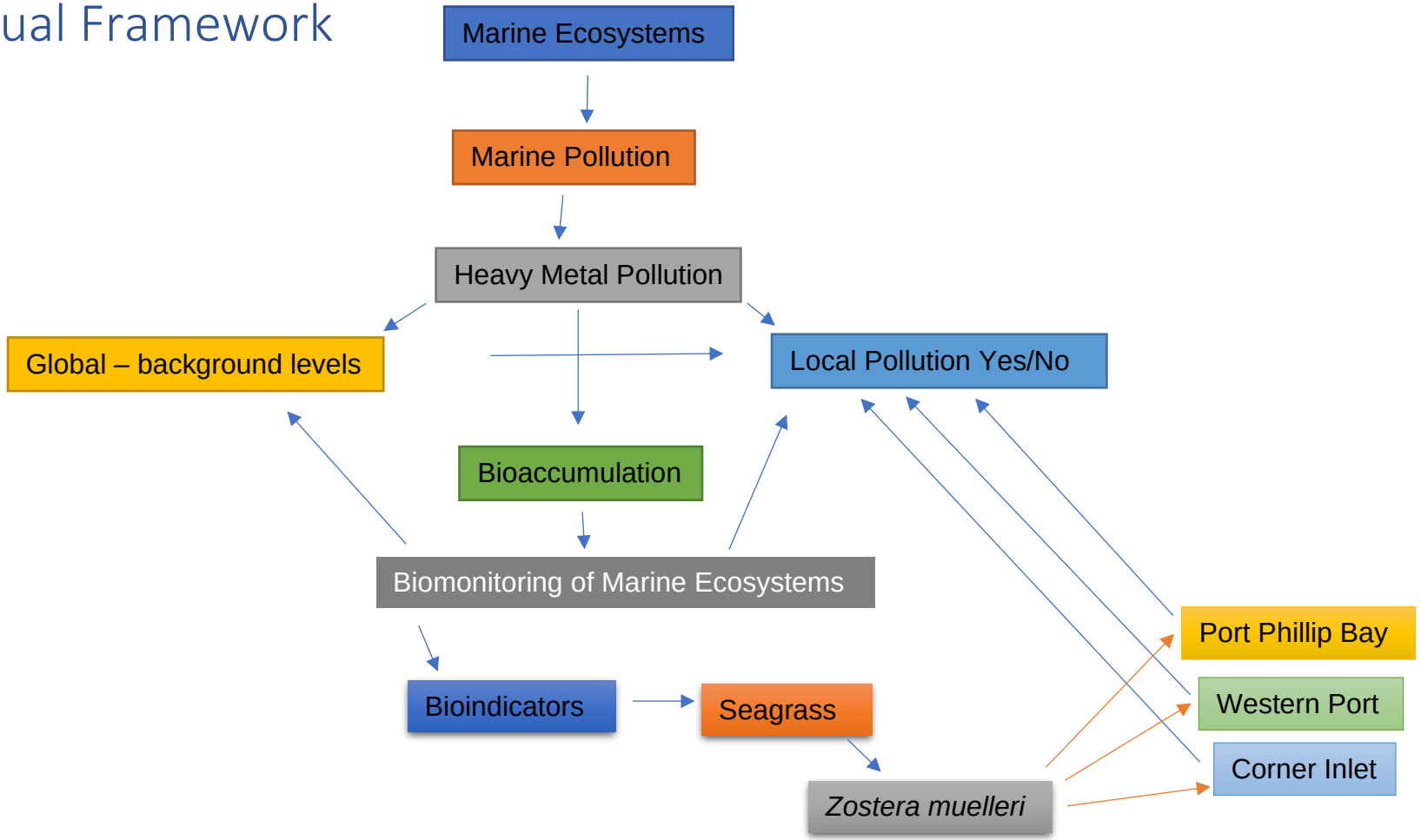
TDS: Total dissolved solids

USFDA: United States Food and Drug Administration

WP: Western Port:

Z. muelleri: Zostera muelleri

Conceptual Framework



This schematic provides an overview of the project.

Chapter 1 - Introduction

1.1: Marine ecosystems

Marine ecosystems are the largest of Earth's ecosystems and are distinguished by waters that have a high salinity of ~ 35 parts per thousand (ppt). Seawater covers more than 70% of the surface of the Earth, accounting for more than 97% of the Earth's water supply and 90% of habitable space (UNESCO 2017). Marine ecosystems are characterised by their physical environment and their associated biological community of organisms. Marine ecosystems include nearshore systems, such as salt marshes, mudflats, seagrass meadows, mangroves, rocky intertidal systems and coral reefs. They also extend outwards from the coast to include offshore systems, such as the ocean surface, pelagic ocean waters, the deep sea, oceanic hydrothermal vents and the seafloor.

In addition to providing many benefits to the natural world, marine ecosystems also provide social, economic, and biological ecosystem services to humans. Pelagic marine systems regulate the global climate, contributing to the water cycle, maintaining biodiversity, provide food and energy resources, and create opportunities for recreation and tourism (Hassan et al. 2005). Economically, marine systems support billions of dollars' worth of fisheries, aquaculture, offshore oil and gas, and trade and shipping. Ecosystem services fall into multiple categories, including supporting services, provisioning services, regulating services and cultural services (The Nature Conservancy 2020).

The Earth's marine ecosystems, that provide such essential ecosystem services, face various threats. Such threats include human exploitation and development, marine contamination, invasive species and climate change. This project will focus on investigating the extent and assessment of heavy metal contamination in the marine environment, specifically in the vicinity of seagrass meadows.

1.2: Marine pollution

Marine pollution occurs when detrimental effects resulting from the entry into the ocean of chemicals, particles, noise, waste from industry, agriculture/aquaculture or residential waste, or via the spread of invasive organisms. Eighty percent of marine pollution originates from land (Sheppard 2019). In addition, air pollution is also a contributing factor by transporting pesticides and/or foreign materials into the water. These kinds of pollution have proven to be harmful to marine life and ecosystems (NOAA 2020). Furthermore, marine pollution often comes from non-point sources (multiple and diffuse) such as agricultural runoff, wind-blown debris, and dust. Many potentially toxic chemicals adhere to tiny particles, which are then taken up by zooplankton, and benthic organisms, most of which are either deposit or filter feeders. In this way, toxins maybe bioaccumulated within aquatic food webs. Heavy metals can also be introduced into marine food webs. When this happens, it causes changes to tissue matter, biochemistry, behaviour, reproduction and suppresses growth of marine life. In addition, since many animal feeds have a high percentage of fishmeal, heavy metals can be transferred to land animals, which can contaminate meat and dairy products. In order to protect the marine ecosystems from pollution, policies have been developed internationally. Thus there are many different ways for marine ecosystems to be polluted and overtime there have been multiple laws, policies, and treaties put into place.

Although marine pollution has a long history, significant international laws to counter them were not enacted until the twentieth century. Marine pollution was a concern during several United Nations Conventions on the Law of the Sea beginning in the 1950s. At that time most scientists believed that the oceans were so vast that they had unlimited ability to dilute, and thus render pollution harmless. In the late 1950s and early 1960s, there were several controversies about dumping radioactive waste off the coast of the United States by companies licensed by the Atomic Energy Commission, into the Irish Sea from the British reprocessing facility at Windscale, and into the Mediterranean Sea by the French Commissariat à l'Energie Atomique. In relation to the latter, Jacques Cousteau a famous oceanographer became an important international figure in campaigning to stop marine pollution. Marine pollution has also made further international headlines after the 1967 crash of the oil tanker Torrey

Canyon, and after the 1969 Santa Barbara oil spill off the coast of California. Marine pollution was a major area of discussion during the 1972 United Nations Conference on the Human Environment, held in Stockholm. Also, that year saw the ratification of the Convention on the Prevention of Marine Pollution by Dumping of Wastes and Other Matter, also known as the London Convention. The London Convention did not ban marine pollution, but it established black and grey lists for substances to be banned (black) or regulated by national authorities (grey). Cyanide and high-level radioactive waste, for example, were put on the blacklist. The London Convention applied only to waste being dumped from ships, and thus did nothing to regulate waste discharged as liquids from pipelines (Hamblin 2008)

There are many ways to categorize and examine the inputs of pollution into marine ecosystems. Inputs of pollution into the ocean maybe divided into three main types: the direct discharge of waste into the oceans, runoff into the waters due to rain and pollutants released from the atmosphere. A common path of entry by contaminants to the sea are rivers. The evaporation of water from the oceans exceeds precipitation and the balance is restored by rain over the continents entering rivers and subsequently being returned to the sea.

An example of this ‘river effect’ is the Hudson River in the State of New York and the Raritan River in the State of New Jersey, which drain at the northern and southern ends of Staten Island, respectively. These are sources of mercury (Hg) contamination of copepods, a type of zooplankton in the open ocean. The highest concentration in the filter-feeding copepods is not at the mouths of these rivers but 110 kilometres south, near Atlantic City, New Jersey, because the water flows close to the coast. It takes a few days for the mercury to start being bioaccumulated by the copepods (Gerlach 1975).

Pollution is often classified as point source or non-point source pollution. Point source pollution occurs when there is a single, identifiable, localized source of the pollution. An example is the direct discharge of sewage and industrial waste into an aquatic ecosystem. This kind is common in developing nations. Non-point source pollution occurs when the pollution comes from uncertain and diffuse sources. This kind of

pollution can be difficult to regulate and examples of this are agricultural runoff and windblown debris.

There are several different pathways for marine pollution to occur. Namely direct discharge, land runoff, ship pollution, atmospheric pollution and deep sea mining. The first pathway of marine pollution is direct discharge. Examples of direct discharge are sewerage, industrial waste, and mining. These pollutants enter rivers and marine waters directly from urban sewerage and industrial waste discharges, sometimes in the form of hazardous and toxic wastes, or in the form of plastics. Another source of direct discharge into marine ecosystems is inland mining for copper (Cu), gold (Au), and other precious metals. This contamination occurs when contaminated soil ends up in rivers flowing to the ocean. Such metals, discharged during mining of can cause significant environmental problems, for example copper, a common industrial pollutant, can interfere with the life history and development of coral polyps (Young 2003).

The second pathway of marine pollution is land runoff. This is also known as surface runoff or urban runoff. Examples of runoff pollution is from stormwater and nutrient pollution, which causes eutrophication. Surface runoff from farming, as well as urban runoff and runoff from the construction of roads, buildings, ports, channels, and harbours, can carry soil and particles laden with carbon (C), nitrogen (N), phosphorus (P) and other 'nutrient' chemicals. This nutrient rich water can cause microalgae and other phytoplankton to explode in coastal areas in a phenomenon known as an algal bloom. When an algal bloom occurs, it creates hypoxic conditions by depleting the available oxygen. For example, off the coast of southwest Florida, harmful algal blooms have existed for over 100 years. These algal blooms have been a cause of decrease in the species richness (the number of different species represented in an ecological community, landscape or region) in fish, turtles, dolphins, and shrimp and have caused harmful effects on humans who swim in the water (Weis & Butler 2009). Polluted runoff from roads and highways can be a significant source of water pollution in coastal areas. For example, about 75% of the toxic chemicals that flow into Puget Sound in the State of Washington are carried by stormwater that runs off paved roads and driveways, rooftops, yards, and other developed land (Washington State Department of Ecology 2008). While in California, there are many rainstorms that

runoff into the ocean. These rainstorms occur from October to March, and these runoff waters contain may petroleum, heavy metals and other pollutants from emissions (Holt et al. 2017). In China, there is a large coastal population that pollutes the ocean through land runoff. This includes sewage discharge and pollution from urbanization and land use. In 2001, more than 173000 km^2 of the Chinese coastal ocean waters were rated less than Class I of the Sea Water Quality Standard of China. Much of this pollution came from Silver (Ag), Copper (Cu), Cadmium (Cd), Lead (Pb), Arsenic (As), Dichlorodiphenyltrichloroethane (DDT) and Polychlorinated biphenyl (PCBs), which occurred from contamination, via land runoff (Li & Dag 2004).

The third pathway of marine pollution is ship pollution. Ships can pollute waterways and oceans in many ways. This can occur by oil spills, cargo discharge, ballast water discharge and invasive species. Oil spills can have devastating effects. While being toxic to marine life, polycyclic aromatic hydrocarbons (PAHs), found in crude oil, are very difficult to clean up and last for years in the sediment and marine environment (Panetta 2003). Discharge of cargo residues from bulk carriers can pollute ports, waterways, and oceans. In many instances, ships intentionally discharge illegal wastes despite foreign and domestic regulation prohibiting such actions. An absence of national standards provides an incentive for some cruise liners to dump waste in places where the penalties are inadequate (Schulkin 2002). It has been estimated that container ships lose over 10,000 containers at sea each year usually during storms (Podsadam 2001). Ships also create noise pollution that disturbs natural wildlife, and water from ballast tanks can spread harmful algae and other invasive species. Ballast water taken up at sea and released in port is a major source of unwanted exotic marine life. The invasive freshwater zebra mussels (*Dreissena polymorpha*), native to the Black, Caspian, and Azov seas in Europe, were probably transported to the Great Lakes via ballast water from a transoceanic vessel. Invasive species can take over once occupied areas which can facilitate the spread of new diseases, introduce new genetic material, alter underwater seascapes, and jeopardise the ability of native species to obtain food. Invasive species are responsible for about $US138 billion annually in lost revenue and management costs in the United States of America alone (Pimentel et al. 2005).

The fourth pathway of marine pollution is atmospheric pollution. This form of pollution occurs when windblown dust and debris, including plastic bags, are blown seaward from landfills and other areas. For example, dust from the Sahara moving around the southern periphery of the subtropical ridge moves into the Caribbean and Florida during the warm season as the ridge builds and moves northward through the subtropical Atlantic. Dust can also be attributed to global transport from the Gobi and Taklamakan deserts in China across Korea, Japan, and the Northern Pacific to the Hawaiian Islands (Duce 1980). Since 1970, dust outbreaks have worsened due to periods of drought in Africa. However, the flux is greater during positive phases of the North Atlantic Oscillation (Prospero & Nees 1986). Climate change is raising ocean temperatures and raising levels of carbon dioxide (CO_2) in the atmosphere. These rising levels of carbon dioxide are acidifying the oceans (Doney 2006). This, in turn, is altering aquatic ecosystems and modifying fish distributions, with impacts on the sustainability of fisheries and the livelihoods of the communities that depend on them. Healthy ocean ecosystems are also important for the mitigation of climate change.

Another example of atmospheric pollution affection marine ecosystems is smelting. Smelting is a process of applying heat and a chemical reducing agent to an ore to extract a desired base metal product. In Port Pirie, South Australia the upper Spencer Gulf has the world's largest single stream Pb-Zn smelter (Lafratta et al. 2019). This smelter has caused environmental and health issues related to elevated metal concentrations in the surrounding environment. In addition the region has a *Posidonia australis* seagrass meadows, occupying an area greater than 4000 km^2. There was a 9 fold increase of Pb, Zn and Cd concentrations following the beginning of smelter operations in the 1880s, and the stable Pb isotopic signatures confirmed the smelter has been the main source of lead pollution in the seagrass soils until present (Lafratta et al. 2019). Preliminary estimates suggest that over the past 15 years seagrass meadows within 70km^2 of the smelter bioaccumulated about 7–15% of the smelter emissions in their soils (Lafratta et al. 2019).

The final pathway of marine pollution is deep sea mining. Deep sea mining is a relatively new mineral retrieval process that takes place on the ocean floor. Ocean mining sites are usually around large areas of polymetallic nodules (Manganese) or active and extinct hydrothermal vents at about 1400 to 3700 meters below the ocean's

surface (Ahnert & Borowski 2000). The vents create sulphide deposits, which contain precious metals and high value such as silver (Ag), gold (Au), copper (Cu), cobalt (Co), and zinc (Zn) (Halfar & Fujita 2007; Glasby 2000). The deposits are mined using either hydraulic pumps or bucket systems that take ore to the surface to be processed. As with all mining operations, deep sea mining raises questions about environmental damages to the surrounding areas. Because deep sea mining is a relatively new field, the complete consequences of full-scale mining operations are unknown. However, experts are certain that removal of parts of the sea floor will result in disturbances to the benthic layer, increased toxicity of the water column, and sediment plumes from tailings (Halfar & Fujita 2007). Removing parts of the sea floor disturbs the habitat of benthic organisms, possibly, depending on the type of mining and location, causing permanent disturbances (Ahnert & Borowski 2000). Aside from direct impact of mining the area, leakage, spills, and corrosion would alter the mining area's chemical makeup. Among the impacts of deep sea mining, sediment plumes could have the greatest impact. Plumes are caused when the tailings from mining (usually fine particles) are resuspended back into the ocean, creating a cloud of particles floating in the water. Two types of plumes occur: near bottom plumes and surface plumes (Ahnert & Borowski 2000). Near-bottom plumes occur when the tailings are pumped back down to the mining site. The floating particles increase the turbidity, or cloudiness, of the water, clogging filter-feeding apparatuses used by benthic organisms (Sharma 2005). Surface plumes cause a more serious problem. Depending on the size of the particles and water currents, the plumes could spread over vast areas (Ahnert & Borowski 2000; Nath & Sharma 2000). The plumes could affect zooplankton and light penetration, in turn affecting the food web of the area (Ahnert & Borowski 2000; Nath & Sharma 2000). Another example of residual metal contamination caused by the dumping of mine tailings is in Portman Bay Southeastern Spain during the 20th century. Concentrations of Hg, Cd, Pb, Cu, Zn, and As in wild mussels (*Mytilus galloprovincialis*) were found during the period 1991–2005. The results show significant downward trends in the concentrations of Hg, Cu, Pb, and Zn (Benedicto et al. 2008). Nevertheless, trace metal concentrations in mussels from Portman in 2005 was higher than the reference concentrations established for the coast of Murcia and the Spanish Mediterranean littoral, the only exceptions being Cu and As. Red mullets (*Mullus barbatus*) caught at Portman in 1990 and 2004 presented higher levels of Hg, Cd, and Pb, in comparison with other areas of the south and southeastern coast of

Spain. The results indicate that Portman Bay is still one of the areas most heavily contaminated by Pb and Cd along the Mediterranean coast of Spain 15 years after the cessation of mining activities. Also in Spain the Tinto river drains into the Rio Tinto mining district, which comprises the world's largest known massive sulphide deposits; these orebodies have been mined from the third millennium BC to the present. The Tinto river is strongly acidic with a pH, 1.5–2.5 when it floods events, it transports a sandy material, including abundant detrital pyrite grains (Leblanc 2000). The metal association of Pb, Ba, As, Cu, Zn, Sn, Tl, Cd, Ag, Hg, Au is typical of that of the Rio Tinto pyritic ore.

According to the New South Wales EPA, a pollution event is 'an incident or set of circumstances during or as a consequence of which there is or is likely to be a leak, spill or other escape or deposit of a substance, as a result of which pollution has occurred, is occurring or is likely to occur'. It includes an incident or set of circumstances in which a substance has been placed or disposed of on premises, but it does not include an incident or set of circumstances involving only the emission of any noise (NSWEPA 2018). An example of this is the dumping of waste products that contain heavy metals.

1.3: Heavy metal pollution

Many types of pollution affect marine ecosystems. These also include ocean acidification, eutrophication, plastic debris including microplastics, toxins and underwater noise. One specific type of pollution is so-called 'heavy metals'. Heavy metals are metallic chemical elements that have a relatively high density (greater than 5g/cm^3) and are toxic or poisonous at low concentrations (Ali et al. 2019). Examples are mercury (Hg), lead (Pb), nickel (Ni), arsenic (As), and cadmium (Cd) (Rahman & Singh 2019). Such toxins can accumulate in the tissues of many species of aquatic life in a process called bioaccumulation. They are also known to accumulate in benthic environments, such as estuaries and bay muds. There are several examples of heavy metals affecting marine ecosystems. Due to their high position in the food chain and the subsequent accumulation of heavy metals from their diet, mercury levels can be high in tuna species such as the Pacific Bluefin tuna (*Thunnus orientalis*) and Albacore tuna (*Thunnus alalunga*). As a result, in March 2004 the United States Food and Drug

Administration (USFDA) issued guidelines recommending that pregnant women, nursing mothers and children limit their intake of tuna and other types of predatory fish (USFDA 2021). Some shellfish and crabs can survive polluted environments, accumulating heavy metals or toxins in their tissues. For example, Chinese mitten crabs (*Eriocheir sinensis*) have a remarkable ability to survive in highly modified aquatic habitats, including polluted waters (Gollasch 2009). The farming and harvesting of such species needs careful management if they are to be used as a food source (Hui et al. 2005; Silvestre et al. 2004).

Heavy metal pollution is contamination by any relatively dense metal or metalloid that is noted for its potential toxicity (Pourret & Hursthouse 2019), especially in environmental contexts (Zhang et al. 2019; Srivastava & Goyal 2010). The term has particular application to cadmium (Cd), mercury (Hg) and lead (Pb) (Brathwaite & Rabone 1985), all of which appear in the World Health Organization's list of 10 chemicals of major public concern (WHO 2020). Other examples include manganese (Mn), chromium (Cr), cobalt (Co), nickel (Ni), copper (Cu), zinc (Zn), silver (Ag), antimony (Sb) and thallium (Tl). Heavy metals are found naturally in the earth. They become concentrated because of human activities and can enter plant, animal, and human tissues via inhalation, diet, and manual handling. Subsequently, they can bind to and interfere with the functioning of vital cellular components such as structural proteins, enzymes, and nucleic acids, and interfere with their functioning (Landis 2017). Symptoms and effects can vary according to the metal or metal compound, and the dose involved. Long term exposure to heavy metals can have carcinogenic effects, affecting the central and peripheral nervous systems and circulatory systems. The toxic effects of arsenic (As), mercury (Hg), and lead (Pb) have been known to ancient cultures, but methodical studies of the toxicity of certain heavy metals did not appear until around 1868 (Wanklyn et al. 1868). In humans, heavy metal poisoning is generally treated by the administration of chelating agents. Some elements otherwise regarded as toxic heavy metals are essential, in small quantities, for human health (Pourret 2018). These essential elements include vanadium (V), manganese (Mn), iron (Fe), cobalt (Co), copper (Cu), zinc (Zn), selenium (Se), strontium (Sr) and molybdenum (Mo) (Bánfalvi 2011).

Heavy metals are found naturally in the earth, and become concentrated because of human activities, or, in some cases geochemical processes, such as accumulation in peat soils that are then released when drained for agriculture (Qureshi et al. 2003). Common sources of heavy metal pollution are mining, industrial wastes, vehicle emissions, lead acid batteries, fertilisers, paints, treated woods, aging water supply infrastructure (Harvey et al. 2015) and microplastics floating in the world's oceans (Cole et al. 2011).

Arsenic (As), cadmium (Cd) and lead (Pb) may be present in children's toys at levels that exceed regulatory standards. Pb can be used in toys as a stabiliser, colour enhancer, or anti-corrosive agent. Cd is sometimes employed as a plastic stabiliser, or to increase the mass and lustre of jewellery. As is used to be in with colouring dyes such as blue, yellow, and magenta dyes. Furthermore, arsenic was not the only hazardous element used in 19th century fabrics. (Finch et al. 2015). Regular drinkers of illegally distilled alcohol may be exposed to arsenic or Pb poisoning the source of which is As-contaminated Pb used to solder the distilling apparatus. Rat poison used in grain and mash stores may be another source of the arsenic. Lead is the most prevalent heavy metal contaminant (Di Maio 2001). In the form of tetraethyl lead, $(CH_3CH_2)_4Pb$, this compound was used extensively in petrol during the 1930s to the 1970s (Lovei 1998). Pb levels in the aquatic environments of industrialized societies have been estimated to be two to three times those of pre-industrial levels (Perry & Vanderklein 1996). Although the use of leaded petrol was largely phased out in Australia by 1 January 2002, soils next to roads built before this time still retain high lead concentrations (Houlton 2014).

Heavy metals enter plant, animal and human tissues via air inhalation, diet, and manual handling. Vehicle emissions are a major source of airborne contaminants including arsenic (As), cadmium (Cd), cobalt (Co), nickel (Ni), lead (Pb), antimony (Sb), vanadium (V), zinc (Zn), platinum (Pt), palladium (Pd) and rhodium (Rh) (Balasubramanian et al. 2009). Water sources (groundwater, lakes, streams and rivers) can be polluted by heavy metals leaching from industrial and consumer waste; acid rain can exacerbate this process by releasing heavy metals trapped in soils (Worsztynowicz & Mill 1995). Transport through soils can be facilitated by the presence of preferential flow paths such as soil particles greater than 0.08mm in

diameter (macropores) and dissolved organic compounds (Camobreco 1996). Plants are exposed to heavy metals through the uptake of water, which in are consumed by turn animals. Ingestion of plant and animal-based foods are the largest sources of heavy metals in humans (Radojevic & Bashkin 1999). Absorption through skin contact, for example from contact with soil, or metal containing toys and jewellery (Guney & Zagury 2014) is another potential source of heavy metal contamination (Qu et al. 2014). Heavy metals can bioaccumulate in organisms, as they are hard to metabolise (Pezzarossa et al. 2011).

In a global review of heavy metal pollution in coastal wetlands over the past three decades by Li et al. 2022 it was found that mercury (Hg), cadmium (Cd), and copper (Cu) were the most widely studied heavy metal elements globally, but patterns differed geographically, with Hg being most widely examined in the Americas, Cd in China and India, and lead (Pb) in the western Europe and Australia. Among different types of coastal wetlands, salt marshes, mangrove forests, and estuaries were the most widely studied, in contrast to seagrass beds and tidal flats. As for ecosystem components, soils/sediments and plants were most extensively investigated, while algae, microbes, and animals were much less examined.

In Victoria, Australia, sources of heavy metal pollution can include former mining operations, which has led to increase levels of mercury in the Lerderderg and Goulburn Rivers (EPA Victoria 2017). In addition, there are additional sources of heavy metal pollution in Western Port Bay, which includes several industrial complexes, including the BlueScope steel processing works and the major Royal Australian Navy training base, HMAS Cerberus. In addition, Holden Australia's proving ground is located just east of Western Port Bay at Lang Lang where Holden Cars are tested for safety. Finally Westernport Oil Refinery was operated by BP at Crib Point from 1966 to 1985.

Another example of a Victorian heavy metal pollution source is the legacy gold mining in Maldon, Victoria, Australia (Abraham 2018). This site is a source of the heavy metals Mn, Zn, As, Cr, Cu, Pb, Ni, Co, Hg and Cd. In an environment of climate fluctuation, with increased storm events and forest fires, such heavy metals contaminants maybe mobilised and pose a real threat to the environment and the community.

In humans, heavy metal poisoning is generally treated by the administration of chelating agents. These are chemical compounds, such as calcium disodium ethylenediaminetetraacetate ($CaNa_2$ EDTA) that convert heavy metals to chemically inert forms that can be excreted without further interaction with the body. Chelates are not without side effects and can also remove beneficial metals from the body. Vitamin and mineral supplements are sometimes co-administered for this reason (Blann & Ahmed 2014).

Soils contaminated by heavy metals can be remediated by isolation, immobilisation, toxicity reduction, physical separation, or extraction. Isolation involves the use of caps, membranes or below-ground barriers in an attempt to quarantine the contaminated soil. Immobilisation aims to alter the properties of the soil to hinder the mobility of the heavy metal contaminants. Toxicity reduction attempts to oxidise or reduce the heavy metal ions, via chemical or biological means into less toxic or mobile forms. Physical separation involves the removal of the contaminated soil and the separation of the metal contaminants by mechanical means. Extraction is an on or offsite process that uses chemicals, high temperature, volatilisation or electrolysis to extract contaminants from soils. The process or processes used will vary according to contaminant and the characteristics of the site (Evanko & Dzombak 1997). A final method where soils contaminated by heavy metals can also be bioremediated by plants. Bioremediation is the process wherein a biological system (typically bacteria, microalgae, fungi, and plants also known as phytoremediation), living or dead, is employed for removing environmental pollutants from the air, water, soil, flue/exhale gasses or industrial effluents, in natural or artificial settings (Yuvraj 2022). In the case of phytoremediation is defined as the use of green plants and the associated microorganisms, along with proper soil amendments and agronomic techniques to either contain, remove or render toxic environmental contaminants harmless (Das et al. 2018).

In Victoria**,** heavy metal testing in marine waters has been done mainly by the EPA Victoria. In a 2010 study, this testing took place from March 2008 and February 2009. The EPA does this by sampling 36 sites around Port Phillip Bay, with collection occurring once a week on a Monday morning (EPA Victoria 2010). Heavy metal pollution surface water samples are collected using a 125 mL plastic bottle. These

sample bottles for trace metals are not rinsed before sample collection. At each beach, samples are collected at the same approximate location each time, at wading depth, 30 cm below the surface. At each beach, a range of on-site measurements was also taken, and observations were recorded. The EPA also measured water temperature, conductivity, turbidity, light intensity and wind speed and recorded them on a field sheet, along with general conditions at the time of sampling.

For the laboratory analysis, unfiltered heavy metal samples were analysed using the Total Metals in Saline Water Suite A using Inductively Coupled Plasma Mass Spectrometry (ORC-ICP-MS). While mercury was analysed using the Total Mercury by the Flow Injection Mercury System (FIMS) method. The preparation method for heavy metal analysis is Digestion for Total Recoverable Metals — ORC (Method 3005, APHA 2005). These results were recorded in µg/l.

However, there are shortcomings to using water to track heavy metals in the environment. The shortcomings of detecting heavy metals directly from water include measuring low concentrations accurately, getting representative or “average” samples when heavy metal concentrations fluctuate over time, and isolating the bioavailable fraction of the contaminant from unavailable forms (Roditi et al. 2000). An alternative method for assessing heavy metals in the environment, especially in marine ecosystems, is via biomonitoring using plant bioindicators such as seagrass, which bioaccumulate heavy metals overtime.

1.4: Bioaccumulation

Bioaccumulation is the gradual accumulation of chemicals, such as pesticides or heavy metals, in an organism (Alexander 1999). Bioaccumulation occurs when an organism absorbs a chemical at a rate faster than that at which the chemical is lost or eliminated by catabolism and excretion. Thus, the longer the biological half-life of a toxic chemical, the greater the risk of chronic poisoning, even if environmental levels of the toxin are not very high. (Bryan et al. 1979). Bioaccumulation refers to uptake from all sources combined (e.g., water, food, air, etc.) while bioconcentration refers to uptake and accumulation of a substance from water alone.

Examples of bioaccumulation in marine ecosystems are coastal fish such as the smooth toadfish (*Tetractenos glaber*) and seabirds such as the Atlantic puffin (*Fratercula arctica*) are often monitored for heavy metal bioaccumulation. Methylmercury gets into freshwater systems through industrial emissions and rain. As its concentration increases up the food web, it can reach dangerous levels for both fish and the people who rely on fish as a food source (IISD Experimental Lakes Area 2017). Naturally, produced toxins can also bioaccumulate. The marine algal blooms known as "red tides" can result in local filter-feeding organisms such as mussels and oysters becoming toxic; coral reef fish can be responsible for the poisoning known as ciguatera when they accumulate a toxin called ciguatoxin from red algae. In some eutrophic aquatic systems, biodilution can occur. This trend is a decrease in a contaminant with an increase in trophic level and is due to higher concentrations of algae and bacteria to "dilute" the concentration of the pollutant. Wetland acidification can raise the chemical or metal concentrations which lead to an increased bioavailability in marine plants and freshwater biota. Plants situated there which includes both rooted and submerged plants can be influenced by the bioavailability of metals (Albers and Camardese 1993). This is especially true for seagrass as they have high resistance to high levels of pollution make them ideal sentinels, together with their bioaccumulation capacity. All of these examples need to be monitored to prevent deleterious effects in the environment.

1.5: Aquatic biomonitoring

Aquatic biomonitoring is the science of inferring the ecological condition of aquatic ecosystems by examining the organisms that live there (Vandewalle et al. 2010). Biomonitoring typically takes three different approaches/tools. The first is using bioassays, the second community assessments and the third online biomonitoring devices. Bioassays are test organisms that are exposed to an environment and their response is measured. Typical organisms used in bioassays are fish, water fleas (Daphnia), and frogs. Community assessment, also called biosurveys, is where an entire community of organisms is sampled to see what types of taxa remain. In aquatic ecosystems, these assessments often focus on invertebrates, algae, macrophytes (aquatic plants), fish, or amphibians (Karr 1981). While online biomonitoring devices or toximeter, using the ability of animals to permanently taste their environment. This

operates on real time basis and consists of a living organism with a sensitive behavioural or physiological response to chemical stressors as the "sensor", a quantitative recording unit of this response and a PC unit with specialized software to detect behavioural differences and to interpreted and classify them as water quality alarm. Different types of animals are used for this purpose either in the lab or in the field. The study of the opening and closing activity of clams' valves is an example of one possible way to monitor in-situ the quality of fresh and coastal waters (MolluSCAN Eye 2023). Aquatic invertebrates have the longest history of use in biomonitoring programs (Barbour et al. 1999). In typical unpolluted temperate streams of Europe and North America, certain insect taxa predominate. Mayflies (Ephemeroptera), caddisflies (Trichoptera), and stoneflies (Plecoptera) are the most common insects in these undisturbed streams (Lawrence et al. 2010). In contrast, in rivers disturbed by urbanization, agriculture, forestry, and other disturbances, flies (Diptera) especially midges (family Chironomidae), predominate (Lawrence et al. 2010). Aquatic invertebrates are also responsive to climate change (Lawrence et al. 2010; Filipe et al. 2013).

Aquatic biomonitoring is important in assessing marine life forms and their ecosystems. Monitoring aquatic life, from which life on land evolved, can also be beneficial in understanding land ecosystems (Maine DEP 2019). Aquatic biomonitoring can reveal the overall health and status of the environment, can detect environmental trends and how different stressors will affect those trends, and can interpret the effect that various environmental activities will have on the overall health of the environment. Pollution and general stresses to aquatic life can have a major impact on the environment. The main sources of pollution to oceans, rivers, and lakes are sewage, oil spills, land runoff, littering, ocean mining, and nuclear waste. Pollution greatly upsets marine life and can endanger species that live in or close to water. Because many aquatic animals serve as a main food source for many land animals, when aquatic species are affected, it causes a ripple effect in land species. Biomonitoring can help mitigate such problems through monitoring all forms of life and conditions in different bodies of water, both in fresh and marine water. A challenge in aquatic biomonitoring is to simplify data and make data easier for all to understand, especially investigators in the health and environmental fields.

A challenge in aquatic biomonitoring is to simplify data and make data easier for all to understand, especially investigators in the health and environment fields.

Water quality is tested on its appearance and chemical content (New York State Department of Environmental Conservation 2021). Thus, parameters that are tested include turbidity, pH, conductivity, algal cell count and what chemicals are in the sample. Algal cell counts is a biological assessment to assess water quality. Changes in these factors can impact the overall aquatic environment and can severely affect aquatic life. Indeed, some contaminants, such as metals and certain organic wastes, can be lethal to individual creatures and could ultimately lead to the extinction of certain species (Bartram & Balance 1996). This could affect both aquatic and land ecosystems and cause disruption in other biomes and ecosystems. Currently, there are three methods employed in aquatic biomonitoring: monitoring and assessing aquatic species and ecosystems, monitoring the behaviour of certain aquatic species and assessing any changes in species behaviour and looking at contaminants in the water and their effect on marine life (Bartram & Balance 1996). In this PhD study, the first method will be employed, namely using a plant based bioindicator seagrass.

1.6: Plant bioindicators

A biological indicator or bioindicator is any species (i.e., indicator species) or group of species whose function, population, or status can reveal the qualitative status and/or quantitative of its environment (Siddig et al. 2016). For example, copepods and other small water crustaceans that are present in many water bodies can be monitored for changes (chemical/biochemical, physiological, behavioural) that may indicate a problem within their ecosystem. Bioindicators can tell us about the cumulative effects of different pollutants in the ecosystem and about how long a problem may have been present, which immediate and chemical testing cannot (Karr 1981).

A good bioindicators should have the following traits:

1. Provides a measurable response to expose, without perishing during exposure.
2. Its response reflects the whole population/community/ecosystem response.
3. Its response is proportional to the severity of contamination.

4. The species is abundant and widely distributed, and stable despite moderate climate and environmental variability.
5. Its lifespan is long enough to compare different life stages.
6. It is easy and inexpensive to sample and survey.
7. It has a relevant role on the ecosystem (food chain, public interest) (Carignan and Villard, 2002).

Seagrass is a good bioindicator as its provides a measurable response to expose, without perishing during exposure. It response to an environmental stressor is reflective of the whole population/community/ecosystem. The seagrass is abundant and widely distributed throughout the world and has stable growth despite moderate climate and environmental variability. Finally it has a relevant role on the ecosystem (Govers et al. 2014).

A biological monitor or biomonitor is an organism that provides quantitative information on the quality of the environment around it on a temporal basis (NCSU Water Quality Group 2016). Therefore, a good biomonitor will indicate the relative level of a pollutant and can be used in an attempt to provide additional information about the amount and intensity of the exposure. These organisms (or communities of organisms) can be used to deliver information on alterations in the environment or the quantity of environmental pollutants by changing in one of the following ways, which include physiologically, chemically, or behaviourally. The information can be deduced through the study of: (Fleishman et al. 2005)

1. The content of certain elements or compounds
2. Their morphological or cellular structure
3. Metabolic biochemical processes
4. Behaviour
5. Population structures

The importance/relevance of biomonitors, rather than people made measuring equipment, is justified by the observation that the best indicator of the status of a species or system is itself (Tingey 1989). Bioindicators can reveal indirect biotic effects of pollutants where as many physical or chemical measurements cannot. Through

bioindicators, scientists need to observe only the single indicating species to check on the environment rather than monitor the whole community. The use of a biomonitor is described as biological monitoring and is the use of the properties of an organism to obtain information on certain aspects of the biosphere. Biomonitoring of air pollutants can be passive or active. Scientists use passive methods to observe plants growing naturally within the area of interest. Active methods are used to detect the presence of air pollutants by placing test plants of known response and genotype into the study area. The use of a biomonitor is described as biological monitoring. This refers to the measurement of specific properties of an organism to obtain information on the surrounding physical and chemical environment (USEPA 2016). Bioaccumulative indicators are frequently regarded as biomonitors. Depending on the organism selected and their use, there are several types of bioindicators.

The presence or absence of certain plant or other vegetative life in an ecosystem can provide important clues about the health of the environment (Diekmann 2023). There are several types of plant biomonitors, including mosses, lichens, tree bark, bark pockets, tree rings, and leaves. Fungi too may be useful as indicators. Lichens are organisms comprising both fungi and algae. They are found on rocks and tree trunks, and they respond to environmental changes in forests, including changes in forest structure, air quality, and climate. The disappearance of lichens in a forest may indicate environmental stresses, such as high levels of sulphur dioxide (SO_2), sulphur-based pollutants, and nitrogen oxides. The composition and total biomass of algal species in aquatic systems serve as an important metric for organic water pollution and nutrient loading such as nitrogen (N) and phosphorus (P). There are genetically engineered organisms that can respond to toxicity levels in the environment. For example, there is a type of genetically engineered Thale cress (*Arabidopsis thaliana*) a model organism that turns red in the presence of Nitrogen dioxide (NO_2) in the soil (Halper 2006).

Research into seagrass as a bioindicator for heavy metal pollution originated with Schroeder & Thorhaug (1980) and has continued to the present day (Zhang et al. 2021) in an upward trend **(Figure 1.1)**. Most of these studies have been based in the Mediterranean Sea and used Neptune grass (*Posidonia oceanica*) as the bioindicator. In Victoria, Australia, at the time of writing, there are no known published studies on

this topic. However, there are studies in Western Australia (Fraser & Kendrick 2017, Serrano et al. 2020), South Australia (Ward & Hutching 1996), New South Wales (Barwick and Maher 2003, Birch et al. 2018 A and B, Schneider et al. 2018) and Tasmania (Farias et al. 2018).

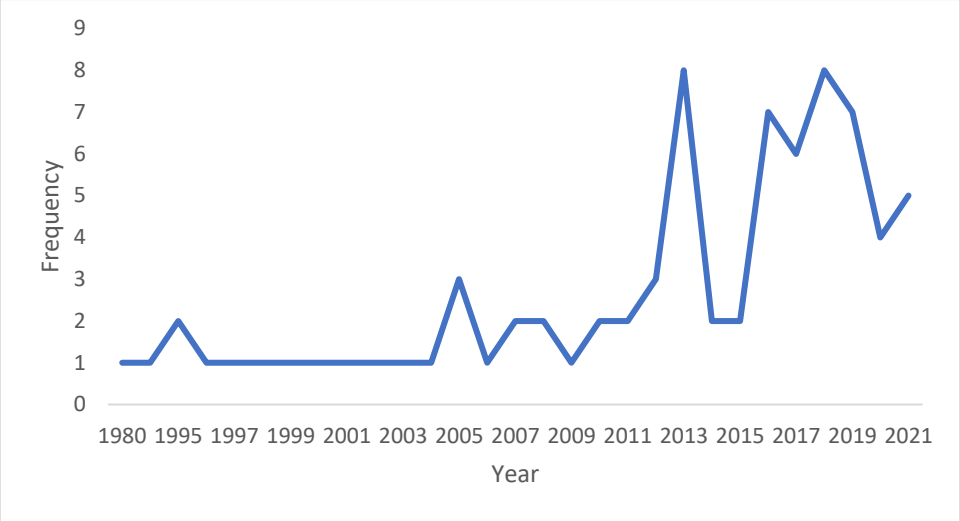


Figure 1.1: Number of articles published in the peer reviewed scientific literature from 1980 to the present, on heavy metal accumulation in seagrass. This Figure was produced by doing a frequency count of how many studies in a year a study was published.

The use of plants and other organisms as bioindicators is well recognised and utilised in fields as diverse as disease detection, ecosystem health and the monitoring and measurement of a range of pollutants, particularly heavy metals. This information could include the detection of anthropogenic stressors that may not be immediately detectable by other methods such as episodic contamination or pollution. More importantly, the information gleaned from bioindicators could quantify the adverse impacts that the stressors are having on the environment (Chang et al. 2009). Indeed, there are many advantages to using bioindicators. For example, the use of bioindicators to measure the response of organisms or communities to a stressor under natural conditions where biotic and abiotic factors are integrated. This reduces the need to make assumptions regarding the bioavailability of a pollutant (Chaphekar 1991). In addition, the use of bioindicators could help to identify effects caused by anthropogenic stressors versus those caused by natural forces (Mhatre 1991). Plants used as bioindicators can also provide historical information regarding past

environmental conditions (Fränzle 2006). The use of plant bioindicators can provide a cost-effective approach for monitoring long-term impacts when compared to water and sediment analysis that requires periodic analysis, since plants accumulate pollutants over time.

A program to update and redefine strategic priorities in seagrass research was considered at the Australian Marine Sciences Association (AMSA) Conference, July 2015, Geelong, Victoria. Here, a knowledge gap was identified as being related to bioindicators. Namely, discussions revolved around which species are suitable as early warning bioindicators of loss of richness and diversity of an ecosystem and whether these species should be incorporated into existing monitoring programs (York et al. 2017). Whilst specific bioindicators were not identified at the time, it was suggested that the use of 'early warning' bioindicators at appropriate temporal (time) and spatial (space) scales should be incorporated into existing monitoring programs. Furthermore, it was determined that bioindicators should be incorporated via a pre-determined set of criteria or thresholds for pollutants such as heavy metals which, if exceeded, are capable of triggering a management response (McMahon et al. 2013). This research project will be using seagrass meadows to test for heavy metal pollution.

1.7: Seagrass meadows

Seagrasses are angiosperms or flowering plants not to be confused with seaweed, which do not form flowers, fruits, and seeds to reproduce – although both grow in marine environments. There are about 70 species of seagrass known to science belonging to four families of the order Alismatales - a class of monocotyledons (Tomlinson & Vargo 1966).

Seagrasses evolved from terrestrial plants, which recolonised marine environments 70 to 100 million years ago. The name seagrass stems from the many species with long and narrow leaves, which grow by rhizome extension and often spread across large "meadows" resembling grasslands; many species superficially resemble terrestrial grasses of the family Poaceae. Like all autotrophic plants, seagrasses photosynthesize, in the submerged photic zone, and most occur in shallow and sheltered coastal waters anchored in sandy or muddy bottoms (Hogarth 2015).

Most species undergo submarine pollination and complete their life cycle underwater. Seagrass meadows can be either monospecific (made up of a single species) or in mixed beds. In temperate areas, usually one or a few species dominate (like the Common Eelgrass (*Zostera marina*) in the North Atlantic), whereas tropical meadows are usually more diverse, with up to thirteen species recorded in the Philippines.

Seagrasses are found all over the world, in both tropical and temperate locations. Seagrasses live in shallow seas on the continental shelf of all continents except Antarctica. It is believed that seagrasses cover 125,000 km^2 around the world, but other estimates suggest that this number might be a lot higher with suggestions that seagrasses may cover up to 600,000 km^2 of the world's oceans (Cullen-Unsworth et al. 2018).

Seagrass meadows are diverse and productive ecosystems can harbor hundreds of associated species from all phyla, for example, juvenile and adult fish, epiphytic and free-living macroalgae and microalgae, molluscs, bristleworms, and nematodes. Few species were originally considered to feed directly on seagrass leaves (partly because of their low nutritional content), but scientific reviews and improved working methods have shown that seagrass herbivory is an important link in the food chain, feeding hundreds of species, including green turtles (*Chelonia mydas*), dugongs (*Dugong dugon*), manatees, fish (cod, flatfish and seahorses) geese, swans, sea urchins and crabs. Some fish species that visit and/or feed on seagrass raise their young in adjacent mangroves or coral reefs (Hogarth 2015). Seagrass traps sediment and slows down water movement, causing suspended sediments to settle out. Trapping sediment benefits coral by reducing sediment loads, improving photosynthesis for both coral and seagrass (Statton et al. 2017).

Seagrass meadows form in maximum depths of up to 60 meters, depending on water quality and light availability, and can include up to 12 different species in one meadow (Duarte 2011). These seagrass meadows are highly productive habitats that provide many ecosystem services, including sediment stabilisation, habitat and biodiversity, better water quality, and carbon and nutrient sequestration (Greiner 2013).

Seagrass are considered ecosystem engineers (Jones et al. 1994; Papenbrock 2012; Orth et al. 2006). This means that seagrass can alter the ecosystem around them. This adjusting occurs in both physical and chemical forms. Many seagrass species produce an extensive underground network of roots and rhizomes, which stabilise sediment and reduces coastal erosion (William & Moffler 1987). This system also assists in oxygenating the sediment, providing a hospitable environment for sediment-dwelling organisms (Jones et al. 1994). Seagrass also enhances water quality by stabilising heavy metals, pollutants, and excess nutrients (Darnell & Dunton 2016; Papenbrock 2012; Orth et al. 2006). The long blades of seagrasses slow the movement of water, which reduces wave energy and offers further protection against coastal erosion and storm surges. Furthermore, because seagrass are underwater plants, they produce significant amounts of oxygen, which oxygenate the water column. These meadows account for more than 10% of the ocean's total carbon storage.

Per hectare, it holds twice as much carbon dioxide as rain forests and can sequester about 27.4 million tons of CO_2 annually (Macreadie et al. 2013). The storage of carbon is an essential ecosystem service as society moves into a period of elevated atmospheric carbon levels. Seagrass meadows also provide physical habitat in areas that would otherwise be bare of any vegetation. Due to these three dimensional structures in the water column, many species occupy seagrass habitats for shelter and foraging.

It is estimated that 17 species of coral reef fish spend their entire juvenile life stage solely in seagrass meadows (Nagelkerken et al. 2002). These habitats also act as nursery grounds for commercially and recreationally valued fishery species, including the gag grouper (*Mycteroperca microlepis*), red drum (*Sciaenops ocellatus*), common snook (*Centropomus undecimalis*) and many other species (Nordlund et al. 2018; Unsworth et al. 2019A). Some fish species utilise seagrass meadows and various stages of the life cycle. Furthermore, many commercially important invertebrates also reside in seagrass habitats including bay scallops (*Argopecten irradians*), horseshoe crabs, and shrimp. Charismatic fauna can also be seen visiting these seagrass habitats. These species include West Indian manatees (*Trichechus manatus*), green sea turtles, and various species of sharks. The high diversity of marine organisms that

can be found on seagrass habitats promotes them as a tourist attraction and a significant source of income for many coastal economies along the Gulf of Mexico and in the Caribbean.

Natural disturbances, such as grazing, storms, ice scouring and desiccation, are an inherent part of seagrass ecosystem dynamics. Seagrasses display a high degree of phenotypic plasticity (changes in an organism's behaviour, morphology and physiology in response to a unique environment), adapting rapidly to changing environmental conditions.

Seagrasses are in global decline, with some 30,000 km^2 having been lost during recent decades (Waycott 2009). The main cause is human disturbance, most notably eutrophication, mechanical destruction of habitat and overfishing. Excessive inputs of nutrients such as nitrogen (N) and phosphorus (P) is directly toxic to seagrass, and more importantly, stimulates the growth of epiphytic and free-floating macro and microalgae (Laffoley & Baxter 2019). This blocks out sunlight, reducing the photosynthesis that nourishes the seagrass and its primary production. Decaying seagrass leaves and algae bodies fuel increasing algal blooms resulting in a positive feedback loop. This can cause a complete regime shift from seagrass to algal dominance. Accumulating evidence also suggests that overfishing of apex predators could indirectly increase algal growth by reducing grazing control performed by mesograzers, such as crustaceans and gastropods, through a trophic cascade (McGlathery 2001).

Macroalgal blooms cause the decline and eradication of seagrasses. Known as nuisance species, macroalgae grow in filamentous and sheet like forms and forms thick unattached mats over seagrass, occurring as epiphytes on seagrass leaves. Eutrophication leads to the forming of a bloom, causing the attenuation of light in the water column, which eventually leads to anoxic conditions for the seagrass and organisms living in and around the plants. In addition to the direct blockage of light to the plant, benthic macroalgae have low carbon and nitrogen content, causing their decomposition to stimulate bacterial activity, leading to sediment resuspension, an increase in water turbidity and further light attenuation (Fox et al. 2010).

When people drive boats over shallow seagrass meadows, sometimes the propeller blade can damage the seagrass. In addition, fishing methods that rely on heavy nets that are dragged across the sea floor put this important ecosystem at serious risk (Fusi & Daffonchio 2019). The most used methods to protect and restore seagrass meadows include nutrient and pollution reduction, marine protected areas and restoration using seagrass transplanting. Seagrass is not seen as resilient to the impacts of future environmental change (Unsworth et al. 2015). In various locations, communities are attempting to restore seagrass meadows that were lost to human action, including in the US states of Virginia, Florida (FFWCC 2021), and Hawaii, as well as the United Kingdom (Unsworth et al. 2019B). Such reintroductions have been shown to improve ecosystem services (van Katwijk et al. 2015). The seagrass species that will be used in this PhD project is *Zostera muelleri*.

1.8: *Zostera muelleri* ecology

Zostera muelleri **(Figure 1.2)** is a southern hemisphere temperate species of seagrass native to the seacoasts of South Australia, Southern Queensland (Edgar 2019), New South Wales, Victoria, and Tasmania. Today, *Z. muelleri* can be found in regions of Australia, New Zealand, and Papua New Guinea (Short et al. 2010) as well as areas of the eastern Indian Ocean, and the southwest and western central Pacific Ocean. *Z. muelleri* is a marine angiosperm and is commonly referred to as eelgrass or garweed. It is a fast growing and readily colonising species that serves as a feeding ground for wading birds and aquatic animals, and a breeding ground for juvenile fish and shrimp species (Waycott et al. 2014) *Z. muelleri* is a perennial species, meaning populations of it endure year-round (Dos Santos & Matheson 2017). They are mostly found in places such as littoral or sublittoral sand flats, sheltered coastal embayments, soft, muddy, sandy areas near reefs, estuaries, shallow bays, and in intertidal shores. They are not common on reefs because there is little space and nutrients for them to grow there. *Z. muelleri* is a marine species, but it can tolerate some freshwater inputs. It mostly occurs in mono-specific meadows, but it can grow alongside *Ruppia* (widgeon grass), *Halophila* (Tapegrass), and *Lepilena*. This species has long strap-shaped leaves, rounded leaf tip and thin rhizomes that are less than 3mm in diameter (Edgar 2012). There are visible cross-veins in the leaf. The rhizomes are either dark brown or yellow. Young rhizomes are typically yellow, but the leaves of this plant can turn red if

they are exposed to high sunlight (Bull 2014). Threats to this species include coastal development, eutrophication, (Pernice et al. 2016) boat mooring, dredging, agricultural/urban runoff, and sedimentation. Meadows of *Z. muelleri* have been lost in areas of Port Phillip Bay and New Zealand due to habitat disturbance, sedimentation, and turbidity (Waycott et al. 2014). During the 1960s, a wasting disease affected meadows of *Z. muelleri* in New Zealand. Because it is less tolerant to heat than other tropical species, climate change may be a threat to meadows of this species in tropical regions. There are currently no conservation measures for this species as it is a Least-concern species by the IUCN (Short et al. 2010).

Figure 1.2*:* Photograph of seagrass species *Zostera muelleri* – Source http://www.marinelife.ac.nz/species/1187

1.9: Port Phillip Bay History and Ecology

Port Phillip Bay or Port Phillip is a 1,930km^2 saline, permanent, natural bay with an average depth of 8 meters on the central coastline of southern Victoria, Australia, and flows into the Bass Strait through a narrow channel known as the Rip. Most of the bay is navigable, although it is extremely shallow for its size with the deepest portion being 24 metres. The species of seagrass found in the bay are Eelgrass (*Zostera muelleri)*, *Zostera tasmanica*, *Zostera nigricaulis*, paddle weed (*Halophila australis*) and wire

weed (*Amphibolis antarctica*) (Edgar 2012). Warry and Hindell (2009) in their Review of Victorian Seagrass Research, identified climate change (as well as population pressures along the coast) as a significant threat to seagrass habitats. Seagrass habitat is important to the enhancement of key fish stocks in the Bay and maintaining the value of fishing. Seagrass meadows provide important nurseries for many fish species, including species fished commercially and recreationally. Analysis by Blandon and Ermgassen (2014) estimated that seagrass habitat enhances the stock of King George whiting (*Sillaginodes punctatus*) at a rate of 5 kilograms/hectare/year, which for commercial fishing has an economic value of $5.6 million per year. The value of other target species such as Australasian snapper (*Chrysophrys auratus*) is also enhanced by seagrass habitat. The bay is surrounded mostly by the metropolitan city of Melbourne in its main eastern portion north of the Mornington Peninsula, and the city of Geelong in the much smaller western portion north of the Bellarine Peninsula. Port Phillip Bay is surrounded by the settlements of Melbourne, Geelong, Frankston, Mornington, Queenscliff, and Sorrento. The Primary inflows are the Yarra River, Patterson River, Werribee River, Little River and Kororoit Creek. Before European settlement, the area around Port Phillip was divided between the territories of the Wathaurong (to the west), Wurundjeri (north) and Boonwurrung (south and east) Nations. Its waters and coast are home to seals, whales, dolphins, corals and many kinds of seabirds and migratory waders. The first Europeans to enter the bay were the crews of HMS Lady Nelson, commanded by John Murray and, ten weeks later, HMS Investigator commanded by Matthew Flinders, in 1802. Subsequent expeditions into the bay took place in 1803 to establish the first settlement in Victoria, near Sorrento, but was abandoned in 1804. Thirty years later, settlers from Tasmania returned to establish Melbourne, now the state's capital city, at the mouth of the Yarra River in 1835 and Geelong at Corio Bay in 1838. Today Port Phillip is the most densely populated catchment in Australia with an estimated 4.5 million people living around the bay; Melbourne's suburbs extend around much of the northern and eastern shorelines, and the city of Geelong sprawls around Corio Bay, in the bay's western arm (Marine and Coasts Vic Gov 2023).

The region has an oceanic climate with warm summers possessing occasional very hot days due to northerly winds and mild winters. Annual rainfall, which is evenly distributed throughout the year, shows considerable variation due to the Otway

Ranges to the southwest. The northwestern shore of the bay is the driest part of southern Victoria and almost approaches a semi-arid climate with a mean annual rainfall as low as 425 mm, whilst the eastern shores are less shielded by the Otways receives as much as 850 mm. Summer temperatures average around 25 °C during the day and 14 °C at night, but occasional northerly winds can push temperatures over 40 °C, whilst in winter a typical day will range from 6 °C to 14 °C (Beer 1996).

Port Phillip hosts many beaches, most of which are flat, shallow, and long, with very small breaks making swimming quite safe. This attracts many tourists, mostly families, to the beaches of Port Phillip during the summer months and school holidays. In addition, stand up paddle boarding, kite surfing and wind surfing are very popular. Most sandy beaches are located on the bay's northern, eastern and southern shorelines, while the western shorelines host a few sandy beaches; there mostly exists a greater variety of beaches, swampy wetlands and mangroves. The occasional pebble beach and rocky cliffs can also be found, mostly in the southern reaches. Major beaches include St. Kilda Beach, Brighton Beach, Sandringham Beach, and Dromana Beach. Port Phillip Bay is one of Victoria's most popular tourist destinations. Many residents of Melbourne take holidays on the shorelines of the bay, particularly the Bellarine and Mornington Peninsulas, most annually, camping in either tents, caravans, or villas in caravan parks, sharing rental houses or staying in holiday homes. Port Phillip is home to 36 yacht clubs. It also hosts the Melbourne to Hobart and Melbourne to Launceston Yacht Races. Port Phillip is also home to several marinas, including large marinas at St Kilda, Geelong and Brighton. For the 1956 Summer Olympics, it hosted the sailing events (LA84 Foundation 1958). There are many lifesaving clubs in Port Phillip, especially on the east coast from Altona to Frankston. These clubs provide volunteer lifesaving services and conduct sporting carnivals. Port Phillip is also known as a temperate water scuba diving destination. The shore dives from beaches and piers around the Bay providing a wide variety of experiences on day and night dives. Boat diving in Port Phillip provides access to a remarkable variety of diving environments including wrecks, reefs, drift dives, scallop dives, seal dives and wall dives. With three Marine Sanctuaries and easily accessible piers, Port Phillip is also popular for recreational snorkelling.

Jellyfish are a familiar sight in Port Phillip Bay, and its waters are home to species such as Australian fur seals (*Arctocephalus pusillus*), bottlenose dolphins, common dolphins, humpback whales (*Megaptera novaeangliae*), and southern right whales (*Eubalaena australis*). Many other cetacean species may also migrate off the areas. The smooth toadfish is one of the most common fish in muddy areas (Museum Victoria, 2006). The bay has many endemic species including the Eastern bluedevil fish (*Paraplesiops bleekeri*) and sponge walls on the Lonsdale wall in the heads of the bay. It also hosts breeding colonies of Australian fur seals. Occasionally, Australian sea lions (*Neophoca cinerea*), New Zealand fur seals (*Arctocephalus forsteri*), and subantarctic fur seals (*Arctocephalus tropicalis*) may come into the bay as well. Certain individual southern elephant seals (*Mirounga leonina*) may also frequent the bay. Swan Bay, adjacent to Queenscliff, is an important feeding ground for waterbirds and migratory waders. The Mud Islands, off Sorrento, are an important breeding habitat for white-faced storm petrels (*Pelagodroma marina*), silver gulls (*Chroicocephalus novaehollandiae*), Australian pelicans (*Pelecanus conspicillatus*) and Pacific gulls (*Larus pacificus*). Salt marshes in the northwestern sections of the bay, such as that in the Werribee Sewage Farm and the adjacent Spit Nature Conservation Reserve, are within the Port Phillip Bay's western shoreline and Bellarine Peninsula Ramsar Site, which are listed as wetlands of international importance under the Ramsar Convention, and is the wintering grounds of the critically endangered, orange-bellied parrot (*Neophema chrysogaster*). A variety of seabirds, such as Australasian gannets (*Morus serrator*) (Pyk et al. 2007), nest on artificial structures in the bay. Port Phillip contains three Marine Sanctuaries managed by Parks Victoria to protect and conserve the bay's biodiversity, ecological processes, and the natural and heritage features.

Like the Yarra River, which flows into it, Port Phillip faces the environmental concerns of pollution and water quality. Litter, silt and toxins can affect the beaches to the point where they can be shut down by the EPA. It should be noted that Port Phillip Bay location 2 (PPB2) has crude oil storage, which is being used by Shell Refining, located at Refinery Road, Corio (Geelong Advertiser 2015). Today, the Port of Melbourne has grown to become Australia's busiest commercial port, serving Australia's second largest city and handling an enormous number of imports and exports into and out of the country. The Port of Geelong also handles a large volume of dry bulk and oil, while nearby the Port of Hastings on Western Port handles steel and oil products. In 2004,

the Victorian Government launched the Port Phillip Channel Deepening Project to deepen the existing shipping channels and the lower Yarra to accommodate deeper draft vessels. The lower Yarra sediments were identified as likely to be contaminated with toxic chemicals and heavy metals and were to be contained within a sealed berm barrier clear of the shipping channels south of the Yarra entrance. Fifty two environmental groups, recreational fishing groups, and divers' groups formed the "Blue Wedges" group to oppose the proposed channel deepening and dredging with organised protests carried out, culminating in the group taking action in the Federal Court in January 2008 against the Federal Government to stop it signing off on the project (Lucas 2007). On 15 January 2008 it was announced that the Blue Wedges appeal was dismissed (Lucas 2008), with dredging starting soon after. The government announced the completion of the works in November 2009.

1.10: Western Port History and Ecology

Western Port Bay or Western Port is a 680km^2 tidal bay with an average depth of 6 meters in southern Victoria, Australia, opening into Bass Strait making it the second largest bay in the state. The species of seagrass found in the bay are Eelgrass (*Zostera muelleri)*, *Zostera tasmanica*, *Zostera nigricaulis*, paddle weed (*Halophila australis*) and wire weed (*Amphibolis antarctica*) (Edgar 2012).

A survey in 1994 found that 5000 ha of seagrass had regrown, mostly in the south-eastern section of the inlet, but that there had been little recovery in the north-eastern region (Stephens 1995). The role of excess epiphyte, macroalgal or phytoplankton growth in shading seagrass leaves and negatively affecting seagrass health is generally agreed to be a prevalent mechanism in seagrass decline worldwide (Walker and McComb 1992, Walker et al. 2006, Duarte et al. 2008). Morris et al. (2007) carried out nutrient addition experiments at three sites in Western Port. The addition of Nitrogen Phosphorus and Potassium (NPK) fertiliser increased the ash-free dry weight of seagrass leaves and loose algae at two of the three sites studied. There was also an increase in gammarid amphipod densities at the Crib Point. Shepherd et al. (2009) reported on a long-term analysis of algae in Western Port. The algal assemblage on Crawfish Rock in northern Western Port was surveyed in 1967–1971 and in 2002–2006. During the 1980s, water quality declined following the large-scale seagrass loss.

In 1971 Crawfish Rock had a rich algal flora with 138 recorded species, including 97 species of Rhodophyta. The biomass and cover of canopy and understorey species were measured at sites of strong and slight current on a depth gradient. In 1971, fucoid or laminarian canopy species were dominant from about 1–8 m depth, and an algal understorey extended from the intertidal zone to 12–13 m depth. By 2002–2006 the canopy species extended to only 3 m depth and the algal understorey to about 4 m depth, and 66% of the algal species had disappeared, although a few additional species were present. Persistent, sediment-tolerant species included several phaeophycean canopy species, some chlorophytes (Caulerpa spp.) and a few rhodophytes. These findings suggest that a long term shift in light climate in Western Port had taken place, with reduced light availability, increased sedimentation, and unfavourable conditions for photosynthetic organisms. Geographically the bay is dominated by two large islands, French and Phillip Islands. Contrary to its name, it lies to the east of the larger Port Phillip Bay and is separated from it by the Mornington Peninsula. Australian fur seals, whales, and dolphins, as well as many migratory waders and seabirds visit Western Port. The primary inflows into this bay are Bunyip River, Lang, Bass River, Cardinia Creek, Redbill Creek, Mosquito Creek, Brella Creek and Tankerton Creek. Over a 10 year period approximately 70% of the total area of seagrass and macroalgae was lost in Western Port (Blake and Ball 2001). Mapping from 1994 identified a subsequent increase in the cover of seagrass and macroalgae, with the total area having increased from approximately 59 km^2 in 1983-84 to 93 km^2 in 1994 (Blake and Ball 2001). A further increase was observed in Western Port during the present study with the area covered by seagrass and macroalgae increasing in 1999 to 154.5 km^2 (Blake and Ball 2001). The bay is surrounded by the towns of Hastings, Tooradin, San Remo, and Cowes. Western Port was designated a RAMSAR site on 15 December 1982 (Australia DAWE 2019). The area around the bay and the two main islands were originally part of the Boonwurrung nation's territory prior to European settlement. Western Port was first seen by Europeans in 1798 when an exploration crew in a whaleboat led by George Bass, journeyed south from Sydney to explore Australia's southeastern coastline. Due in most part to a lack of food, the expedition was halted, spending two weeks in Western Port before returning to Sydney. As it was the westerly charted point at the time, it was named Western Port; however, it actually lies in the eastern half of Victoria and to the east of the larger Port Phillip and the city of Melbourne. The bay is home to three Marine National Parks,

French Island, Churchill Island and Yaringa, while the land adjacent to the north is largely used for farming purposes including cattle and wineries (Parks Victoria 2003). Today the bay is mostly used for recreation; however, there is also a military base (HMAS Cerberus), shipping and oil production facilities adjoining the bay. Western Port is a one hour drive from Melbourne with a small number of holiday towns with sandy swimming beaches lie on its shores. Fishing, pleasure boating and yachting are some of the popular pastimes on the bay. Deep channels lead from Bass Strait into the western section of the bay, giving access to the region's port facilities. The town of Hastings is the main boat landing in the bay with the Yaringa Marina at Somerville also offering boat harbor facilities.

Western Port supports a mosaic of habitat types including underwater seagrass meadows, intertidal rock platforms, sandy beaches, intertidal mudflats, tidal channels, saltmarshes, and mangroves. The coastline around Phillip Island is one of state significance because of its remnant coastal tussock grasslands and dune scrub, a rare vegetation community in Victoria. Western Port consists of rocky platforms, sandy beaches, and marine habitats. It is home to a diverse range of invertebrates including colonial ascidians, sponges and corals. Mudflats and mangrove swamps around the northern end of the bay support many invertebrates that are an important food source for waders and visiting migratory birds. French Island is home to migratory waders, Australian pelicans (*Pelecanus conspicillatus*), short-tailed shearwater (*Ardenna tenuirostris*) rookeries, and many other significant fauna species. The bay has been identified as a 623km^2 Important Bird Area as it regularly supports small numbers of critically endangered, orange-bellied parrots (*Neophema chrysogaster*), over 1% of the world’s populations of Far Eastern curlews (*Numenius madagascariensis*), red-necked stints (*Calidris ruficollis*) and pied oystercatchers (*Haematopus longirostris*), and declining numbers of vulnerable fairy terns (*Sternula nereis*). Phillip Island Penguin Reserve has the largest colony of little penguins (*Eudyptula minor*) in Victoria as well as a major colony of short-tailed shearwaters (*Ardenna tenuirostris*), with breeding hooded plovers (*Thinornis cucullatus*) and peregrine falcons (*Falco peregrinus*). Seal Rock off Phillip Island is home to the largest colony of Australian fur seals and a breeding site of kelp gulls (*Larus dominicanus*) and sooty oystercatchers (*Haematopus fuliginosus*). San Remo's marine community is a rich assemblage of marine biota listed under the State Flora and Fauna Guarantee Act, 1988. The Moonlit

Sanctuary Wildlife Conservation Park situated near the northwestern corner of the bay in Pearcedale offers a close up look at some of the indigenous mammals and birds of the region. In recent years, numbers of whale sightings mostly southern right (*Eubalaena australis*) and humpback (*Megaptera novaeangliae*) have increased, and local institutes conduct research of their presence in the bay along within vicinity to the bay and Port Phillip Bay, have been asking public to report sightings (Preserve Western Port Action Group 2020; Strachan 2015; ABC News 2016).

Western Port has several industrial complexes, including a BlueScope steel processing works and the major Royal Australian Navy training base, HMAS Cerberus. Holden Australia's proving grounds were located just east of Western Port at Lang Lang where Holden Cars are tested for safety. In addition, Westernport Oil Refinery was operated by BP at Crib Point from 1966 to 1985.

Current development plans for the Port at Hastings would see it become the major shipping port for container and bulk freight in and out of Victoria. For the protection of the marine environment, a number of activities are prohibited within the boundaries of Victoria's Marine National Parks and Marine Sanctuaries. These include no fishing, netting, spearing, taking, or killing of marine life. In addition, all methods of fishing, from the shore or sea, are prohibited. There is also a rule of no taking or damaging of animals, plants and objects or artefacts. There are strong penalties under the National Parks Act 1975 for fishing in Marine National Parks and Sanctuaries (enforced by the Australian Federal Government's Department of Climate Change, Energy, Environment and Water). However, there is an exception where finfish can be carried on board a boat within park boundaries if the fish was caught outside the parks and a person may also carry but not use a fishing rod or spear gun. In addition, a person may also have abalone or rock lobster associated equipment on board the boat if they are travelling straight through the park by the shortest practicable route.

1:11: Corner Inlet History and Ecology

Corner Inlet is 600 km^2 bay located 200 kilometres southeast of Melbourne in the South Gippsland region of Victoria, Australia. It contains intertidal mudflats, mangroves, salt marshes and seagrass meadows, sheltered from the surf of Bass

Strait by a complex of 40 sandy barrier islands. The primary inflows are the Albert River and the Agnes River. The species of seagrass found in the bay are Eelgrass (*Zostera muelleri)*, *Zostera tasmanica*, *Zostera nigricaulis*, paddle weed (*Halophila australis*) and wire weed (*Amphibolis antarctica*), ribbon weed (*Posidonia australis*) (Edgar 2012). Corner Inlet Marine National Park contains a representative area of the only extensive Broad-leaf Seagrass meadows in Victoria. This seagrass community supports the most diverse fauna of all marine habitats in the Corner Inlet and Nooramunga area (ECC 2000). The extensive 'dieback' of Broad-leaf Seagrass beds in Corner Inlet since the 1970s is of particular concern (Poore 1978; Roob et al. 1998). Although little dieback has occurred within the park, the potential for it to occur is a significant threat to the park's natural values. Dieback is still occurring within Corner Inlet; however the cause or causes are relatively unknown. A greater understanding of the ecology of seagrass communities in Corner Inlet, and of natural influences such as climatic cycles on species such as Broad-leaf Seagrass which has a limited range, is required. The park is susceptible to a number of other threatening processes, including pollution (e.g. oil spills), marine pests, and physical disturbance (e.g. anchoring and propeller scouring). Prior to the proclamation of the park, most visitors anchoring in the area were recreational anglers. As fishing is no longer permitted, the potential for anchoring impacts is much lower. Nevertheless, visitor use of the park and associated impacts of anchoring on seagrass communities will need to be monitored over time. Sensitive seagrass communities within the park are being damaged by vessels (including personal water craft) operating in shallow waters, particularly at low tide. Scouring of the seabed by boat propellers has been observed within the park (Stevenson, J. pers. comm. 2004). Impacts to seagrass communities from the direct physical disturbance of vessels, in particular propeller scour, can be severe and long-term. Broad-leaf Seagrass is particularly susceptible to disturbance as it very slow to re-establish in disturbed areas (O'Hara et al. 2002). The inlet was designated a 1,550 hectare (15.5 km^2) RAMSAR site on 15 December 1982 (Ramsar Sites Information Service 1998). The site is called the Nooramunga and Corner Inlet Marine and Coastal Parks. In addition, Corner Inlet Marine National Park is found here as well. The inlet adjoins Wilsons Promontory in the west, extends to Ninety Mile Beach in the east, and supports large numbers of migratory waders and other birds as well as a rich marine flora and fauna. Corner Inlet lies within the traditional lands of the Brataolong clan of the Gunai nation. In the early 1840s, European settlers

moved into the area and established agricultural, mining and forestry enterprises. Commercial fishing became established in the 1860s. The surrounding land was originally covered by forest, which has mostly since been cleared. It has become a popular tourist destination for recreational boating and fishing (Parks Victoria 2008). 720 km^2 of land and water covering Corner Inlet has been recognised as an important bird area. Containing the most extensive intertidal mudflats in Victoria, it supports over 1% of the world's populations of chestnut teals (*Anas castanea*) (Norman and Chambers 2010), Far Eastern curlew, red-necked stint, pied and sooty oystercatchers and the hooded plover. The critically endangered, orange-bellied parrot has occasionally been seen here as well.

1.12: Aims and hypotheses

This research aims to further develop and refine our knowledge of the potential of the seagrass species *Zostera muelleri* (Eelgrass) as a biomonitor of heavy metal contamination in Victorian coastal areas. In a previous study (Lee 2016), it was found that by using *Z. muelleri* as a bioindicator, significant differences in concentration levels were found between the areas of Port Phillip Bay, Western Port and Corner Inlet for the metals aluminium (Al), barium (Ba), cobalt (Co), chromium (Cr), copper (Cu), iron (Fe), manganese (Mn), nickel (Ni), strontium (Sr) and zinc (Zn). This has led to the main hypothesis is that Eelgrass can be developed as a useful tool, when compared to more traditional methods such as testing of water and/or sediment (Ohls & Bogdain 2016), for identifying differences in heavy metal contamination within and between marine areas such as Port Phillip Bay, Western Port and Corner Inlet - arising from natural and/or anthropogenic sources. Other minor hypotheses that will be addressed during this study are:

- Is seagrass a reliable bioindicator of heavy metal levels in the global marine environment? To answer this question, some benchmarking or control measures are required. This involves a global audit of all such studies reported in the scientific literature across a range of species and geographical locations. Such an audit requires a scrutiny of the "circumstances" of each study. For example, is the study in an area that is known to be polluted? Of interest here is whether the global data lies within a defined magnitude range that may

represent a “background” and whether a departure from this magnitude range can indicate “contamination”?

- Can such a "normal"; - i.e., background versus "polluted" range be defined across different species, geographical locations, seasons, method variations etc.? How can this be ascertained?

- How does the local (temporal) data for Victoria’s bays compare with the global data? Are there significant departures from "normal"; - typical background levels between the three bays being investigated in Victoria and between the different sampling periods?

- How do we justify the identification of any departures from the global norm (contamination events)?

- Which sampling matrices (seagrass whole plants or organs tissues) are preferential for the quantification of which metals?

- Which of the metals measured in this study are of most concern with respect to the health of the bays?

- What sampling protocol would be suitable to survey long-term metal contamination within the bays?

Chapter 2 - Materials and Methods

A range of techniques were employed to collect and process the data required for this project. **Figure 2.1**, below, gives a broad schematic overview of the project, which is divided into two parts.

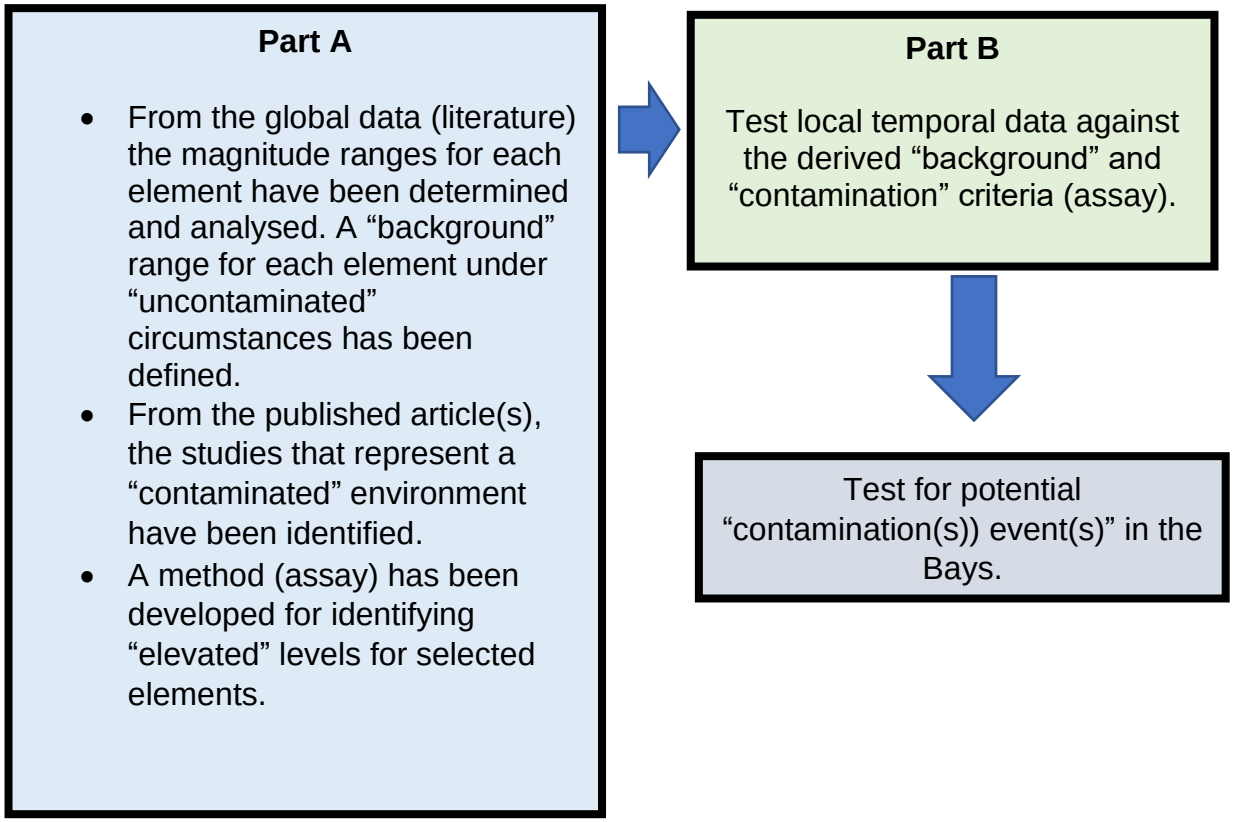


Figure 2.1: Project Overview

2.1 - Preamble

Part A involves an analysis of the magnitude ranges of elements in seagrass, worldwide. This involves an exhaustive and detailed search of the literature where such studies have been undertaken, **Figure 1.1**. This part of the project is intended to define a background "standard" for levels of potentially polluting heavy metals in the marine environment. Standard literature searching techniques have been employed here.

Part B involves a local temporal case study of Port Phillip Bay, Western Port and Corner Inlet, Victoria, Australia, **Figure 2.4**, using the seagrass species *Zostera melleri,* **Figure 1.2**, as a bioindicator for heavy metals. This has been referenced to Part A in order to test the "magnitude range" concept and to identify potential elevated levels or contamination events in the Bays. The ultimate aim here is to establish a rapid "assay" in order to identify a contamination event.

Part B involved the sampling of seagrass material and the subsequent chemical analysis of this material for heavy metals over the five-year period, 2015 – 2019, utilizing Inductively Coupled Plasma (ICP-AES) spectroscopy. Other environmental parameters associated with free and interstitial water (water in the pore spaces of soil or rocks) and substrates have also been (incidentally) measured. Field sampling utilized a randomized ecological survey using quadrats, where a range of ecological parameters were also incidentally[1] measured and recorded, including pH, conductivity, turbidity, dissolved oxygen, total dissolved solids, salinity, specific gravity and water depth.

Part A - A review of magnitude ranges of chemical elements in seagrass throughout the world

One intention of this study was the collection and use of the presently disparate 'grey' literature and the linking of this data directly to the findings of the specific testing of seagrass and the sites where they originated. However, at present, few such data sources are maintained centrally in relation to the sources of potential heavy metal pollution in the environment. Instead, a global meta-analysis of magnitude ranges of chemical elements in seagrass, as reported in the peer reviewed international literature, was carried out. This allowed the comparison of various levels of chemical contamination, irrespective of the geographical location and species type - given various rational assumptions, *vide infra*. Thus, existing heavy metal pollution levels were obtained from peer reviewed journal articles located in various databases, such as Victoria University's Library Catalogue, Google Scholar, Scopus® and

[1]This data, although outside of the scope of this thesis with respect to detailed analysis, has been documented in the Appendix and has been subjected to a preliminary Principal Component Analysis.

ScienceDirect. Papers were sought worldwide with no restrictions as to year or geographical location, from 1980 to the present, **Figure 1.1**. Keywords used for the search included: seagrass, seagrasses, metal, trace, chemical, pollution, bioaccumulation, bio indicator and bio monitor. Concentrations levels reported in both seagrass whole plants and/or organs were tabulated (PPM) for the reported elements, including Ag, Al, As, B, Ba, Be, Bi, Ca, Cd, Co, Cr, Cu, Fe, K, Hg, Li, Mg, Mn, Mo, Na, Ni, P, Pb, Rb, S, Sb, Si, Se, Sn, Sr, Ti, Tl, U, V, W, Zn and Zr. A subset of these elements, namely **As, Cd, Co, Cr, Cu, Hg, Mn, Ni, Pb and Zn**, was chosen from this series to represent and have been selected to reflect "anthropogenic impacts" and for the definition and construction of the "assay". Several other metals, i.e. Al and Fe were also closely examined to assist in the development of this methodology. This data is fully documented and discussed in **Chapter 3**.

To assess the measured magnitude ranges for each relevant element, scatter/dot-plots were initially employed. These plots arranged the data from the lowest concentration to the highest and allowed an arbitrary identification of "background", "elevated" and "polluted (or highly impacted)" levels for each element. This was also informed by the site information provided in the relevant publication. An example of such a plot (for Cu) is shown in **Figure 2.2**. For example, a suggested *subjective* cut-off point for a background range may be as indicated by the arrow in this plot.

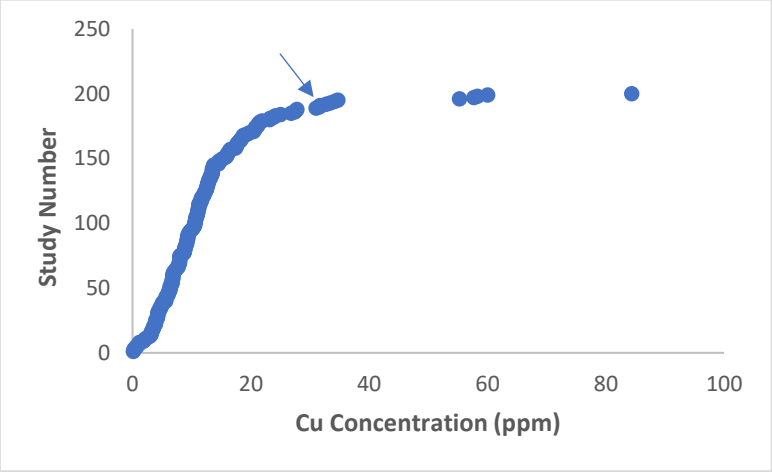


Figure 2.2: Representative scatter/dot-plot for the element Cu across 200 different studies. Arrow represents a possible cut-off for the "Background" data.

A complimentary, more mathematical, way of deriving such cutoffs, utilizing box-plot methodology, has also been used as follows:

From Boxplot analyses of the raw data, the 1st quartile (Q_1) and 3rd quartile (Q_3) were established in Microsoft Excel. An Interquartile range (IQR) was then obtained using the following formula:

$$\boldsymbol{IQR = Q3 - Q1}$$

Equation 1: Interquartile range (IQR): IQR= Interquartile range; Q1= 1st quartile ;Q3= 3rd quartile.

From the IQR, an outlier value can be obtained by the following formula. Any value greater than this is considered an outlier.

$$\boldsymbol{Q3 + (1.5\ X\ IQR)}$$

Equation 2: Outlier formula: Q3= 3rd quartile; IQR = Interquartile range.

The term “background” reflects the levels of ambient water/sediment contamination to which the seagrass is chronically exposed. The category of “elevated” reflects increased levels of water/sediment contamination to which the seagrass study is chronically exposed. The category “polluted” or “highly impacted” reflects water/sediment contamination where the seagrass has been chronically exposed to serious pollution. In this scenario, the latter is also referred to as a “contamination event”. A schematic overview of this methodology is represented by the schematic in **Figure 2.3**.

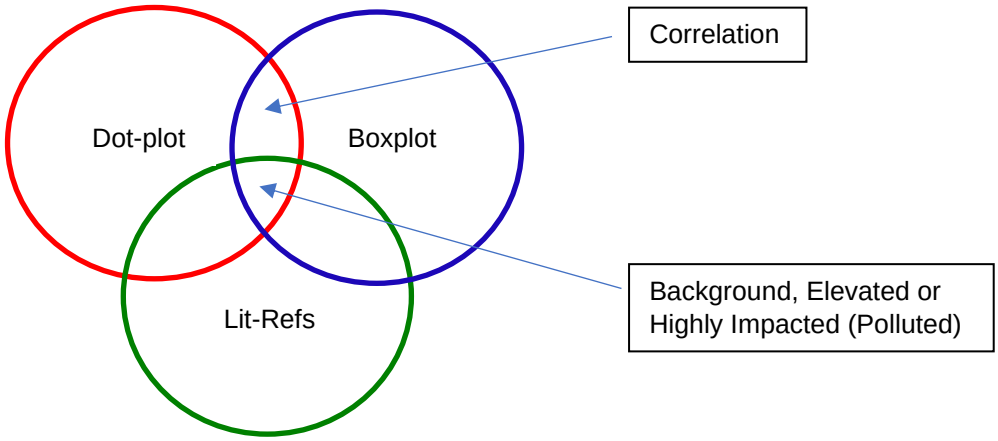


Figure 2.3: Schematic of the method for determining the magnitude ranges of chemical elements in seagrass worldwide.

In summary, the background/elevated cutoff is established mathematically (from the box plots) and validated subjectively (from the dotplots), whereas the elevated/highly impacted cutoff is established subjectively with reference to the journal article itself.

In order to attempt exploiting the global database of seagrass heavy metal levels via establishing a useable magnitude range, the following assumptions are made.

- Each different species of seagrass absorbs metals at a similar rate and up to a similar level with respect to ambient environmental concentrations.

- Geographical location and/or season does not affect the level of heavy metal accumulation, *per se*.

- Although variation due to the analysis of different plant organs is to be expected, and indeed is found in the literature, this is not sufficient to corrupt this “background” range of values.

Please note that seagrass organs not only accumulate different levels of contaminants but that their lifespan is different with e.g., leaves lasting up to 1 year and roots and rhizomes more than a decade depending on the species. The bioaccumulation term is linked to the lifespan of the plant organ. Thus, this approach to establishing a useable assay is based on the following important overarching assumption:

The variances in element levels due to different seagrass species, geographical locations and/or season, different analytical techniques and the analysis of different plant parts, are subsumed into the concept of a "magnitude range" of values and it is reasonably assumed that a significant pollution event that is reflected in seagrass heavy metal levels will result in magnitudes that will be well outside of this range. This will enable elevated or highly elevated levels and hence pollution events to be readily identified. This forms the basis for a convenient assay.

Part B: Sampling the Victorian Bays

The sampling sites that are used in this PhD program are located along the southern coast of Victoria, Australia. Namely, within Port Phillip Bay (S1), Western Port Bay (S2) and Corner Inlet (S3), **Figure 2.4 and 2.5**. The nine locations within these bays are also indicated in this figure and are also listed, and fully coded, in **Table 2.1**. The sampling periods are given in **Table 2.2**. Given that the data was collected over a five year period, that included the pandemic, some variation in logistics was inevitable. However, the method is deemed to be adequately consistent.

These sites were originally chosen because of ease of access and their anticipated differing levels of heavy metal contamination - since they are expected to be impacted by anthropogenic activity with respect to heavy metals in the order S1 > S2 > S3. Indeed, S3 is considered to be "pristine" in this regard (Ramsar Sites Information Service 1998). Note that all three bays flow out to Bass Strait.

The following convention was devised to describe the sampling protocol, refer to **Table 2.2** and **Figure 2.4 and 2.5**.

- Sampling site is denoted by the letter S (S1 to S3)
- Sampling period is denoted by the letter P (P1 to P6)
- Sampling location within a site is denoted by the letter L (L1 to L3)
- Replicate sample is denoted by the letter R (R1 to R3)
- PPB = Port Phillip Bay

- WP = Western Port
- CI = Corner Inlet

Note, with respect to **Figure 2.4**, L1 = PPB1; L2 = PPB2; L3 = PPB3; L4 = WP1; L5 = WP2; L6 = WP3; L7 = CI1; L9 = CI8; L9 = CI3, i.e., there are 3 locations within each of the 3 sites. From within each of the nine locations, three replicate samples, R, were taken. Thus, the data may[2] be represented by: **SxPyLzRn**, where: Sx = 1 – 3; Py = 1 – 6; Lz = 1 – 9; Rn = 1 – 3. For example, **S1P1L1R1** represents a sample taken from Port Phillip Bay (PPB - S1) (see **Figure 2.4** and **Table 2.1**) over the summer period of 2015/16 (P1) (see **Table 2.2**) from location L1 (see **Table 2.1**) and is the first of 3 replicate samples (R1) taken from L1.

[2] Although this specific convention is not extensively utilized in this thesis, it represents an example of how a potentially useful convention can be established for such a complex study.

Table 2.1: The seagrass sampling sites of Port Phillip Bay (S1), Western Port (S2) and Corner Inlet (S3) and the GPS coordinates of the locations (L1 to L9) within those sites, refer to **Figure 2.4.**

Site	Site Code	Site Address and GPS Location	Location Code
Port Phillip Bay	PPB – S1	Esplanade, Altona VIC 3018 (37°52'30.17"S 144°48'53.32"E)	L1 – PPB1
		Limeburner's Bay, Corio VIC 3214 (38° 4'16.32"S 144°24'25.98"E)	L2 – PPB2
		Sandringham Harbour, Hampton VIC 3188 (37°56'35.38"S 144°59'53.16"E)	L3 – PPB3
Western Port Bay	WP – S2	C786, Crib Point VIC 3919 (38°22'33.40"S 145°13'24.48"E)	L4 – WP1
		The Esplanade, Cape Woolamai VIC 3925 (38°31'31.42"S 145°20'35.95"E)	L5 – WP2
		Back Beach Rd, San Remo VIC 3925 (38°31'41.21"S 145°22'11.19"E)	L6 – WP3
Corner Inlet	CI – S3	245 Foley Rd, Yanakie VIC 3960 (38°48'37.98"S 146°16'7.13"E)	L7 – CI1
		188-189 Foster Beach Rd, Foster VIC 3960 (38°41'47.26"S 146°14'51.46"E)	L8 – CI2
		Toora Jetty Rd, Toora VIC 3962 (38°41'26.18"S 146°20'13.36"E)	L9 – CI3

Table 2.2: Seagrass sampling periods

SAMPLING PERIOD	CODE
Summer of 2015/16	P1
Winter of 2018	P2
Spring of 2018	P3
Summer of 2018/19	P4
Autumn of 2019	P5
Winter of 2019	P6

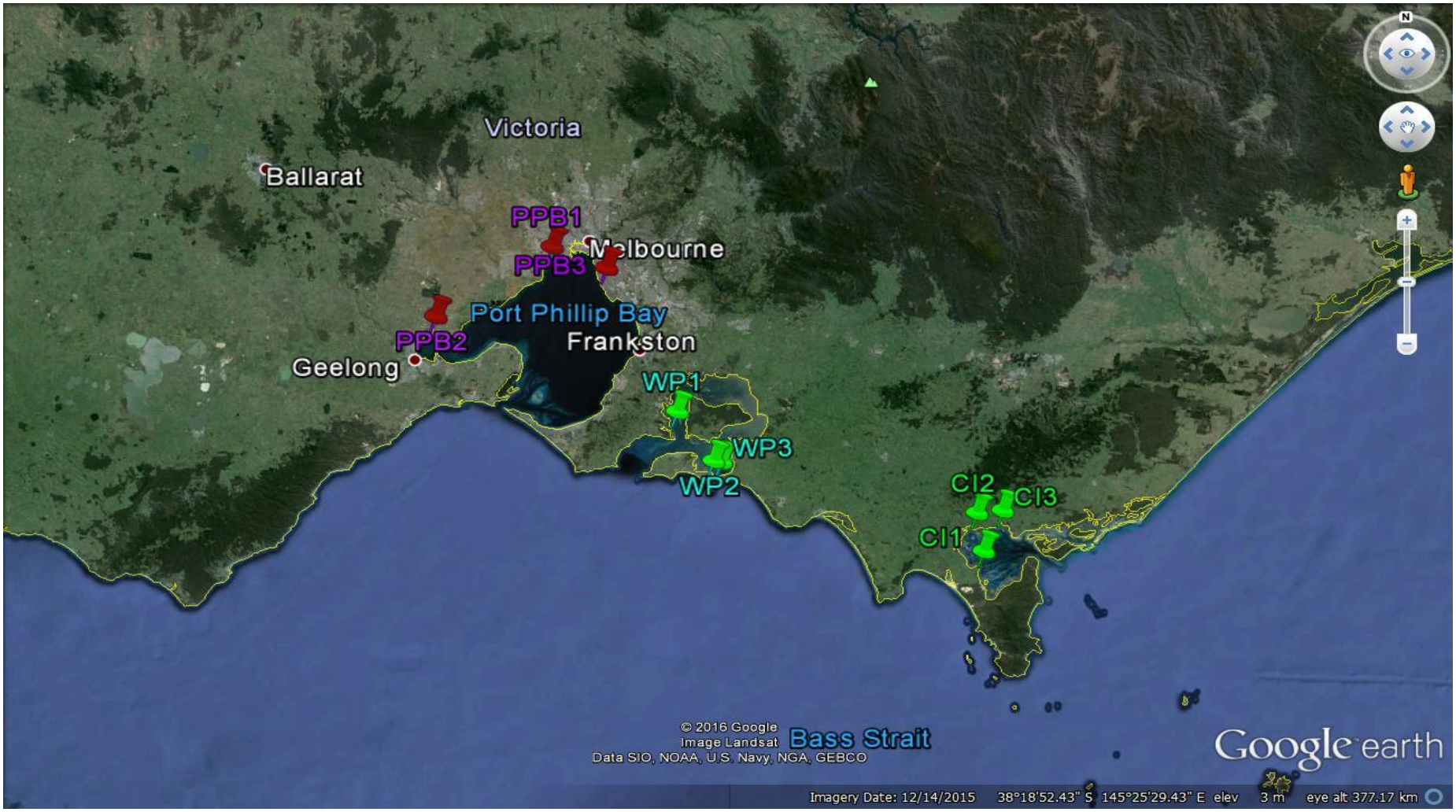


Figure 2.4: Overview map of all three sampling areas along the southern coast of Victoria, Australia. Namely, Port Phillip Bay (PPB), Western Port (WP) and Corner Inlet (CI). Sampling sites and GPS coordinates are provided in **Table 2.1.** Source Google Earth.

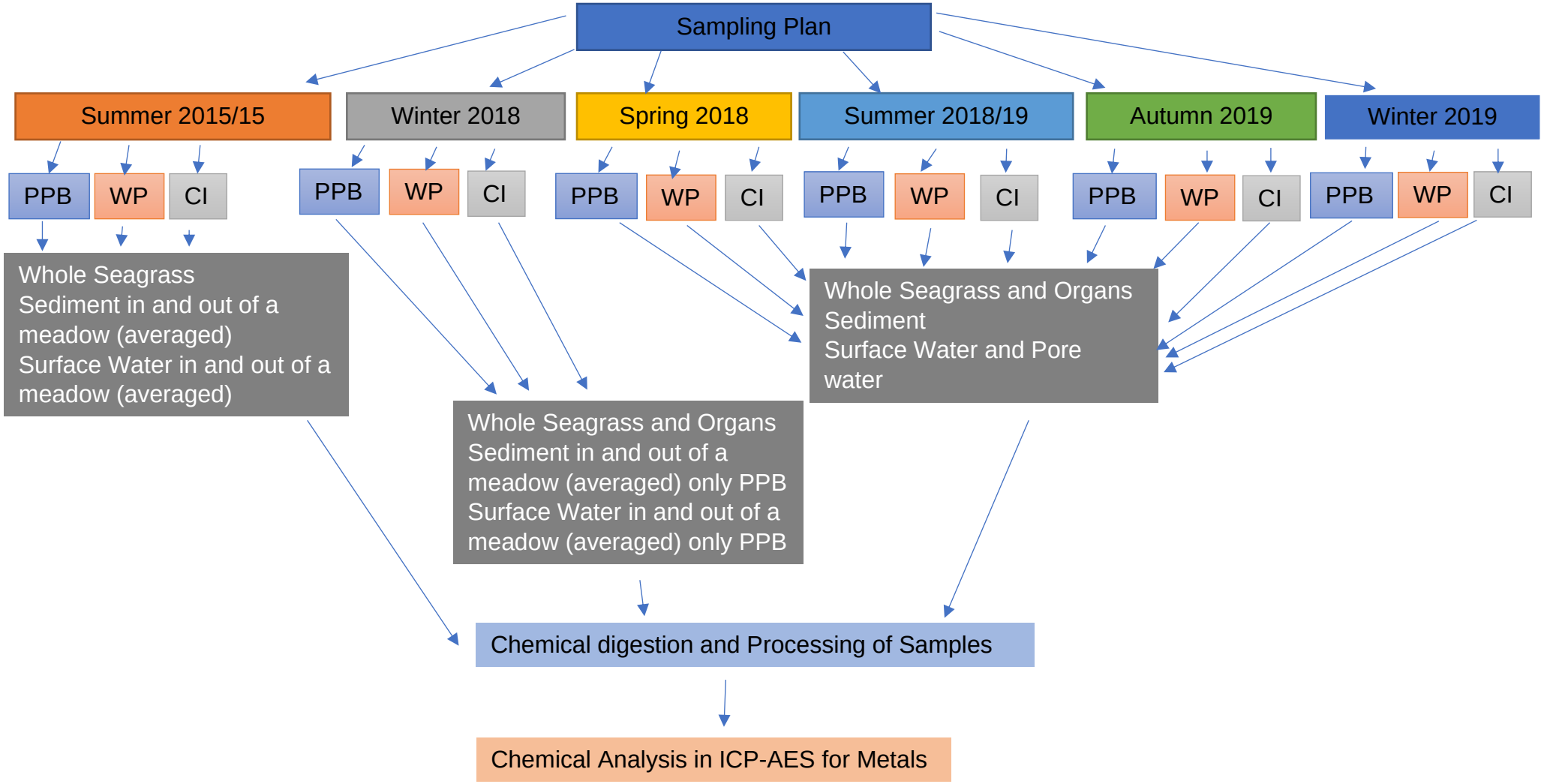


Figure 2.5*:* Schematic of the sampling plan

2.2: Sampling methodology

Patches of seagrass were sampled within meadows that were randomly selected by blind-tossing a 50 cm by 50 cm quadrat, **Figure 2.6 (a)**. Within each quadrat, samples were taken of whole seagrass plants including the leaves, roots, and rhizomes - as well as incidental samples of sediment, surface and porewater. The seagrass whole plants were separated into component parts. The reason for this step is that the leaves, rhizomes and root components bioaccumulate specific metals into different parts of the plant (Malea & Kevrekidis 2013). All samples were collected by hand within the sampled seagrass meadow during low tide where the water depth would be at or below wading height. To obtain these samples a 10 cm (internal diameter) by 26 cm length of PVC pipe, **Figure 2.6 (b)**, was pushed into the sediment to a depth of 2-3 cm to collect the seagrass cores, which were then placed into labelled zip lock bags and covered in seawater. Sediment and water samples were placed in labelled snap cap plastic vials and, with the seagrass cores, were transported back to the laboratory on ice to be frozen at -18 °C for later analysis at Victoria University's Werribee Campus. Sediment from the seagrass was removed by rinsing with Milli-Q water and any epiphytic material was scraped off using a ceramic scalpel or a glass slide.

For all seagrass, substrate and water samples within seagrass meadows, nine seagrass cores and one sediment core were collected at each location during various seasons to create a timeseries. Additionally, with the sediment, care was taken when removing extraneous roots and non-substrate materials from samples prior to freezing for later processing.

The collection of surface water was carried out in a manner analogous to the collection of sediment. The water samples were immediately stored in sterilised snap cap plastic vials and transported back to the laboratory to avoid contamination. In addition, porewater was obtained by centrifuging the sediment samples and the water was collected from the top. Finally, water abiotic factors were measured using a Horiba U-50 Portable Multi Water Quality Meter, **Figure 2.6 (c)**. This meter measures pH, conductivity (mS/cm), Turbidity (NTU), Dissolved oxygen (DO) mg/L, Total dissolved solids (TDS) g/L, salinity (ppt), specific gravity (s.g.) and water depth (m) were all

recorded on a field sheet. The Horiba was calibrated every time a fieldtrip was undertaken and cleaned with Milli-Q water and soap when finished.

All samples were pooled before chemical digestion. During sampling, blanks and replicates were used as field controls. Samples were obtained from clear open water were possible and care was taken in order not to disturb the sediment.

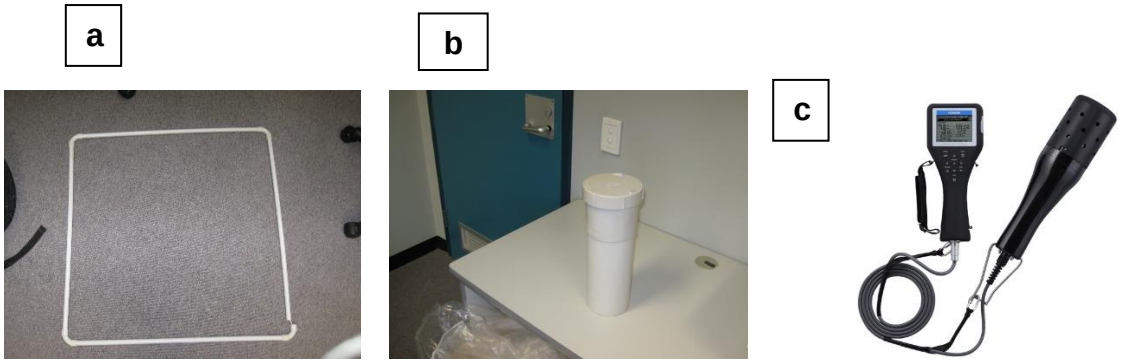


Figure 2.6: (a) 50 cm X 50 cm Quadrat used to randomly find sample – Source: own image; **(b)** 10 cm internal diameter by 26 cm length of PVC pipe used to collect seagrass and sediment sample – Source: own image; **(c)** Horiba U-50 – Source: https://www.horiba.com/en_en/products/detail/action/show/Product/u-50-434/

2.3 Sampling sites characteristics

Sampling sites and methods have been described in **Chapter 2.2, Materials and Methods - Sampling methodology**. The date of sampling for the pilot study was the 23rd of December 2015 for Port Phillip Bay (all sites), the 9th of January 2016 for Crib Point (Western Port), the 13th of January for San Remo (Western Port) and Phillip Island (Western Port) and the 18th of January 2016 for Corner Inlet (all sites). While for the main study, the dates are provided in **Table 2.3**. As seen in **Figure 3.5** shows a sampling plan that was used throughout the study. In addition, seawater abiotic data is also provided in **Table 2.3**. The pH for the sites ranged from 3.05 to 9.39, the dissolved oxygen ranged from 0 to 19.39 mg/L, the water temperature ranged from 9.51 to 30.4^{o}C and the salinity ranged from 32.1 to 39.8 ppt.

Table 2.3: Sampling locations, dates and water abiotic factors of surface water for each location – sampling depth was when the probe was submerged

Location	*pH*	*Dissolved oxygen (DO) mg/L*	*Water Temperature °C*	*Salinity (ppt)*
Corio (23/12/15)	*8.5*	*10.2*	*23*	*37*
Altona (23/12/15)	*8.6*	*9.9*	*25*	*37*
Sandringham (23/12/15)	*8.5*	*9.9*	*23*	*35*
Crib Point (9/1/16)	*8.6*	*10.1*	*24*	*35*
San Remo (13/1/16)	*8.86*	*9.94*	*22.8*	*34.8*
Phillip Island (13/1/16)	*9*	*10.4*	*23.3*	*35*
Toora (18/1/16)	*8.87*	*9.87*	*24.9*	*37.6*
Foster (18/1/16)	*8.7*	*9.6*	*26.7*	*38.8*
Yanakie (18/1/16)	*9.07*	*9.56*	*30.4*	*37.2*
Altona (20/6/18)	*4.01*	*6.26*	*14.13*	*36.7*
Corio (20/6/18)	*6.11*	*4.31*	*9.51*	*9.8*
Sandringham (20/6/18)	*6.44*	*5.53*	*11.66*	*33.1*
Phillip Island (18/7/18)	*6.27*	*7.41*	*11.07*	*36.1*
San Remo (18/7/18)	*6.05*	*4.35*	*11.33*	*35.4*
Crib Point (18/7/18)	*6.38*	*8.33*	*13.22*	*38.8*
Toora (2/8/18)	*8.54*	*19.39*	*11.44*	*32.3*
Foster (2/8/18)	*6.55*	*14.54*	*14.61*	*34.9*
Yanakie (2/8/18)	*6.74*	*7.82*	*14.8*	*35.9*
Corio (19/11/18)	*8.87*	*2.89*	*24.12*	*38.6*
Altona (19/11/18)	*8.56*	*4.11*	*25.15*	*34.3*
Sandringham (19/11/18)	*8.44*	*0*	*23.73*	*36.3*
Phillip Island (29/11/18)	*8.87*	*7.41*	*24.12*	*36.1*
San Remo (29/11/18)	*8.56*	*4.35*	*25.15*	*35.4*
Crib Point (29/11/18)	*8.44*	*8.33*	*23.73*	*38.8*
Toora (6/12/18)	*4.19*	*2.5*	*23.7*	*33.5*
Foster (6/12/18)	*5.55*	*1.47*	*23.48*	*32.8*
Yanakie (6/12/18)	*6.13*	*0.1*	*24.58*	*33.3*
Altona (31/1/19)	*3.05*	*2.18*	*23.9*	*36.7*

Sandringham (31/1/19)	*3.88*	*2.33*	*24.02*	*37.3*
Corio (31/1/19)	*5.82*	*2.24*	*23.13*	*36.6*
Phillip Island (13/2/19)	*8.33*	*0.24*	*17.95*	*37.1*
San Remo (13/2/19)	*3.55*	*1.54*	*17.98*	*36.8*
Crib Point (13/2/19)	*5.46*	*1.77*	*19.79*	*37.7*
Toora (20/2/19)	*8.42*	*0.56*	*14.95*	*39.8*
Foster (20/2/19)	*8.17*	*0.39*	*15.56*	*39.3*
Yanakie (20/2/19)	*8.17*	*0*	*17.12*	*38.9*
Altona (14/5/19)	*8.2*	*N/A*	*15.83*	*35.8*
Sandringham (14/5/19)	*5.06*	*N/A*	*17.52*	*35.7*
Corio (14/5/19)	*5.92*	*N/A*	*17.08*	*37.5*
Phillip Island (21/5/19)	*7.64*	*N/A*	*14.13*	*36.8*
San Remo (21/5/19)	*7.6*	*N/A*	*14.79*	*36.5*
Crib Point (21/5/19)	*7.65*	*N/A*	*15.77*	*36.5*
Toora (8/6/19)	*4.92*	*N/A*	*10.06*	*34.9*
Foster (8/6/19) - sampled from different source from seagrass but same location	*4.09*	*N/A*	*10.75*	*34.3*
Yanakie (8/6/19)	*4.56*	*N/A*	*13.66*	*36.5*
Altona (13/8/19)	*9.08*	*N/A*	*9.95*	*35.4*
Sandringham (13/8/19)	*9.29*	*N/A*	*10.91*	*33.6*
Corio (13/8/19)	*6.75*	*N/A*	*11.31*	*33.4*
Phillip Island (21/8/19)	*3.75*	*N/A*	*13.49*	*35.3*
San Remo (21/8/19)	*8.16*	*N/A*	*11.68*	*34.5*
Crib Point (21/8/19)	*8.25*	*N/A*	*10.79*	*35.5*
Toora (27/8/19)	*8.23*	*N/A*	*14.24*	*33*
Foster (27/8/19)	*3.16*	*N/A*	*13.39*	*32.1*
Yanakie (27/8/19)	*4.49*	*N/A*	*14.12*	*34.4*

Weather and tide information was collected from Bureau of Meteorology (BOM) website and is provided in **Appendix 8**.

2.4: Chemical digestion

Samples of seagrass and sediment were digested using the method described by Finger et al. (2015, 2016, and 2017). This method was also used to acidify surface and pore-water samples. Before the chemical digestion, the frozen samples were thawed to room temperature in zip lock bags on a tray in the fume hood.

Seagrass cores were separated into organs (leaves, rhizomes, and roots). Each seagrass organ plus a whole plant sample and sediment were placed on watch glasses. The watch glasses were placed into an oven (Memmert Oven – Selby, **Figure 2.7**) at a constant temperature of 70 °C for at least 48 hours.

All of the organic matter was ground using an IKEA A11 Micromill. 50 mL DigiTubes PP with lid (SCP Science) and were labelled with a unique sample number according to the datasheet on the side of the DigiTube. Each sample was prepared in triplicate. However, duplicates were sometimes used when supply was limited.

Approximately 0.5 g of 'dry' sample was placed into the DigiTube using a plastic spatula and the exact wet weight of the sample was recorded. This was also done for reference materials (AGAL-6, BCR-679 and AQA 16-12 S2 Cabbage leaves, AGAL-10 Hawkesbury River Sediment and CASS-4 nearshore seawater) and blanks, which contained Milli-Q water.

Cabbage leaves were selected as the SRM for seagrass because they were a botanical reference material available from the Australian National Measurement Institute (NMI). Each sample had a constant weight with +/- 0.5 mg. Using a separate Eppendorf Automatic Pipette, 3 mL of 65% Nitric acid (HNO_3) (Merck SUPRAPUR) and 0.5 mL of 37% Hydrochloric acid (HCl) (EMSURE Merck) was added to each DigiTube.

This combination of acids is colloquially known as "aqua regia". Aqua regia, also known as 'regal water' or 'king's water', is a yellow orange, sometimes red, fuming liquid, so named by alchemists because it can dissolve the noble metals, gold, and platinum. The lids were placed back tightly on all DigiTubes and the samples were

mixed thoroughly using the Vortex Mixer for about 3 seconds each. The lids of the DigiTubes were loosely placed (thread engaged but not fully closed so built-up pressure during digestion can escape) and placed onto a dry block heater at 95^{o}C for 60 minutes.

This process was repeated three time (whole process takes 3 hours) with a vortex mix in-between. Each DigiTube with a tightly closed lid was placed onto the Vortex Mixer for a few seconds. The DigiTubes in the rack were placed into an ice bath under the fume hood allowing the samples to cool for 15 minutes. 14 mL Falcon tubes with screw tops were labelled corresponding to each DigiTube. Approximately 1 mL of Milli-Q water was added to contents of each DigiTube, recapped tightly, and placed on the Vortex Mixer for a few seconds.

A syringe filter (28 mm diameter, 0.45-micrometer pore size, non-sterile) was attached to a 10 mL Terumo syringe with Luer lock and the plunger removed. While holding the filter over the falcon tube the content was transferred into the matching DigiTube using a disposable transfer pipette. The DigiTube was then carefully rinsed by adding about 2 mL of Milli-Q water with the lid on top and gently shaking the DigiTube. The contents of the DigiTube were carefully transferred into the syringe using the same pipette. The plunger is inserted into the syringe and was carefully pushed into the solution through the filter into the falcon tube. This was then topped up with Milli-Q water to 14 mL using a transfer pipette.

The samples were centrifuged on a Beckman Coulter Model Avanti J-26S XPI at 4000 RPM for 20 minutes at 20 ºC to remove any solids not removed by filtering. The samples were split in half by taking out 7 mL of sample and placing it into a new 14 mL labelled falcon tube. All samples were topped up with Milli-Q water back to 14 mL using a transfer pipette. The lid was screwed on tightly onto the falcon tube and sealed with parafilm to be stored at room temperature until analysis.

Figure 2.7: Selby Memmert Oven – used to Dry Sample – Source: own image

2.5: Chemical analysis

To test the metal concentrations, a Shimadzu ICPE-9000 Multitype ICP was used, **Figure 2.8**. The Shimadzu ICPE-9000 is an Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES). This technique is also referred to as inductively coupled plasma optical emission spectrometry (ICP-OES). For further detailed information on how ICP-AES works[3] please go to **Appendix 6**.

Figure 2.8: Shimadzu ICPE-9000 Multitype ICP used to analysis samples– Source: https://shimadzu.com.au/icpe-9000-multitype-icp

[3]It is appreciated that readers of this thesis might have more of an ecological than a chemistry background. Therefore, some summary information of ICP-AES and AAS has been provided for convenience in the Appendix. Similarly, some guidance has been provided in this Chapter on how to approach using the typical software packages.

To use the Shimadzu ICPE-9000 Multitype ICP it is necessary to master its software package. For this thesis, tis was ICPEsolution Launcher. The method is as follows: click Analysis then select QuanBase.iem as the method. Under the Method menu select Measurement Conditions. Under Rinse Setting, select 60 seconds for Solvent Rinse. The Rinse used was Milli-Q water. For Sample Rinse, 60 seconds was also selected. These times were selected based on the length of the tube from the autosampler to the nebulizer/spray chamber. Next, Select Analysis Element and Wavelength Registration; Analysis/quantitative was chosen from the menu.

The wavelengths that were used for testing were: Aluminium: 257.510nm; Arsenic: 193.759nm; Barium: 455.403nm; Cadmium: 226.502nm; Chromium: 205.552nm; Cobalt: 228.616nm; Copper: 324.754nm; Iron: 259.940nm; Lead: 220.353nm; Manganese: 257.610nm; Nickel: 231.604nm; Selenium: 196.090nm; Tin: 189.980nm; Strontium: 407.771nm; Vanadium: 292.402nm; Zinc: 213.856nm.

These wavelengths were sourced from USEPA Methods 200.7 and 6010B and Standard Methods for the Examination of Water and Wastewater 3120 B. During this PhD study Ba and Sr were omitted. This was due to both these metals not being detected. Due to wavelength interference, the metals were divided into different groups for analysis as follows: Group 1: As, Cd, Cu, Mn and Se; Group 2: Co and V; Group 3: Al, Cr, Sn and Zn; Group 4: Fe, Ni and Pb.

Standard Registration was selected, and six calibration points were selected. The first calibration point was of a Blank of Milli-Q water. Calibration points 2 - 6 for each of the metals are shown in **Table 2.4**. For quality control the Eppendorf Automatic Pipette was calibrated by doing 10 measurements of a known volume and calculating the average of these measurements.

Table 2.4: Standard solutions concentrations for all metals tested (mg/L or PPM)

Metal	*Calibration 2*	*Calibration 3*	*Calibration 4*	*Calibration 5*	*Calibration 6*
As	*1*	*2.5*	*5*	*7.5*	*10*
Cd	*0.1*	*0.5*	*1*	*1.5*	*2*

Cu	*0.1*	*0.5*	*1*	*1.5*	*2*
Mn	*0.1*	*0.5*	*1*	*1.5*	*2*
Se	*1*	*2*	*3*	*4*	*5*
Co	*0.1*	*0.5*	*1*	*1.5*	*2*
V	*0.1*	*0.5*	*1*	*1.5*	*2*
Al	*1*	*2.5*	*5*	*7.5*	*10*
Cr	*1*	*2*	*3*	*4*	*5*
Sn	*0.1*	*1*	*2*	*3*	*4*
Zn	*1*	*2*	*3*	*4*	*5*
Fe	*1*	*2.5*	*5*	*7.5*	*10*
Ni	*0.1*	*0.5*	*1*	*1.5*	*2*
Pb	*1*	*2.5*	*5*	*7.5*	*10*
Ba	*0.01*	*0.5*	*1*	*N/A*	*N/A*
Sr	*0.01*	*0.5*	*2*	*N/A*	*N/A*

The procedure was as follows: At the start of each experiment activate the Turn on Plasma button. Next, run the instrument calibration using Milli-Q water. Activate the measurement conditions button and select **NO** for qualitative database calibration. For standard calibration, activate Sample Registration and select Add/Insert Standard Sequence.

For the sample run, select Sample Registration and activate Batch Create and enter the sample names into the database. Each sample was measured in triplicate whenever possible. Activate the Start button to start the ICP-AES. This method was used for standard reference materials (AGAL-6, BCR-679 and AQA 16-12 S2) Cabbage leaves, AGAL-10 Hawkesbury River and CASS-4 nearshore seawater) and blanks for Method validation. The Shimadzu ICPE-9000 Multitype ICP calibration curves are found in **Appendix 9-33**.

To assist in the validation of the ICP-AES metal concentrations, a Shimadzu Atomic Absorption Spectrophotometer Model AA-6300 was used, **Figure 2.8**. Further information on how an AAS works is summarised in **Appendix 6**.

Figure 2.9: Shimadzu Atomic Absorption Spectrophotometer AA-6300 used to validate the ICP-AES samples. Source - https://caeonline.com/buy/spectrophotometers/shimadzu-aa-6300/9241714

The procedure for using the Shimadzu Atomic Absorption Spectrophotometer Model AA-6300 involves first opening its software package called WizAArd. From the top tool bar go to Instrument, select and activate Connect. From the tool bar go to File, select and activate New. In the Wizard Selection window, select the Wizard tab. Activate the Element Selection and OK. Activate the Select Elements Icon and the Periodic Table. Select the Element Metal to be analysed. The elements chosen were Co, Mn, Pb and Zn. Click on OK. Click the Using ASC box, then on OK. Click on Next. Click on the Calibration Curve Setup Tab. Selected the concentration unit required under Conc. Unit. The concentration units used were PPM. Under Measurement Sequence for Calibration Curve, input the number of calibration standards including the Blank, click the Update tab. Six standards including a blank containing Milli-Q water was used **(Table 2.4)**. Input the concentration for each standard and its position in the autosampler. Click on the Update tab again, then on OK. Click on the Sample Group Tab. Select the Concentration Unit of the sample required. Input the number of samples that need to be analysed, click onto Update. Click on OK, then on Next. Click on the Finish tab. Click on the Repeated Measurement Conditions tab. Input the number of replicates and Max replicates for the Blank, Standards and Samples required. Only one replicate was run for all samples. Click on the Measure Parameters tab, enter 35 seconds for the Pre-Spray time and 8 seconds Integration Time.

The wavelengths that were used for testing are: Cobalt: 240.7nm with an optimum range of 3-12 µg/mL (PPB); Lead: 283.3nm with an optimum range 10-40 µg/mL; Manganese: 403.1nm with an optimum range 15-60 µg/mL; Zinc: 213.9nm with an optimum range 0.4-1.6 µg/mL. These wavelengths were sourced from Varian 1979.

2.6: Method validation

To validate the data a number of strategies were used. This included the use of a Standard References Material (SRM) and percentage recovery calculations via spiking.

For SRM validation, AGAL-6 (cabbage leaves) provided by the National Measurement Institute (NMI) were selected, being a close botanical reference material that was readily available. Blanks were Milli-Q water.

Spiking of a sample with a known concentration of metal was carried out as follows. Note that if the volume of the added spike solution increased the sample volume by more than 5%, or if a more accurate spike recovery calculation is desired, Equation 4 may be modified by employing a dilution factor, obtained using Equation 5.

$$\%\ Recovery = \frac{(spike\ concentration - sample\ concentration)}{Spike\ X\ Dilution\ Factor}\ X\ 100$$

Equation *3:* Percentage recovery of metals in spiked solution. Spike concentration: the concentration of spike solution; sample concentration: the concentration of sample; spike: the volume of spike added; dilution factor: the ratio of solute to solvent.

$$DF = Sample\ Volume\ \div\ (total\ volume\ of\ the\ sample + \ spike)$$

Equation 4: Dilution Factor (DF).

The spiking method used is analogous to the Matrix Spike test for Fluoride in Ocean Water by Direct ISE (Thermo Fisher Scientific 2011) which is described as follows:

1. The fluoride electrode is calibrated in the range of 1 – 10 mg/L.
2. 25 mL of the unknown ocean water sample is mixed with 25 mL of the TISAB II and analysed. The fluoride concentration is found to be 1.22 mg/L.
3. The appropriate spike concentration for this sample is 1 mg/L, which will approximately double the sample concentration.
4. The Orion 100mg/L Fluoride Standard (Cat. No. 940907) is used as a spiking solution since it is between 50 to 100 times the desired spike concentration.
5. The volume of spiking solution to be added to 25 mL of ocean water sample is calculated as 0.25 mL: Vol spike to add = (1 mg/L (Chosen spike conc.) x 25 mL Sample) 100 mg/L Spiking solution = 0.25 mL spike
6. Add 0.25 mL of 100 mg/L spiking solution to 25 mL of ocean water. Mix with 25 mL of the TISAB II and analyse.
7. Calculate % Recovery. If the concentration of the spiked sample is found to be 2.24 mg/L, %R = (2.24 mg/L spiked result – 1.22 mg/L unspiked result) / 1 mg/L known spike added concentration (see Note below) = 102.0 %R
8. Compare the %R to the published analytical method criteria or the laboratory SOP criteria to determine if the analytical procedure is performing well for this sample (and sample type).

2.7: Statistical analysis

ICP-AES concentrations were transferred to a Microsoft Excel 365 spreadsheet and all concentrations were doubled to account for dilution. This reflects the samples being split in half, by taking out 7mL of the sample and placing it into a new labelled falcon tube and being topped up with Milli-Q water to 14 mL. This was done by using the following equation,

$$True\ sample\ concentration = ((sample\ concentration\ X\ dilution\ factor))/sample\ mass$$

Equation 5: True sample concentration calculation

True sample concentration = Real concentration of sample in mg/kg (PPM); sample concentration = ICP-AES concentration in mg/L; Dilution Factor = Volume of sample 14 mL; Sample mass = mass of sample digested in grams. Sampling was carried out as described previously, *vide supra*.

For the statistical analyses, Microsoft Excel 365 with the XLSTAT add-on was used (Addinsoft 2020). An initial descriptive statistics/univariate analyses was carried out after which a bivariate analysis was undertaken. Histograms of means with 95% Confidence Intervals were used to graphically represent data.

Principal Component Analysis (PCA) of the data was undertaken for the seagrass samples that included the whole plants and various organs, for the sediment samples and for the water samples (including surface and pore water). The statistical strategy employed was similar to that of Vidal et al. 2020. PCA is a popular technique for analysing large datasets containing a high number of dimensions/features per observation, increasing the interpretability of data while preserving the maximum amount of information, and enabling the visualization of multidimensional data. Formally, PCA is a statistical technique for reducing the dimensionality of a dataset. Thus, using the XLSTAT add on, the Analysing Data menu was selected, followed by the Principal Components Analysis option. In the General tab, the data format chosen for the PCA is Observations/Variables. The PCA standardisation chosen was the Pearson's correlation. In the Options tab the data was standardised by picking the "n" standardisation option. The dataset did not contain any supplementary data and therefore no options were selected. In the Data Options tab, the option "estimate missing data to the nearest neighbour" for the missing data was selected. In the Outputs tab, the following options were chosen: Descriptive Statistics – which gives a summary table with the descriptive statistics of the data set; Correlations - both the Test Significance and Bartlett's Sphericity Test, at a significance level of 95%, (which tests the hypothesis that the correlation matrix is an identity matrix) and the Kaiser-Meyer-Olkin test were selected (this tests the suitability of the data set for factor analysis). In the Charts tab, the Variables subtab was selected, and the following options chosen - Correlations Charts, Vectors, Orientate Labels and Coloured Labels. In the Observations subtab the following options were chosen - Observation Charts, Labels, Coloured Labels and Colour by Group. In the Biplots subtab, Biplots was enabled as well as Vectors and Labels. For the Observations option, Labels was enabled. The type of biplot chosen was a Distance Biplot. The Coefficient was set to Automatic. Bootstrap charts were not selected. Click OK to start PCA computations based on data selections and configurations made. The principal component plot with the highest variance was selected. Finally, click done to display the PCA results.

In addition to the PCA a Manhattan analysis was undertaken. A Manhattan analysis is a type of plot, usually used to display data with a large number of data points, many of non-zero amplitude, and with a distribution of higher magnitude values. The plot is commonly used in genome-wide association studies to display significant Single-nucleotide polymorphisms (Gibson 2010). It gains its name from the similarity of such a plot to the Manhattan skyline, a profile of skyscrapers towering above the lower level "buildings" which vary around a lower height.

For this research Grootendorst 2021 Manhattan distance mythology from 9 Distance Measures in Data Science was used. This method is used when the dataset has discrete and/or binary attributes, this method seems to work quite well since it takes into account the paths that realistically could be taken within values of those attributes. For example, take Euclidean distance, it would create a straight line between two vectors when in reality this might not actually be possible.

Although Manhattan distance seems to work okay for high-dimensional data, it is a measure that is somewhat less intuitive than euclidean distance, especially when using in high-dimensional data. Moreover, it is more likely to give a higher distance value than euclidean distance since it does not the shortest path possible.

Chapter 3. Results and Discussion

Part A: A collation of the magnitude ranges of chemical elements in seagrass throughout the world

3.1 The derived heavy metal assay

A review of the international literature has brought together 75 studies (publications in the peer reviewed literature) from 26 different countries, **Figure 3.1**.

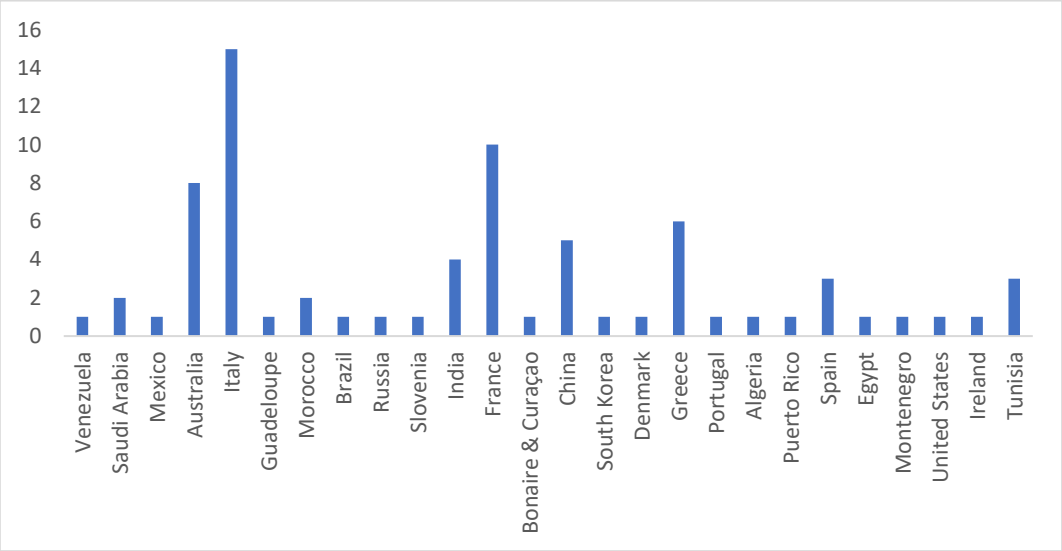


Figure 3.1: The number of studies of the heavy metal analysis of seagrass versus geographical location.

From these publications, a comprehensive set of data has been compiled that is provided as an Excel file within **Appendix 34**. This data set provides the full range of metal concentrations in seagrass species, worldwide, across 38 elements, as published since 1980. Also provided in this data set are all of the specific journal references for these studies. From this data, via the method described in **Chapter 2 PART A -** and described in more detail later on in this Chapter, a general method has been derived for conveniently determining the "industrial" heavy metal pollution status of any marine environment using any seagrass species as a bioindicator. This assay is presented **upfront** in **Table 3.1**, since it is the cornerstone of this thesis and will be

used in subsequent discussions and conclusions. ANZG Guidelines from 'Table 4.3.2 Recommended water quality trigger values (low risk) for heavy metals and metalloids in livestock drinking water', (AZNECC/ARMCANZ, 2000) have been added to Table 3.1 and have no bearing during this PhD study.

Table 3.1 Derived Heavy Metal Assay

Element	Background Range - ppm	Elevated to Highly Impacted Range - ppm (Polluted)	ANZG Guidelines (AZNECC/ARMCANZ, 2000) - ppm
As	0 - 12	13 - 151	0.5
Cd	0 - 5	6 - 3358	0.01
Co	0 - 5	6 - 12	1
Cr	0 - 29	30 - 203	1
Cu	0 - 30	31 - 84	0.4-5
Hg	0 - 1	2 - 21	0.002
Mn	0 -244	245 - 2544	not sufficiently toxic
Ni	0 - 31	32 - 43	1
Pb	0 - 18	10 - 2630	0.1
Zn	0 - 198	199 - 7280	20

Proposed assay table (below) for deciding when a given metal concentration, as determined in a sample of seagrass (any species, any geographical location, whole plant, or plant part/organ), is representative of typical *background levels* worldwide. Values above these levels would be regarded as being from potentially *polluted* areas.

Element	As	Cd	Co	Cr	Cu	Hg	Mn	Ni	Pb	Zn
Background ≤ Concentration (ppm)	**12**	**5**	**5**	**29**	**30**	**1**	**244**	**31**	**18**	**198**

3.2 Details for the derivation of the derived heavy metal assay in Table 3.1

The literature studies cover 10 different genera that are distributed as shown in **Table 3.2**. Also shown in **Table 3.2** is how these studies have been categorized into three different contamination levels, according to the method described previously in **Chapter 2 PART A**. Namely, "Background", which is defined as the lowest level of ambient water/sediment contamination to which the seagrass study is chronically exposed. The second category is "Elevated" which is defined by increased levels of water/sediment contamination to which the seagrass study is chronically exposed and "Highly Impacted" which is defined by water/sediment contamination to which the seagrass study is acutely exposed (e.g., due to a "contamination event").

Table 3.2: The distribution of genera and the assessed levels of pollution.

Genus	Number of entries	Background	Elevated	Highly Impacted
Enhalus	4	2	2	
Phyllospadix	1		1	
Ruppia	1			1
Halodule	4	1	3	
Syringodium	8	6	2	
Halophilia	21	2	19	
Thalassia	16	8	5	3
Zostera	24	7	10	7
Cymodocea	52	20	27	5
Posisdonia	88	66	19	3

3.3 Analysis of the dotplot data

Figures 3.2 to 3.18 show the scatter/dotplot analyses for the elements Al, **As, Cd, Co, Cr, Cu,** Fe, **Hg, Mn, Ni, Pb and Zn**, respectively. The subjective and mathematically derived cutoff values for elevated levels are indicated on each of these plots. The elements Al and Fe are included over and above the basic set of 10 "anthropogenic" assay metals (emboldened) to further illustrate the method. A brief commentary on each of these metals is provided after each figure. Information on the remaining 25 metals, Ag, B, Ba, Be, Bi, Ca, K, Li, Mg, Mo, Na, P, Rb, S, Sb, Si, Se, Sn, Sr, Ti, Tl, U, V, W and Zr, that were measured are provided in **Appendix 35,**

together with their corresponding dot plots. Note that in determining the subjective cutoffs from each these dot plots, the horizontal range is manipulated within Microsoft Excel and the relevant journal publications are consulted within respect to potential elevated or polluted values. Then an informed decision is made as per **Figure 2.3.**

3.3.1 Aluminium (Al)

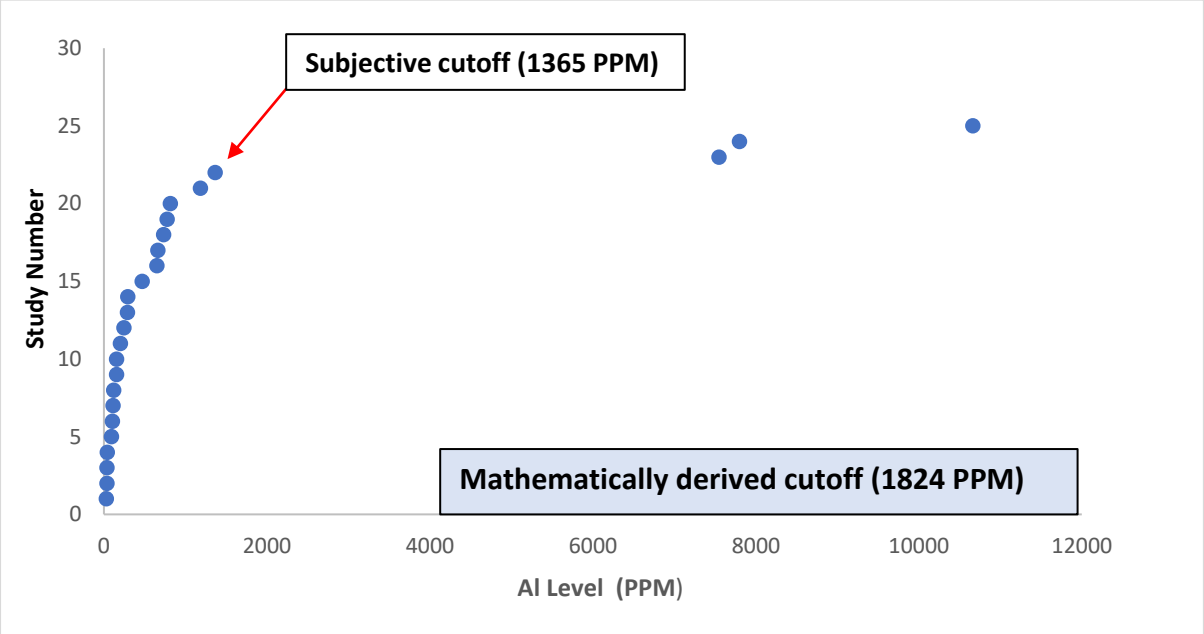


Figure 3.2: Aluminium scatter/dotplot indicating the subjective cut-off. The mathematically derived cut-off is given in the box.

Aluminium is a non-essential plant element. As seen in **Figure 3.2,** the minimum concentration of Al in seagrass throughout the world is 29 PPM while the maximum concentration is 10,622 PPM. The average and median concentrations of Al in seagrass through the world are 1383 and 290 PPM, respectively. The upper subjective background level is 1365 PPM, and the mathematically derived cutoff is 1824 PPM as indicated. Three outliers were identified at 7550, 7799 and 10,662 PPM. These outliers were from a study by Thangaradjou et al. 2013 (Lakshadweep, India) and represent three different species of seagrass *Halodule pinifolia*, *Halodule uninervis*, *Halophila decipiens* respectively. The Lakshadweep islands are under increasing anthropogenic pressure due to rapid developments in infrastructure and tourism that could exert pressure and cause pollution in the fragile ecosystems of these islands.

3.3.2 Arsenic (As)

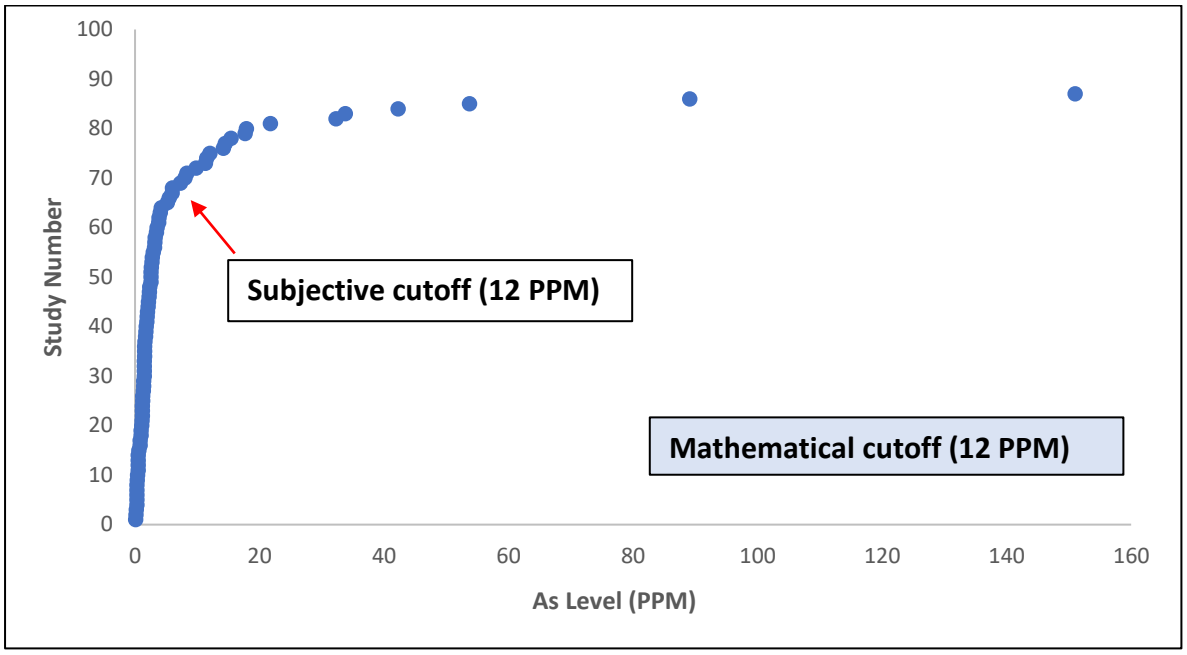


Figure 3.3: Arsenic scatter/dotplot indicating the subjective cut-off. The mathematically derived cut-off is given in the box on the right.

Arsenic is a non-essential plant element. As seen in **Figure 3.3** the minimum concentration of As in seagrass throughout the world is 0.12 PPM while the maximum concentration is 151 PPM. The average and median concentrations of As in seagrass throughout the world are 8 and 2 PPM, respectively. Both the upper subjective background level and the mathematically derived cutoff are identical at 12 PPM, as indicated. Outliers were identified at 14, 15, 18, 22, 32, 34, 42, 54, 89 and 151 PPM. The following references follow the order of these outliers in the following studies. Vizzini et al. 2013 commented that identified outliers of overall trace element levels were comparable with the ranges found in “slightly and moderately contaminated” Mediterranean lagoons. Bonanno & Borg 2018 reported urban seaside resorts sites that are affected by trace element inputs due to untreated municipal wastewaters and pollution spills from marine traffic. Farias et al. 2018 reports poor water quality, industrial discharges, sewage treatment plants, aquaculture and a recorded history of heavy metals. The Bonanno & Martino 2016 site was similar to Bonanno & Borg 2018. The Serrano et al. 2019 site is considered to be highly impacted; 18 and 42 PPM from Birch et al. 2018B is considered highly impacted. 22 and 32 PPM from Birch et al. 2018A is similar to Birch et al. 2018B pollution profile. Lin et al. 2016 is considered

highly impacted. Lin et al. 2018 is similar to Lin et al. 2016. Serrano et al. 2011 is considered elevated despite the 89 PPM which puts it in the highly impacted category; Ward & Hutching 1996 is considered highly impacted.

3.3.3 Cadmium (Cd)

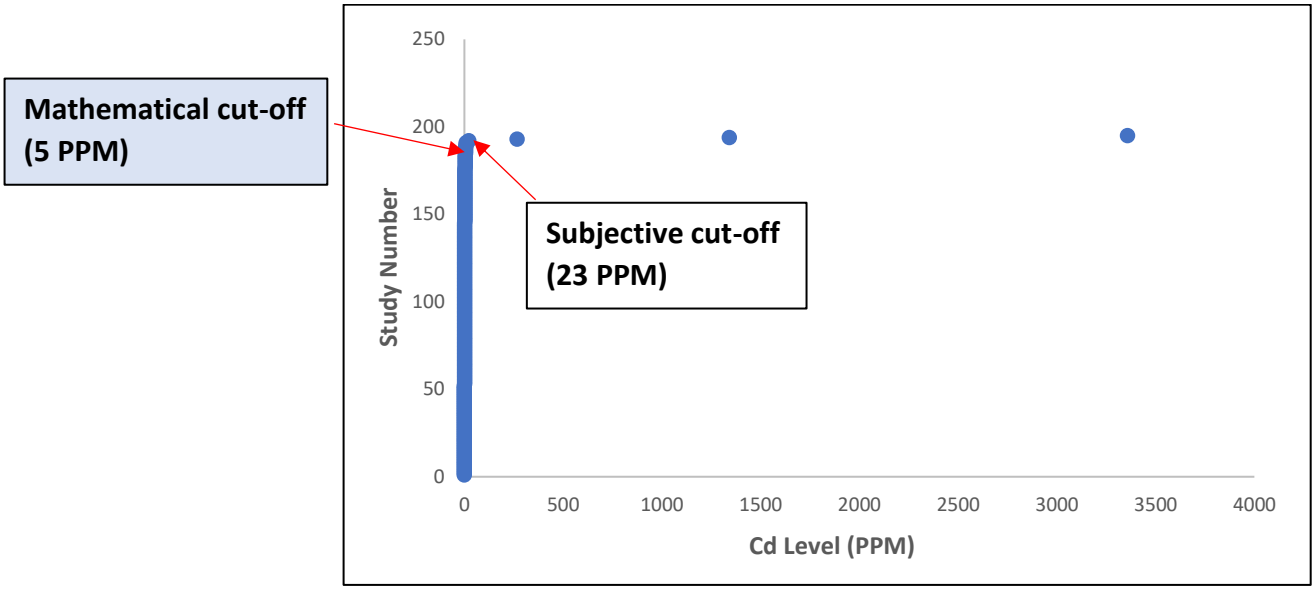


Figure 3.4: Cadmium scatter/dotplot indicating the subjective cut-off. The mathematically derived cut-off is given in the blue box on the left. Refer to **Figure 3.5** for enlargement of the 0 – 10 PPM section. This illustrates how the horizontal range is manipulated within excel as part of the determination method.

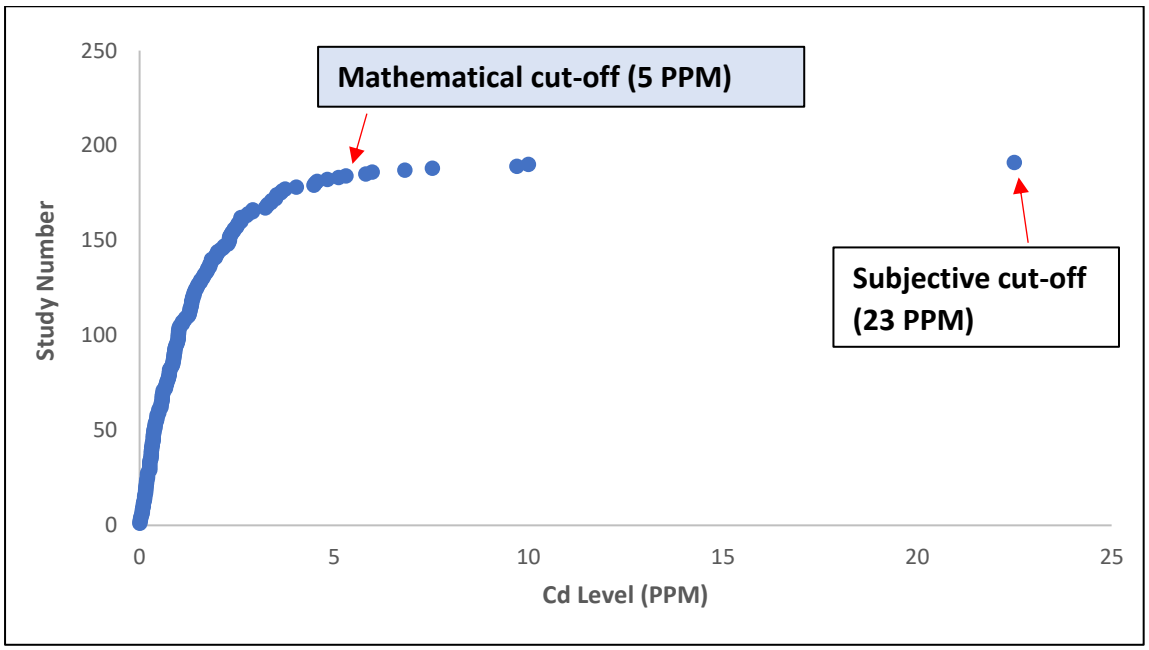


Figure 3.5: Cadmium scatter/dotplot indicating the subjective cutoff and the mathematically derived cut-off. This is an enlargement of the 0 – 10 PPM section of **Figure 3.4.**

Cadmium is a non-essential plant element. As seen in **Figures 3.4, 3.5** and the boxplot in **Figure 3.18** the minimum concentration of Cd in seagrass through the world is close to zero PPM whilst the maximum concentration as high as 3358 PPM. The average and mean concentrations of Cd in seagrass through the world are 27 and 1 PPM, respectively. The upper subjective background level and the mathematically derived cutoffs are 23 and 5 PPM respectively, as indicated. Eleven outliers were identified. The first eight are considered "Elevated" (5 to 23 PPM) and the final three were designated as "Highly Impacted". These outliers were found in the following studies: (5 to 23 PPM): Hu et al. 2019 is considered elevated; Cozza et al. 2013 at 5.82 is considered elevated. The Conti et al. 2007 claims the site has no sources of seawater contamination and it is supposedly exposed to a very limited anthropogenic impact, both in terms of time and quantity. However with *Posidonia oceanica* leaves have a concentration of 6 PPM just meets the minimal requirements elevated at 5 PPM. Wilkes et al. 2017; Cozza et al. 2013 and Ward & Hutching 1996 are considered highly impacted. Barwick & Maher 2003 supports large commercial and recreational fisheries and is considered elevated. Both Ward & Hutching 1996 (22.5 and 267 PPM) results and Lin et al. 2018 (1341 PPM) are considered highly impacted. Lin et al. 2018 (3358 PPM) is considered highly impacted.

3.3.4 Cobalt (Co)

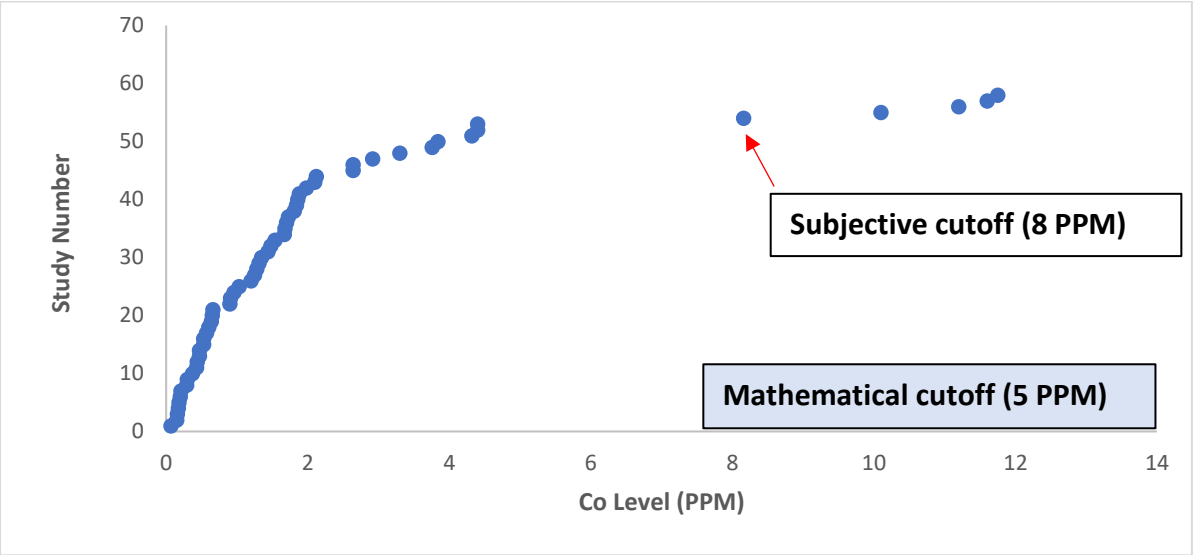


Figure 3.6: Cobalt scatter/dotplot indicating the subjective cutoff. The mathematically derived cut-off is given in the blue box.

Cobalt is an essential plant micronutrient. As seen in **Figure 3.6** and the boxplot in **Figure 3.20** the minimum concentration of Co in seagrass throughout the world is close to 0 PPM while the maximum concentration is 12 PPM. The average and median concentrations of Co in seagrass throughout the world are 2.2 and 1.3 PPM, respectively. The upper subjective background level and the mathematically derived cutoffs are 23 and 5 PPM respectively, as indicated. Five outliers were identified at 8, 10, 11.2, 11.6 and 12 PPM. The first three outliers were from a study by Thangaradjou et al. 2013 (Lakshadweep, India), using the whole plants of *Halodule pinifolia*, *Halophila decipiens* and *Halodule pinifolia,* respectively. The Lakshadweep islands are under increasing anthropogenic pressure due to rapid developments in infrastructure and tourism that could exert pressure and pollution on the fragile ecosystems of the islands (Thangaradjou et al. 2013). The next outlier was from a paper of Schroeder & Thorhaug 1980 (Joyuda, Puerto Rico - using *Thalassia testudinum* roots). This site is considered highly impacted. The final outlier was from Nicolaidou & Nott 1998 (Larymna, Greece - using *Cymodocea nodosa* leaves from a site reported to be highly impacted.

3.3.5 Chromium (Cr)

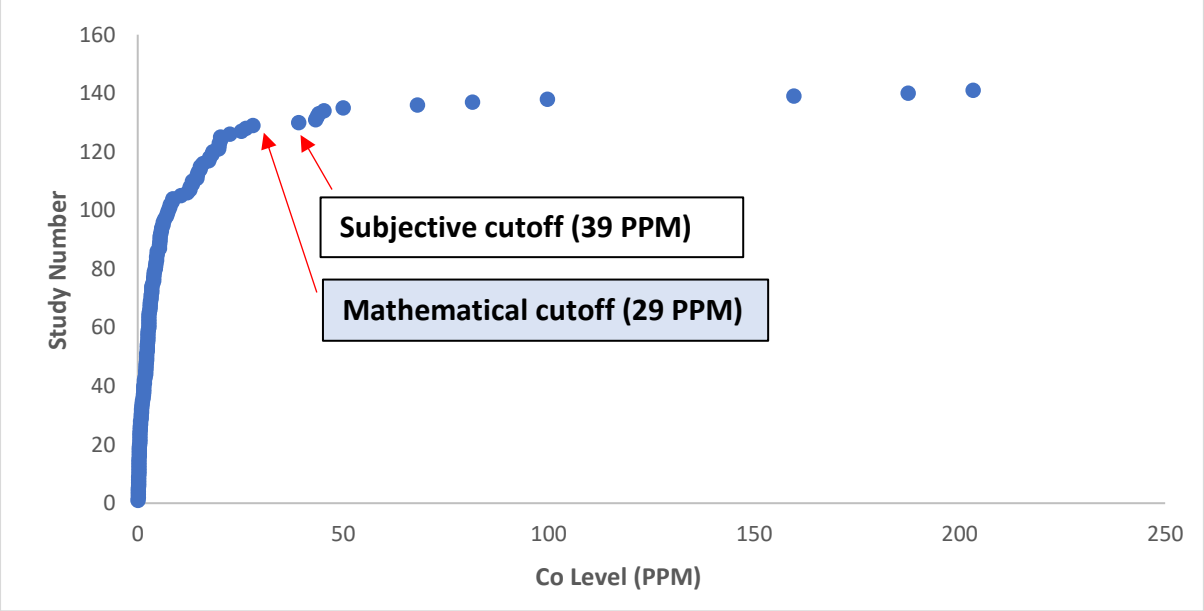


Figure 3.7: Chromium scatter/dotplot indicating the subjective cutoff. The mathematically derived cut-off is given in the blue box.

Chromium is a non-essential plant element. As seen in **Figure 3.7** and the boxplot in **Figure 3.19** the minimum concentration of Cr in seagrass throughout the world is essentially 0 PPM while the maximum concentration is 203 PPM. The average and median concentrations of Cr in seagrass throughout the world are 13 and 3 PPM, respectively. The upper subjective background level and the mathematically derived cutoffs are 39 and 29 PPM respectively, as indicated. Twelve outliers were identified, the first nine values are considered elevated and the final three highly impacted. The elevated levels of 39, 44, 45, 50 and 82 PPM were found in the work of Zhang et al. 2021. This site is considered to be highly impacted due to aquaculture-associated pollution, fast economic development and urbanisation in recent decades, that has caused environmental deterioration primarily due to industrial pollution, agricultural activities and domestic sewage. The elevated levels of 43, 44, 68, 100 PPM were found in Malea et al. 2019A. A highly impacted value of 160 PPM was also found in this paper for the plagiotropic rhizomes. This site is considered highly impacted due to toxic trace elements, originating from human activities. The remaining two highly impacted levels of 187 and 203 PPM were reported in Malea et al. 2019B and Malea & Kevrekidis 2013, respectively. The Malea et al. 2019B site is considered elevated due to agriculture and the Malea & Kevrekidis 2013 site is considered highly impacted due to the coastal area receiving local effluents from a small wastewater treatment plant, direct urban discharges and freshwater inputs from a local stream.

3.3.6 Copper (Cu)

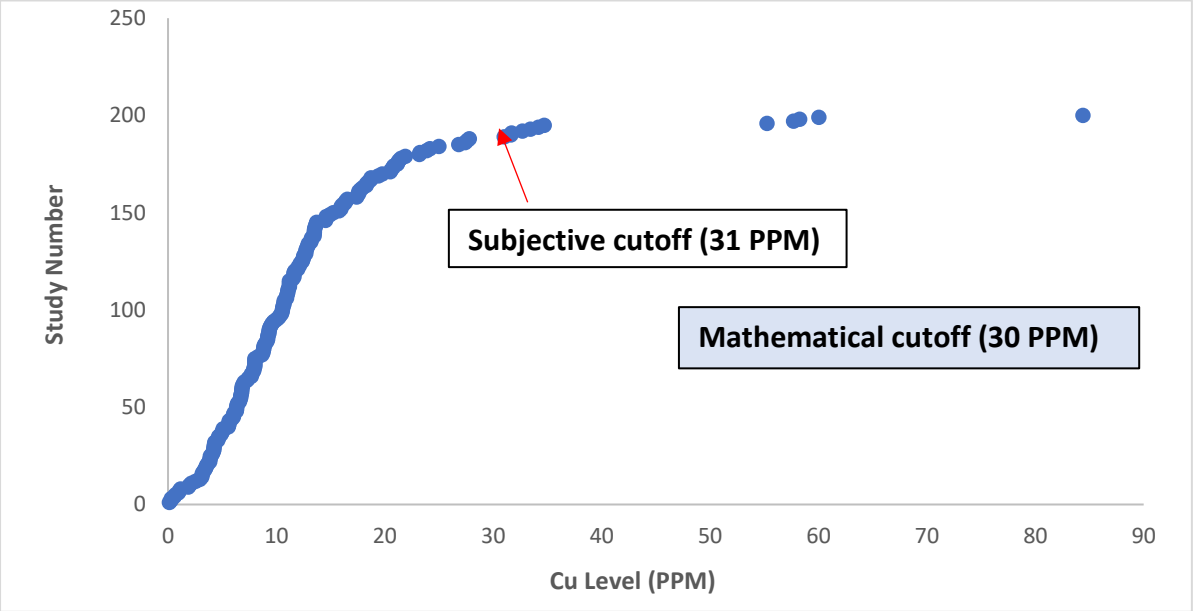


Figure 3.8: Copper scatter/dotplot indicating the subjective cutoff. The mathematically derived cut-off is given in the blue box on the right.

Copper is an essential plant micronutrient. As seen in **Figure 3.8** and boxplot in **Figure 3.20** the minimum concentration of Cu in seagrass through the world is 0 PPM while the maximum concentration is 84 PPM. The average and median concentrations of Cu in seagrass throughout the world are 13 and 11 PPM, respectively. The upper subjective background level and the mathematically derived cutoff are almost the same at 31 and 30 PPM, respectively, as indicated. Twelve outliers were identified at 31, 31.62, 31.7, 32.7, 33.42, 34.18, 34.71, 55.25, 57.7, 58.27, 60.03 and 84.4 PPM. The following reference follow the order of these outliers which are found in the following studies. Ward & Hutching 1996, considered to be a highly impacted site due to the being located in close proximity to a lead smelter Spencer Gulf, South Australia; Bonanno & Raccuia 2018A considered to be highly impacted due to a high level of industrial and municipal wastewaters; Bonanno & Raccuia 2018B has a similar pollution scenario to Bonanno & Raccuia 2018A; Bonanno et al. 2017 is considered elevated; Malea et al. 2019A is considered highly impacted due to toxic trace elements, originating from human activities; Cozza et al. 2013 is considered elevated; Birch et al. 2018B is considered highly impacted; Mishra et al. 2020 site is considered elevated; 57.7 for *Halophila beccarii* Rhizome/Roots and 84.4 their associated leaves

Zhang et al. 2021 site is considered high impacted due to aquaculture-associated pollution, fast economic development and urbanisation in recent decades has caused environmental quality deterioration primarily due to industrial pollution, agricultural activities and domestic sewage (Zhang et al. 2021); 58.27 for *Zostera capricorni* Leaves and 60.03 for their associated rhizomes Birch et al. 2018A has a similar pollution scenario to Birch et al. 2018B.

3.3.7: Iron (Fe)

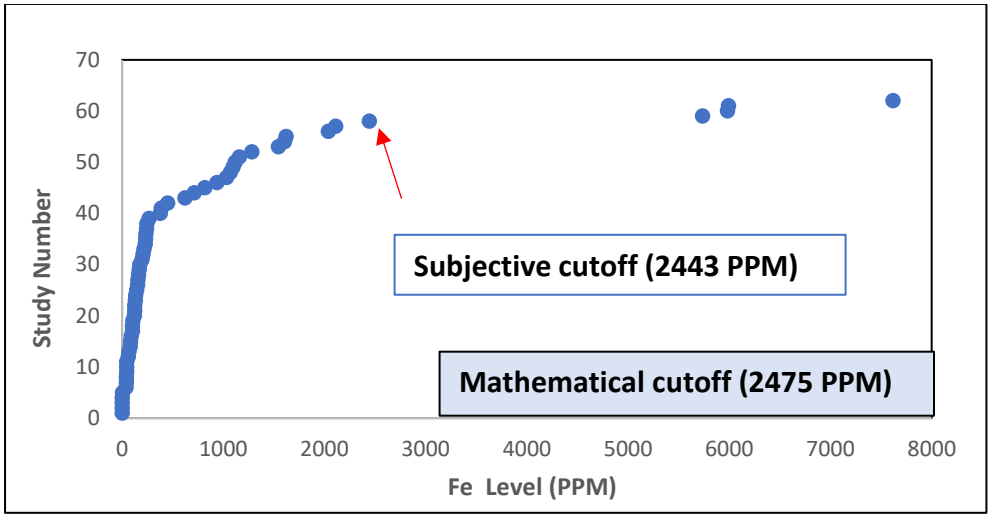


Figure 3.9: Iron scatter/dotplot indicating the subjective cutoff. The mathematically derived cut-off is given in the blue box.

Iron is an essential plant micronutrient. As seen in **Figure 3.9** the minimum concentration of Fe in seagrass through the world is 0.34 PPM while the maximum concentration is 7619 PPM. The average and median concentrations of Fe in seagrass through the world are 845 and 202 PPM, respectively. The upper value for normal distribution subjectively 2443 PPM as seen by the arrow in **Figure 3.9** and the mathematical cut-off is 2475 PPM. Four outliers at 5737, 5982, 5992 and 7618 PPM were identified. The first three were from a study by Thangaradjou et al. 2013 in Lakshadweep, India using *Halodule uninervis, Halophila decipiens* and *Halodule pinifolia* respectively. The Lakshadweep islands are under increasing anthropogenic pressure due to rapid developments in infrastructure and tourism that could exert pressure and pollution on the fragile ecosystems of the islands (Thangaradjou et al. 2013). The fourth outlier was a study by Serrano et al. 2011 using *Posidonia oceanica*

sheaths in Portlligat Bay, Spain which is considered an elevated. This is due to agriculture and fisheries.

3.3.8: Mercury (Hg)

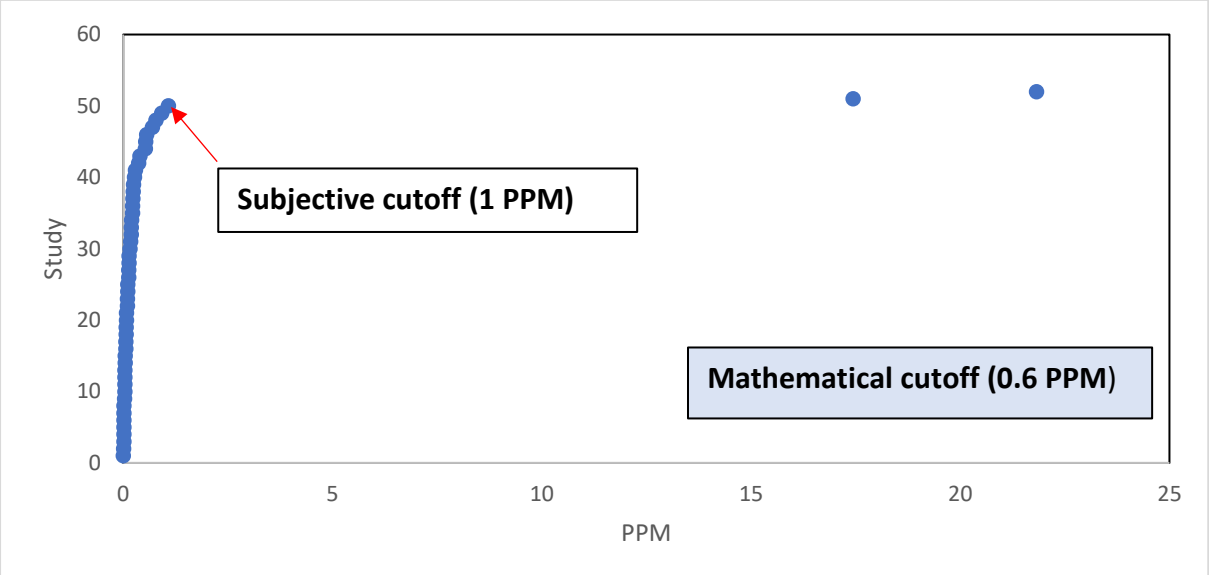


Figure 3.10: Mercury scatter/dotplot indicating the subjective cutoff. The mathematically derived cut-off is given in the blue box on the right. Refer to **Figure 3.11** for enlargement of the 0 – 10 PPM section. Again, this illustrates how the horizontal range is manipulated within excel as part of the determination method.

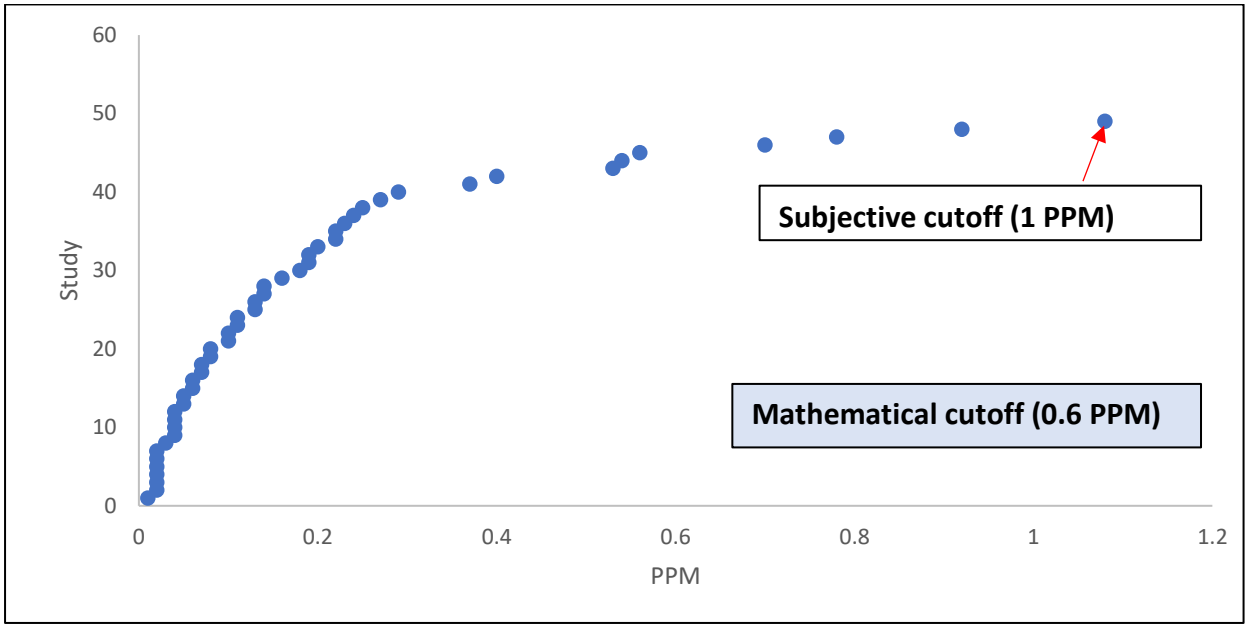


Figure 3.11: Mercury scatter/dotplot indicating the subjective cutoff and the mathematically derived cut-off. This is an enlargement of the 0 – 1.2 PPM section of **Figure 3.10.**

Mercury is a non-essential element. As seen in **Figures 3.10, 3.11** and boxplot in **Figure 3.20** the minimum concentration of Hg in seagrass throughout the world is close to 0 PPM while the maximum concentration is 22 PPM. The average and median concentrations of Hg in seagrass through the world are just under 1 and 0.13 PPM respectively. The upper subjective level is 1.08 PPM, and the mathematically derived cutoff is 0.6 PPM, **Figure 3.11**. Six outliers were identified at, 0.7, 0.78, 0.92, 1.08, 17.44 and 21.82 PPM. The first outlier is from the following study from Montenegro by Stanković et al. 2015 using *Posidonia oceanica* a site considered elevated. This is due human and industrial activities in the coastal areas of the south eastern Adriatic have increased. The next two 0.78 and 1.08 are from a study from the Gulf of Mannar, India by Arisekar et al. 2021 using *Cymodocea serrulate* and *Cymodocea rotundata* respectively. This site is considered pristine due to its location in the Gulf of Mannar Marine National Park. However, at our cutoff at 0.6 PPM this site is considered elevated. The fourth is from Sragnone di Marsla, Sicily by Vizzini et al. 2013 using *Posidonia oceanica* a site considered elevated. Using out cutoff values these four studies are considered elevated while the final two are considered highly impacted. These outliers are reported as being from aboveground biomass such as the leaves and below-ground biomass such as the roots and rhizomes, respectively which, were from a study by Lin et al. 2018 using *Zostera japonica* in the Yellow River, China, which is considered highly impacted due to factory discharges and sewage from fast-expanding cities. Please note that in **Figure 3.11** the zero value and the last two outliers are removed to allow for appropriate scaling.

3.3.9: Manganese (Mn)

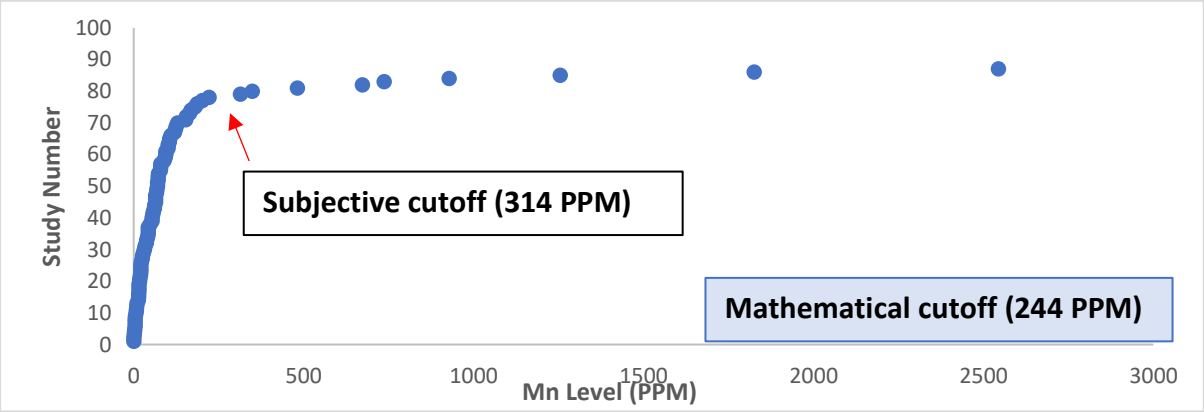


Figure 3.12: Manganese scatter/dotplot indicating the subjective cutoff. The mathematically derived cut-off is given in the blue box.

Manganese is an essential plant micronutrient. As seen in **Figure 3.12** and the boxplot in **Figure 3.18** the minimum concentration of Mn in seagrass through the world is 0.4 PPM while the maximum concentration is 2544 PPM. The average and median concentrations of Mn in seagrass through the world are 160 and 62 PPM, respectively. The upper subjective value is 314 PPM as seen by the arrow and the mathematically derived cutoff is 243 PPM, **Figure 3.12.** Nine outliers were identified at 314, 349, 482, 672, 737, 928, 1255, 1825 and 2544 PPM. The following references follow the order of these outliers for these studies. The Nicolaidou & Nott 1998 site is reported to be a highly impacted. Birch et al. 2018B at 482 and 1825 PPM are at highly impacted sites. Lin et al. 2016 is a highly impacted site; Chernova et al. 2002 is an elevated site; Serrano et al. 2019 is a site considered to be highly impacted. 928.3 and 2544 PPM for Lin et al. 2018 is similar to Lin et al. 2016. Thangaradjou et al. 2013 site is under increasing anthropogenic pressure due to rapid developments in infrastructure and tourism that could exert pressure and pollution on the fragile ecosystems of the islands (Thangaradjou et al. 2013).

3.3.10: Nickel (Ni)

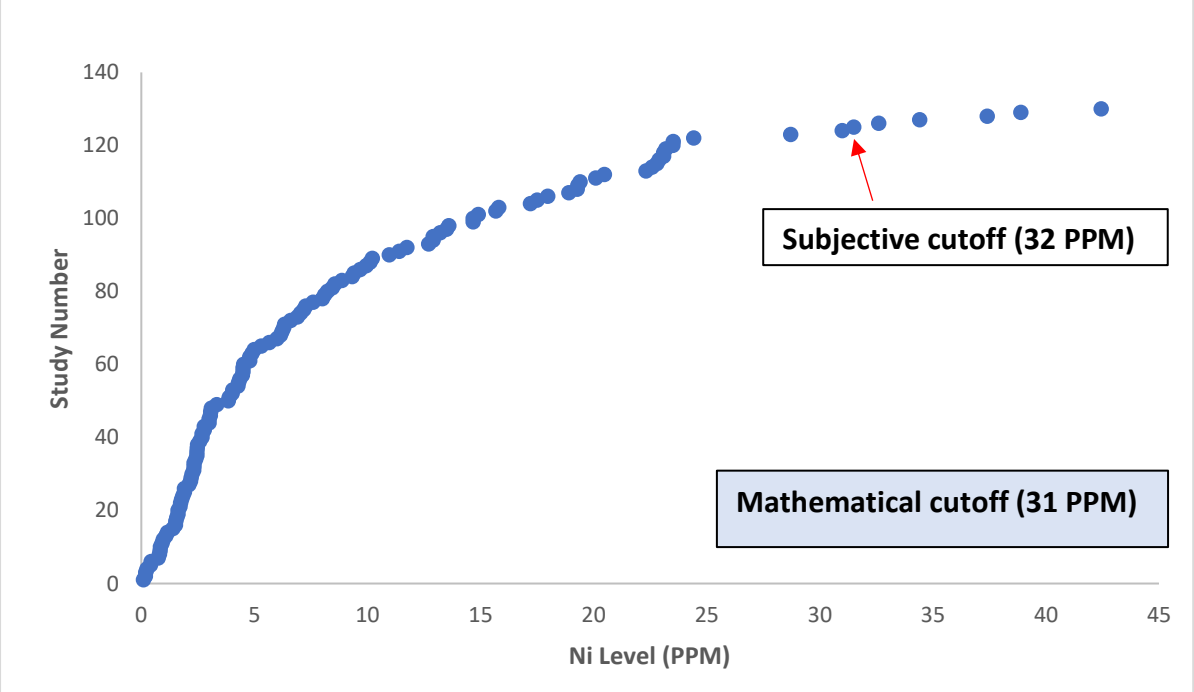


Figure 3.13: Nickel scatter/dotplot indicating the subjective cutoff. The mathematically derived cut-off is given in the blue box.

Nickel is an essential plant micronutrient. As seen in **Figure 3.13** and the boxplot in **Figure 3.20,** the minimum concentration of Ni in seagrass through the world is close to zero PPM while the maximum concentration is 42 PPM. The average and median concentrations of Ni in seagrass through the world are 9 and 5 PPM, respectively. The upper subjective value and the mathematical cutoff levels are very close at 31.5 PPM and 31.18 PPM, respectively. Five outliers were identified at 32, 33, 34, 37, 39 and 42 PPM. These outliers were found in the following studies from Zhelin Bay, China by Zhang et al. 2021, using *Halophila beccarii* leaves. This site is considered to be highly impacted due to aquaculture associated pollution, fast economic development and urbanisation in recent decades that has caused environmental quality deterioration, primarily due to industrial pollution, agricultural activities and domestic sewage. From France, a study by Luy et al 2012 (using *Posidonia oceanica* shoots) considers the site to be elevated. In the northwest Mediterranean, a study by Lopez y Royo et al. 2009 using (*Posidonia oceanica* adult leaves) considers the site to be elevated. From Corsica, a study by Richir et al. 2013 using (*Posidonia oceanica* rhizomes) considers the site to be elevated. Also from Corsica a study by Lafabrie et al. 2007 using *Posidonia oceanica* considers the site to be elevated. Finally, from Larymna, Greece a study by Nicolaidou & Nott 1998 using (*Cymodocea nodosa* leaves) considers the site to be highly impacted.

3.3.11: Lead (Pb)

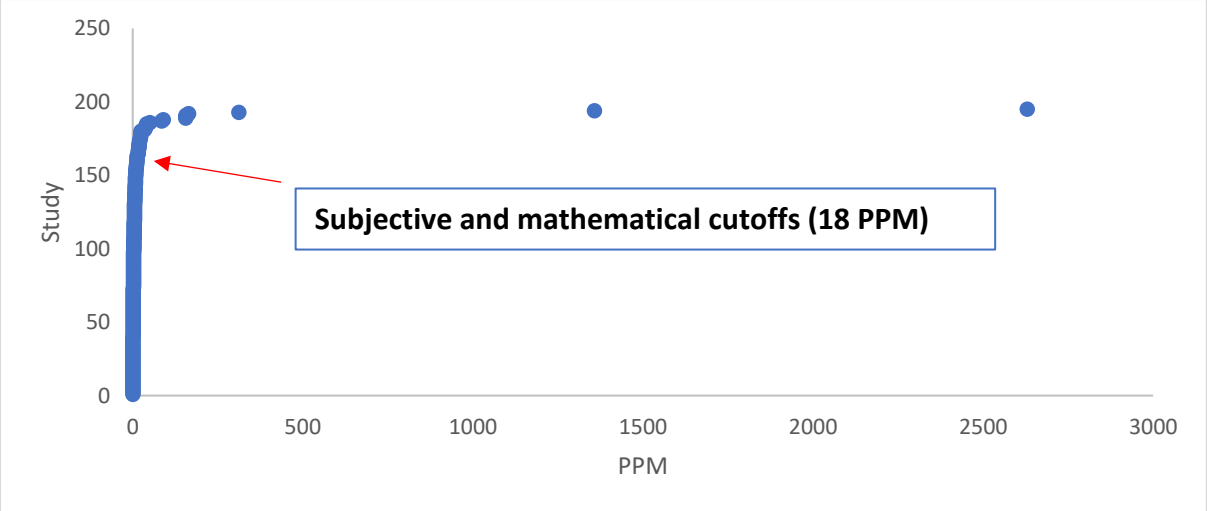


Figure 3.14: Lead scatter/dotplot indicating the subjective cutoff and the mathematically derived cut-off.

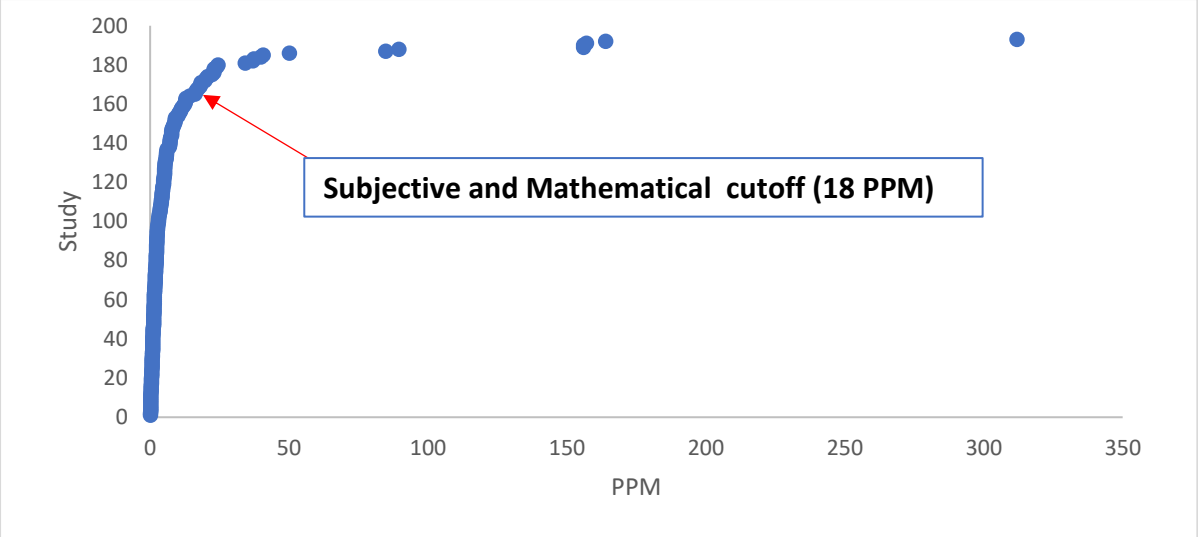


Figure 3.15: Lead scatter/dotplot indicating the subjective cutoff and the mathematically derived cut-off. This is an enlargement of the 0 – 350 PPM section of **Figure 3.14.**

Lead is a non-essential plant element. As seen in **Figure 3.14, 3.15** and the boxplot in **Figure 3.18**, the minimum concentration of Pb in seagrass throughout the world is close to zero PPM while the maximum concentration is 2630 PPM. The average and median concentration of Pb in seagrass through the world are 32 and 3 PPM, respectively. The upper subjective background level and the mathematically derived cutoffs are almost identical at 17.96 and 17.72, respectively, as indicated in **Figure 3.15.** 27 outliers have been identified; the first twelve are elevated while the final fifteen are considered highly impacted. Please note that these outliers have been removed in **Figure 3.15** to obtain appropriate scaling. These outliers were found in the following studies 17.96 PPM for a study by Aljahdali et al. 2020 is a site considered to be elevated. 18.17 PPM a study by Roméo et al. 1995 is a site considered to be highly impacted. 18.37 PPM a study by Vizzini et al. 2013 is a site considered to be elevated. 19.71 PPM a study by Boutahar et al. 2021 is a site considered to be elevated. 20.17 PPM a study by Hu et al. 2019 is a site considered to be elevated. 20.73 PPM a study by Malea et al. 2019A is a site that is highly impacted due to toxic trace elements, originating from human activities. 22.27, 24.46 and 84.81 PPM from Birch et al. 2018A is a site which is considered highly impacted. 22.9 and 40.7 PPM from Zhang et al. 2021 a site considered high impacted due to aquaculture associated pollution, fast economic development and urbanisation in recent decades has caused environmental quality deterioration primarily due to industrial pollution, agricultural activities and domestic sewage (Zhang et al. 2021). 23 PPM from a study by Schroeder & Thorhaug

1980 this site is considered highly impacted. 23.1 PPM from a study by Thangaradjou et al. 2013 this site is under increasing anthropogenic pressure due to rapid developments in infrastructure and tourism that could exert pressure and pollution on the fragile ecosystems of the islands (Thangaradjou et al. 2013). 23.98 and 34.29 PPM is from a study by Lei & Xiaoping 2012 a site that is considered highly impacted due to anthropogenic activities. 37 and 89.5 PPM from a study by Farias et al. 2018 a site that has poor water quality, industrial discharges, sewage treatment plants, the presence of aquaculture and a recorded history of heavy metal (Farias et al. 2018). 37.3 PPM from a study by Lin et al. 2016 is a highly impacted site due to study being located in the Yellow River, China, which is a major area of terrigenous pollution or sediments from terrestrial sources. 39.89 and 50.14 PPM from a study by Birch et al. 2018B is similar to Birch et al. 2018A; 156, 312 and 2630 PPM from a study by Ward & Hutching 1996 a highly impacted site. 156 157 and 164 PPM from a study by Malea & Haritonidis 1999 is a site considered to be elevated. 1358 PPM from a study by Serrano et al. 2019 is a site considered to be highly impacted.

3.3.12: Zinc (Zn)

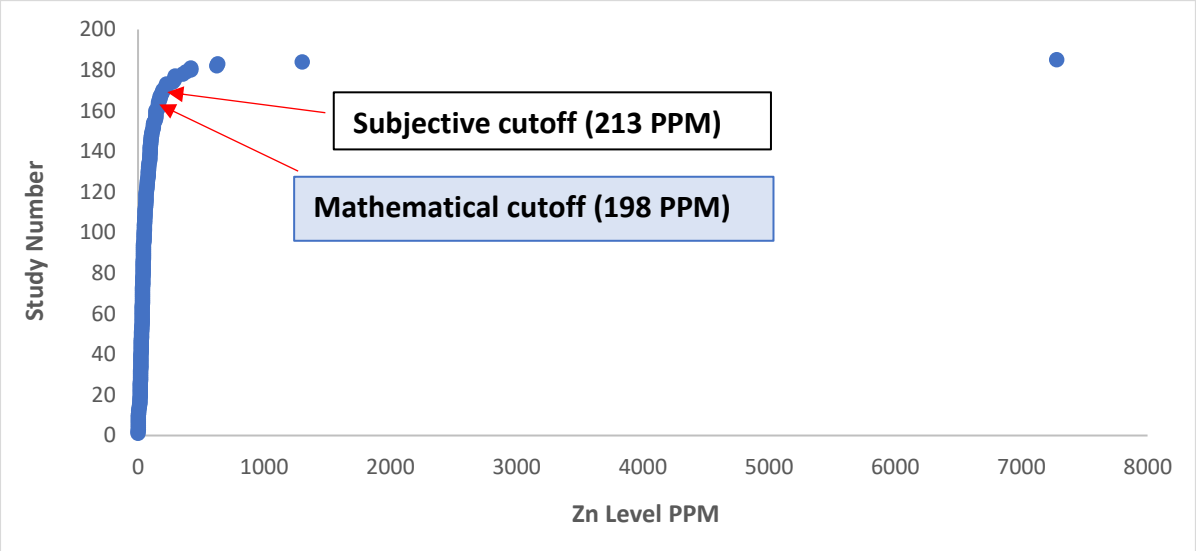


Figure 3.16: Zinc scatter/dotplot indicating the subjective cutoff and the mathematically derived cut-off.

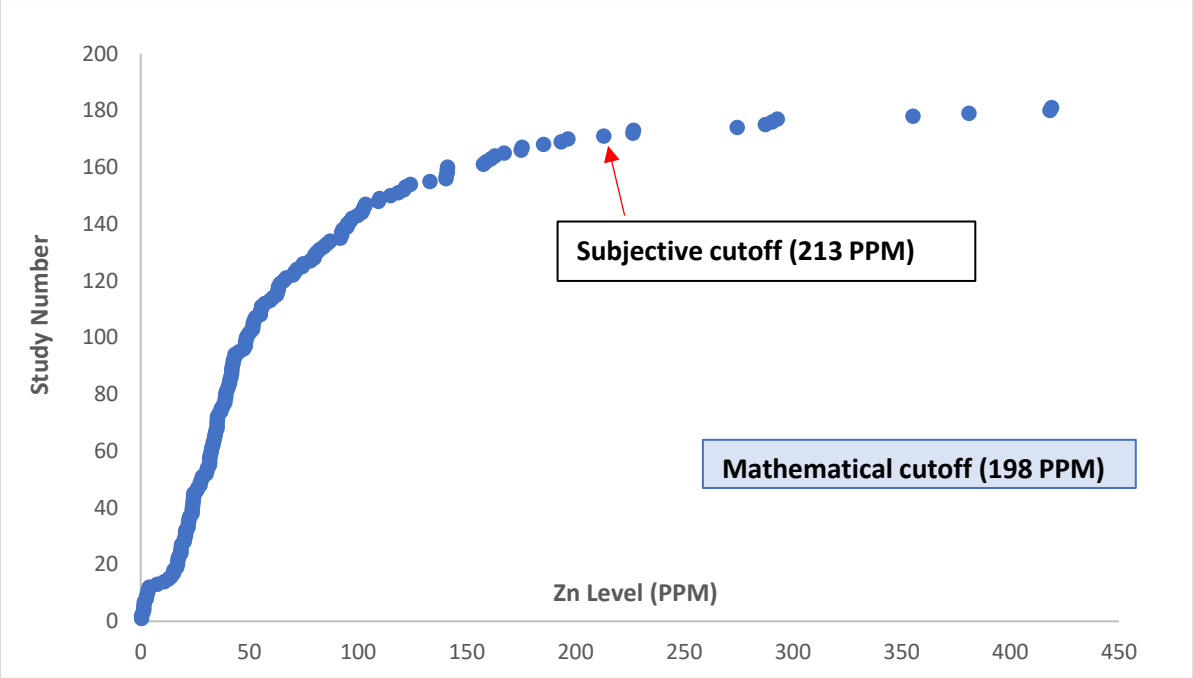


Figure 3.17: Zinc scatter/dotplot indicating the subjective cutoff and the mathematically derived cut-off. This is an enlargement of the 0 – 350 PPM section of **Figure 3.16.** Again, this illustrates how the horizontal range is manipulated within Excel as part of the determination method.

Zinc is an essential plant micronutrient. As seen in **Figure 3.16, 3.17** and the boxplot in **Figure 3.18**, the minimum concentration of Zn in seagrass throughout the world is near 0 PPM while the maximum concentration is 7280 ppm. The average and median concentrations of Zn in seagrass through the world are 123 and 44 PPM, respectively. The upper subjective background level and the mathematically derived cutoffs are 213 and 198 PPM, respectively, as indicated in **Figure 3.17.** 15 outliers were identified at 213, 226.38, 226.67, 274.41, 287.4, 290.5, 292.75, 355.25, 381, 418.32, 418.98, 624, 631, 1300 and 7280 PPM. These outliers were found from the following studies: 213 and 287.4 PPM from Conti et al. 2007 has no sources of seawater contamination and it is supposedly exposed to a very limited anthropogenic impact, both in terms of time and quantity. However, the *Posidonia oceanica* (leaf tips) concentration was reported at 287 PPM which is considered elevated. 226.38 PPM from a study from Birch et al. 2018A which is considered to be highly impacted. 226.67, 290.5, 292.72 and 355.25PPM is from a study by Tranchina et al. 2005 a site considered to be elevated. 274.41 PPM from a study by Shabaka et al. 2021 is a site considered to be elevated. 381 and 624 PPM from a study by Farias et al. 2018 is a site that has poor water

quality, industrial discharges, sewage treatment plants, the presence of aquaculture and a recorded history of heavy metal (Farias et al. 2018). 418.32 PPM is from a study by Faganeli et al. 1997 a site considered to be highly impacted. 418.98 from a study by Serrano et al. 2019 a site considered to be highly impacted; 631, 1300 and 7280 PPM is from a study by Ward & Hutching 1996 a highly impacted site. Note the last four outliers as seen in **Figure 3.17** were omitted to allow for scaling.

3.4 Boxplot data

Figures 3.18-3.20 show the boxplots themselves that were used to determine the mathematically derived cut-offs according to the method described in **Chapter 2, PART A.**

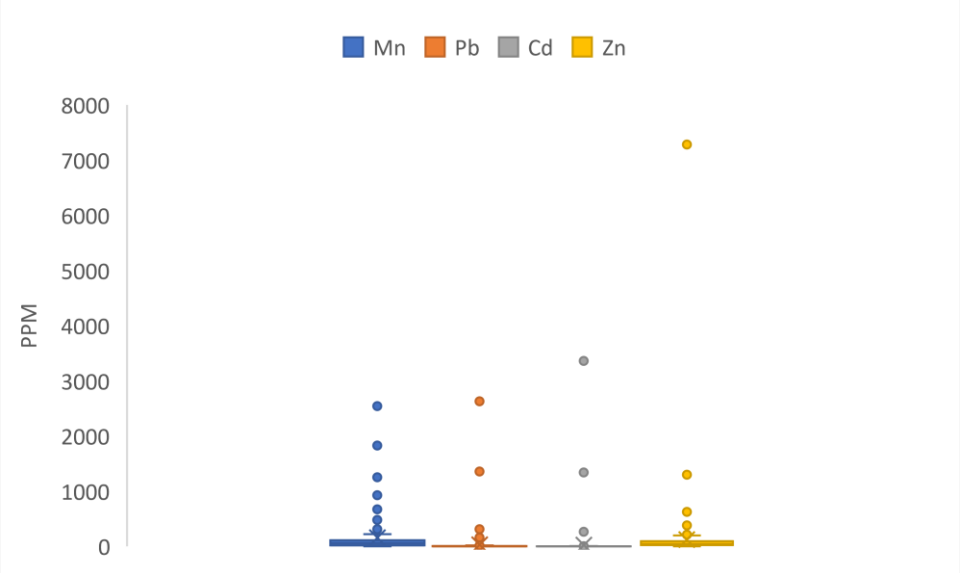


Figure 3.18*:* Mn, Pb, Cd and Zn magnitude ranges in seagrass units in PPM

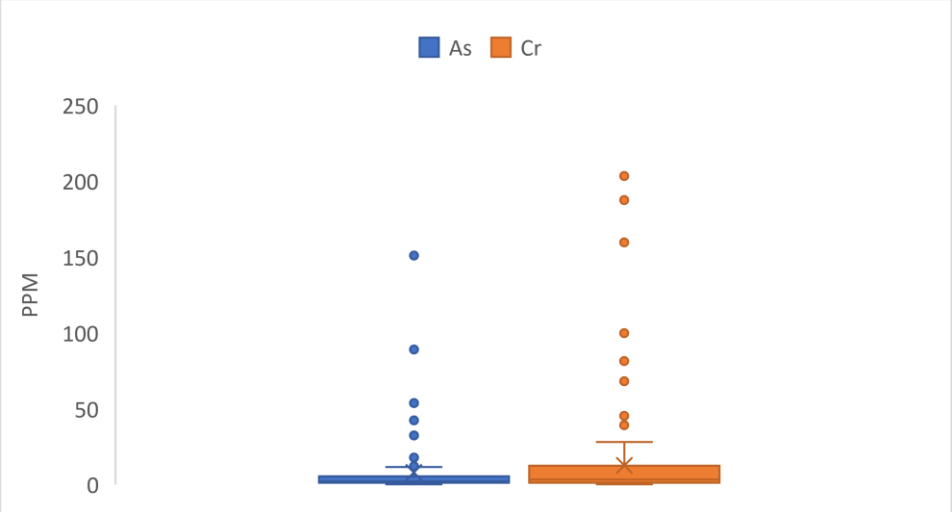


Figure 3.19*:* As and Cr magnitude ranges in seagrass units in PPM

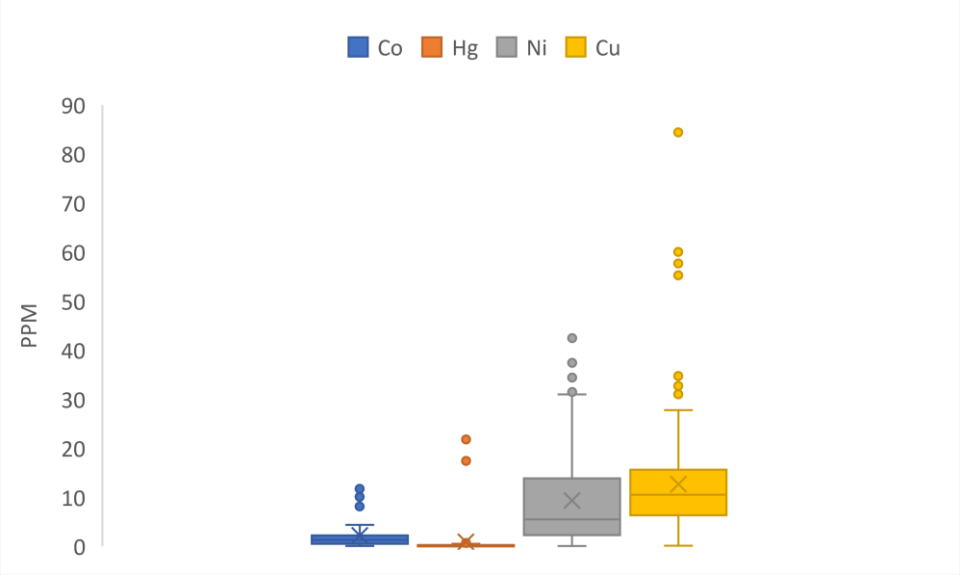


Figure 3.20*:* Co Hg, Ni, Cu magnitude ranges in seagrass units in PPM

3.5 Validation between the subjective (dotplot) and mathematically derived (boxplot) cutoffs

The cut-off values for the subjective (dotplot) (**Figures 3.2 - 3.17**) and mathematical (boxplots) (**Figure 3.18 - 3.20**) parameters have been successfully correlated, **Figure 3.21**, as a means of validation; this data is also represented in **Table 3.3**. It is important to note that this method was also guided by a detailed scrutiny of each individual paper in order to identify sites that were categorized by the authors as “polluted”, as described in the commentaries.

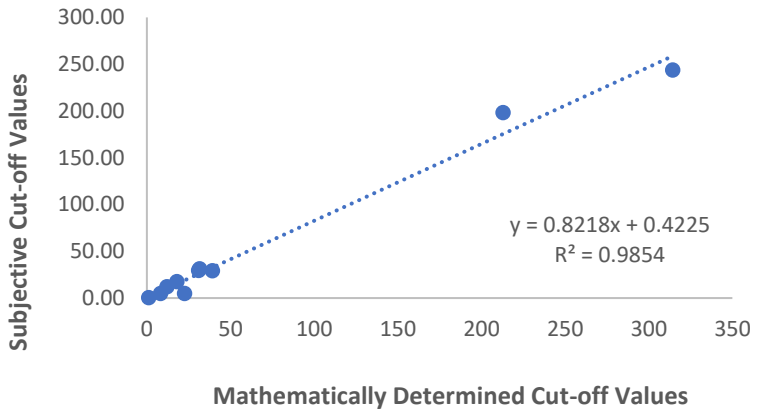


Figure 3.21: Correlation between "subjective" and "mathematically determined" (i.e., via boxplots) background/elevated cut-off values for the "industrially relevant metals As, Cd, Co, Cr, Cu, Hg, Mn, Ni, Pb and Zn.

Table 3.3*: "Subjective" (from dot-plots) cut-off values versus "Mathematical" (from boxplots) cut-off values, correlated in Figure 3.21.*

	"Subjective" Cut-offs	"Mathematical" Cut-offs Q3 + (1.5 X IQR)
As	12.04	12.01
Cd	22.5	4.84
Co	8.16	4.85
Cr	39.2	29.04
Cu	31	29.64
Hg	1.08	0.60
Mn	314.45	243.80
Ni	31.5	31.18
Pb	17.96	17.72
Zn	213	198.32

3.6 – ICP-AES method validation

3.6.1 Standard Reference Material

The SRM AGAL-6 (cabbage standard) was used to validate the ICP-AES method (as described in Chapter 2.7) using the metals Ba, Cd, Co, Cr, Cu, Mn and Ni. The results are depicted in **Table 3.4** and **Figure 3.22.**

Table 3.4: Method validation of against SRM AGAL-6 (cabbage leaf).

Element	AGAL-6 SRM (PPM)	ICP-AES Measurement
Ba	47.1	47.5
Cd	0.1	4.7

Co	0.3	0.8
Cr	1.6	1.2
Cu	54	59
Mn	48	42
Ni	0.7	1.6

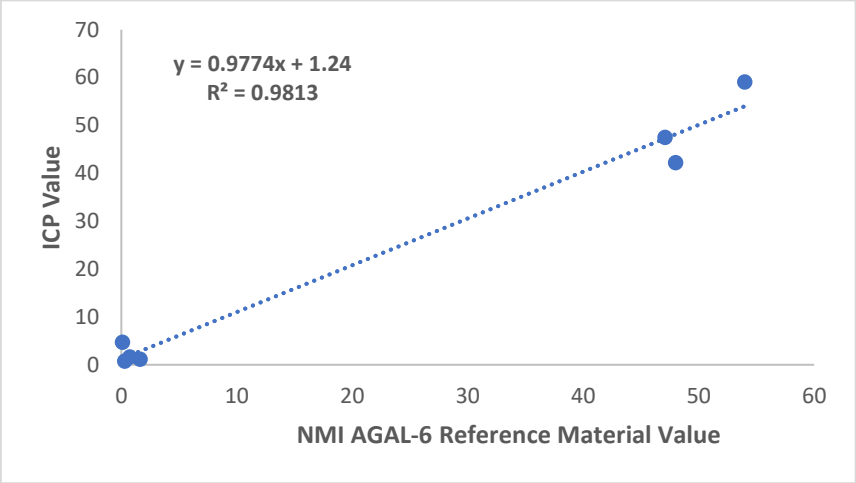


Figure 3.22: Correlation between the ICP-AES method values against the reported AGAL-6 SRM values.

From **Figure 3.22**, the correlation coefficients of 0.98 and 0.9774 are considered satisfactory to validate the ICP-AES method used in this thesis.

3.6.2 Percentage recovery

On the 10th of December 2020, during a break in the COVID-19 pandemic lockdown, a sample was collected at the Altona site (PPB1) for the spiking experiment. The weather for that day was: 25°C, mostly sunny, low tide 4 pm, depth 40 cm. Water abiotic factors were: water temperature 21.4ºC, pH 9.18, dissolved oxygen content 11.28 mg/L, salinity 24.8 ppt. % recoveries were determined for the metals As, Cd, Co, Cr, Cu, Mn, Ni, Pb and Zn be the method described in 2.7. The results are given in Table 3.5.

Table 3.5: Percent recovery of metals in seagrass

Element	As	Cd	Co	Cr	Cu	Mn	Ni	Pb	Zn
% Recovery	118	85	75	81	109	258	108	99	87

The acceptable % recovery range for metals in analytical chemistry is reported to be from 70-120%, Chai et al. 2020. With the exception of Mn, the values ranged from 75 to 118% and this was considered acceptable. The errant reading for Mn could not be explained and limited access to laboratory facilities during the pandemic prevented a follow up on this.

3.6.3: Wavelength Calibration

Calibration curves were preformed every time when a sample run was undertaken. For the Calibration curves results please see **Appendices 9-33**.

3.7: PART B – a case study for the assessment of local temporal data against the derived "background" and "polluted" criteria of PART A.

This section represents a case study for the assay derived in PART A as described in Chapter 2, Part B: Sampling the Victorian Bays.

3.8: Overview of Seagrass, Sediment, Water data

The data set has been split into seasons as seen in **Figure 2.5**. In addition, an overview of the data is provided in the Appendix. Due to the size of the charts, the has been split into in three separate Excel files labelled Seagrass (Appendix 65), Sediment (Appendix 66) and Water (Appendix 67). The Seagrass file contains the whole plant and organ data, while the Sediment contains the sediment data and finally the Water contains the surface and porewater data. Interacting with the charts is the same across the three files. First open the appropriate file then go to the Histogram tab. Clicking on the background of the chart you will see three tabs on the top right side. Choose the third tab from the top labelled Values and Names allows choose what metal and site locations to observe.

3.9: Seagrass order of magnitude comparison

From the international literature database, the order of magnitude for the average (worldwide) concentrations (PPM) of the nine selected "industrial" metals, as reflected in their average bio-accumulated seagrass concentrations, is:

Mn (160.1)< Zn (123.2)< Pb (32.0)<Cd (27.0)< Cu (12.7)< Cr (12.6)< Ni (9.4)< As (8.1)< Co (2.2)

For this study, this the “benchmark ”inequality series, representing “normal” levels of these metals in the worldwide marine environment, as reflected in their average bioaccumulated seagrass concentrations.

For a polluted environment, it would not be unreasonable to expect this order of magnitude to be perturbed (of concern) in some way. Therefore, an examination of order of magnitude series for given locations could provide information about the contamination status of that location. This can be conveniently visualized by arranging such series in a colour-coded matrix as shown in **Table 3.6**.

Table 3.6: From left (highest) to right (lowest), the decreasing order of magnitude of the average concentration levels of ‘industrial metal’ in seagrass plants worldwide, compared with the decreasing order of magnitude of such average concentration levels across the three bays examined in this project (PPB, WP and CI), for the six seasons shown.

	+								-
International literature	Mn	Zn	Pb	Cd	Cu	Cr	Ni	As	Co
Summer 2015/16	Zn	Mn	Cu	Cd	Ni	Cr	Co		
Winter 2018	Zn	As	Cd	Cr	Ni	Co	Pb	Mn	Cu
Spring 2018	As	Zn	Pb	Mn	Cd	Cr	Ni	Cu	Co
Summer 2018/19	As	Zn	Pb	Mn	Cr	Cd	Ni	Co	Cu
Autumn 2019	As	Zn	Pb	Mn	Cr	Cd	Ni	Cu	Co
Winter 2019	As	Zn	Pb	Mn	Cd	Cr	Co	Ni	Cu

From the above Table, the most striking observation is that the As concentration across all three bays is noticeably elevated, for all of the relevant seasons. Also, the series for the Winter of 2018 is grossly perturbed suggesting a major contamination event. This season aside, the rankings of Zn Pb, Mn and Cd appear to be roughly consistent with the international rankings, as do Ni and Co. However, Cu seems to be markedly lower for five of the seasons and elevated for the Summer of 2015/15. These comparisons will be detailed at a more quantitative level in Section 3.6, PART B.

Such a series of inequalities may be further interrogated by a mathematical technique termed a “discrete metric” which is characterized by distances measured on finite dimensional spaces.[4] The utility of this method is demonstrated here by applying one such metric, the “Manhattan” formula, to the measurements upon which **Table 3.7** is based. The relevant general equation is:

$$D(x, y) = \sum_{i=1}^{k} |x_i - y_i|$$

Equation 6: the general “Manhattan” formula

For example, if X = [x(1),….,x(9)] are the “international average background” levels of the 9 metals in **Table 3.5**, in a given order, and Y = [y(1),….,y(9)] are the average measurements of the same 9 metals across the three bays under study, for a given season, and also in the same order, then one can calculate the specific Manhattan metric, D (X, Y), as follows:

$$D(X, Y) = \sum_{i=1}^{9} |x(i) - y(i)|$$

Equation 7: The Manhattan metric for this study

Thus, the D (X, Y) metrics, relative to the international standard (the X series), can be compared for different seasons (different Y series). This quantitatively reveals perturbations in the ordering of the metals.

For example, the data corresponding to the levels of the metals depicted in **Table 3.6** and the calculation of the Manhattan metrics for different seasons are shown in **Tables 3.7 and 3.8.** From these calculations, a quantitative comparison of the Manhattan metrics between the different seasons, with reference to the international background

[4] https://towardsdatascience.com/9-distance-measures-in-data-science-918109d069fa

data, can interrogate the consistency of average heavy metal levels across the bays from season to season, **Figures 3.23 and 3.24.**

Table 3.7: Calculation of the Manhattan metrics (blue values), that compare nine average heavy metal levels across the three bays, over five different seasons.

Metal	Mn	Zn	Pb	Cd	Cu	Cr	Ni	As	Co	D (X, Y)
(International levels)										
x(i), i = 1 to 9	160.1	123.2	32.0	27.0	12.7	12.6	9.4	8.1	2.2	-
(Winter '18 levels)										
y(i), i = 1 to 9	131.3	3159.9	477.9	747.4	23.6	616.2	595.3	1035.3	493.0	
Abs [x(i) – y(i)], i = 1 to 9	28.8	3036.7	445.9	720.4	10.9	490.2	585.9	1027.2	490.8	6836.8
(Spring '18 levels)										
y(i), i = 1 to 9	59.2	89.2	60.9	22.9	11.4	19.1	13.3	301.2	9.9	
Abs [x(i) – y(i)], i = 1 to 9	100.9	34.0	28.9	4.1	1.3	6.5	3.9	293.1	7.7	449.4
(Summer '18/'19 levels)										
y(i), i = 1 to 9	60.8	135.5	86.3	30.6	11.9	51.6	19.3	447.9	16.9	
Abs [x(i) – y(i)], i = 1 to 9	99.3	12.3	54.3	3.6	0.8	39.9	9.9	439.8	14.7	673.7
(Autumn '19 levels)										
y(i), i = 1 to 9	68.3	160.9	104.1	34.3	15.9	34.3	20.4	612.7	11.5	
Abs [x(i) – y(i)], i = 1 to 9	91.8	37.7	72.1	7.3	3.2	21.7	11.0	604.6	9.3	858.7
(Winter '19 levels)										
y(i), i = 1 to 9	59.1	176.4	91.8	37.4	18.0	32.3	19.9	646.4	18.0	
Abs [x(i) – y(i)], i = 1 to 9	101.1	53.2	59.8	10.4	5.3	19.7	10.5	638.3	15.8	914.0

Table 3.8: Calculation of the Manhattan metrics (blue values), that compare six average heavy metal levels across the three bays, over five different seasons. Note that, here, Pb, Cd and As data have been omitted so as to include an extra season, i.e. Summer '15/'16.

Metal	Mn	Zn	Cu	Cr	Ni	Co	D (X, Y)
(International Level)							
x(i), i = 1 to 9	160.1	123.2	12.7	12.6	9.4	2.2	-
(Summer '15/'16)							
y(i), i = 1 to 9	184.1	191.1	56.7	13.8	17.3	3.9	
Abs [x(i) – y(i)], I = 1 to 9	24.0	67.9	44.0	1.2	7.9	1.7	217.8

(Winter ’18 level) y(i), i = 1 to 9 Abs [x(i) – y(i)], I = 1 to 9	131.3 28.3	3159.9 3036.7	23.6 10.9	616.2 490.2	595.3 585.9	493.0 490.8	 4756.9
(Spring ’18 level) y(i), i = 1 to 9 Abs [x(i) – y(i)], I = 1 to 9	59.2 100.9	89.2 34.0	11.4 1.3	19.1 6.5	13.3 3.9	9.9 7.7	 154.3
Summer ’18/’19 y(i), i = 1 to 9 Abs [x(i) – y(i)], I = 1 to 9	60.8 99.3	135.5 12.3	11.9 0.8	51.6 39.9	19.3 9.9	16.9 14.7	 176.0
Autumn ‘19 y(i), i = 1 to 9 Abs [x(i) – y(i)], I = 1 to 9	68.3 91.8	160.9 37.7	15.9 3.2	34.3 21.7	20.4 11.0	11.5 9.3	 174.7
Winter ‘19 y(i), i = 1 to 9 Abs [x(i) – y(i)], I = 1 to 9	59.1 101.1	176.4 53.2	18.0 5.3	32.3 19.7	19.9 10.5	18.0 15.8	 158.3

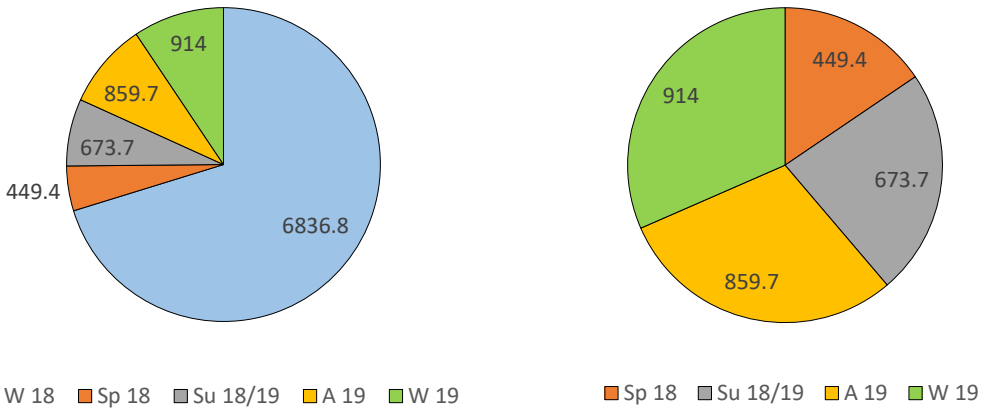


Figure 3.23: Comparison of the relative Manhattan metrics for the average levels of the nine heavy metals, Mn, Zn, Pb, Cd, Cu, Cr, Ni, As and Co, across the three marine bays, for the five seasons, Winter ’18, Spring ’18, Summer ‘18/’19, Autumn ’19 and Winter ’19. The chart on the left includes the “grossly perturbed” Winter ’18 season, where a serious contamination event is suggested. The chart on the right shows the other “normal” seasons and suggests a reasonable consistency of comparative heavy metal levels.

Figures 3.23 and 3.24 demonstrate the utility of employing a discrete metric such as the Manhattan metric in analysing comparative environmental data. The suspected gross contamination event for Winter '18[5], clearly stands out. It may also be confidently stated that there is a reasonable consistency of average heavy metal levels across the bays for all the other seasons. There is obviously many different ways of presenting the data utilizing a discreet metric analysis, that is worthy of a project in its own right. However, this is beyond the scope of this thesis.

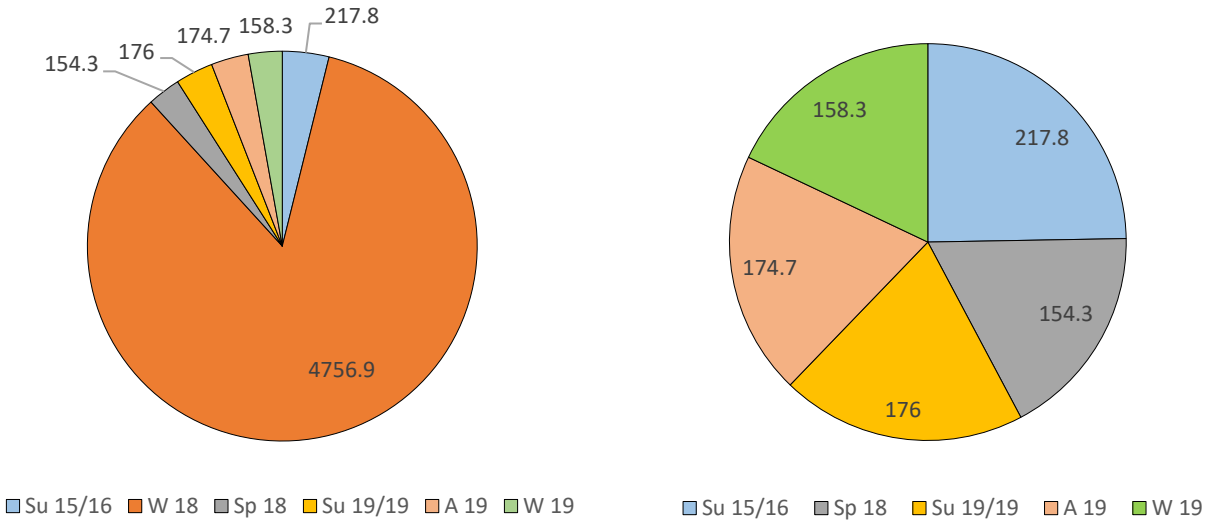


Figure 3.24: Comparison of the relative Manhattan metrics for the average levels of the six heavy metals, Mn, Zn, Cu, Cr, Ni and Co, across the three marine bays, for the six seasons, Winter '18, Spring '18, Summer '18/'19, Autumn '19 and Winter '19. The chart on the left includes the "grossly perturbed" Winter '18 season, where a serious contamination event is suggested. The chart on the right shows the other "normal" seasons and suggests a reasonable consistency of comparative heavy metal levels. Note that, here, Pb, Cd and As data have been omitted so as to include an extra season, i.e. Summer '15/'16

[5] Although instrumental difficulties have not been ruled out for these data.

3.10: Testing the "industrial metal" levels, across the three bays, against the heavy metal assay derived from the totality of the international literature, Table 3.1.

The levels of the metals, As, Cd, Co, Cr, Cu, Mn, Ni, Pb and Zn that were measured in the seagrass samples over the six seasons have been tested against the derived Heavy Metal Assay, Table 3.1. These tests are represented in **Tables 3.9** to **3.17** and **Figures 3.25 to 3.40** below and conclusion are drawn regarding their assessed polluting status. The Tables summarize the average (triplicate) metal concentrations, the 95% confidence intervals and the background levels (as defined by the derived Heavy Metal Assay, **Table 3.1**). To effectively interpret the assay, this data is represented in the corresponding Figures. Within these figures the assay "background level" is represented by an orange horizontal line. Please note 95% confidence intervals are given for the background levels. It should be also noted that this is for the mean value and not the Q_3 of which background level is based on. As the mean and Q_3 are closely aligned the background mean error should be similar to the Q_3 error. Each metal is also represented by two Figures, except for Cu and Mn. The first of such figures include the data for the suspected gross contamination event that was evident in the data for the Winter of 2018. All the metal except Cu and Mn were massively elevated for this period.

3.10.1 - Arsenic

As can be seen in **Table 3.9** and **Figures 3.25 and 3.26**, As was greatly above the international background level in all cases. Excluding the 2018 Winter results As was highest in the Winter of 2019 for Western Port and the lowest for Spring 2018 for Corner Inlet. It should be noted that As is naturally occurring in many Victorian soils and arsenopyrities is commonly associated with gold bearing quartz. The elevated concentrations of As in Port Phillip Bay are attributed to natural sources (Fabris et al. 1999).

Table 3.9: Arsenic levels (PPM) at all sites and background levels (PPM) with 95% conference intervals.

Site/Season	Average As Concentration	95% Confidence Interval	Assay Background Level	95% Confidence Interval
Port Phillip Bay (Winter 2018)	1313.67	258.83	12.01	2.77
Western Port (Winter 2018)	1237.53	551.98	12.01	2.77
Corner Inlet (Winter 2018)	554.57	457.75	12.01	2.77
Port Phillip Bay (Spring 2018)	366.25	76.38	12.01	2.77
Western Port (Spring 2018)	278.56	46.83	12.01	2.77
Corner Inlet (Spring 2018)	258.89	22.16	12.01	2.77
Port Phillip Bay (Summer 2018/19)	486.76	78.18	12.01	2.77
Western Port (Summer 2018/19)	507.41	152.57	12.01	2.77
Corner Inlet (Summer 2018/19)	349.64	82.86	12.01	2.77
Port Phillip Bay (Autumn 2019)	630.45	83.67	12.01	2.77
Western Port (Autumn 2019)	734.61	87.21	12.01	2.77
Corner Inlet (Autumn 2019)	473.12	72.87	12.01	2.77
Port Phillip Bay (Winter 2019)	632.89	129.26	12.01	2.77
Western Port (Winter 2019)	799.81	30.54	12.01	2.77
Corner Inlet (Winter 2019)	506.63	75.83	12.01	2.77

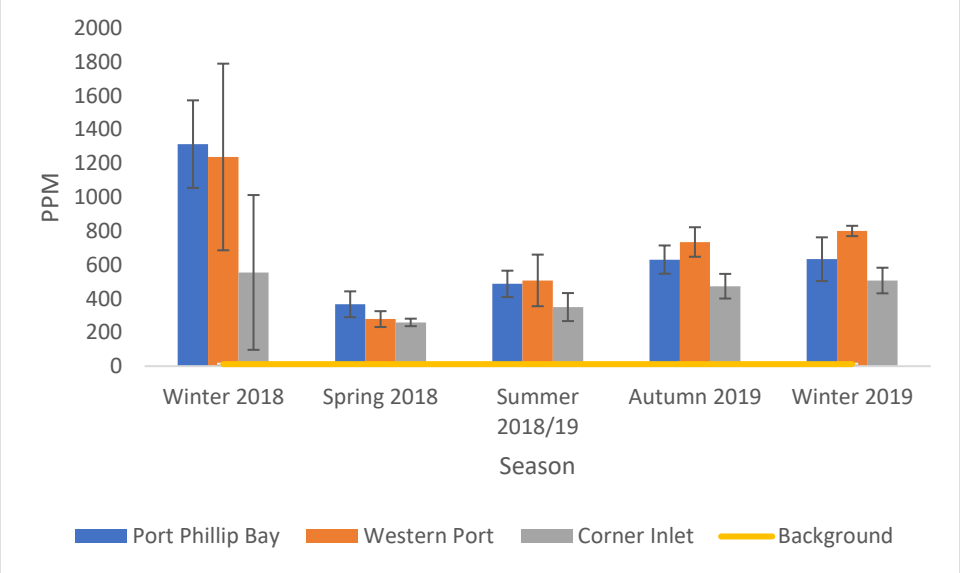


Figure 3.25: Arsenic levels (PPM) with 95% conference intervals for all seasons in comparison with the assay background level - orange horizontal line (PPM). There is a 95% conference interval of 2.77 PPM of the mean for the background level.

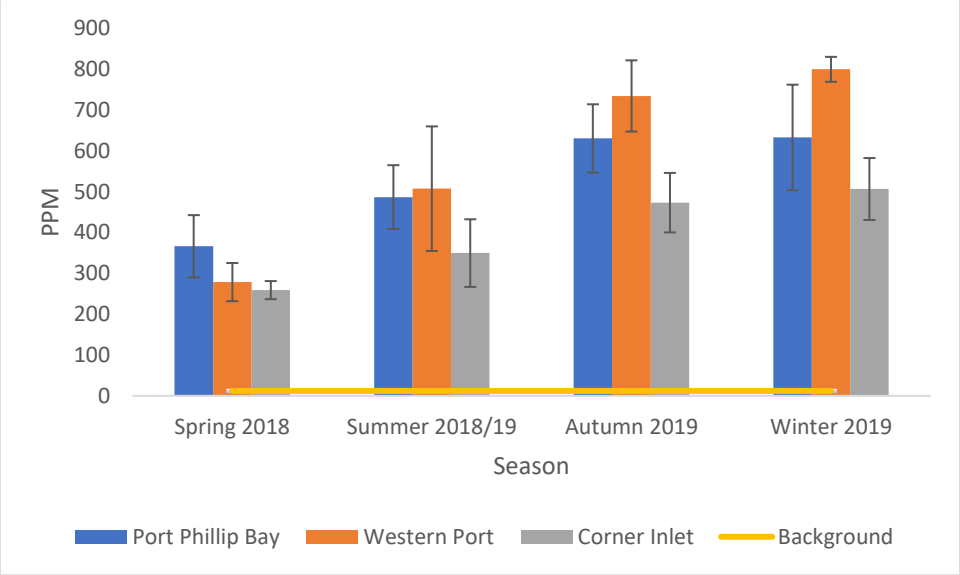


Figure 3.26: Arsenic levels (PPM) with 95% conference intervals for all seasons in comparison with the assay background level - orange horizontal line with (PPM). 2018 Winter data removed.

3.10.2 - Cadmium

As can be seen in **Table 3.10** and **Figures 3.27 and 3.28**, Cd was greatly above the international background level for all cases. Excluding the 2018 Winter results Cd was highest in the Winter of 2019 for Western Port and the lowest for Spring 2018 for Western Port.

Table 3.10: Cadmium levels (PPM) at all sites and background levels (PPM) with 95% conference intervals.

Site/Season	Average Cd concentration	95% Confidence Interval	Assay Background Level	95% Confidence Interval
Port Phillip Bay (Summer 2015/16)	24.27	11.44	4.84	35.8
Western Port (Summer 2015/16)	26.42	16.64	4.84	35.8
Corner Inlet (Summer 2015/16)	28.24	4.02	4.84	35.8
Port Phillip Bay (Winter 2018)	77.90	40.30	4.84	35.8
Western Port (Winter 2018)	1003.76	402.50	4.84	35.8
Corner Inlet (Winter 2018)	1160.38	454.78	4.84	35.8
Port Phillip Bay (Spring 2018)	25.05	5.76	4.84	35.8
Western Port (Spring 2018)	18.64	1.97	4.84	35.8
Corner Inlet (Spring 2018)	25.04	1.92	4.84	35.8
Port Phillip Bay (Summer 2018/19)	34.07	2.95	4.84	35.8
Western Port (Summer 2018/19)	33.94	4.94	4.84	35.8
Corner Inlet (Summer 2018/19)	23.83	5.84	4.84	35.8
Port Phillip Bay (Autumn 2019)	39.29	3.17	4.84	35.8

Western Port (Autumn 2019)	33.65	3.91	4.84	35.8
Corner Inlet (Autumn 2019)	29.96	4.09	4.84	35.8
Port Phillip Bay (Winter 2019)	35.44	5.03	4.84	35.8
Western Port (Winter 2019)	42.06	1.97	4.84	35.8
Corner Inlet (Winter 2019)	34.64	3.95	4.84	35.8

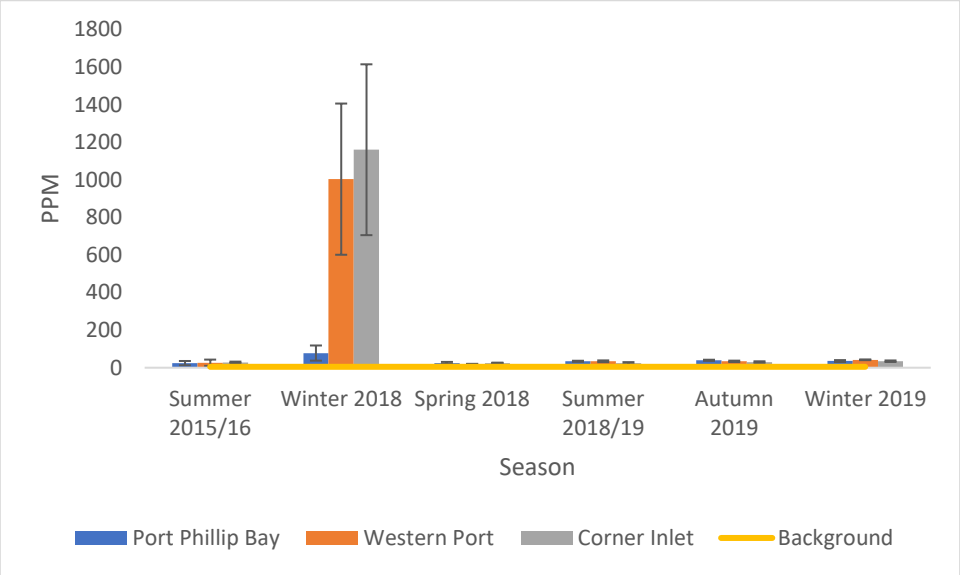


Figure 3.27: Cadmium levels (PPM) with 95% conference intervals for all seasons in comparison with the assay background level - orange horizontal line (PPM). There is a 95% conference interval of 35.8 PPM of the mean for the background level

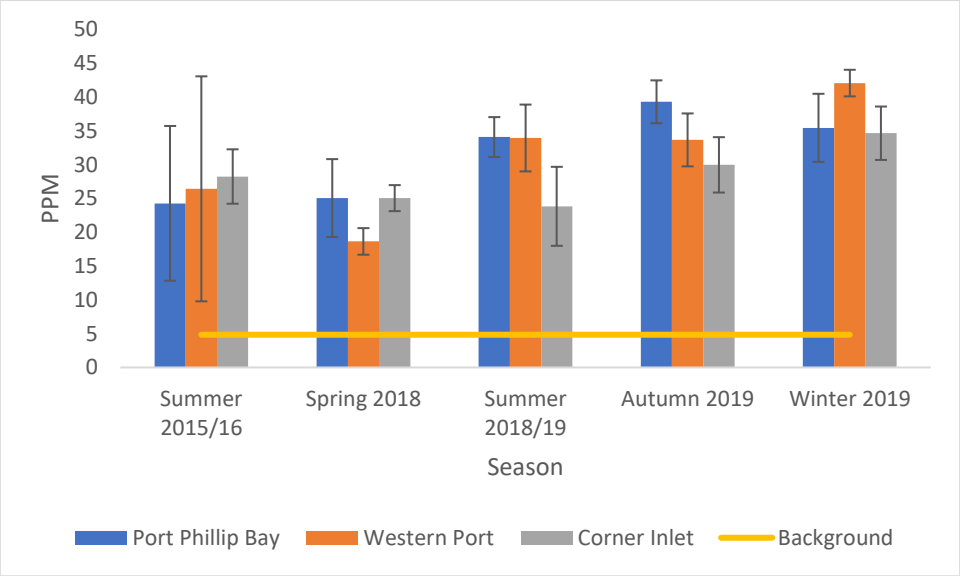


Figure 3.28: Cadmium levels (PPM) with 95% conference intervals for all seasons in comparison with the assay background level - orange horizontal line with (PPM). There is a 95% conference interval of 35.8 PPM of the mean for the background level. 2018 Winter data removed.

3.10.3 - Cobalt

As seen in **Table 3.11** and **Figures 3.29 and 3.30**, Co was significantly higher than the international background level for all cases except for Port Phillip Bay and Corner Inlet Summer of 2015/16 and Port Phillip Bay for Autumn 2019.

Table 3.11: Cobalt levels (PPM) at all sites and background levels (PPM) with 95% conference intervals.

Site/Season	Average Co Concentration	95% Confidence Interval	Assay Background Level	95% Confidence Interval
Port Phillip Bay (Summer 2015/16)	4.23	2.40	4.85	0.39
Western Port (Summer 2015/16)	5.31	3.47	4.85	0.39
Corner Inlet (Summer 2015/16)	2.21	0.74	4.85	0.39
Port Phillip Bay (Winter 2018)	104.73	33.31	4.85	0.39
Western Port (Winter 2018)	598.82	236.93	4.85	0.39
Corner Inlet (Winter 2018)	775.48	300.39	4.85	0.39
Port Phillip Bay (Spring 2018)	11.54	2.33	4.85	0.39
Western Port (Spring 2018)	10.11	1.05	4.85	0.39
Corner Inlet (Spring 2018)	8.04	0.74	4.85	0.39
Port Phillip Bay (Summer 2018/19)	19.58	2.62	4.85	0.39
Western Port (Summer 2018/19)	19.76	2.60	4.85	0.39
Corner Inlet (Summer 2018/19)	11.38	3.00	4.85	0.39
Port Phillip Bay (Autumn 2019)	4.06	0.54	4.85	0.39
Western Port (Autumn 2019)	21.63	1.87	4.85	0.39
Corner Inlet (Autumn 2019)	8.71	0.67	4.85	0.39
Port Phillip Bay (Winter 2019)	19.97	2.79	4.85	0.39
Western Port (Winter 2019)	23.67	1.42	4.85	0.39
Corner Inlet (Winter 2019)	16.86	1.96	4.85	0.39

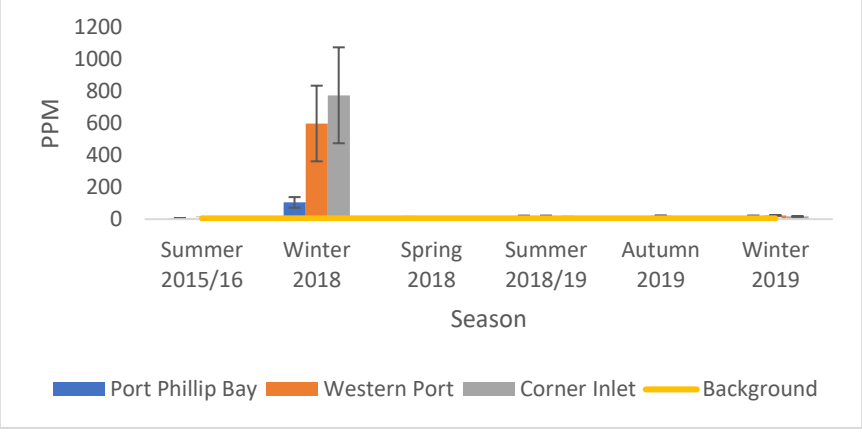


Figure 3.29: Cobalt levels (PPM) with 95% conference intervals for all seasons in comparison with the assay background level - orange horizontal line (PPM). There is a 95% conference interval of 0.39 PPM of the mean for the background level

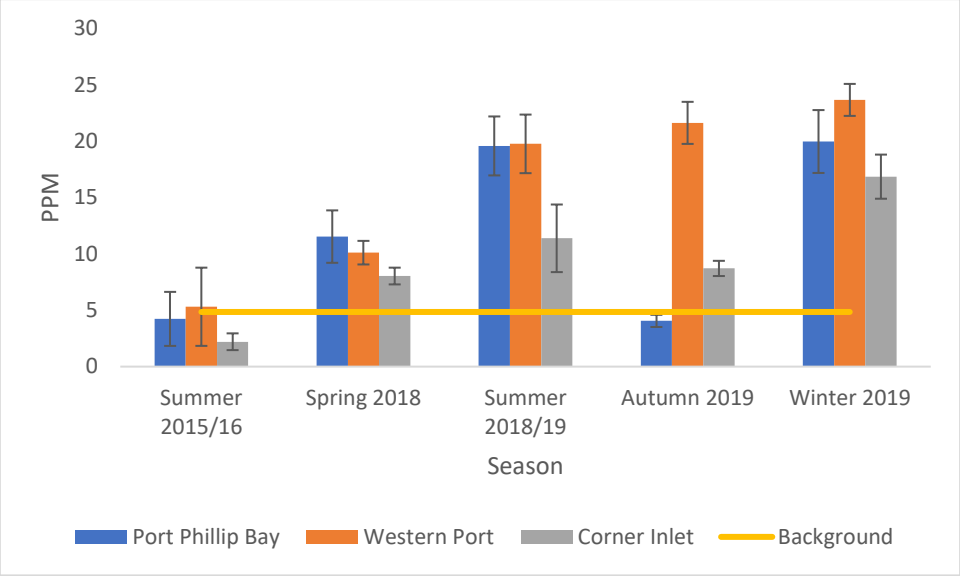


Figure 3.30*:* Cobalt levels (PPM) with 95% conference intervals for all seasons in comparison with the assay background level - orange horizontal line with (PPM). There is a 95% conference interval of 0.39 PPM of the mean for the background level. 2018 Winter data removed.

3.10.4 - Chromium

As seen in **Table 3.12** and **Figures 3.31 and 3.32**, Cr was below background level for Summer 2015/16 for all sites. The level was greatly above background level for Winter 2018. The level was above background level for Port Phillip Bay for the Spring of 2018. The Level was above background level for Western Port and Corner Inlet for the Summer of 2018/19. The level was above background level for Port Phillip Bay and Western Port for the Autumn of 2019. Finally for Winter 2019 Cr was above background level for Western Port.

Table 3.12: Chromium levels (PPM) at all sites and background levels (PPM) with 95% conference intervals.

Site/Season	Average Cr Concentration	95% Confidence Interval	Assay Background Level	95% Confidence Interval
Port Phillip Bay (Summer 2015/16)	9.94	2.93	29.04	4.08
Western Port (Summer 2015/16)	24.18	14.52	29.04	4.08
Corner Inlet (Summer 2015/16)	7.13	2.39	29.04	4.08
Port Phillip Bay (Winter 2018)	92.50	17.46	29.04	4.08
Western Port (Winter 2018)	779.19	320.55	29.04	4.08
Corner Inlet (Winter 2018)	976.77	382.57	29.04	4.08
Port Phillip Bay (Spring 2018)	37.35	18.31	29.04	4.08
Western Port (Spring 2018)	14.26	3.13	29.04	4.08

Corner Inlet (Spring 2018)	5.80	1.08	29.04	4.08
Port Phillip Bay (Summer 2018/19)	26.65	4.50	29.04	4.08
Western Port (Summer 2018/19)	70.55	8.90	29.04	4.08
Corner Inlet (Summer 2018/19)	57.58	7.79	29.04	4.08
Port Phillip Bay (Autumn 2019)	45.24	4.53	29.04	4.08
Western Port (Autumn 2019)	37.23	7.09	29.04	4.08
Corner Inlet (Autumn 2019)	20.48	2.06	29.04	4.08
Port Phillip Bay (Winter 2019)	28.90	6.54	29.04	4.08
Western Port (Winter 2019)	37.87	2.61	29.04	4.08
Corner Inlet (Winter 2019)	30.15	5.26	29.04	4.08

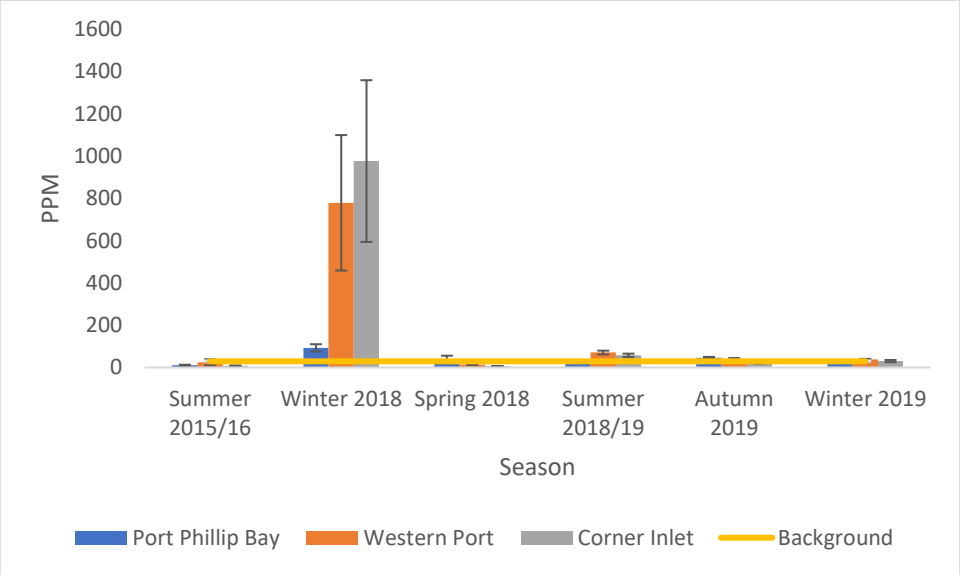


Figure 3.31: Chromium levels (PPM) with 95% conference intervals for all seasons in comparison with the assay background level - orange horizontal line (PPM). There is a 95% conference interval of 4.08 PPM of the mean for the background level

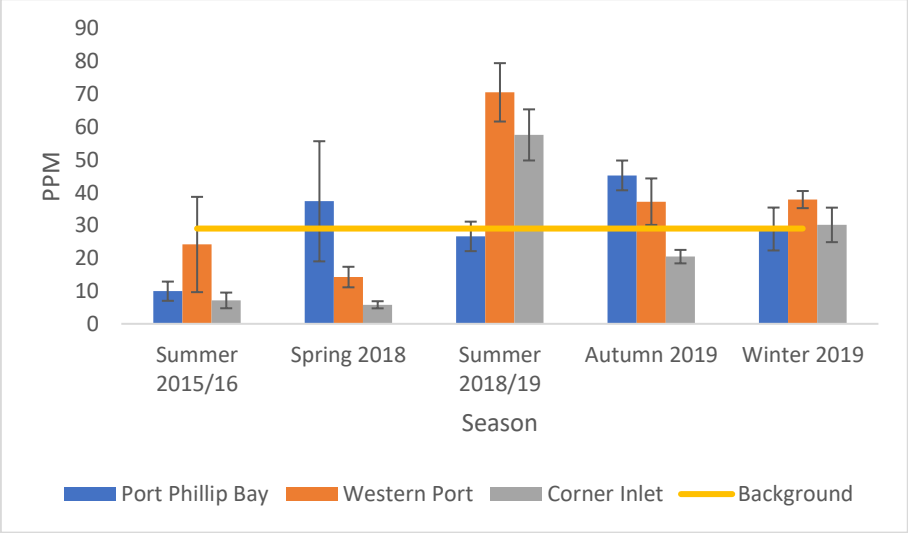


Figure 3.32: Chromium levels (PPM) with 95% conference intervals for all seasons in comparison with the assay background level - orange horizontal line with (PPM). There is a 95% conference interval of 4.08 PPM of the mean for the background level. 2018 Winter data removed.

3.10.5 - Copper

As seen in **Table 3.13** and **Figure 3.33**, Cu was above the international background level for Summer 2015/16 and Port Phillip Bay for Winter of 2018. While Cu was below background level for all other cases. In general Cu was highest in Port Phillip Bay and lowest in Corner Inlet. This can be attributed to anthropogenic pressures decreasing the further the population centres are.

Table 3.13: Copper levels (PPM) at all sites and background levels (PPM) with 95% conference intervals.

Site/Season	Average Cu Concentration	95% Confidence Interval	Assay Background Level	95% Confidence Interval
Port Phillip Bay (Summer 2015/16)	77.35	33.04	29.64	1.52
Western Port (Summer 2015/16)	37.90	26.25	29.64	1.52
Corner Inlet (Summer 2015/16)	54.77	21.39	29.64	1.52
Port Phillip Bay (Winter 2018)	43.88	14.48	29.64	1.52
Western Port (Winter 2018)	20.08	11.06	29.64	1.52
Corner Inlet (Winter 2018)	6.81	3.69	29.64	1.52
Port Phillip Bay (Spring 2018)	23.83	10.17	29.64	1.52
Western Port (Spring 2018)	7.24	1.10	29.64	1.52
Corner Inlet (Spring 2018)	3.21	0.29	29.64	1.52
Port Phillip Bay (Summer 2018/19)	13.80	6.79	29.64	1.52
Western Port (Summer 2018/19)	14.26	3.32	29.64	1.52
Corner Inlet (Summer 2018/19)	7.60	1.92	29.64	1.52
Port Phillip Bay (Autumn 2019)	29.63	10.25	29.64	1.52
Western Port (Autumn 2019)	10.97	1.50	29.64	1.52
Corner Inlet (Autumn 2019)	7.20	1.03	29.64	1.52
Port Phillip Bay (Winter 2019)	28.76	13.69	29.64	1.52
Western Port (Winter 2019)	13.84	3.58	29.64	1.52
Corner Inlet (Winter 2019)	11.37	2.24	29.64	1.52

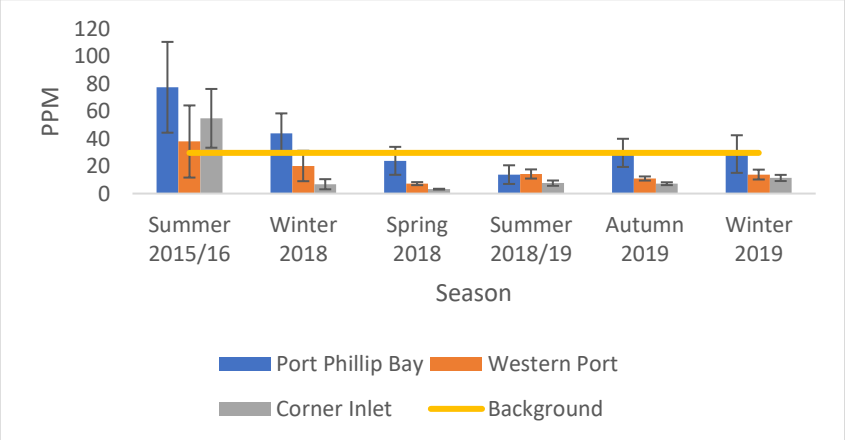


Figure 3.33: Copper levels (PPM) with 95% conference intervals for all seasons in comparison with the assay background level - orange horizontal line with (PPM). Includes 2018 Winter data. There is a 95% conference interval of 1.52 PPM of the mean for the background level

3.10.6 Manganese

As seen in **Table 3.14** and **Figure 3.34**, Mn was below the international background level for all cases. Mn was highest in Corner Inlet for the Winter 2018. While it was lowest in Spring 2018 for Western Port.

Table 3.14*:* Manganese levels (PPM) at all sites and background levels (PPM) with 95% conference intervals.

Site/Season	Average Mn Concentration	95% Confidence Interval	Assay Background Level	95% Confidence Interval
Port Phillip Bay (Summer 2015/16)	205.6	122.8	243.8	51.42
Western Port (Summer 2015/16)	200.0	143.6	243.8	51.42
Corner Inlet (Summer 2015/16)	146.5	21.3	243.8	51.42
Port Phillip Bay (Winter 2018)	115.5	65.2	243.8	51.42
Western Port (Winter 2018)	45.6	23.3	243.8	51.42
Corner Inlet (Winter 2018)	232.9	123.2	243.8	51.42
Port Phillip Bay (Spring 2018)	100.5	41.3	243.8	51.42
Western Port (Spring 2018)	24.2	14.6	243.8	51.42
Corner Inlet (Spring 2018)	52.9	30.7	243.8	51.42
Port Phillip Bay (Summer 2018/19)	92.8	75.5	243.8	51.42
Western Port (Summer 2018/19)	41.8	19.7	243.8	51.42
Corner Inlet (Summer 2018/19)	47.7	15.5	243.8	51.42
Port Phillip Bay (Autumn 2019)	90.5	58.4	243.8	51.42
Western Port (Autumn 2019)	32.6	16.1	243.8	51.42
Corner Inlet (Autumn 2019)	81.6	39.1	243.8	51.42
Port Phillip Bay (Winter 2019)	61.3	15.0	243.8	51.42
Western Port (Winter 2019)	31.1	9.5	243.8	51.42
Corner Inlet (Winter 2019)	84.8	44.4	243.8	51.42

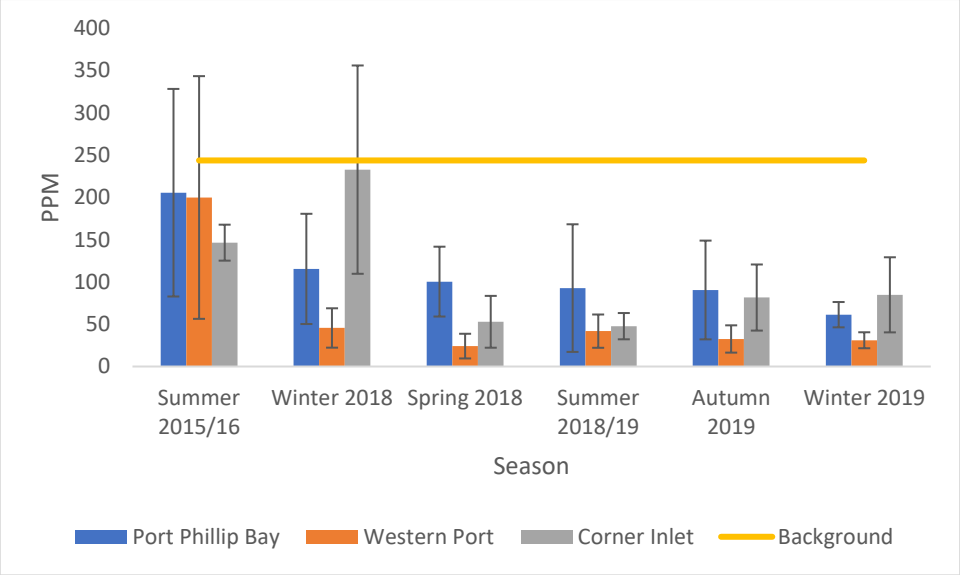


Figure 3.34*:* Manganese levels (PPM) with 95% conference intervals for all seasons in comparison with the assay background level - orange horizontal line with (PPM). Includes 2018 Winter data. There is a 95% conference interval of 51.42 PPM of the mean for the background level

3.10.7 - Nickel

As seen in **Table 3.15** and **Figures 3.35 and 3.36** Ni was below the international background level for all cases except Winter 2018. Exuding the 2018 Winter 2018 results Ni was highest at Western Port Autumn 2019. While Ni was lowest at Corner Inlet 2018.

Table 3.15: Nickel levels (PPM) at all sites and background levels (PPM) with 95% conference intervals.

Site/Season	Average Ni Concentration	95% Confidence Level	Assay Background Level	95% Confidence Level
Port Phillip Bay (Summer 2015/16)	20.11	8.14	31.18	1.31
Western Port (Summer 2015/16)	18.69	10.35	31.18	1.31
Corner Inlet (Summer 2015/16)	13.12	2.19	31.18	1.31
Port Phillip Bay (Winter 2018)	20.93	17.03	31.18	1.31
Western Port (Winter 2018)	785.63	311.59	31.18	1.31
Corner Inlet (Winter 2018)	979.26	378.63	31.18	1.31
Port Phillip Bay (Spring 2018)	16.26	2.76	31.18	1.31
Western Port (Spring 2018)	15.05	3.24	31.18	1.31
Corner Inlet (Spring 2018)	8.58	0.88	31.18	1.31
Port Phillip Bay (Summer 2018/19)	20.54	3.24	31.18	1.31
Western Port (Summer 2018/19)	24.42	1.87	31.18	1.31
Corner Inlet (Summer 2018/19)	13.01	2.42	31.18	1.31
Port Phillip Bay (Autumn 2019)	21.31	2.72	31.18	1.31

Western Port (Autumn 2019)	25.31	3.99	31.18	1.31
Corner Inlet (Autumn 2019)	14.46	1.26	31.18	1.31
Port Phillip Bay (Winter 2019)	20.16	5.27	31.18	1.31
Western Port (Winter 2019)	22.95	2.67	31.18	1.31
Corner Inlet (Winter 2019)	16.63	2.65	31.18	1.31

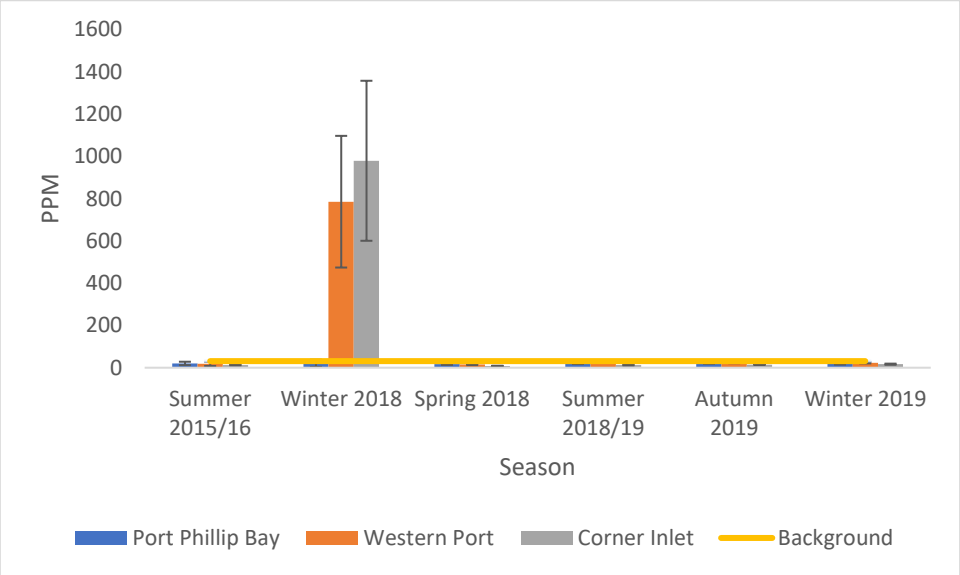


Figure 3.35: Nickel levels (PPM) with 95% conference intervals for all seasons in comparison with the assay background level - orange horizontal line (PPM). There is a 95% conference interval of 1.31 PPM of the mean for the background level

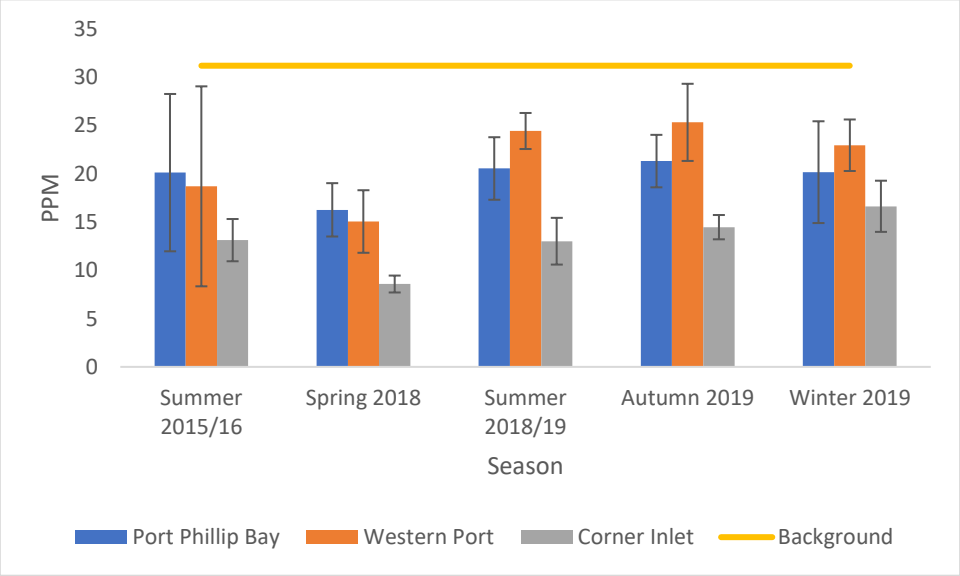


Figure 3.36*:* Nickel levels (PPM) with 95% conference intervals for all seasons in comparison with the assay background level - orange horizontal line with (PPM). There is a 95% conference interval of 1.31 PPM of the mean for the background level. 2018 Winter data removed.

3.10.8 - Lead

As seen in **Table 3.16** and **Figures 3.37 and 3.38**, Pb was significantly above the international background level for all cases. Excluding the 2018 winter results Pb was highest at Western Port Autumn 2019. While Pb was lowest at Corner Inlet for the Spring of 2018.

Table 3.16*:* Lead levels (PPM) at all sites with and background levels (PPM) 95% conference intervals.

Site/Season	Average Pb Concentration	95% Confidence Interval	Assay Background Level	95% Confidence Interval
Port Phillip Bay (Winter 2018)	333.82	77.31	17.72	29.43
Western Port (Winter 2018)	431.90	240.54	17.72	29.43
Corner Inlet (Winter 2018)	667.92	288.30	17.72	29.43
Port Phillip Bay (Spring 2018)	66.62	11.26	17.72	29.43
Western Port (Spring 2018)	70.08	13.25	17.72	29.43
Corner Inlet (Spring 2018)	45.87	4.42	17.72	29.43
Port Phillip Bay (Summer 2018/19)	100.83	12.80	17.72	29.43
Western Port (Summer 2018/19)	99.83	9.06	17.72	29.43
Corner Inlet (Summer 2018/19)	58.09	10.26	17.72	29.43
Port Phillip Bay (Autumn 2019)	106.70	15.98	17.72	29.43
Western Port (Autumn 2019)	124.43	20.79	17.72	29.43
Corner Inlet (Autumn 2019)	81.18	6.05	17.72	29.43
Port Phillip Bay (Winter 2019)	90.31	23.40	17.72	29.43
Western Port (Winter 2019)	106.68	7.69	17.72	29.43
Corner Inlet (Winter 2019)	78.29	11.36	17.72	29.43

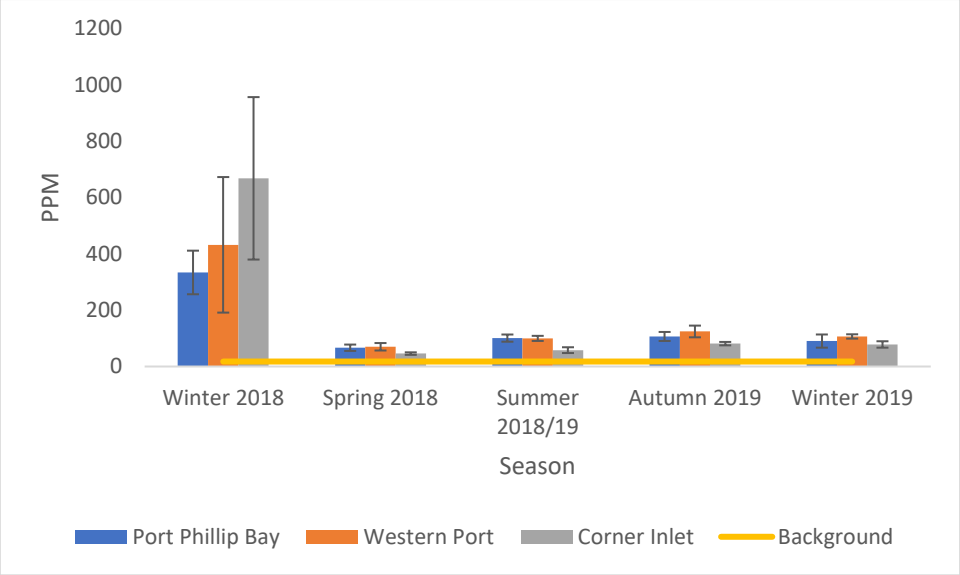


Figure 3.37: Lead levels (PPM) with 95% conference intervals for all seasons in comparison with the assay background level - orange horizontal line (PPM). There is a 95% conference interval of 29.43 PPM of the mean for the background level.

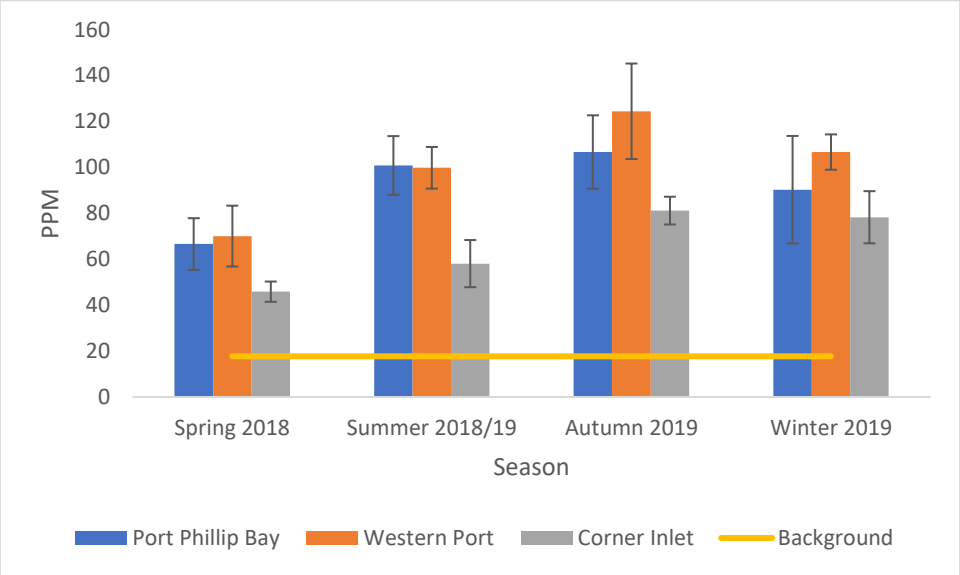


Figure 3.38: Lead levels (PPM) with 95% conference intervals for all seasons in comparison with the assay background level - orange horizontal line with (PPM). There is a 95% conference interval of 29.43 PPM of the mean for the background level. 2018 Winter data removed.

3.10.9 - Zinc

As can be seen in **Table 3.17** and **Figures 3.41 and 3.42**, Zn was above the international background level for Port Phillip Bay for the Summer 2015/16, greatly above the background level for the Winter 2018, below the background level for Spring 2018, Summer 2018/19 and Autumn 2019. Finally for Winter 2019 Zn was above background level for Western Port.

Table 3.17: Zinc levels (PPM) at all sites and background levels (PPM) with 95% conference intervals.

Site/Season	Average Zn Concentration	95% Confidence Interval	Assay Background Level	95% Confidence Interval
Port Phillip Bay (Summer 2015/16)	363.59	162.72	198.32	75.32
Western Port (Summer 2015/16)	118.58	80.46	198.32	75.32
Corner Inlet (Summer 2015/16)	91.14	23.97	198.32	75.32
Port Phillip Bay (Winter 2018)	1843.63	723.03	198.32	75.32
Western Port (Winter 2018)	3888.55	1477.74	198.32	75.32
Corner Inlet (Winter 2018)	3747.53	1425.12	198.32	75.32
Port Phillip Bay (Spring 2018)	57.42	15.79	198.32	75.32
Western Port (Spring 2018)	98.01	4.56	198.32	75.32
Corner Inlet (Spring 2018)	112.09	2.96	198.32	75.32
Port Phillip Bay (Summer 2018/19)	150.02	19.67	198.32	75.32
Western Port (Summer 2018/19)	169.79	11.50	198.32	75.32
Corner Inlet (Summer 2018/19)	86.80	10.19	198.32	75.32
Port Phillip Bay (Autumn 2019)	105.17	5.93	198.32	75.32
Western Port (Autumn 2019)	196.62	25.89	198.32	75.32
Corner Inlet (Autumn 2019)	180.84	26.85	198.32	75.32
Port Phillip Bay (Winter 2019)	147.61	14.99	198.32	75.32
Western Port (Winter 2019)	231.70	17.27	198.32	75.32
Corner Inlet (Winter 2019)	149.77	17.30	198.32	75.32

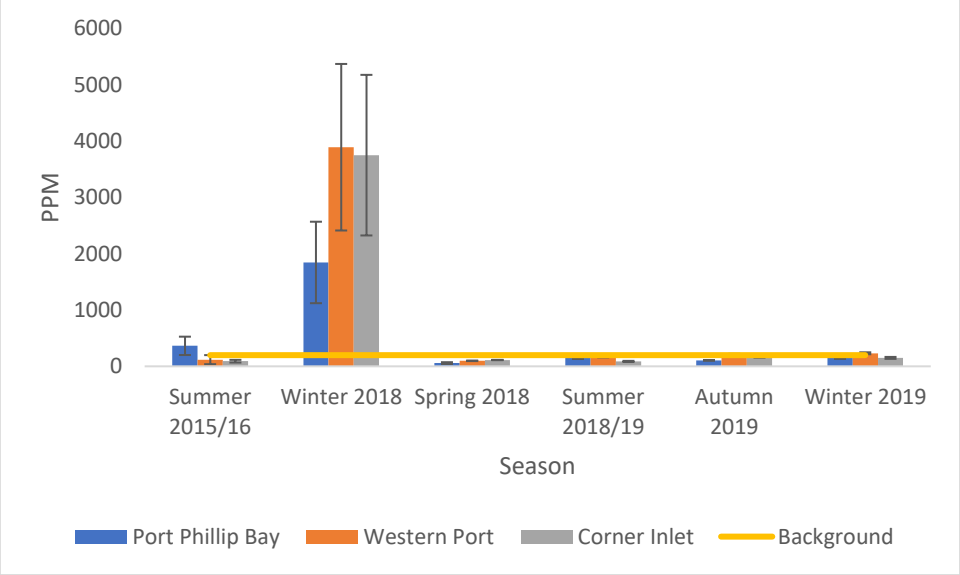


Figure 3.39: Zinc levels (PPM) with 95% conference intervals for all seasons in comparison with the assay background level - orange horizontal line (PPM). There is a 95% conference interval of 75.32 PPM of the mean for the background level

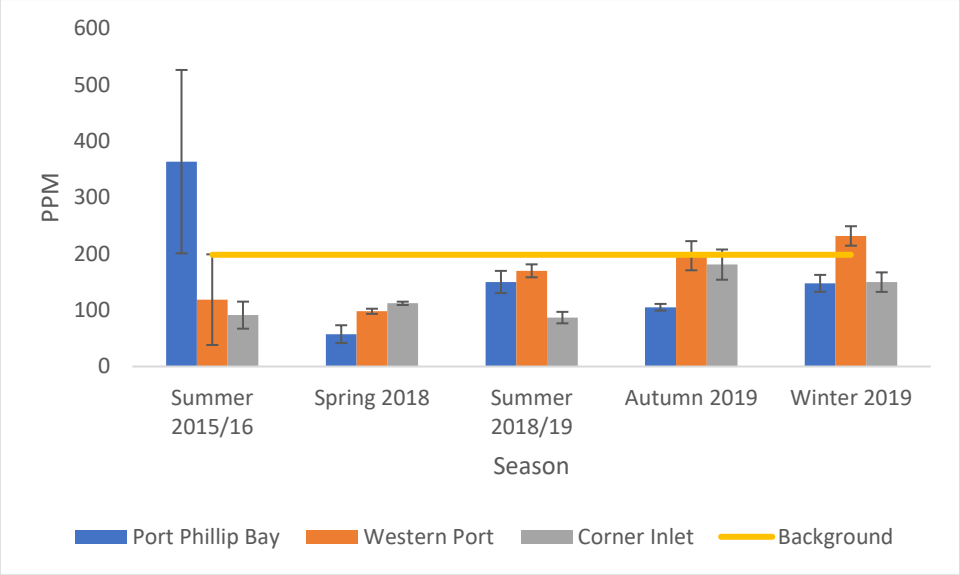


Figure 3.40: Zinc levels (PPM) with 95% conference intervals for all seasons in comparison with the assay background level - orange horizontal line with (PPM). There is a 95% conference interval of 75.32 PPM of the mean for the background level 2018 Winter data removed.

3.11: Principal Component Analysis (PCA) – Seagrass, Sediment and Water

PCA of the data was undertaken for seagrass samples including (whole plants and various organs) the sediment samples and water samples (including surface and pore water). This PCA is a popular technique for analysing large datasets containing a high number of dimensions/features per observation. PCA increases the interpretability of the data while preserving the maximum amount of information and enables the visualization of multidimensional data.

Due to the enormity of the data set, please refer to the attached Excel file - labelled PCAs (Appendix 209); for seagrass PCA data, please select the “Seagrass” tab. The Bartlett's sphericity test compares an observed correlation matrix to the identity matrix. Essentially, it checks to see if there is a certain redundancy between the variables that can be summarise with a minimum number of factors. When the PCA for seagrass data was carried out, the Bartlett's sphericity test could not be computed because of multicollinearity between the selected variables. Thus, a multicollinearity analysis was carried out to identify the problem. However, the problem could not be identified and a covariance PCA was carried out instead. As a result, the computed p-value is lower than the significance level of 0.05; therefore, the null hypothesis H_0 of the “season” making no difference was rejected and the alternative hypothesis H_a was accepted.

The Kaiser-Meyer-Olkin (KMO) test is a measure of how suited the data is for Factor Analysis. KMO values between 0.8 and 1 indicate the sampling is adequate (Statistics How To, 2020). The Kaiser-Meyer-Olkin measure of sampling adequacy was 0.86. As seen in **Figures 3.41 and 3.42** the total variance for seagrass and associated organs was 74.97%. The includes the amount of variability in a data set that can be attributed to each individual principal component. Thus, F1 had the most variance with 62.86% and F2 with 12.11%. In general, “season” did show variation. This is due to “winter 2018 ” being grossly elevated due to a suspected major potential contamination event. The metals that had the most influence on the PCA were Al, Cu, Fe and Mn.

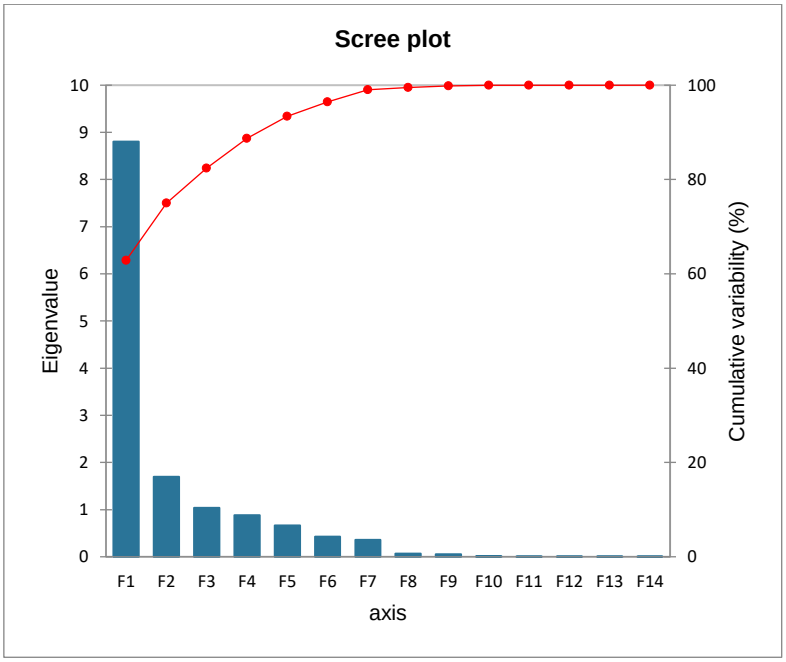


Figure 3.41*:* Seagrass Scree plot - Total variance explained with 14 components heavy metals tested

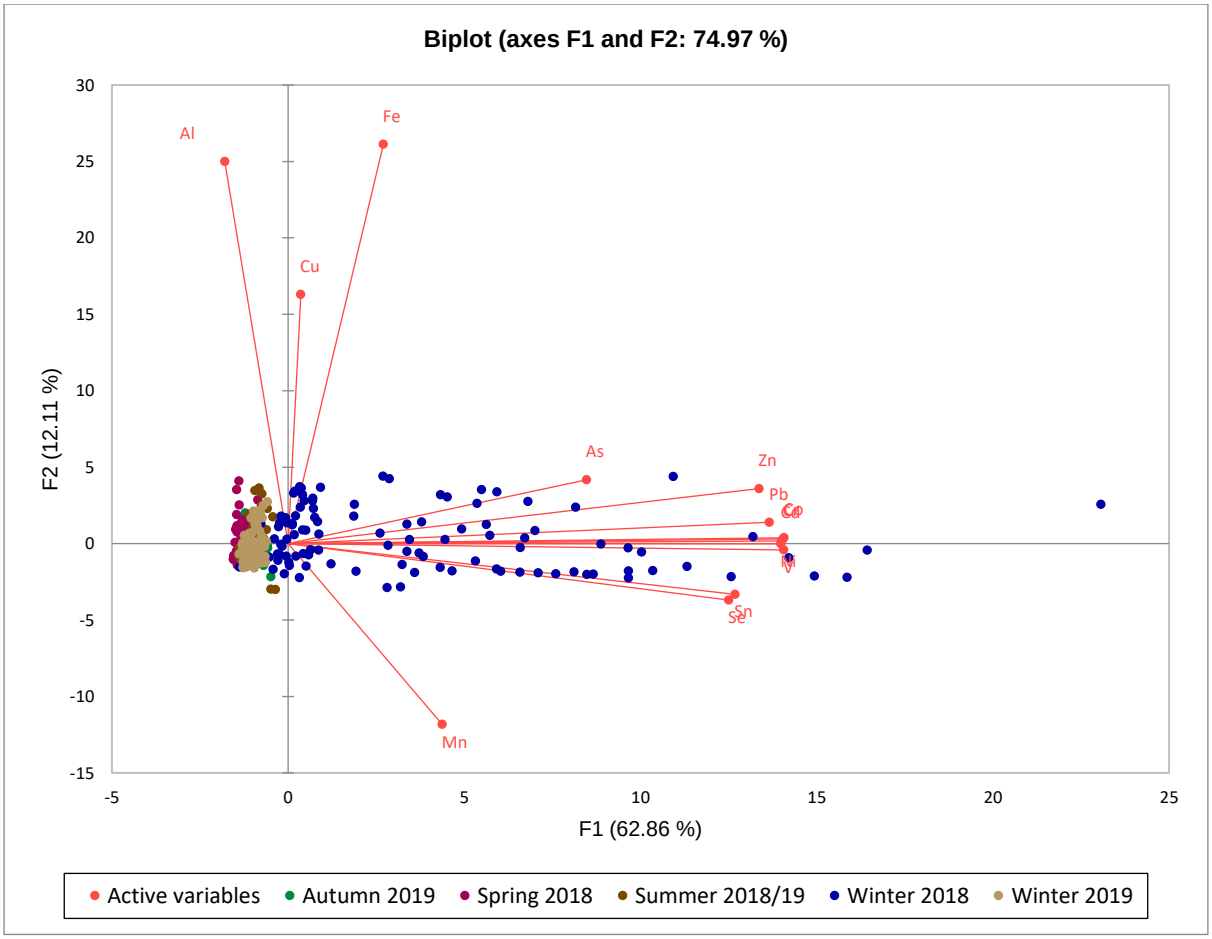


Figure 3.42*: Seagrass Biplot*

For the “sediment” PCA data, please refer to the “Sediment” tab (Excel file – Appendix 209). The Bartlett's sphericity test came back with a computed p-value that is lower than the significance level of 0.05. Therefore the null hypothesis H_0 of “season” having no difference on sediment was rejected, and the alternative hypothesis H_a was accepted. The Kaiser-Meyer-Olkin measure of sampling adequacy was 0.723. This value is considered average (Statistics How To, 2020). As seen in **Figures 3.43 and 3.44** the total variance for “Sediment” was 67.98%. F1 had the most variance with 42.5% and F2 with 25.48%. In general “season” did not show much variation for sediment. However, “winter” did, which could be due to the 2018 winter data being grossly elevated due to a potential contamination event. It should be noted that none of the metals tested affected the variability the PCA analysis.

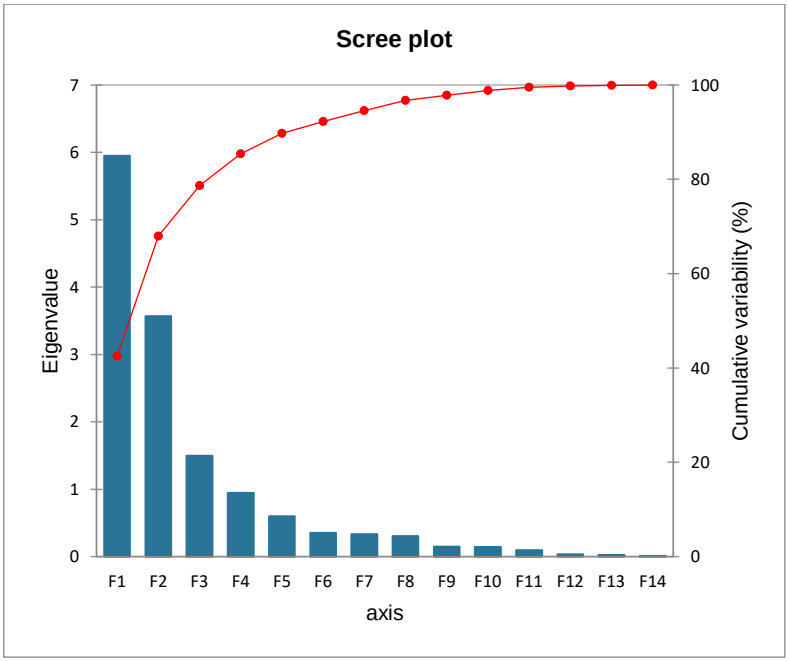


Figure 3.43: Sediment Scree plot - Total variance explained with 14 components / heavy metals tested

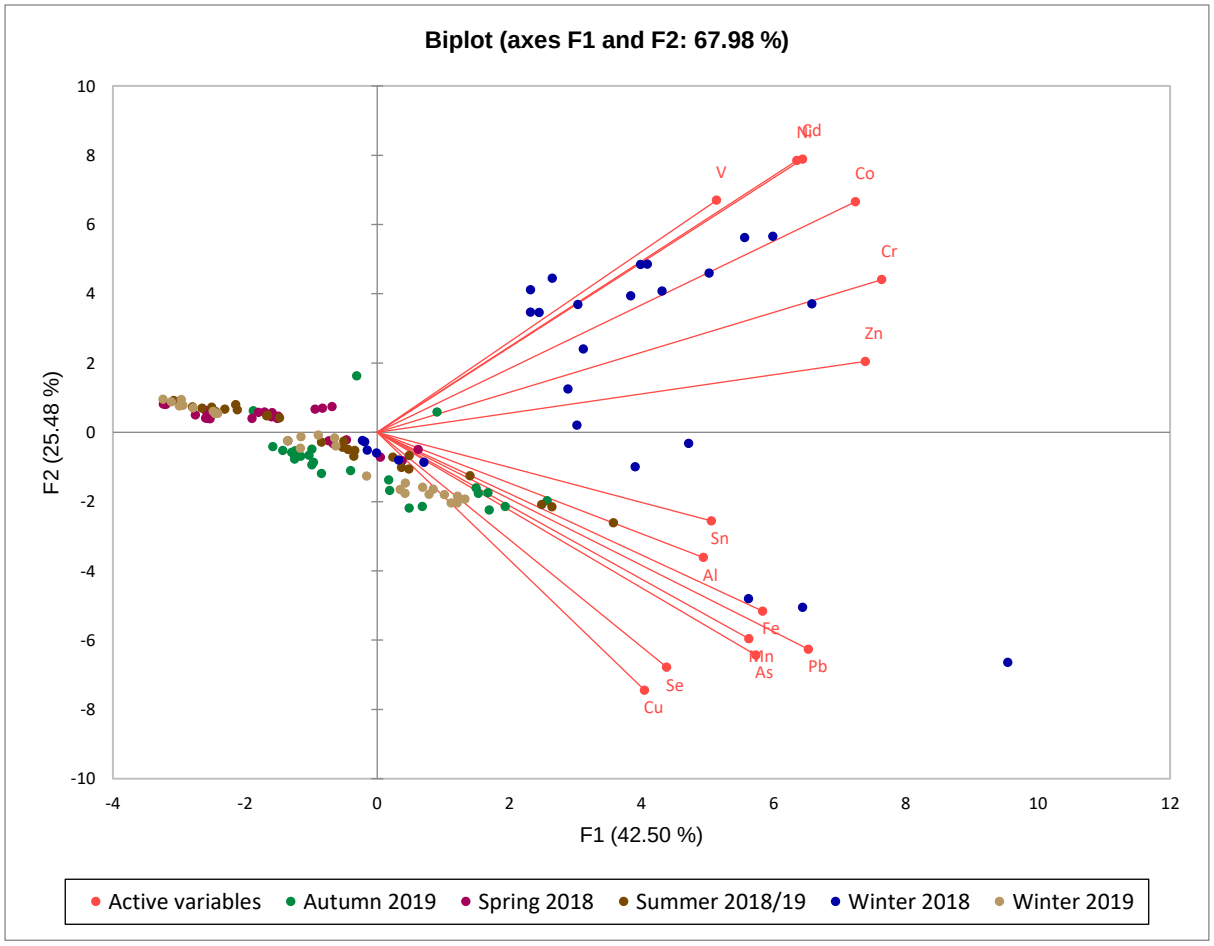


Figure 3.44: Sediment Biplot

For “water” PCA data please refer to the “Water” tab (Excel file – Appendix 209). When the water PCA was conducted, the Bartlett's sphericity test could not be computed because of multicollinearity between the selected variables. A multicollinearity analysis was carried out in an attempt to identify the problem. However, the problem could not be identified and a covariance PCA was carried out instead. As a result, the computed p-value is lower than the significance level of 0.05, therefore the null hypothesis H_0 of season making no difference to surface and porewater was rejected, and the alternative hypothesis H_a was accepted. The Kaiser-Meyer-Olkin measure of sampling adequacy came back 0.72. This value is considered adequate (Statistics How To, 2020). As seen in **Figures 3.45 and 3.46** the total variance explained for surface and porewater was 73.37%. F1 had the most variance at 51.48% and F2 at 21.9%. In general season did not show that much variation on surface and porewater. However winter did, which could be due to the 2018 winter data being grossly elevated due to a suspected major contamination event. In addition, the autumn of 2019 showed some variance. This could be because Victoria experienced a period of unusually high rainfall in summer and autumn, potentially exacerbating of pollution entering waters from the surrounding catchment, especially after extended periods of low catchment flows due to the lack of regular flushing (EPA Victoria 2020). The metals that had the most influence were As, Cu, and Se.

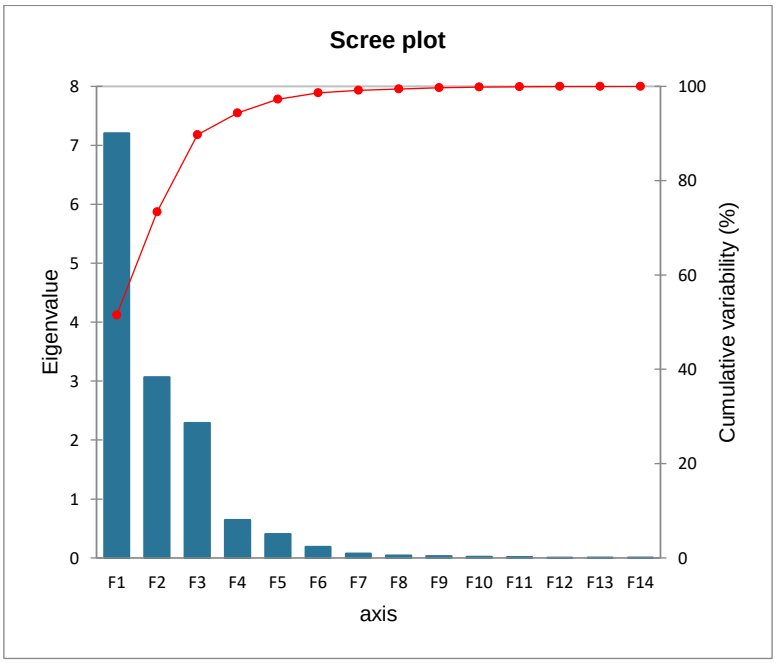


Figure 3.45: Water Scree plot - Total variance explained with 14 components / heavy metals tested

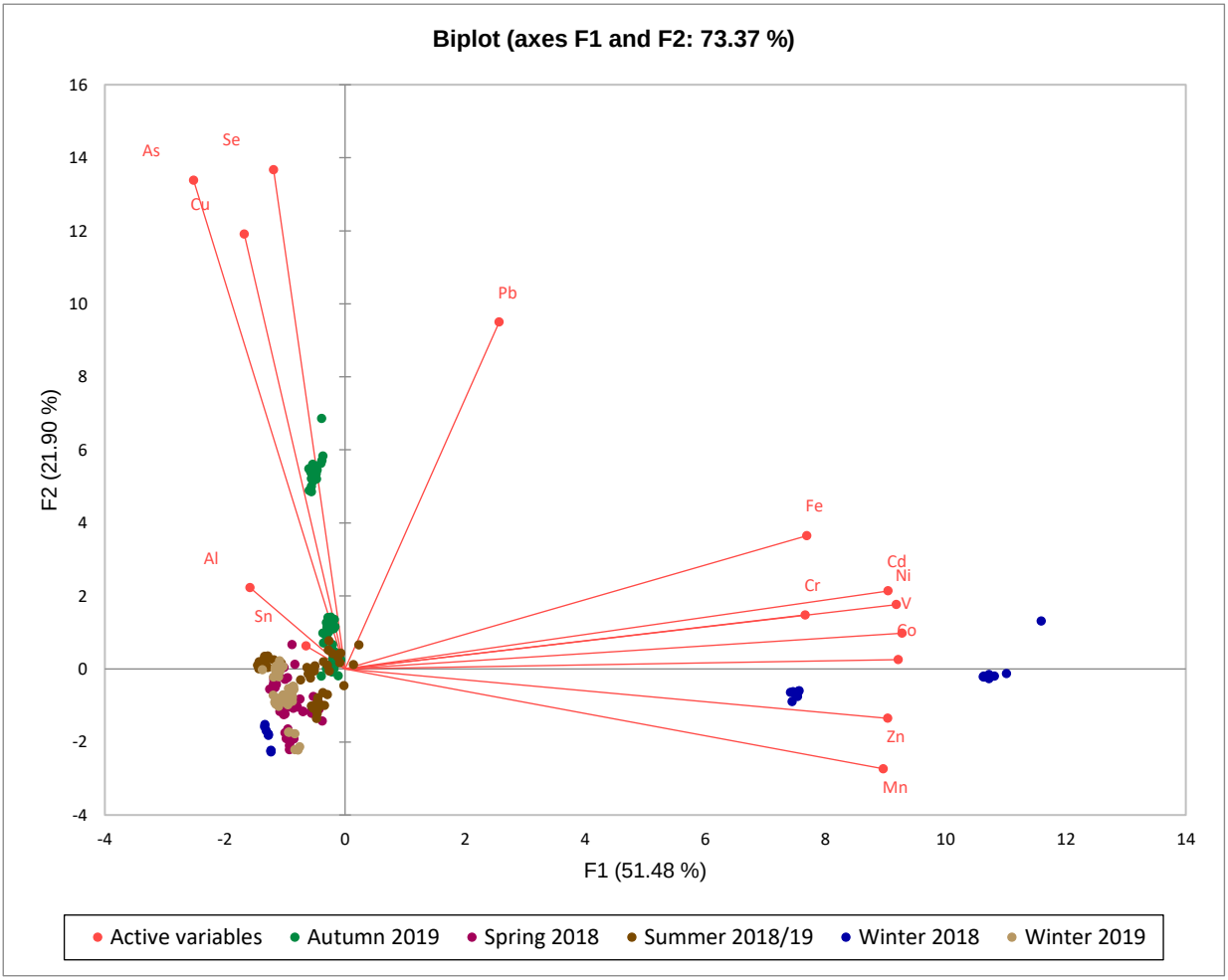


Figure 3.46: Water Biplot

3.8: Heavy metals analysis for seagrass across the three bays

Figure 3.47 compares the average levels across all three bays for Mn, Zn, Cd, Cu, Cr, Ni and Co between the five relevant seasons (the aberrant season Winter '18, has been omitted). This data is generally consistent with the more detailed analysis based on the derived assay and provided in the Conclusions section. This comparison and serves to highlight how the derives assay provides more finesse by factoring out the international data from sites that are subject to pollution. For example, Cd is shown here to be comparable with background levels when in fact it is deemed to be elevated according to the derived assay. This is because the international average levels given in Figure 3.47 also include "polluted" data. However, plots such as this do provide useful insights into inter seasonal changes and trends across the bays. For example, for Mn, Zn and Cu, Summer '15'/'16 show "spikes" in these metal across the bays. The increasing trend for Zn from Spring 2018 through to Winter 2019 is also evident, as is

a similar but less pronounced trend for Cd. Also evident is a “spike” for Cr for Summer ‘18/’19, significant at the 95% level.

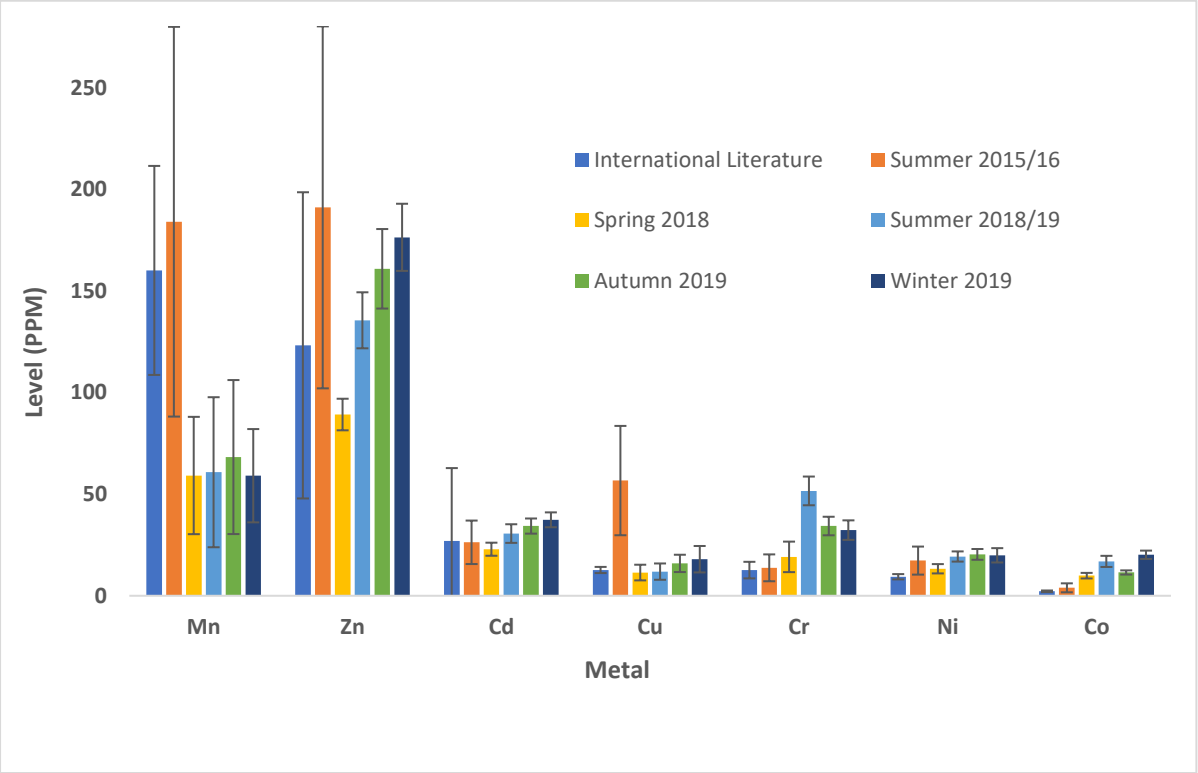


Figure 3.47: Average metal levels over Port Phillip Bay, Western Port Bay and Corner Inlet (combined), compared to the international average. Error bars are 95% confidence intervals.

3.12: Seagrass as a biomonitor

This study has mainly focused on the interpretation of heavy metal concentrations in *Zostera muelleri* as an indicator for heavy metal pollution. However, it should be noted that he bioaccumulation of metals may also affect seagrass health. Many heavy metals are naturally abundant in seagrass meadows (Prange and Dennison, 2000); but at higher concentrations they may become toxic. This toxicity to seagrass meadows can also be indicative to the poisoning of other coastal organisms (Macinnis-Ng and Ralph, 2002). Chronic toxicity of heavy metals can have significant impacts on ecosystems that are dominated by seagrass, due to heavy metals bioaccumulating in the food web becoming more toxic at higher trophic levels (Ikem and Egiebor, 2005). In addition, heavy metal accumulation in plants is often associated with changes in the rate of

photosynthesis (Macfarlane and Burchett, 2001) which can inhibit metabolic activity (Ralph and Burchett, 1998). This may lead to decreased growth rates or even result in plant die-offs (Clijsters and Van Assche, 1985). Heavy metal bioaccumulation in seagrasses can be used as a first level measurement to assess the contamination of a specific marine environment (Prange and Dennison 2000). Bioaccumulation can magnify in the food web and heavy metals in "primary producers" such as seagrasses may indicate serious trace metal pollution in the whole food web. Thus, there is a need for knowledge on the concentration and distribution of heavy metals in marine environments. This will lead to a better understanding of the behaviour of heavy metals in the aquatic environment and in the detecting pollution (Forstner and Wittmann 1979). During the course of this study there have been 1000s of studies of the ecotoxicological effects of heavy metals on aquatic organisms around the world. For example in Victoria, Australia these studies include Ward et al. 2011 on Harpacticoid Copepod, Freshwater Microalga by Franklin et al. 2007, fish by Miranda et al. 2010, amphipods by Dong et al. 2020, Molluscs by Taylor and Maher 2015 and crustaceans by Hose et al. 2019. For seagrass these studies suggest that elevated concentrations of heavy metals can inhibit seagrass growth (Li et al. 2023). With increasing urbanization, there are increasing amounts of heavy metals entering estuaries (Deycard et al. 2014) and intertidal sediments may become seriously contaminated if there is no effective management taken to tackle this issue. Understanding the ecophysiological responses of seagrass would further our understanding of how metal contaminants influence the coastal environment. Thus, while it is necessary to identify the presence and location of pollutants, it is also important to understand the extent to which heavy metals may kill, permanently damage, or cause ecological stress to seagrass. It is also important to detect spatial variation in anthropogenic pressures on coastal ecosystems, and to locate and respond to sources of pollution at both local and global scales. Due to the heterogeneity of sediment, and the often accidental nature of contaminating processes, concentrations of heavy metals can vary dramatically over very short distances (Luo et al. 2007).

This thesis tests the hypothesis that *Zostera muelleri* (Eelgrass) can be a useful tool, when compared to more traditional high-cost methods, for identifying differences in heavy metals pollution status between Port Phillip Bay, Western Port and Corner Inlet as a result of heavy metal levels that originate from natural and/or anthropogenic

sources. The collected data suggests that during the time period of the summer 2016 to the winter of 2018 there was a severe pollution event affecting Western Port and Corner Inlet involving the metals Zn, Mn, Cd, Co, Cr, Ni, Pb, Se, Sn and V. According to the New South Wales EPA, a pollution event is defined by an incident or set of incidents during or as a consequence of which there is or is likely to be a leak, spill or other escape or deposit of a chemical, as a result of which pollution has occurred, is occurring or is likely to occur. It includes an incident or set of incidents in which a chemical has been placed or disposed of on premises (EPA New South Wales, 2018).

3.13: Contamination Events

During a literature review to identify potential pollution sources that might affect these bays, it was discovered that the Lang and Bass Rivers, which are sources of water for Western Port and the Albert, and Agnes Rivers, which are sources for Corner Inlet, have a common source i.e. the Strzelecki Ranges. Gold was discovered in the Strzelecki Ranges in the town of Walhalla in 1862 (Morgan 1997). In addition, the mining of large coal deposits in the La Trobe Valley began in the 1920s, in the towns of Wonthaggi and Yarram. These anthropogenic activities strongly influenced the pattern of later settlement across Gippsland. Logging of the Strzelecki Ranges' Mountain ash (*Eucalyptus regnans*) began in the 1880s. It was not until the establishment the Forests Commission of Victoria (FCV) in 1918 that logging became highly organized (Noble 1976). The FCV played an important role in the development of the forest industry by providing infrastructure and capital to support activities such as sawmilling and perhaps, more importantly, developing long term strategies to manage and sustain Victoria's forest resources (Moulds 1991).

In 2019, the author attended the Royal Australian Chemical Institute (RACI) Health, Safety & Environment Group Victoria Symposium. A presentation by Mr Jamie Twiddle entitled: "Industrial, Waste and Chemical Fires", identified three recent potential sources of pollution. These were chemical fires located in Broadmeadows in 2016 (ABC 2016); Coolaroo 2017 (ABC 2017) and Tottenham 2018 (Vedelago et al. 2018). With this new information in mind, another search of the literature revealed (EPA Victoria) that there was illegal dumping of chemical waste in the town of Kaniva, West Wimmera on the 1 July 2018 (Vedelago et al. 2019). This stockpile of chemical waste

was contained in steel drums and plastic containers entombed in trenches, pits just 60 meters above the water table that is used to irrigate farms and supplies the water for communities near the border of Victoria and South Australia as seen in **Figure 3.48**.

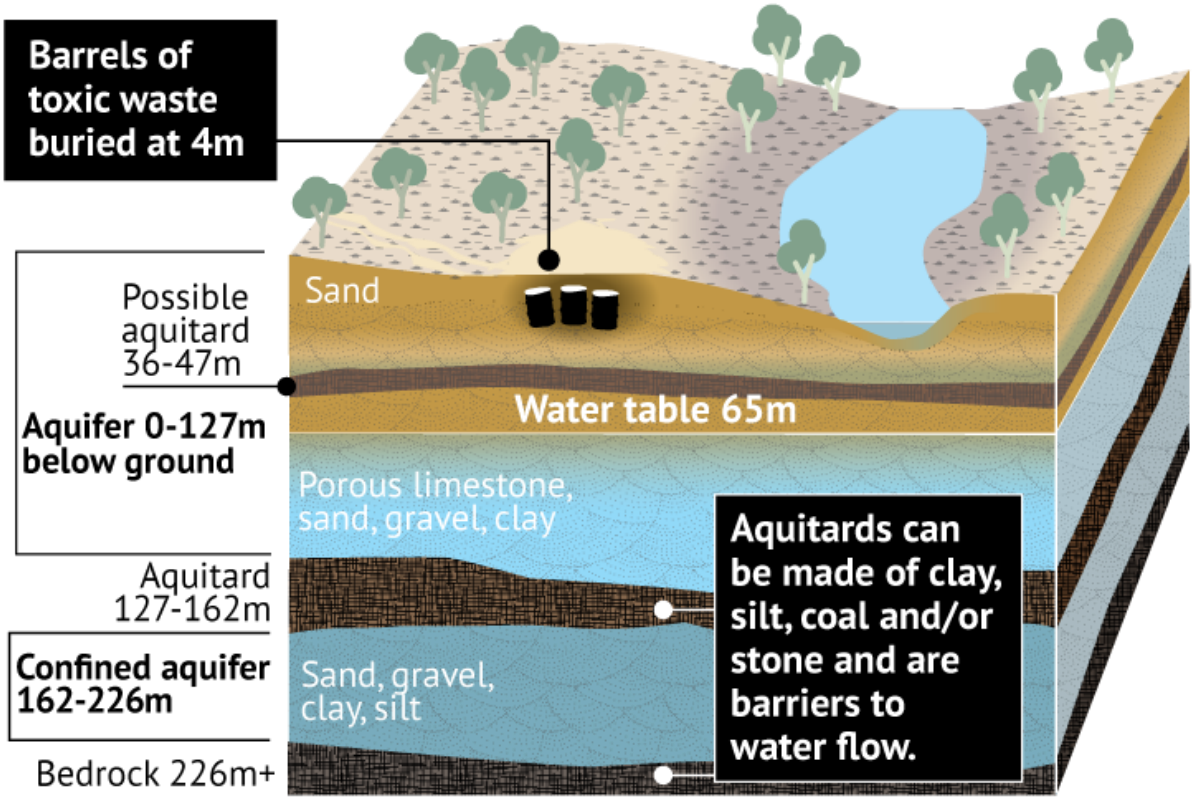


Figure 3.48*: Groundwater aquifers for West Wimmera (Source Vedelago et al. 2019)*

The candidate also attended another presentation by the First Friends of Dandenong Creek entitled: “Stormwater - Australia's great environmental dilemma” by Dr David Sharley of Bio2Labs, which identified another pollution event in 2018 in Heathmont (**Figure 3.49**). At the time, the pollution source was not identified. As for the chemical, it was thought to be a detergent, which is a manmade product, unlike soap, which is created by mixing lipids with a base (Butt 2018) It should be noted that this incident occurred ten times that year. The contamination occurred from Marie Wallace Park in Bayswater to Boronia Road in Vermont. The potential source could have been the Bayswater industrial estate, where a stormwater drains feeds into the creek. When a detergent enters a freshwater system, it has many negative impacts. One of these impacts is that it strips the external mucus layers on fish skin exposing them to pathogens (Dash et al. 2018).

Figure 3.49: Unknown White Foam Dandenong Creek, Heathmont (Source First Friends of Dandenong Creek)

Even though these two contamination events do involve on heavy metals in relation to the Victorian bays, they demonstrate that pollution in all its forms, especially heavy metals, can end up in the bays from dumping into surface water or being leached into ground water. The extent of such dumping activities is unknown and should be better investigated and monitored.

Another potential source of heavy metal pollution is the coal mining activities in the Latrobe Valley, Victoria, Australia (Schneider et al. 2020). For example, this study showed there was an increase in As and Se in the sediment. This could account for the elevated As levels since the La Trobe Valley is in the proximity of the Corner Inlet Marine National Park.

3.14: The Future

The heavy metal assay methodology developed for this project could be used for other bioindicators. Examples of this are Finger et al. 2015; 2016; 2017 using the blood, feathers and faeces of the Little penguin (*Eudyptula minor*). Other examples include green sea turtle blood (*Chelonia mydas*) (Villa et al. 2017), fish such as mudskippers

and southern sand flathead (*Platycephalus bassensis*) (Gagnon et al. 2016), molluscs such as The Mediterranean mussel (*Mytilus galloprovincialis*) (Richir and Gobert 2014), invertebrates such as the purple sea urchin (*Paracentrotus lividus*) (Warnau et al. 1995) and crabs. Note that all of these example if done at Victoria University or any University in Australia would require animal ethics approval. According to the Prevention of Cruelty to Animals Act 1986 and Regulations 2019, an “animal” is a live member of a vertebrate species including any fish, amphibian; reptile, bird or mammal, that has passed the mid-point of gestation for the particular species; other than any human being; or a live adult decapod crustacean, that is a lobster; crab; crayfish; or a live adult cephalopod including an octopus; squid; cuttlefish; or a nautilus.

If animal ethics is an issue, plants such as the macroalgae sea lettuce (*Ulva lactuca*) (Bonanno et al. 2020), smooth cordgrass (*Spartina alterniflora*) (Negrin et al. 2019), reed plants (*Phragmites australis*) (Bonanno and Lo Giudice 2010), seaweeds, mangroves and terrestrial plants such as apple tree leaves, could be used instead.

During this research program, consideration was given to growing seagrass in a mesocosm. However this was not feasible due the “wet lab” at Victoria University being decommissioned. A “mesocosm” is any replicate outdoor experimental system that examines the natural environment under controlled conditions. In this way, mesocosm studies provide a link between field surveys and highly controlled laboratory experiments. Mesocosms tend to be medium sized to large (e.g., aquatic mesocosm range: 1 to > 10,000 L) and contain multiple trophic levels of interacting organisms Hosokawa et al. (2016).

In contrast to laboratory experiments, mesocosm studies are often conducted outdoors to incorporate natural variation. Mesocosm studies may be conducted in either an enclosure that is small enough that key variables can be brought under control or by field-collecting key components of the natural environment for further experimentation. Extensive mesocosm studies have been conducted to evaluate how organisms or communities might react to environmental change, through deliberate manipulation of environmental variables, such as increased temperature, carbon dioxide or pH levels.

More understanding of the uptake of heavy metals by seagrass could be achieved by a mesocosm where by the controlled up take of heavy metals could be characterized under various conditions.

[6]Another potential study to track the origins of a heavy metal pollution event is the use Pb isotopes to fingerprint the origin of pollution. Stable Pb isotopes provide a powerful tool that can be used to separate anthropogenic Pb from natural Pb derived from mineral weathering. Pb present in the environment has four stable isotopes: 204Pb, 206Pb, 207Pb, 208Pb. While 204Pb is non-radiogenic with a constant abundance on earth in time (Komarek et al., 2008), isotopes 206Pb, 207Pb, and 208Pb are radiogenic and produced by the radioactive decay of 238U, 235U, and 232Th, respectively. Because the isotopic composition of Pb is not significantly affected by physico-chemical fractionation processes associated with smelting, refining and manufacturing (Ettler et al., 2004), each source of Pb can have distinct or sometimes overlapping isotopic ratio ranges from mixing of local/natural Pb with anthropogenic Pb sources. Investigations of Pb isotope compositions have been well-established in geochemistry and are increasingly used in environmental science (Monna et al., 2000, Komarek et al., 2008). The isotopic composition of Pb has been used as an indicator of anthropogenic contribution in many ecosystems, such as sediments, to investigate the impact of recent Pb smelting and/or mining activities on the surrounding environment (Renberg et al., 2002, Monna et al., 1999). This technique could have been used in winter 2018, for instance to trace if the pollution events recorded linked to gold or coal mining within Strzelecki Ranges or La Trobe Valley, or other potential pollution activities discussed in the thesis. Finally it would be good to evaluate whether the perceived increase in metals in the three bays is a genuine event or from artifacts from the methods used in this thesis.

It is also of relevance to note that seagrass is being used as an alternative or complementary medicine, in the “management” of an array of pathological disorders such as muscle aches, wounds, abdominal pain, indigestion, hangover, and mental disorders (Kim et al. 2021). There is also the possibility of seagrass, as an example of

[6] The author would like to thank Dr Oscar Serrano and Professor Vincent Pettigrove for the suggestions of a future studies using Pb isotopes to fingerprint the origin of pollution and to evaluate the perceived increase metals due to methodology or an actual pollution event.

a nonconventional protein rich plant foods to be a functional alternatives for human consumption (Coria-Monter and Durán-Campos 2020). For these application the seagrass species in question must obviously have acceptable levels of toxic heavy metals.

Although metal contamination has been a traditional topic in seagrass ecology, an understanding of the fate of metals in seagrass ecosystems is far from adequate. Seagrass displays highly variable metal concentrations in nature. More study on different species and ecotypes is required to fully understand metal accumulation in seagrass worldwide, especially for species growing in Australia and the Asia-Pacific region. Information on metal contamination in seagrass associated organisms such as epiphytes, seagrass grazers, invertebrates, and fish are also limited, and this aspect should be further explored.

Furthermore even less is known about the accumulation and toxic effects of multivalent metals such as Hg, As, and Cr in seagrasses. Seagrass ecosystems are increasingly under pressure from a variety of global and local anthropogenic stressors, including higher seawater temperatures due to climate change, more acidified oceans caused by elevated carbon, and more eutrophic coastal seawaters due to the increasing release of nutrients from human activities (Li et al. 2023). How these changes are affecting metal bioaccumulation in seagrasses is still a mystery. Additionally, more efforts are needed to a gain better insight into the contributions of seagrass ecosystems to global metal cycles under our changing climate conditions.

A complete understanding of metal variability in seagrass must include a knowledge of the geochemical and physiological processes governing metal kinetics and the mechanisms of metal detoxification. Future research priorities should include the following directions:

All the above may be suggested as future research priorities and directions. Although metal contamination has long been a traditional topic in seagrass ecology. Yet our knowledge of the ecophysiological impacts of heavy metals on seagrass is still limited and the mechanisms underlying seagrass response to metal challenges are far from being understood. Despite several reviews (Li et al. 2022; Li et al. 2023) on metal

contamination in seagrasses, these studies are mainly confined to simply reporting the metal concentrations and evaluating the utility of seagrass as potential biomonitors of metal pollution.

Chapter 4 - Conclusions

This research aims to further develop and refine our knowledge of the potential of the seagrass species *Zostera muelleri* (Eelgrass) as a biomonitor of heavy metal contamination in Victorian coastal areas. In a previous study (Lee 2016), it was found that by using *Z. muelleri* as a bioindicator, significant differences in concentration levels were found between the areas of Port Phillip Bay, Western Port and Corner Inlet for the metals aluminium (Al), barium (Ba), cobalt (Co), chromium (Cr), copper (Cu), iron (Fe), manganese (Mn), nickel (Ni), strontium (Sr) and zinc (Zn). This has led to the main hypothesis is that Eelgrass can be developed as a useful tool, when compared to more traditional methods such as testing of water and/or sediment (Ohls & Bogdain 2016), for identifying differences in heavy metal contamination within and between marine areas such as Port Phillip Bay, Western Port and Corner Inlet - arising from natural and/or anthropogenic sources. Other minor hypotheses that will be addressed during this study include:

- Is seagrass a reliable bioindicator of heavy metal levels in the global marine environment? To answer this question, some benchmarking or control measures are required. This involves a global audit of all such studies reported in the scientific literature across a range of species and geographical locations. Such an audit requires a scrutiny of the "circumstances" of each study. For example, is the study in an area that is known to be polluted? Of interest here is whether the global data lies within a defined magnitude range that may represent a “background” and whether a departure from this magnitude range can indicate “contamination”?

- Can such a "normal"; - i.e., background versus "polluted" range be defined across different species, geographical locations, seasons, method variations etc.? How can this be ascertained?

- How does the local (temporal) data for Victoria's bays compare with the global data? Are there significant departures from "normal"; - typical background levels between the three bays being investigated in Victoria and between the different sampling periods?

- How do we justify the identification of any departures from the global norm (contamination events)?

- Which sampling matrices (seagrass whole plants or organs tissues) are preferential for the quantification of which metals?

- Which of the metals measured in this study are of most concern with respect to the health of the bays?

- What sampling protocol would be suitable to survey long-term metal contamination within the bays?

In conclusion, the use of seagrass magnitude ranges to identify pollution hotspots is a useful tool. By using boxplots and range curves background, elevated and highly impacted pollution levels can be defined. Background concentrations of heavy metals and metalloids should be documented in the different environmental media around the world, for later use as a reference. Efforts should be made to minimize heavy metal contamination in aquatic and terrestrial ecosystems to safeguard the biota and the health of their consumers. In addition, the levels of potentially toxic heavy metals and metalloids in water, sediments, soils, and the resident biota should be assessed and monitored regularly. The public should be educated about the harmful effects of toxic heavy metals on human health and the environment.

Differences between Port Phillip Bay, Western Port and Corner Inlet in terms of the amount of oceanic heavy metal levels in *Zostera muelleri* was extensively investigated. This seagrass species was shown to be able to reveal such differences and trends at a high level of significance for all metals tested. It should be euphemised that testing seagrass is continuous and testing sediment, surface seawater and porewater is not. This means that seagrass accumulates metals over a period of time (cumulative),

whist sediment, surface seawater and porewater can only reveal the concentration at that point of time. Overall, it was found that heavy metal levels in Port Phillip Bay and Western Port were generally higher than in Corner Inlet. This is not unexpected and is due to industry around Port Phillip Bay and Western Port, with hardly any industry around Corner Inlet, that is considered to be “pristine”.

The derived assay, **Table 3.1**, based on the totality of the international data reported in the peer reviewed literature, proved to be a useful tool for creating a temporal heavy metal pollution profile for three neighbouring marine bays along the southern coastline of Victoria, Australia; namely Port Phillip Bay (PPB), Western Port (WP) and Corner Inlet (CI). For ten metals, As, Cd, Pb, Co, Cr, Cu, Mn, Ni and Zn, considered to be affected by industrial/anthropogenic activities, the following general conclusions may be drawn from these studies.

- As was found to be highly elevated across the three bays, over all seasons. PPB and WP were comparably affected (WP slightly more so), with CI less affected overall. This could be attributed to natural sources such leaching from basalt rocks (Smith et al 2003) or from anthropogenic sources such as mining sites, e.g., historical gold mining sites in the area. However, the lesser impact on CI suggests an anthropogenic source since CI is expected to be less impacted in this regard than PPB and WP. Notably, there appears to be a gradual increase across all bays from Spring 2018 to Winter 2019. This trend has also been noted for Co and Zn (see below) and warrants some further investigation. For example, it could be related to increasing rainfall over this period.

- Cd was elevated across all three bays over all seasons, with all the bays being comparably affected. The source of the increased level of this pollutant is not clear, although its comparable preponderance across all three bays suggests that a non-anthropogenic source could be a major contributor.

- Co was generally elevated across all the bays over all seasons, except for (all three bays) for Summer 2015/16 and PPB for Autumn 2019. Co pollution is

usually attributed to industrial activity, and this is consistent with CI being significantly less affected overall. Notably, like As and Zn, there appears to be a gradual increase across all bays from Summer 2015/16 to Winter 2019, bar PPB and CI for Autumn 2019.

- Cr was not generally elevated across the three bays from Summer 2015/16 through to Winter 2019, except for a modest elevation for WP and CI for Summer 2018/19, a slight elevation for PPB and WP for Autumn 2109 and for WP for Winter 2019. Elevations in PPB and WP could be attributed to industrial/anthropogenic sources such as the BlueScope Steel processing works and the major Royal Australian Navy training base, HMAS Cerberus. Also, Holden Australia's proving ground is located just east of Western Port. However, the elevation at CI for Summer 2018/19 is more difficult to explain but could be due to the unusually high rainfall over this period. The generally lower levels for CI are consistent with industrial/anthropogenic factors.

- Cu levels were within background across all the bays for all seasons except for Summer 2015/16 where a slight elevation was observed for PPB and CI. Generally, the Cu levels were in the order PPB >> WP > CI, which is consistent with the degree of industrial/anthropogenic activity across these bays. Although the slight elevation in PPB for Summer 2015/16 can be attributed to industrial/anthropogenic pollution, that in CI is more difficult to explain, although it is barely significant in terms of the assay. Although Cu occurs at trace levels in all ecosystems, elevated Cu levels are often attributed to mining activities or the legacy thereof (Keller et al. 1992, Couillard et al. 1993, Garceau et al., 2010).

- Mn was well within background across the three bays over all seasons and tended to be consistently lower in WP than in PPB or CI. Mn appears to be unique in this regard, although the reason for this is unclear.

- Ni was well within background across all three bays and over all seasons with levels for PPB and WP being comparable and the levels for CI being

consistently lower. This is consistent with CI being more “pristine” and less exposed to industrial/anthropogenic pollution that PPB or WP.

- Pb was elevated across all three bays and over all seasons. PPB and WP were comparably affected with CI less so. Widespread Pb contamination in Victoria has been attributed to historical mining (Fabris et al, 1999). However, industrial activity could also be responsible and the consistently lower impact on CI suggests an anthropogenic source.

- Zn was below background across the three bays over all seasons with evidence of a slight elevation in PPB for Summer 2015/16. Notably, Zn levels showed a progressive increase for WP from Spring 2018 to Winter 2019 although the reason for this is unclear.

In summary, according to the derived assay, the high elevation of As across all three bays and over all seasons, is of concern. The source of this is not clear. Similarly, Cd and Pb were found to be elevated although not to the same extent. The metals Co, Cr, Cu, Mn, Ni and Zn were all considered to be comparable to international background levels.

For the Winter of 2018, there was evidence of a very large pollution event with respect to the metals As, Cd, Cr, Co, Ni, Pb and Zn, primarily affecting WP and CI. However, the metals Cu and Mn remained within background levels. In terms of individual polluting metals, As was not as pronounced overall and less so for CI. Also, Cd, Cr, Co, Ni and Zn pollution levels were not as pronounced for PPB and Pb was not as pronounced overall. The origin of this event is unknown and could well be related to illegal discharge in remote areas surrounding WP and CI. This clearly warrants further investigation.

From the international literature database, the order of magnitude for the average (worldwide) concentrations of the nine selected “industrial” metals, as reflected in their bioaccumulated seagrass concentrations is:

$$Mn < Zn < Pb < Cd < Cu < Cr < Ni < As < Co$$

This may be considered as a ‘benchmark series of inequalities that represents “normal” levels of these metals in the worldwide marine environment. For a polluted environment, it would not be unreasonable to expect this order of magnitude to be perturbed in some way. Therefore, an examination of such a series of inequalities for a given location can provide information about the pollution status of that location. Therefore, a complementary method was trialled for comparing the average relative magnitudes of the metals of interest from one season to another, benchmarked against the average relative magnitudes expected from the international database. This was first done qualitatively using colour coding and revealed the potential of this technique.

A more quantitative flavour has been imparted to this approach by invoking a mathematical technique that utilizes a “discrete metric” - specifically the “Manhattan metric”. This method has been shown to dramatically reveal the Winter 2018 contamination event and demonstrates that there is a reasonable consistency in the average heavy metal levels across the bays for all the other seasons. It is suggested that this method could find more extensive application in the analysis of environmental data in general.

From the PCA analysis of seagrass and its organs in general, “season” did not show significant variation, and this is consistent with the assay analysis and the Manhattan analysis results However, “winter” did - and this could be due the 2018 winter data being grossly elevated due to a potential contamination event. The metals that had the most influence according to the PCA were Al, Cu, Fe and Mn.

In association with the use of seagrass as a heavy metal bioindicator, the derived assay, that exploits the entirety of the literature data, has been demonstrated to be a very useful and simple tool for the identification of elevated heavy metals in marine environments. This method is also useful for comparing the environmental status of different sites and for the identification and interpretation of trends. From the case study of this project, it was concluded that the concentration levels in the three Victorian embayments are generally consistent with international levels, with the exception of As, Cd and Pb, that are significantly elevated. There is evidence for a

major contamination event affecting all of the bays in the period leading up to the Winter of 2018.

Continuous monitoring programs are necessary to collect evidence of the efficiency of the regulatory controls on pollution discharges into the environment and to define the relative health status of ecosystems. For example wastewaters from industries should be treated effectively before their discharge into the natural water bodies. Biomonitoring programs that measure contaminants in the tissues of aquatic organisms have been established, in part to overcome the difficulties and shortcomings of measuring metals directly in water. The difficulties of detecting heavy metals directly from water include measuring low concentrations accurately, getting representative or “average” samples when heavy metal concentrations fluctuate over time, and isolating the bioavailable fraction of the contaminant from unavailable forms (Roditi et al. 2000). To be used effectively as a bioindicator, an organism must accumulate contaminants proportionately to its exposure and have other demographic and physiological characteristics. This includes a sufficiently broad geographical distribution, ease of collection and an ability to tolerate elevated contaminant concentrations. Seagrass is a good biomonitor for all these reasons. In this project using the seagrass species *Zostera muelleri*, a major contamination event for the Winter ‘18 that affected all the bays but particularly in Western Port and Corner Inlet was detected. This is of obvious concern since it is unlikely to be due to normal anthropogenic discharge and could be related to illegal dumping. This finding highlights the importance of scientific research on the environmental assessment of toxic chemicals including toxic heavy metals and metalloids since such events would not be readily detected by other means. This requires the allocation of appropriate funds to ensure the ongoing protection of human health and the environment.

In conclusion, the use of seagrass magnitude ranges to identify pollution hotspots is a useful tool. By using boxplots and range curves background, elevated and highly impacted pollution levels can be defined. Background concentrations of heavy metals and metalloids should be documented in the different environmental media around the world, for later use as a reference. Efforts should be made to minimize heavy metal contamination in aquatic and terrestrial ecosystems to safeguard the biota and the health of their consumers. In addition, the levels of potentially toxic heavy metals and

metalloids in water, sediments, soils, and the resident biota should be assessed and monitored regularly. The public should be educated about the harmful effects of toxic heavy metals on human health and the environment.

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Abstract - executive summary

Having an economical and reliable method for determining heavy metal pollution in marine environments is critical for the management of marine resources. In this regard, so-called bioindicators such as plants, animals or organisms can reveal, through bioaccumulation, the qualitative and quantitative status of their environment, including heavy metal levels. More specifically, seagrass, an often-abundant species in coastal zones, that provides ecosystem "goods and services", such as substrate stabilization and the maintenance of fisheries, have been shown by previous researchers, over four decades, to be a potentially useful bioindicator of heavy metal pollution, in various marine environments. Despite the large data base resulting from these studies to date there has been no method for exploiting this data *in its entirety* for the monitoring of sites in general and for identifying pollution events. The research presented here has devised and tested a simple, standard method to identify heavy metal pollution in different marine environments. This assay is based on the benchmarking of any given set of analytical data for heavy metal levels in whole-plant seagrass tissue samples against heavy metal concentration magnitude ranges that have been derived from the totality of the available international data. Notably, this subsumes the broad range of geographical locations, climatic conditions, different seagrass species and the different plant organs analysed, that are inherent in the available data sets. An important consideration in the development of this method has involved the scrutiny of each individual publication of literature data to characterize the particular site/location as "normal", "elevated" or "polluted".

Thus, magnitude criteria have been established for "normal" (background) versus "elevated" and "polluted" levels for the ten metals, As, Cd, Co, Cr, Cu, Hg, Mn, Ni, Pb and Zn, that are representative of anthropogenic pollution. Concurrently, an experimental case study was devised and carried out to implement and test the devised assay. This study has involved the assessment of the environmental health of three marine embayments along the southern coast of Victoria, Australia. Employing the regional seagrass species *Zostera muelleri* (Eelgrass) as a bioindicator, seagrass samples were collected from locations in Port Phillip Bay (PPB), Western Port (WP)

and Corner Inlet (CI) and were subsequently processed and analysed for their heavy metal content by Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES). As well as the ten metals that were considered as representatives of anthropogenic pollution, several other elements, including Fe, Al, Ba and Sr, were also simultaneously determined. Thus, heavy metal data from various sites were collected over a five-year period from 2015 to 2020, across six seasons. Namely, Summer 2015/16, Winter 2018, Spring 2018, Summer 2018/19, Autumn 2019, and Winter 2019. Since the assay is intended to be as simple and efficient as possible, only data from an analysis of the whole plant was utilized, although some analyses for specific plant organs was carried out, based on scientific curiosity. Simultaneously, seasonal data was also collected and documented for the heavy metal content of sediment, surface seawater and porewater across the three bays, but a full analysis of this data is outside of the scope of this thesis, although a preliminary Principal Components Analysis has been carried out on this data.

According to the derived assay, the concentration levels found in the case studies carried out for the three bays, were able to be compared to the totality of the international database, with respect to the following nine metals (omitting Hg): As, Cd, Co, Cr, Cu, Mn, Ni, Pb and Zn. Notably, of the six seasonal periods considered, Winter 2018 data (except for the metals Cu and Mn) showed evidence of a gross heavy metal contamination event, affecting all bays. Therefore, the data for the other seasons have been compared separately from that of the aberrant Winter 2018 season. This study has found that for all seasons and across all bays, As was very highly elevated and Cd and Pb were significantly elevated. For As and Pb, PPB and WP were comparable with CI being less affected for both metals. For Cd, the pollution levels were essentially comparable across all three bays. For all seasons, Co was also generally elevated across the bays, except for (all bays) Summer 2015/16 and PPB Autumn 2019, with CI being less affected overall. Generally, over all seasons and bays, Cr levels were not highly elevated. However, a slight elevation was observed for WP and CI Summer 2019/18, PPB and WP Autumn 2019 and WP Winter 2019. PPB and WP appear more affected than CI. Cu levels were within background for all seasons with the exception of Summer 2015/2016, where a slight elevation was observed for PPB and CI. Generally, the Cu levels for PPB > WP > CI. For all seasons, Mn levels were well within background across all the bays and tended to be lower in WP than in PPB or

CI. Across all seasons, Ni levels were well within background across all the bays, with the levels for PPB and WP being comparable and the levels for CI being consistently lower. For all seasons, Zn levels across all the bays were essentially within background, with evidence of a slight elevation in PPB Summer 2015/16. There was a progressive increase in Zn levels for WP from Spring 2018 to Winter 2019, with a slight elevation being evident for the latter season.

A complementary method has also been trialled for comparing the average relative magnitudes of the metals of interest from one season to another, benchmarked against the average relative magnitudes expected from the international database. This is based on the premise that for a polluted environment, such a series of inequalities would be noticeably perturbed. This problem has been addressed by invoking a mathematical technique that utilizes a “discrete metric” - specifically the “Manhattan metric”. This method has been shown to dramatically reveal the Winter 2018 pollution event and also shows that there is a reasonable consistency in the average heavy metal levels across the bays for all the other seasons. It is suggested that this method could find more extensive application in the analysis of environmental data in general.

In summary, an assay has been postulated for determining whether heavy metal levels in a marine environment, as reflected in their bioaccumulation in seagrass, are representative of normal background levels or are suggestive of heavy metal pollution. This assay has been derived from the totality of such data reported in the international literature over the last forty years. The derived assay has been tested against a concurrent five-year temporal study of seagrass heavy metal levels across three neighbouring marine bays in Victoria, Australia. This study has revealed a useful temporal heavy metal pollution profile of these bays with respect to nine heavy metals that are influenced by anthropogenic activities.

Appendices

Appendix 1 – Poster for ECSA 57

Please see attach PDF entitled YHL ECSA 57 Poster.

Appendix 2 – Presentation for CHEERS 2019

Please see attached PDF entitled CHEERS 2019 YHL.

Appendix 3 – 3MT Slide and Script

Please see attached PDF entitled YHL 3MT.

Appendix 4 – Presentation for ISILC HDR Student Conference 2020

Please see attached PDF entitled ISILC HDR STUDENT CONFERENCE YHL 2020

Appendix 5 - Presentation for ISILC HDR Student Symposium 2022

Please see attached PDF entitled ISILC HDR STUDENT CONFERENCE YHL 2022

Appendix 6 – Inductively coupled plasma atomic emission spectroscopy Information (ICP-AES)

ICP-AES is an analytical technique used for the detection of chemical elements. It is a type of emission spectroscopy that uses the inductively coupled plasma to produce excited atoms and ions that emit electromagnetic radiation at wavelengths characteristic of a particular element. The plasma is a high temperature source of ionised source gas (often argon). The plasma is sustained and maintained by inductive coupling from cooled electrical coils at megahertz frequencies. The source temperature is in the range from 6000 to 10,000 K. The intensity of the emissions from various wavelengths of light are proportional to the concentrations of the elements within the sample (Stefansson et al. 2007).

Examples of the application of ICP-AES include the determination of metals in wine, (Aceto et al. 2002) arsenic (As) in food, (Benramdane et al. 1999) and trace elements bound to proteins (Ma et al. 2004). ICP-AES is widely used in minerals processing to provide the data on grades of various streams and for the construction of mass balances. ICP-AES is often used for analysis of trace elements in soil, and it is for that reason it is often used in forensics to ascertain the origin of soil samples found at crime scenes or on victims. Taking a sample from a control and determining the metal composition and taking the sample obtained from evidence and determine that metal composition allows a comparison to be made. While soil evidence may not stand alone in court, it certainly strengthens other evidence. It is also fast becoming the analytical method of choice for the determination of nutrient levels in agricultural soils. This information is then used to calculate the amount of fertilizer required to maximise crop yield and quality. ICP-AES is also used for engine oil analysis. Analysing used engine oil reveals a great deal, about how the engine is operating. Parts that wear in the

engine will deposit traces in the oil, which can be detected with ICP-AES. ICP-AES analysis can help to determine whether parts are failing. In addition, ICP-AES can determine what amount of certain oil additives remain and therefore indicate how much service life the oil has remaining. Fleet managers or automotive enthusiasts who have an interest in finding out as much about their engine's operation as possible often use oil analysis. ICP-AES is also used during the production of engine oils (and other lubricating oils) for quality control and compliance with production and industry specifications. This method is a common technique to test heavy metals in organic samples (APHA 2005; USEPA 1996; USEPA 2001).

Appendix 7 – Atomic Absorption Spectroscopy Information (AAS)

AAS is a spectro-analytical procedure for the quantitative determination of chemical elements using the absorption of optical radiation (light) by free atoms in the gaseous state. Atomic absorption spectroscopy is based on absorption of light by free metallic ions. In analytical chemistry the technique is used for determining the concentration of a particular element (the analyte) in a sample. AAS can be used to determine over 70 different elements in solution. AAS is used in pharmacology, biophysics, archaeology and toxicology research. AAS has many uses in different areas of chemistry such as clinical analysis of metals in biological fluids and tissues such as whole blood, plasma, urine, saliva, brain tissue, liver, hair and muscle tissue. AAS can be used in qualitative and quantitative analysis.

The technique makes use of the atomic absorption spectrum of a sample in order to assess the concentration of specific analytes within it. It requires standards with of known concentration to establish the relation between the measured absorbance and the analyte concentration and relies therefore on the Beer–Lambert law. The Beer-Lambert law relates the attenuation (a reduction in the intensity) of light to the properties of the material through which the light is travelling.

In order to analyse a sample for its atomic constituents, it has to be atomised. The atomisers most commonly used nowadays are flames and electrothermal (graphite tube) atomisers. A flame based atomiser was used for this project. The atoms are then irradiated by optical radiation, and the radiation source is an element-specific line radiation source or a continuum radiation source. Here, element-specific line of radiation was employed. The radiation then passes through a monochromator in order to separate the element-specific radiation from any other radiation emitted by the radiation source, which is finally measured by a detector.

The flame atomiser is the oldest and most commonly used atomiser in AAS. The flames are principally an air-acetylene flame with a temperature of about 2300 °C or a N2O-acetylene flame with a temperature of about 2700°C (Koirtyohann 1991). An air-acetylene flame atomiser was used in this project. Liquids or dissolved samples are typically used with flame atomisers. The sample solution is aspirated by a pneumatic analytical nebuliser, which is transformed into an aerosol and is introduced into a spray chamber, where it is mixed with the flame gases and conditioned in a way that only

the finest aerosol droplets (< 10 µm) enter the flame. This conditioning process reduces interference, but only about 5 % of the aerosolized solution reaches the flame. On top of the spray chamber is a burner head that produces a flame that is usually 5 – 10 cm) long and only a few millimetres deep. The radiation beam passes through this flame at its longest axis, and the flame gas flow rates may be adjusted to produce the highest concentration of free atoms. The burner height may also be adjusted, so that the radiation beam passes through the zone of highest atom cloud density in the flame, resulting in the highest sensitivity. The processes in a flame include the stages of drying in which the solvent is evaporated and the dry sample nanoparticles remain, vaporisation (transfer to the gaseous phase) in which the solid particles are converted into gaseous molecule, atomisation in which the molecules are dissociated into free atoms, and ionisation where (depending on the ionisation potential of the analyte atoms and the energy available in a particular flame) atoms may be in part converted to gaseous ions. Each of these stages includes the risk of interference in case the degree of phase transfer is different for the analyte in the calibration standard and in the sample. Ionisation is generally undesirable, as it reduces the number of atoms that are available for measurement, i.e., the sensitivity. In flame based AAS a steady-state signal is generated during the time period when the sample is aspirated. This technique is typically used for determinations in the mg/L−1 range and may be extended down to a few µg/L−1 for some elements.

For the radiation source hollow cathode lamps (HCL) were used. HLC is the most common radiation source in AAS. Inside the sealed lamp, which is filled with argon or neon gas at low pressure, is a cylindrical metal cathode containing the element of interest and an anode. A high voltage is applied across the anode and cathode, resulting in an ionisation of the fill gas. The gas ions are accelerated towards the cathode and, upon impact on the cathode, sputter cathode material that is excited in the glow discharge to emit the radiation of the sputtered material, i.e., the element of interest. In the majority of cases single element lamps are used, where the cathode is pressed out of predominantly compounds of the target element. Multi-element lamps are available with combinations of compounds of the target elements pressed in the cathode. Multi element lamps produce slightly less sensitivity than single element lamps and the combinations of elements have to be selected carefully to avoid spectral interferences.

The relatively small number of atomic absorption lines when compared to atomic emission lines and their narrow width of a few picometres (pm), make spectral overlap rare. There are only few examples known that an absorption line from one element will overlap with another. Molecular absorption, in contrast, is much broader, so that it is more likely that some molecular absorption bands will overlap with atomic lines. This kind of absorption might be caused by un-dissociated molecules of concomitant elements of the sample or by flame gases. This phenomena, molecular absorption and radiation scattering, can result in artificially high absorption and an improperly high (erroneous) calculation for the concentration or mass of the analyte in the sample. There are several techniques available to correct for background absorption, and they are significantly different for line source (LS) AAS. In LS AAS background absorption

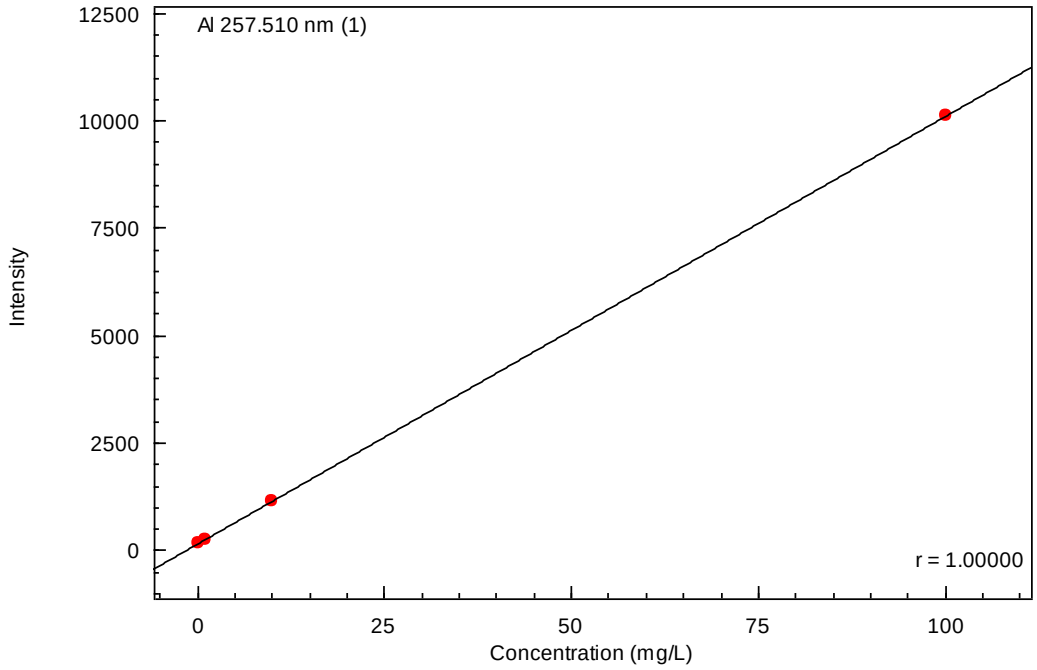
can only be corrected using instrumental techniques, and all of them are based on two sequential measurements (Preedy 2015). First is total absorption (atomic plus background). While the second is background absorption only. The difference between the two measurements gives the net atomic absorption. Because of this, and because of the use of additional devices in the spectrometer, the signal-to-noise ratio of background-corrected signals is always significantly inferior compared to uncorrected signals. It should also be pointed out that in LS AAS there is no way to correct for (the rare case of) a direct overlap of two atomic lines.

Appendix 8 – Table of Weather and Tide conditions during the sampling period

Location	Weather	Low Tide Time and Depth (cm)
Corio (23/12/15)	25ºC was partly cloudy with light winds	6:19am and 43 cm
Altona (23/12/15)	25ºC was partly cloudy with light winds	6:34am and 47 cm
Sandringham (23/12/15)	25ºC was partly cloudy with light winds	6:16am and 45 cm
Crib Point (9/1/16)	25ºC mostly sunny	6:13pm and 67 cm
San Remo (13/1/16)	41ºC hot sunny	9:54am and 79 cm
Phillip Island (13/1/16)	41ºC hot sunny	9:54am and 79 cm
Toora (18/1/16)	36ºC mostly sunny with a late cool change	2:30pm and 27 cm
Foster (18/1/16)	36ºC mostly sunny with a late cool change	2:30pm and 27 cm
Yanakie (18/1/16)	36ºC mostly sunny with a late cool change	2:30pm and 27 cm
Altona (20/6/18)	14ºC partly cloudy	1:45pm and 30 cm
Corio (20/6/18)	14ºC partly cloudy	1:47pm and 30 cm
Sandringham (20/6/18)	14ºC partly cloudy	1:49pm and 34 cm
Phillip Island (18/7/18)	14ºC partly cloudy	10:10am and 42 cm
San Remo (18/7/18)	14ºC partly cloudy	10:10am and 42 cm
Crib Point (18/7/18)	14ºC partly cloudy	10:13am and 43 cm
Toora (2/8/18)	13ºC partly cloudy	10:30am and 73 cm
Foster (2/8/18)	13ºC partly cloudy	10:30am and 73 cm
Yanakie (2/8/18)	13ºC partly cloudy	10:30am and 73 cm
Corio (19/11/18)	31ºC Mostly Sunny	6:22pm and 42 cm
Altona (19/11/18)	31ºC Mostly Sunny	5:54pm and 43 cm
Sandringham (19/11/18)	31ºC Mostly Dunny	5:56pm and 38 cm
Phillip Island (29/11/18)	22ºC Partly Cloudy	11:54am and 74 cm
San Remo (29/11/18)	22ºC Partly Cloudy	11:54am and 74 cm
Crib Point (29/11/18)	22ºC Partly Cloudy	11:55am and 79 cm
Toora (6/12/18)	33ºC Sunny	6:48pm and 46 cm
Foster (6/12/18)	33ºC Sunny	6:48pm and 46 cm
Yanakie (6/12/18)	33ºC Sunny	6:48pm and 46 cm
Altona (31/1/19)	21ºC Showers	6:04pm and 30 cm
Sandringham (31/1/19)	21ºC Showers	6:02pm and 26 cm
Corio (31/1/19)	21ºC Showers	6:31pm and 20 cm
Phillip Island (13/2/19)	21ºC Partly Cloudy	12:56pm and 42 cm
San Remo (13/2/19)	21ºC Partly Cloudy	12:56pm and 42 cm
Crib Point (13/2/19)	21ºC Partly Cloudy	1:30pm and 58 cm

Toora (20/2/19)	20ºC Partly Cloudy	8:01pm and 32 cm
Foster (20/2/19)	20ºC Partly Cloudy	8:01pm and 32 cm
Yanakie (20/2/19)	20ºC Partly Cloudy	8:01pm and 32 cm
Altona (14/5/19)	18ºC Possible Showers	3:46pm and 32 cm
Sandringham (14/5/19)	18ºC Possible Showers	3:36pm and 32 cm
Corio (14/5/19)	18ºC Possible Showers	4:08pm and 37 cm
Phillip Island (21/5/19)	19ºC Partly Cloudy	8:02am and 29 cm
San Remo (21/5/19)	19ºC Partly Cloudy	8:02am and 29 cm
Crib Point (21/5/19)	19ºC Partly Cloudy	7:56 am and 48 cm
Toora (8/6/19)	15ºC Partly Cloudy and Windy	10:30am and 32 cm
Foster (8/6/19)	15ºC Partly Cloudy and Windy	10:30am and 32 cm
Yanakie (8/6/19)	15ºC Partly Cloudy and Windy	10:30am and 32 cm
Altona (13/8/19)	14ºC Afternoon Showers	6:41am and 24 cm
Sandringham (13/8/19)	14ºC Afternoon Showers	6:41am and 18 cm
Corio (13/8/19)	14ºC Afternoon Showers	6:56am and 28 cm
Phillip Island (21/8/19)	17ºC Showers and Windy	10:07am and 70 cm
San Remo (21/8/19)	17ºC Showers and Windy	10:07am and 70 cm
Crib Point (21/8/19)	17ºC Showers and Windy	10:07am and 90 cm
Toora (27/8/19)	13ºC Showers	2:36pm and 106 cm
Foster (27/8/19)	13ºC Showers	2:36pm and 106 cm
Yanakie (27/8/19)	13ºC Showers	2:36pm and 106 cm

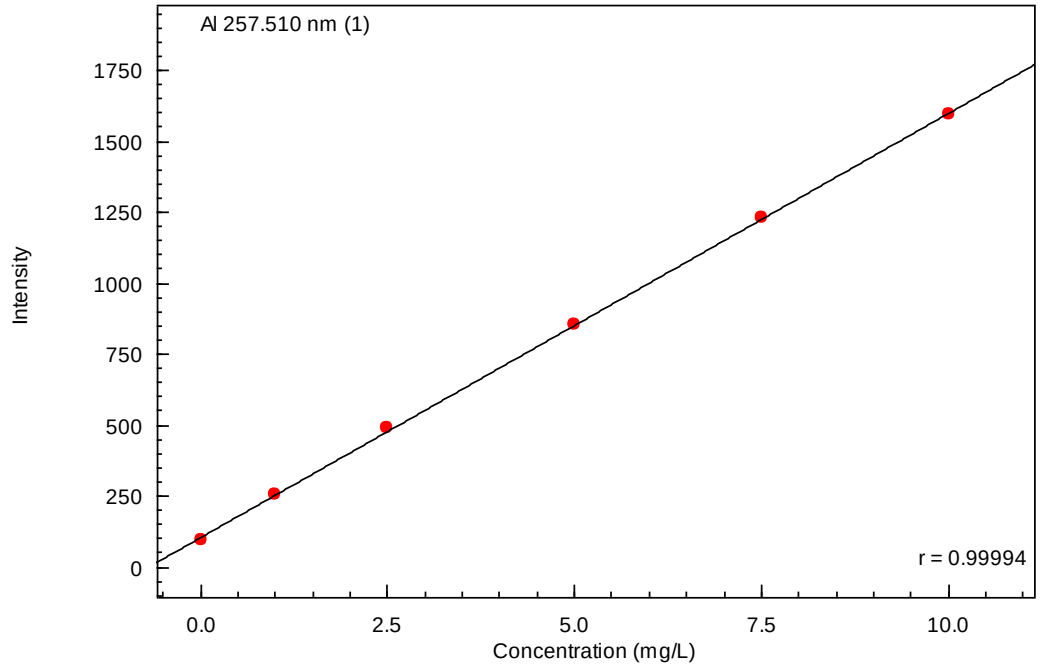
Appendix 9 – Al 257.510 nm Honours Calibration



Equation: Conc = a * I ^ 3 + b * I ^ 2 + c * I + d

Factor: a = 0.0000000 c = 0.0100411 Weight: None
b = 0.0000000 d = -1.702432 Origin: None

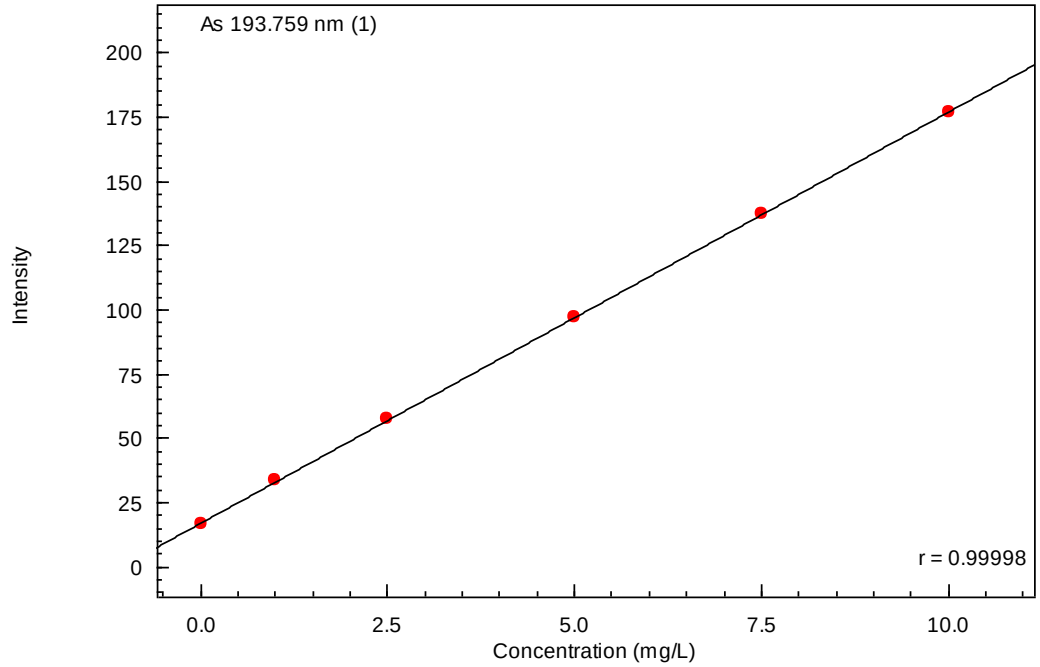
Appendix 10 – Al 257.510 nm PhD Calibration



Equation: Conc = a * I ^ 3 + b * I ^ 2 + c * I + d

Factor:	a = 0.0000000	c = 0.0066914	Weight: None
	b = 0.0000000	d = -0.7169076	Origin: None

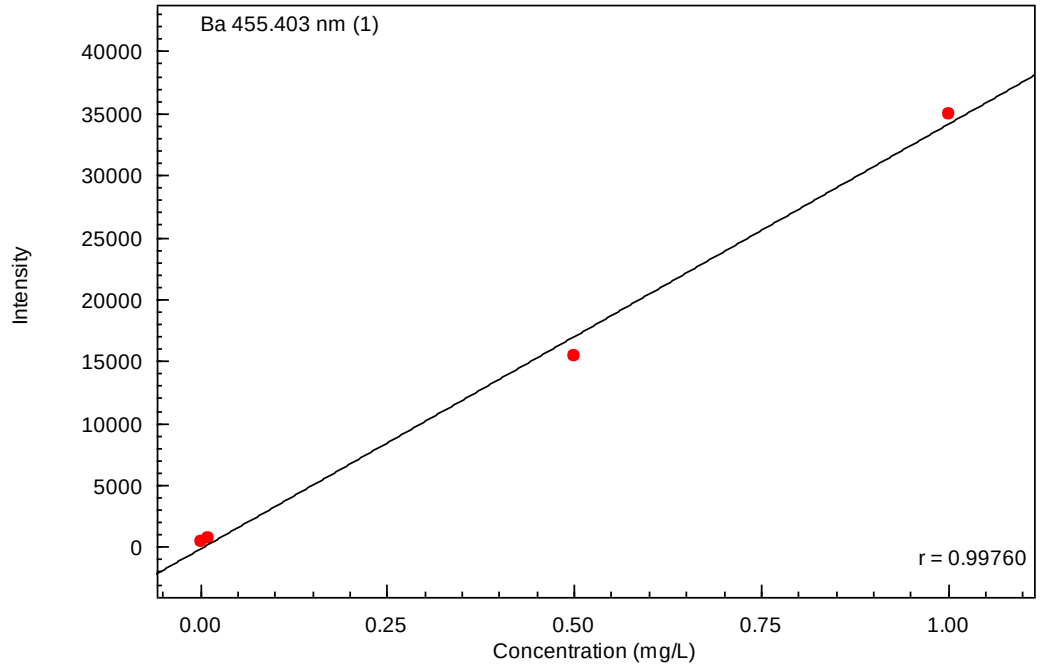
Appendix 11 – As 193.759 nm PhD Calibration



Equation: Conc = a * I ^ 3 + b * I ^ 2 + c * I + d

Factor:	a = 0.0000000	c = 0.0624950	Weight: None
	b = 0.0000000	d = -1.077196	Origin: None

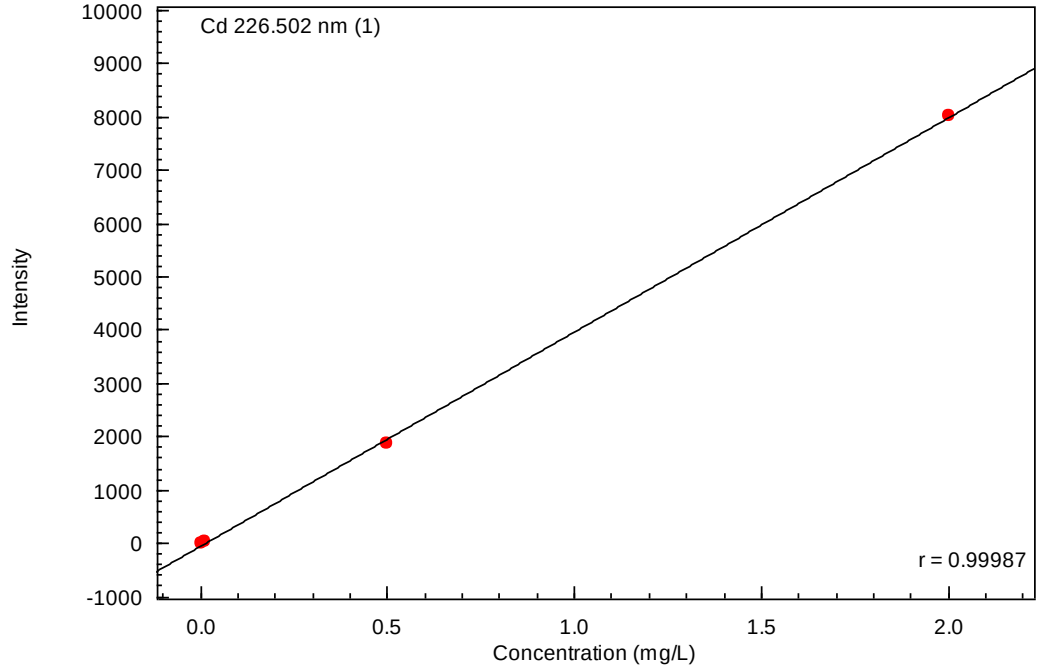
Appendix 12 – Ba 455.403 nm Honours Calibration



Equation: Conc = a * I ^ 3 + b * I ^ 2 + c * I + d

Factor:	a = 0.0000000	c = 2.917159e-005	Weight: None
	b = 0.0000000	d = 0.0012674	Origin: None

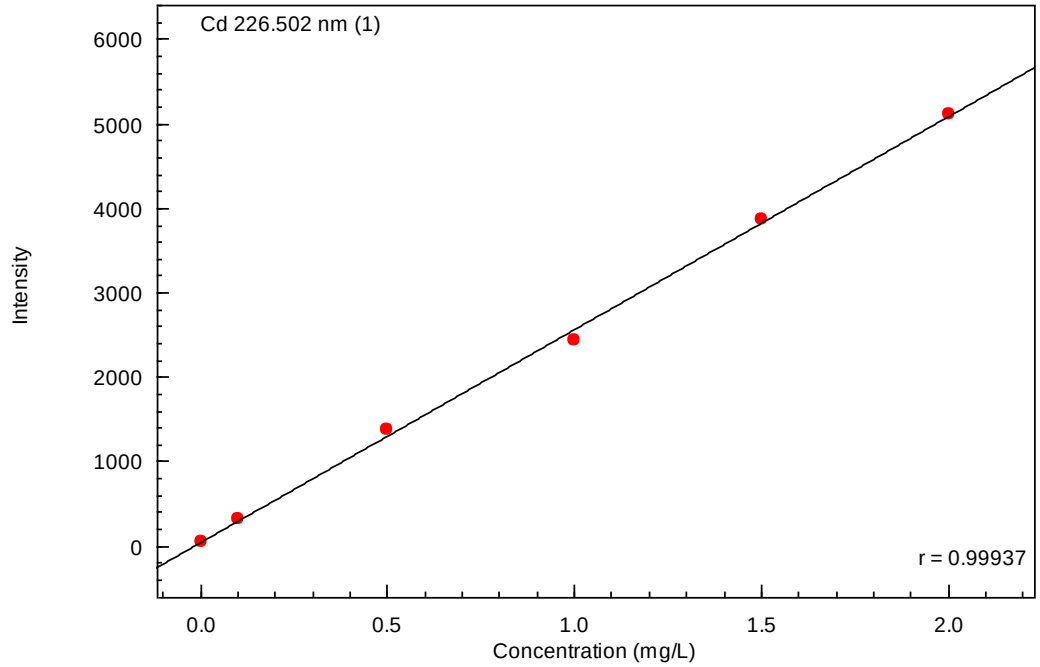
Appendix 13 – Cd 226.502 nm Honours Calibration



Equation: Conc = a * I ^ 3 + b * I ^ 2 + c * I + d

Factor:	a = 0.0000000	c = 2.487773e-004	Weight: None
	b = 0.0000000	d = 0.0089918	Origin: None

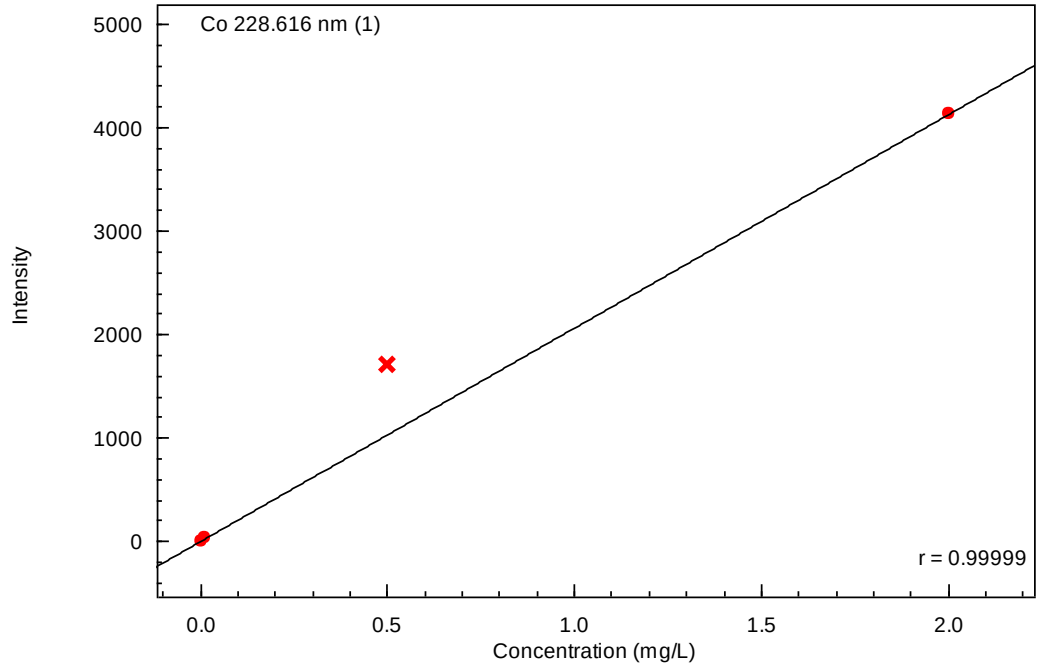
Appendix 14 – Cd 226.502 nm PhD Calibration



Equation: Conc = a * I ^ 3 + b * I ^ 2 + c * I + d

Factor:	a = 0.0000000	c = 3.964315e-004	Weight: None
	b = 0.0000000	d = -0.0209316	Origin: None

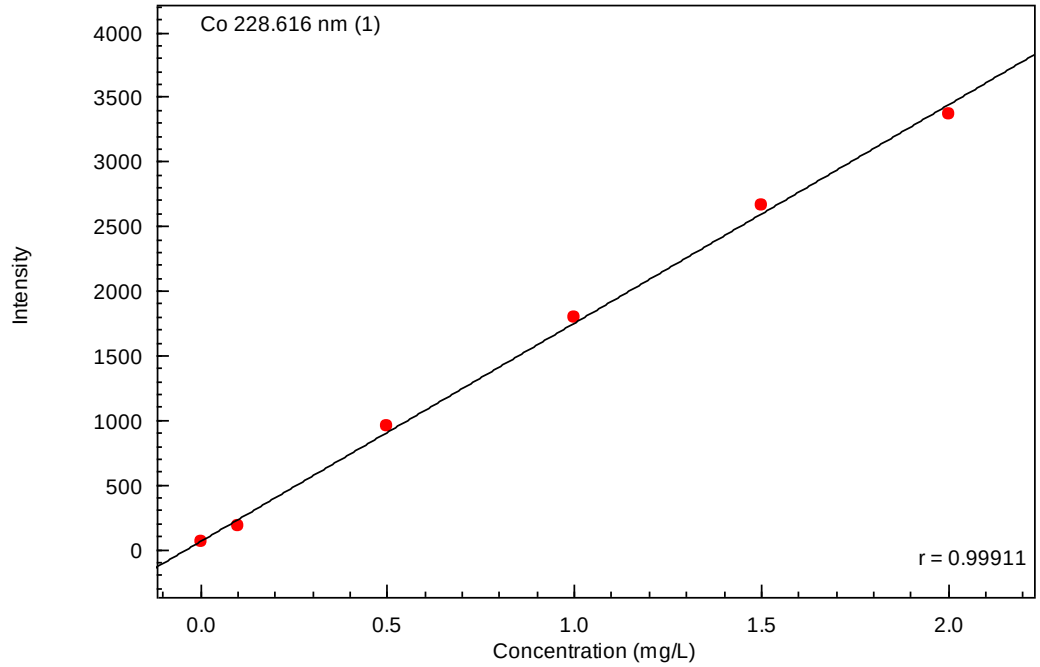
Appendix 15 – Co 228.616 nm Honours Calibration



Equation: Conc = a * I ^ 3 + b * I ^ 2 + c * I + d

Factor:	a = 0.0000000	c = 4.845561e-004	Weight: None
	b = 0.0000000	d = -0.0040727	Origin: None

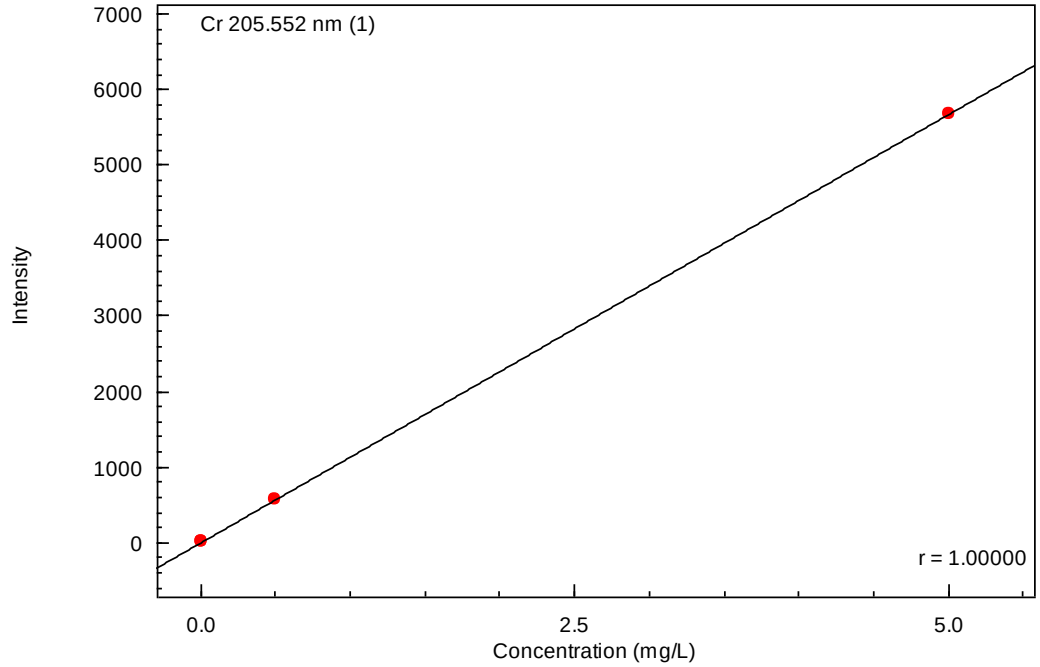
Appendix 16 – Co 228.616 nm PhD Calibration



Equation: Conc = a * I ^ 3 + b * I ^ 2 + c * I + d

Factor:	a = 0.0000000	c = 5.917168e-004	Weight: None
	b = 0.0000000	d = -0.0412390	Origin: None

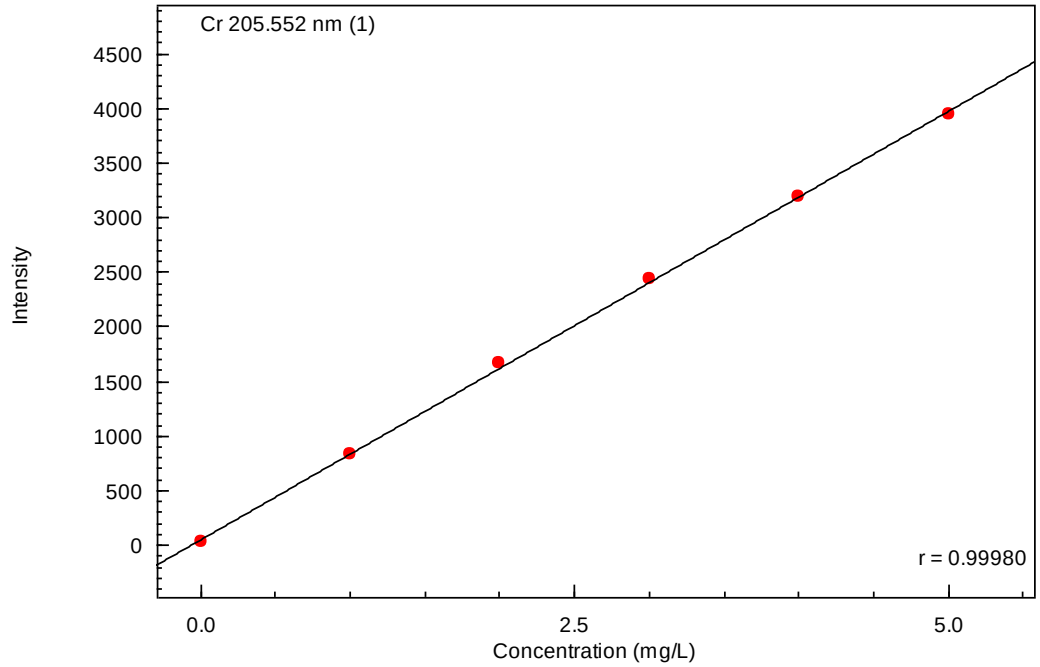
Appendix 17 – Cr 205.552 nm Honours Calibration



Equation: Conc = a * I ^ 3 + b * I ^ 2 + c * I + d

Factor:	a = 0.0000000	c = 8.815069e-004	Weight: None
	b = 0.0000000	d = -0.0059989	Origin: None

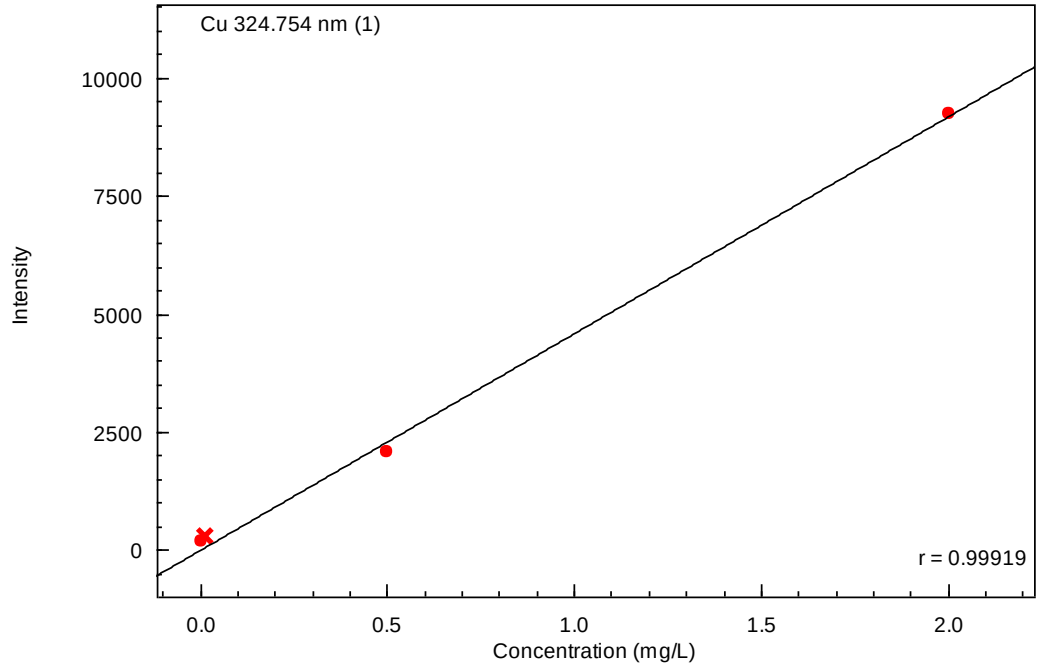
Appendix 18 – Cr 205.552 nm PhD Calibration



Equation: Conc = a * I ^ 3 + b * I ^ 2 + c * I + d

Factor:	a = 0.0000000	c = 0.0012735	Weight: None
	b = 0.0000000	d = -0.0727938	Origin: None

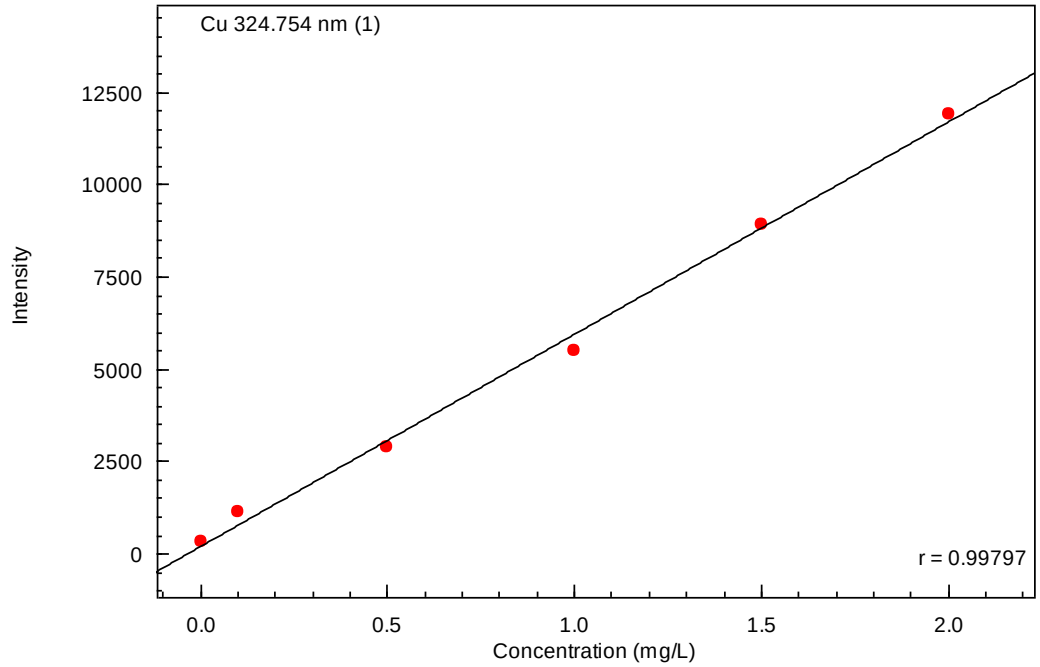
Appendix 19 – Cu 324.754 nm Honours Calibration



Equation: Conc = a * I ^ 3 + b * I ^ 2 + c * I + d

Factor:	a = 0.0000000	c = 2.178914e-004	Weight: None
	b = 0.0000000	d = -0.0048148	Origin: None

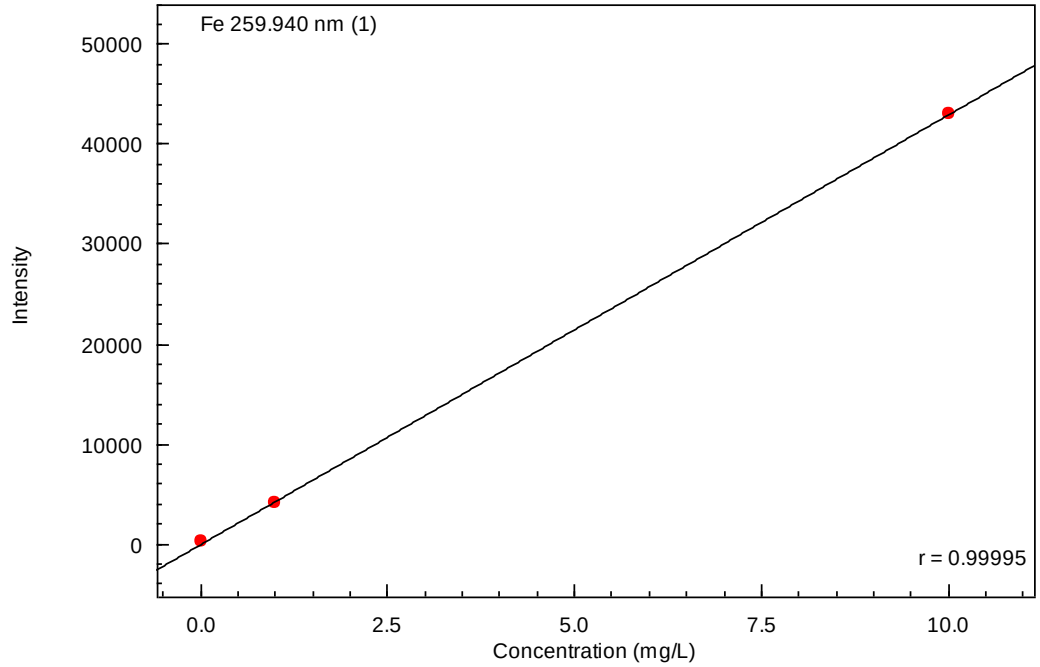
Appendix 20 – Cu 324.754 nm PhD Calibration



Equation: Conc = a * I ^ 3 + b * I ^ 2 + c * I + d

Factor:	a = 0.0000000	c = 1.739022e-004	Weight: None
	b = 0.0000000	d = -0.0392582	Origin: None

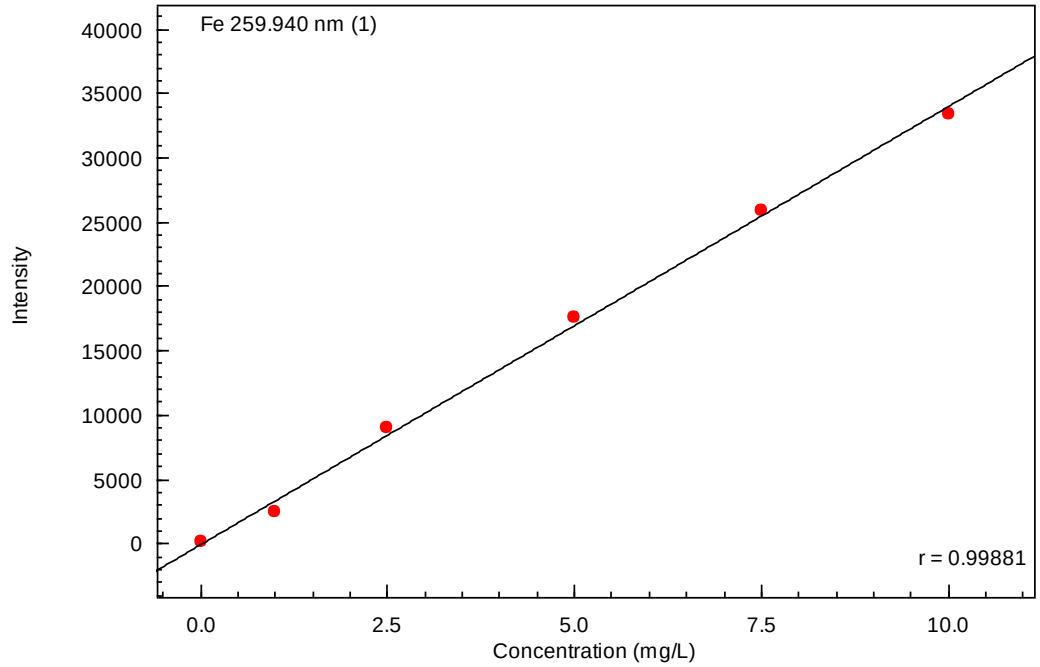
Appendix 21 – Fe 259.940 nm Honours Calibration



Equation: Conc = a * I ^ 3 + b * I ^ 2 + c * I + d

Factor:	a = 0.0000000	c = 2.324371e-004	Weight: None
	b = 0.0000000	d = -0.0035182	Origin: None

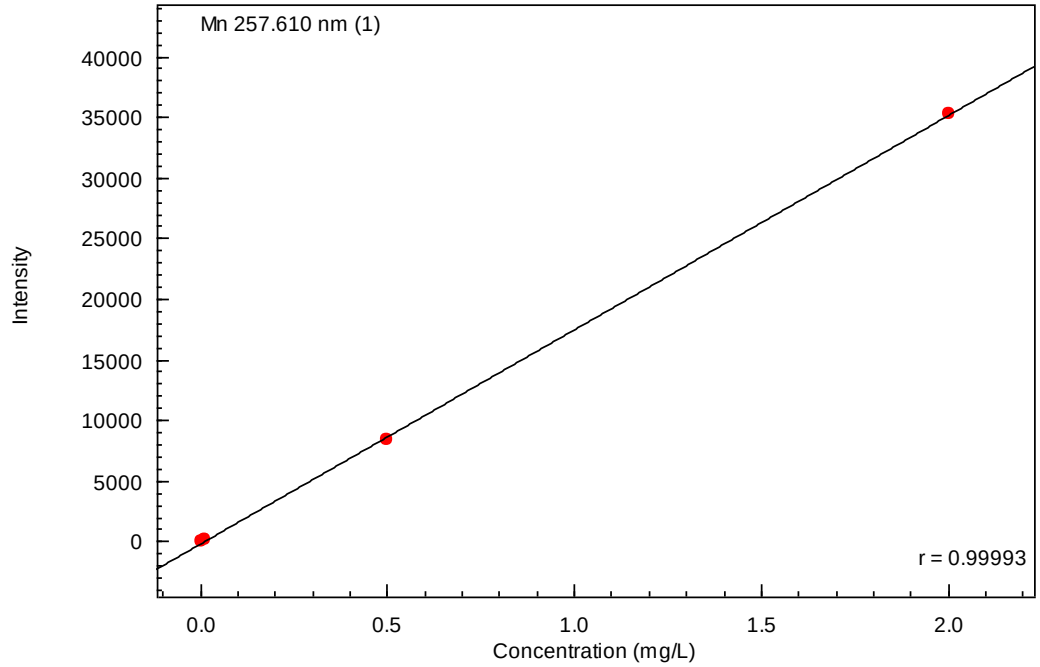
Appendix 22 – Fe 259.940 nm PhD Calibration



Equation: Conc = a * I ^ 3 + b * I ^ 2 + c * I + d

Factor:	a = 0.0000000	c = 2.934727e-004	Weight: None
	b = 0.0000000	d = 0.0019095	Origin: None

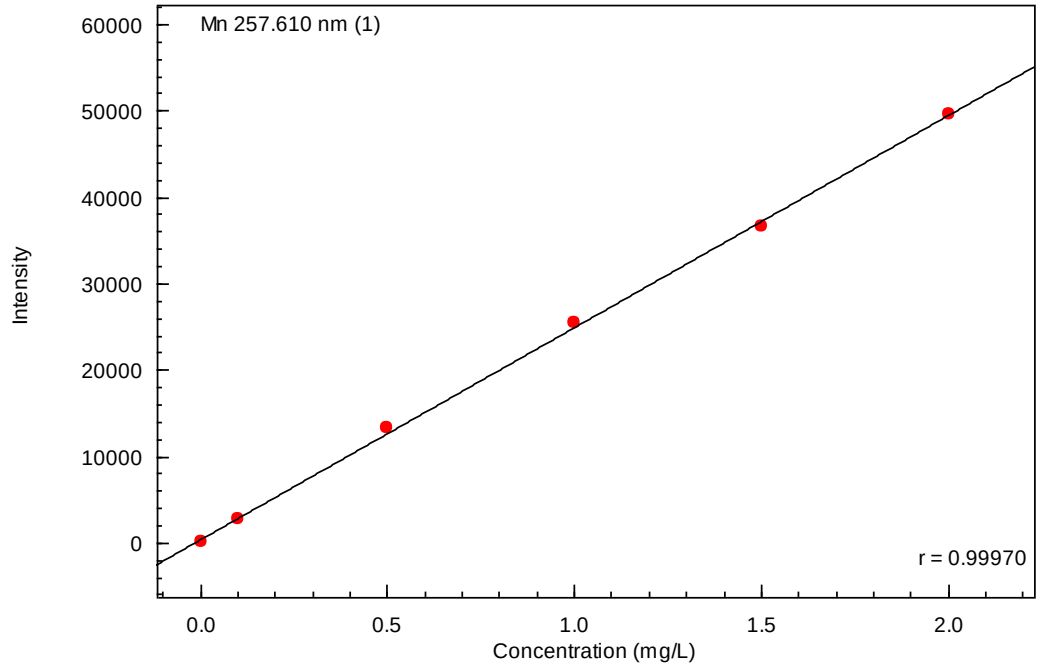
Appendix 23 – Mn 257.610 nm Honours Calibration



Equation: Conc = a * I ^ 3 + b * I ^ 2 + c * I + d

Factor:	a = 0.0000000	c = 5.660775e-005	Weight: None
	b = 0.0000000	d = 0.0053470	Origin: None

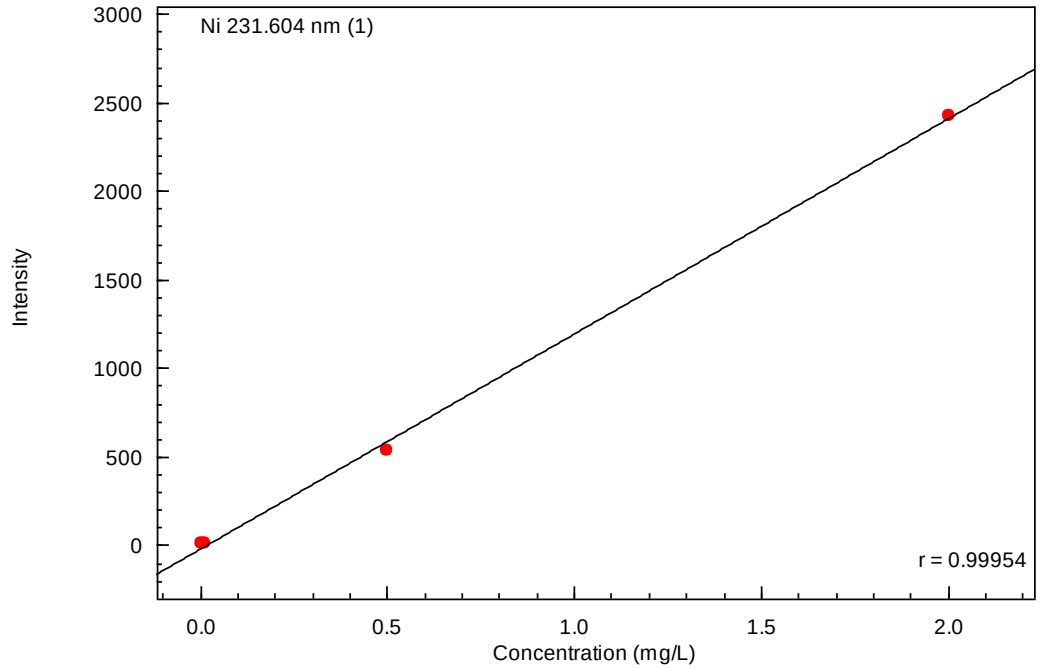
Appendix 24 – Mn 257.610 nm PhD Calibration



Equation: Conc = a * I ^ 3 + b * I ^ 2 + c * I + d

Factor:	a = 0.0000000	c = 4.070424e-005	Weight: None
	b = 0.0000000	d = -0.0194727	Origin: None

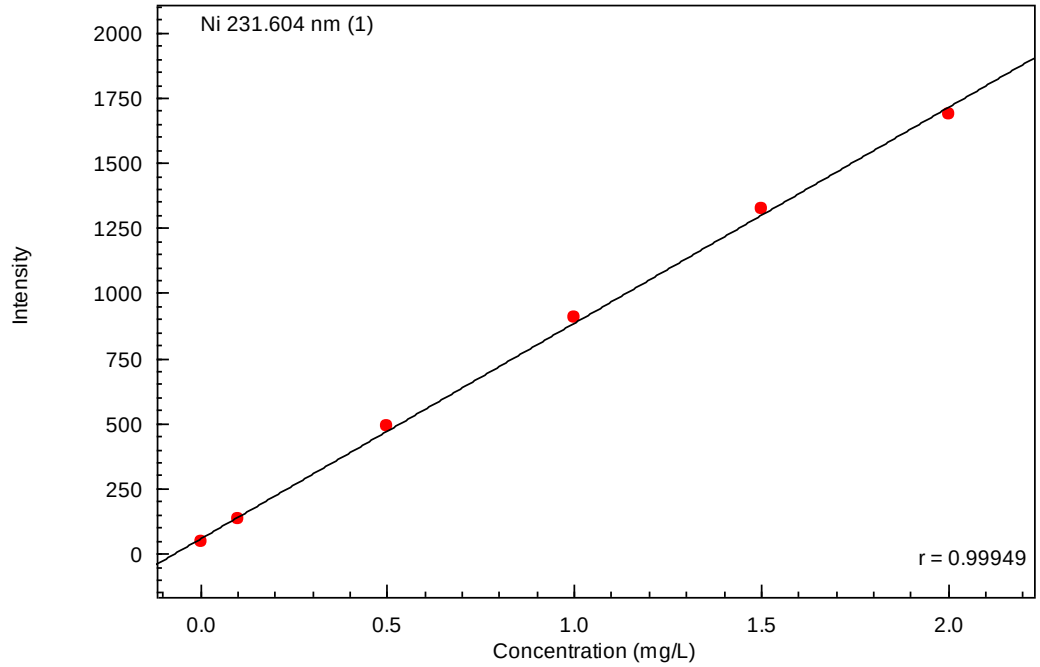
Appendix 25 – Ni 231.604 nm Honours Calibration



Equation: Conc = a * I ^ 3 + b * I ^ 2 + c * I + d

Factor:	a = 0.0000000	c = 8.230129e-004	Weight: None
	b = 0.0000000	d = 0.0112291	Origin: None

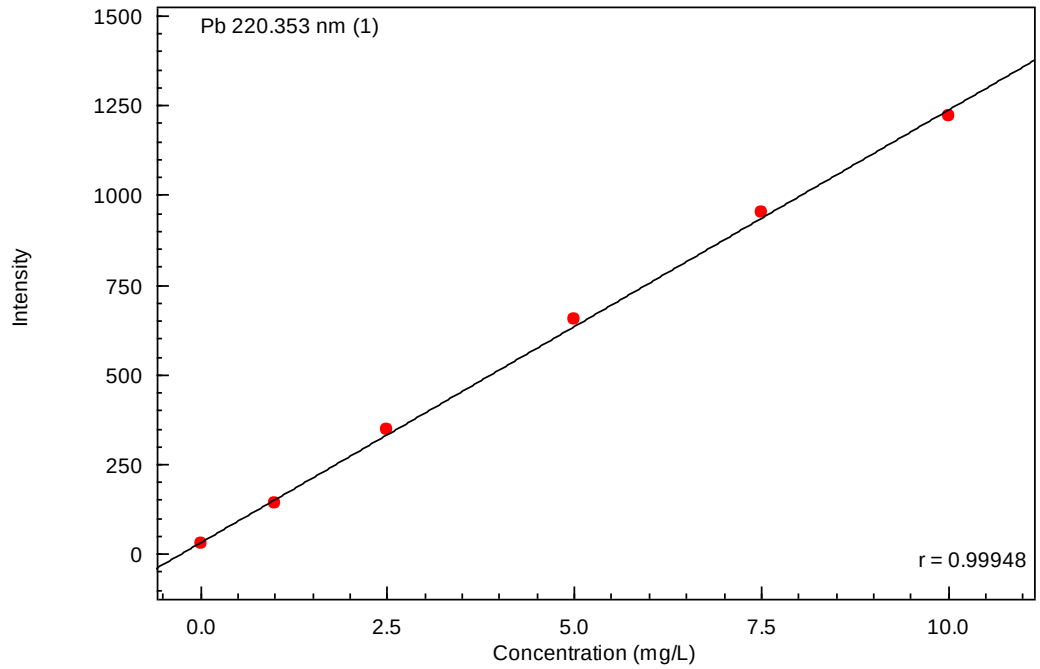
Appendix 26 – Ni 231.604 nm PhD Calibration



Equation: Conc = a * I ^ 3 + b * I ^ 2 + c * I + d

Factor:	a = 0.0000000	c = 0.0012068	Weight: None
	b = 0.0000000	d = -0.0742103	Origin: None

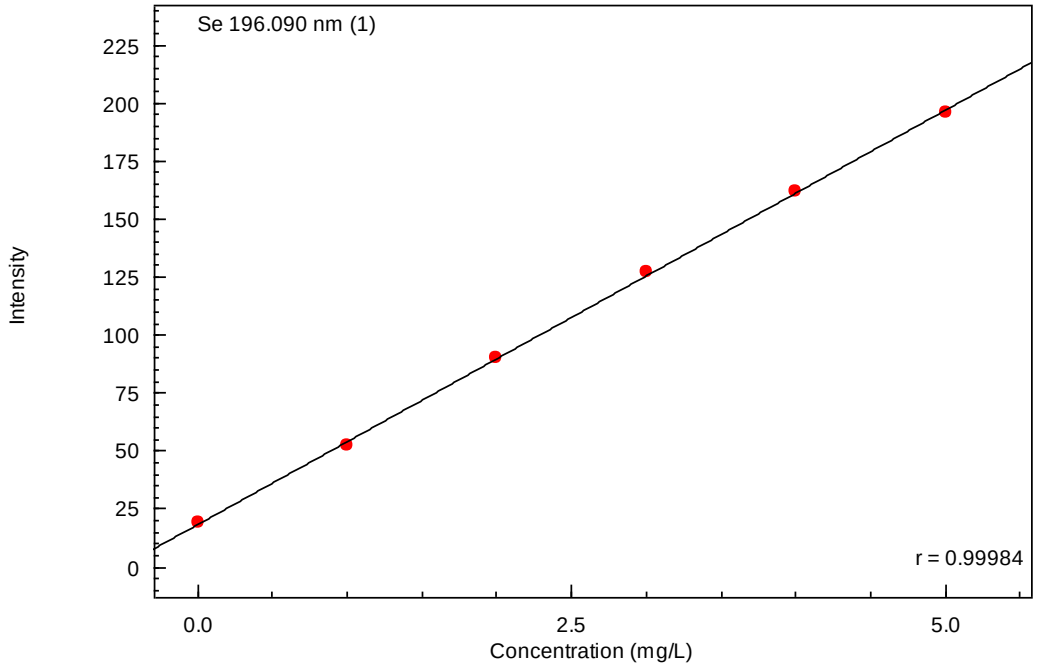
Appendix 27 – Pb 220.353 nm PhD Calibration



Equation: Conc = a * I ^ 3 + b * I ^ 2 + c * I + d

Factor:	a = 0.0000000	c = 0.0082943	Weight: None
	b = 0.0000000	d = -0.2868709	Origin: None

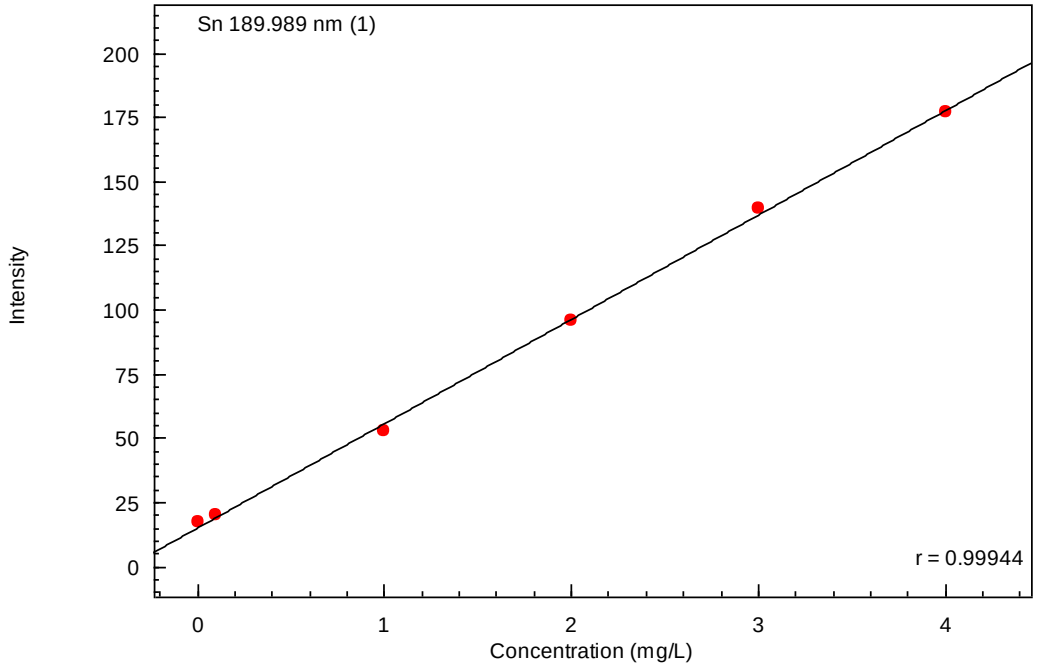
Appendix 28 – Se 196.090 nm PhD Calibration



Equation: Conc = a * I ^ 3 + b * I ^ 2 + c * I + d

Factor:	a = 0.0000000	c = 0.0279928	Weight: None
	b = 0.0000000	d = -0.5237660	Origin: None

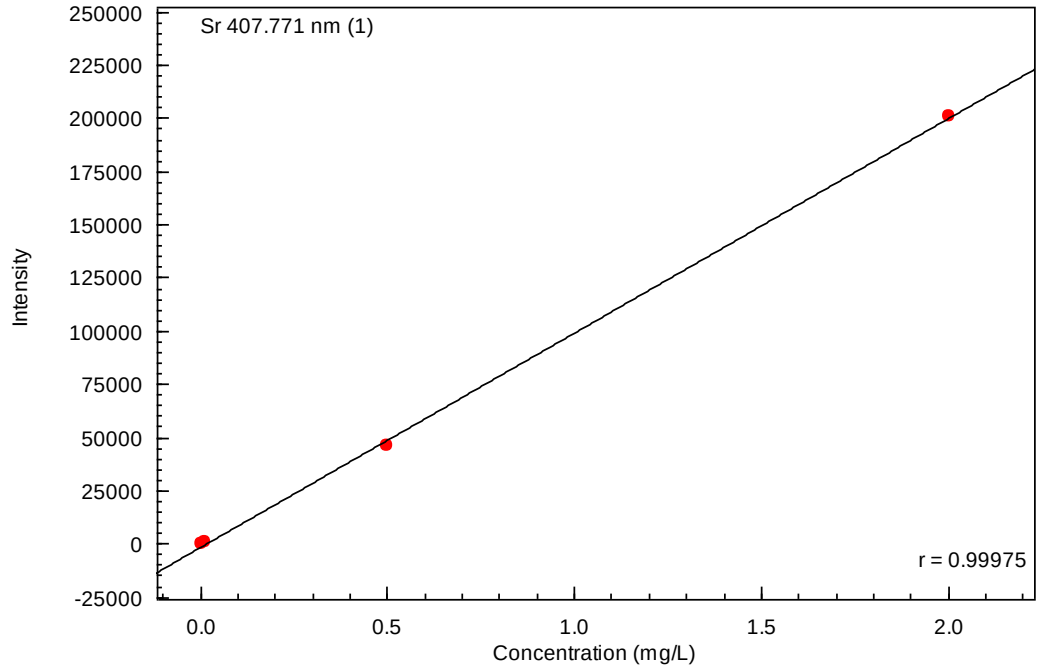
Appendix 29 – Sn 189.989 nm PhD Calibration



Equation: Conc = a * I ^ 3 + b * I ^ 2 + c * I + d

Factor:	a = 0.0000000	c = 0.0246072	Weight: None
	b = 0.0000000	d = -0.3801567	Origin: None

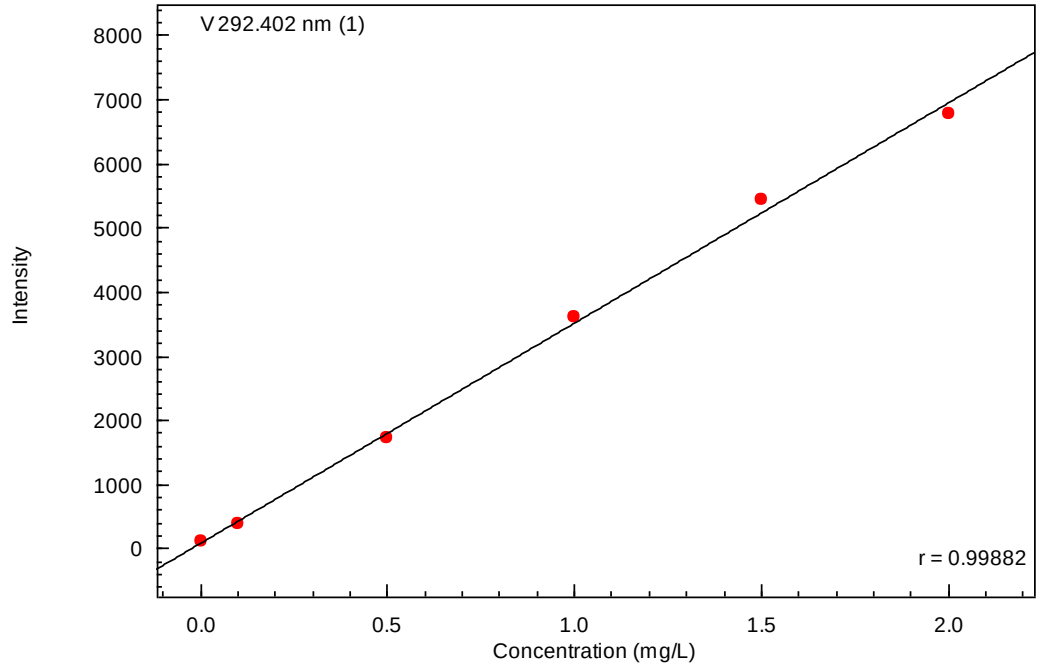
Appendix 30 – Sr 407.771 nm Honours Calibration



Equation: Conc = a * I ^ 3 + b * I ^ 2 + c * I + d

Factor:	a = 0.0000000	c = 9.919310e-006	Weight: None
	b = 0.0000000	d = 0.0118989	Origin: None

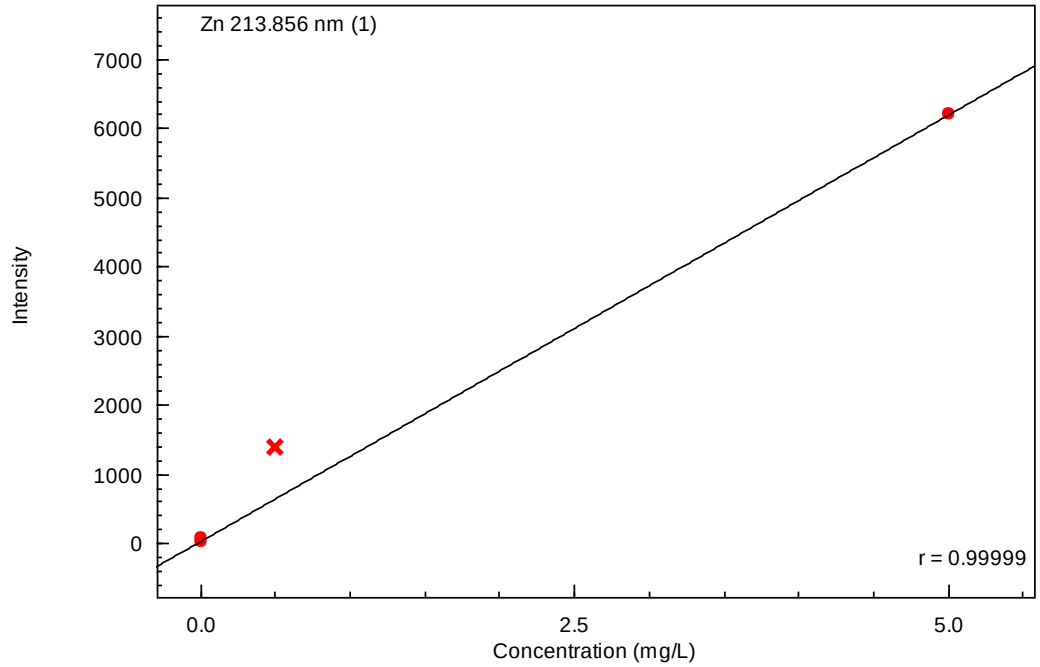
Appendix 31 – V 292.402 nm PhD Calibration



Equation: Conc = a * I ^ 3 + b * I ^ 2 + c * I + d

Factor:	a = 0.0000000	c = 2.913460e-004	Weight: None
	b = 0.0000000	d = -0.0294824	Origin: None

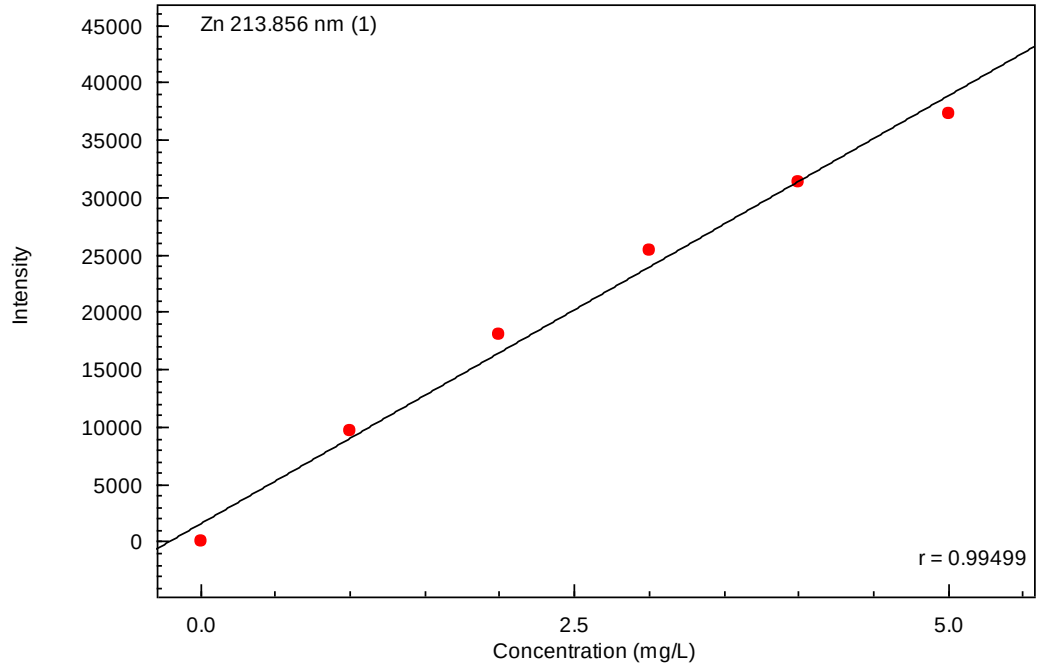
Appendix 32 – Zn 213.856 nm Honours Calibration



Equation: Conc = a * I ^ 3 + b * I ^ 2 + c * I + d

Factor: a = 0.0000000 c = 8.104002e-004 Weight: None
b = 0.0000000 d = -0.0337044 Origin: None

Appendix 33 – Zn 213.856 nm PhD Calibration



Equation: Conc = a * I ^ 3 + b * I ^ 2 + c * I + d

Factor: a = 0.0000000 c = 1.340118e-004 Weight: None
b = 0.0000000 d = -0.2234020 Origin: None

Appendix 34 – Part A raw data

Please see attach Excel spreadsheet labelled Review under the tab raw.

Appendix 35 – Part A Chemical Elements Comments in Seagrass Magnitude Ranges

Silver (Ag)

Silver is a non-essential plant element. The minimum concentration of Ag in seagrass through the world is 0.34 PPM while the maximum concentration is 8.66 PPM. The average concentration of Ag in seagrass through the world is 1.72 PPM. The median concentration of Ag in seagrass through the world is 0.83 PPM. The upper value for normal distribution is 1.46 PPM. From this, two outliers were identified. The first outlier at 4.91 PPM was a study by Richir et al. 2013 in Corsica using *Posidonia oceanica* rhizomes. While the second at 8.66 PPM was a study by Tovar-Sánchez et al. 2010 in the Balearic Archipelago, Spain also using *Posidonia oceanica* rhizomes. For the corresponding magnitude curve and boxplot please go to **Appendix 35 and 55**.

Boron (B)

Boron is an essential plant micronutrient. The minimum concentration of B in seagrass through the world is 304.1 PPM while the maximum concentration is 1438 PPM. The average concentration of B in seagrass through the world is 598.14 PPM. The median concentration of B in seagrass through the world is 335.4 PPM. For the corresponding magnitude curve and boxplot please go to **Appendix 36 and 52**.

Barium (Ba)

Barium is a non-essential plant element. The minimum concentration of Ba in seagrass through the world is 13.92 PPM while the maximum concentration is 169.9 PPM. The average concentration of Ba in seagrass through the world is 126.75 PPM. The median concentration of Ba in seagrass through the world is 143.25 PPM. An outlier of 13.92 PPM was identified using *Halodule wrightii* in Santos Bay, Bahia, Brazil. For the corresponding magnitude curve and boxplot please go to **Appendix 37 and 54**.

Beryllium (Be)

Beryllium is a non-essential plant element. The minimum concentration of Be in seagrass through the world is 0.007 PPM while the maximum concentration is 0.01 PPM. The average concentration of Be in seagrass through the world is 0.0085 PPM. The median concentration of Be in seagrass through the world is 0.0085 PPM.

Bismuth (Bi)

Bismuth is a non-essential plant element. The minimum concentration of Bi in seagrass through the world is 0.005 PPM while the maximum concentration is 0.02 PPM. The average and median concentration of Bi in seagrass through the world is

0.01 PPM. For the corresponding magnitude curve and boxplot please go to **Appendix 38 and 56**.

Calcium (Ca)

Calcium is an essential plant macronutrient. The minimum concentration of Ca in seagrass through the world is 1.1 PPM while the maximum concentration is 20689 PPM. The average concentration of Ca in seagrass through the world is 2902.93 PPM. The median concentration of Ca in seagrass through the world is 526.5 PPM. The upper value for normal distribution is 1930 PPM. From this an outlier of 20689 PPM was identified from a study from Morocco by Boutahar et al. 2019 using *Zostera noltei*. For the corresponding magnitude curve and boxplot please go to **Appendix 39 and 51**.

Potassium (K)

Potassium is an essential plant macronutrient. The minimum concentration of K in seagrass through the world is 208 PPM while the maximum concentration is 26493 PPM. The average concentration of K in seagrass through the world is 9386.97 PPM. The median concentration of K in seagrass through the world is 5515.5 PPM. For the corresponding magnitude curve and boxplot please go to **Appendix 40 and 51**.

Lithium (Li)

Lithium is non-essential plant element. In a study by Boutahar et al. 2019 using *Zostera noltei* whole plants recorded a concentration of 0.68 PPM.

Magnesium (Mg)

Magnesium is an essential plant macronutrient. The minimum concentration of Mg in seagrass through the world is 0.9 PPM while the maximum concentration is 10368PPM. The average concentration of Mg in seagrass through the world is 5376.64 PPM. The median concentration of Mg in seagrass through the world is 5774.3 PPM. For the corresponding magnitude curve and boxplot please go to **Appendix 41 and 51**.

Molybdenum (Mo)

Molybdenum is an essential plant micronutrient. The minimum concentration of Mo in seagrass through the world is 0.3 PPM while the maximum concentration is 93.31 PPM. The average concentration of Mo in seagrass through the world is 10.17 PPM. The median concentration of Mo in seagrass through the world is 1.94 PPM. The upper value for normal distribution is 14.02 PPM. From this, two outliers were identified at 17.48 and 93.31. These outliers were from a study by Wilkes et al. 2017 using *Zostera noltei*. The leaves contained 17.48 PPM while the roots contained 93.31 PPM. For the corresponding magnitude curve and boxplot please go to **Appendix 42 and 53**.

Sodium (Na)

Sodium is an essential plant micronutrient. The minimum concentration of Na in seagrass through the world is 1310.67 PPM while the maximum concentration is 42722 PPM. The average concentration of Na in seagrass through the world is 24794.57 PPM. The median concentration of Na in seagrass through the world is 34833 PPM. For the corresponding magnitude curve and boxplot please go to **Appendix 43 and 58**.

Phosphorus (P)

Phosphorus is an essential plant macronutrient. In a study by Serrano et al. 2020 using *Posidonia sinuosa* whole plant recorded a concentration of 365 PPM.

Rubidium (Rb)

Rubidium is a non-essential plant element. In a study by Serrano et al. 2020 using *Posidonia sinuosa* whole plant recorded a concentration of 3.86 PPM.

Sulphur (S)

Sulphur is an essential plant macronutrient. In a study by Serrano et al. 2020 using *Posidonia sinuosa* whole plant recorded a concentration of 3448 PPM.

Antimony (Sb)

Antimony is a non-essential plant element. The minimum concentration of Sb in seagrass through the world is 0.03 PPM while the maximum concentration is 48.6 PPM. The average concentration of Sb in seagrass through the world is 8.81 PPM. The median concentration of Sb in seagrass through the world is 1.06 PPM. The upper value for normal distribution is 33.7 PPM. From this An outlier of 48.6 PPM was identified from a study South Australian by Ward & Hutching 1996 using a species of Zostera. For the corresponding magnitude curve and boxplot please go to **Appendix 44 and 54.**

Silicon (Si)

Silicon is an essential plant micronutrient. In a study by Serrano et al. 2020 using *Posidonia sinuosa* whole plant recorded a concentration of 11703 PPM.

Selenium (Se)

Selenium is an essential plant micronutrient. The minimum concentration of Se in seagrass through the world is 0.24 PPM while the maximum concentration is 1.99 PPM. The average concentration of Se in seagrass through the world is 0.81 PPM. The median concentration of Se in seagrass through the world is 0.65 PPM. For the corresponding magnitude curve and boxplot please go to **Appendix 45 and 53**.

Tin (Sn)

Tin is a non-essential plant element. The minimum concentration of Sn in seagrass through the world is 0.05 PPM while the maximum concentration is 1.45 PPM. The average concentration of Sn in seagrass through the world is 0.44 PPM. The median concentration of Sn in seagrass through the world is 0.2 PPM. For the corresponding magnitude curve and boxplot please go to **Appendix 46 and 55**.

Strontium (Sr)

Strontium is a non-essential plant element. The minimum concentration of Sr in seagrass through the world is 67.25 PPM while the maximum concentration is 297.2 PPM. The average concentration of Sr in seagrass through the world is 113.98 PPM. The median concentration of Sr in seagrass through the world is 80.25 PPM. The upper value for normal distribution is 240.9 PPM. From this an outlier of 297.2 PPM was identified in a study by Boutahar et al. 2019 using *Zostera noltei* in Morocco. For the corresponding magnitude curve and boxplot please go to **Appendix 47 and 54.**

Titanium (Ti)

Titanium is an essential plant micronutrient. The minimum concentration of Ti in seagrass through the world is 3.73 PPM while the maximum concentration is 26.02 PPM. The average concentration of Ti in seagrass through the world is 12.19 PPM. The median concentration of Ti in seagrass through the world is 6.83 PPM.

Thallium (Tl)

Thallium is a non-essential plant element. The minimum concentration of Tl in seagrass through the world is 0.0009 PPM while the maximum concentration is 0.02 PPM. Both the average and median concentration of Tl in seagrass through the world is 0.01 PPM. For the corresponding magnitude curve and boxplot please go to **Appendix 48 and 56**.

Uranium (U)

Uranium is a non-essential plant element. The minimum concentration of U in seagrass through the world is 0.08 PPM while the maximum concentration is 2.85 PPM. The average concentration of U in seagrass through the world is 0.65 PPM. The median concentration of U in seagrass through the world is 0.21 PPM. The upper value for normal distribution is 2.21 PPM. From this An outlier of 2.85 PPM was identified by a study Boutahar et al. 2019 using *Zostera noltei* in Morocco. For the corresponding magnitude curve and boxplot please go to **Appendix 49 and 55**.

Vanadium (V)

Vanadium is an essential plant micronutrient. The minimum concentration of V in seagrass through the world is 0.63 PPM while the maximum concentration is 20.33 PPM. The average concentration of V in seagrass through the world is 6.43 PPM. The median concentration of V in seagrass through the world is 4.98 PPM. The upper value for normal distribution is 15.42 PPM from this An outlier of 20.33 PPM was identified by a study Boutahar et al. 2019 using *Zostera noltei* in Morocco. For the corresponding magnitude curves and boxplots please go to **Appendices 50 and 53**.

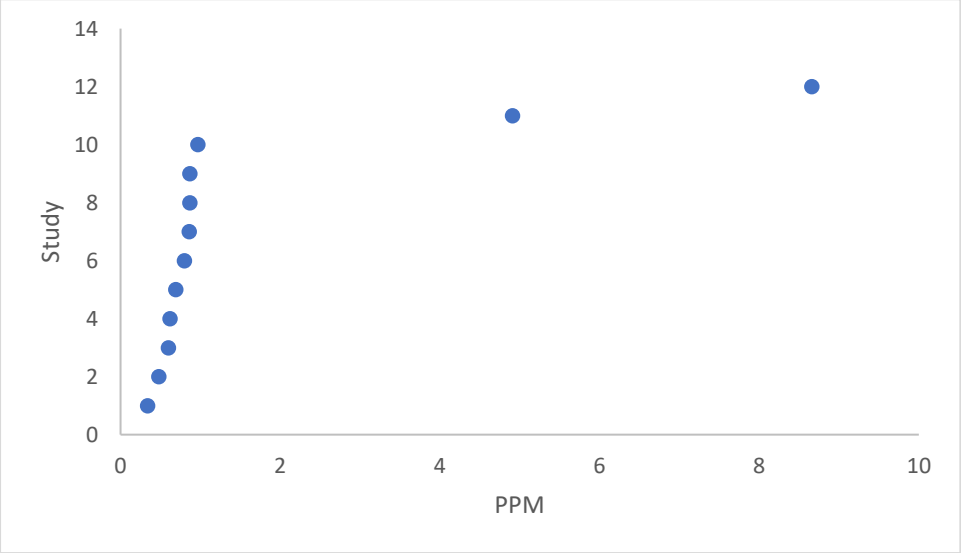
Tungsten (W)

Tungsten is a non-essential plant element. The minimum concentration of W in seagrass through the world is 1.51 PPM while the maximum concentration is 2.39 PPM. The average and median concentration of W in seagrass through the world is 1.95 PPM.

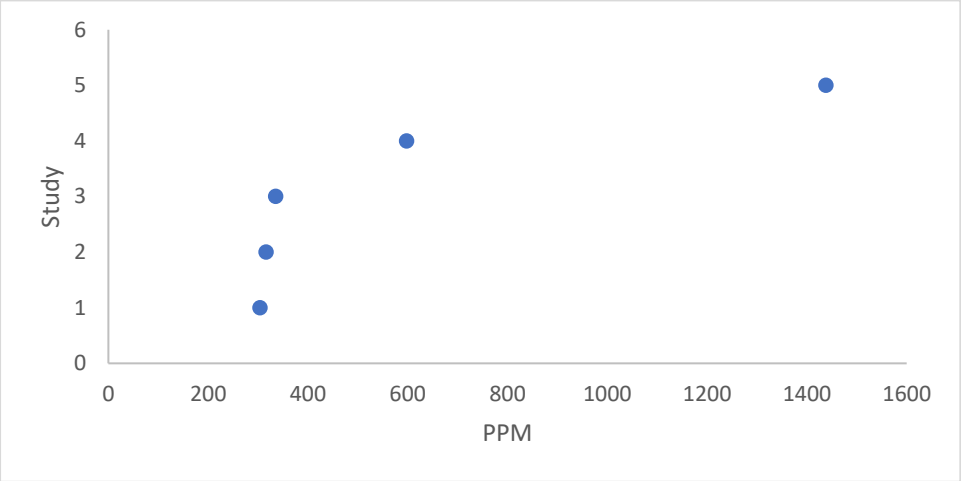
Zirconium (Zr)

Zirconium is a non-essential plant element. In a study by Serrano et al 2020 using *Posidonia sinuosa* whole plant recorded a concentration of 0.51 PPM.

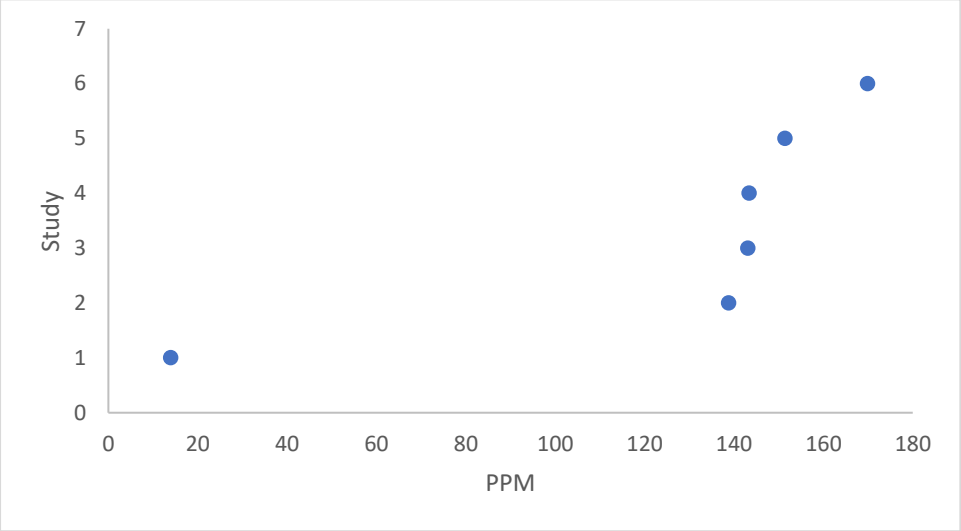
Appendix 36 – Ag Seagrass International magnitude range scatter/dotplot



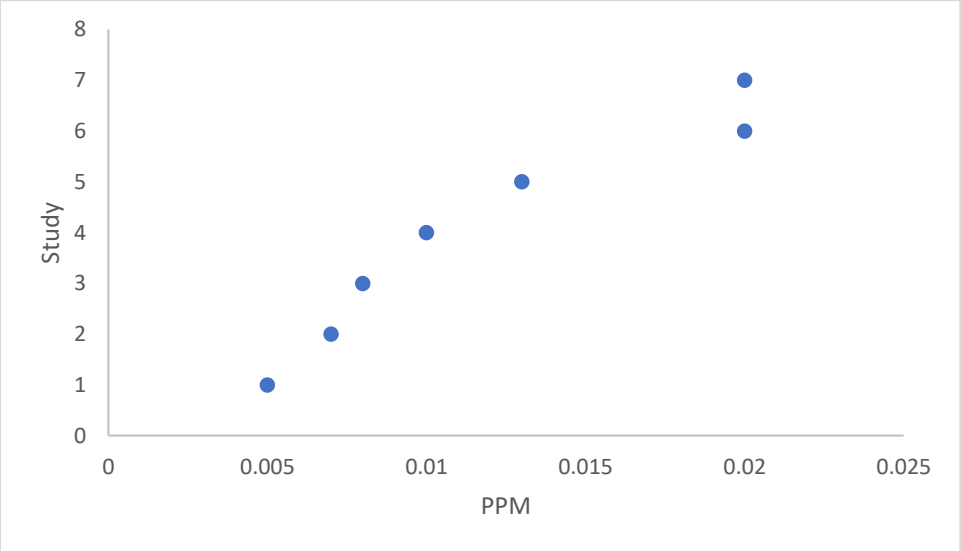
Appendix 37 – B Seagrass International magnitude range scatter/dotplot



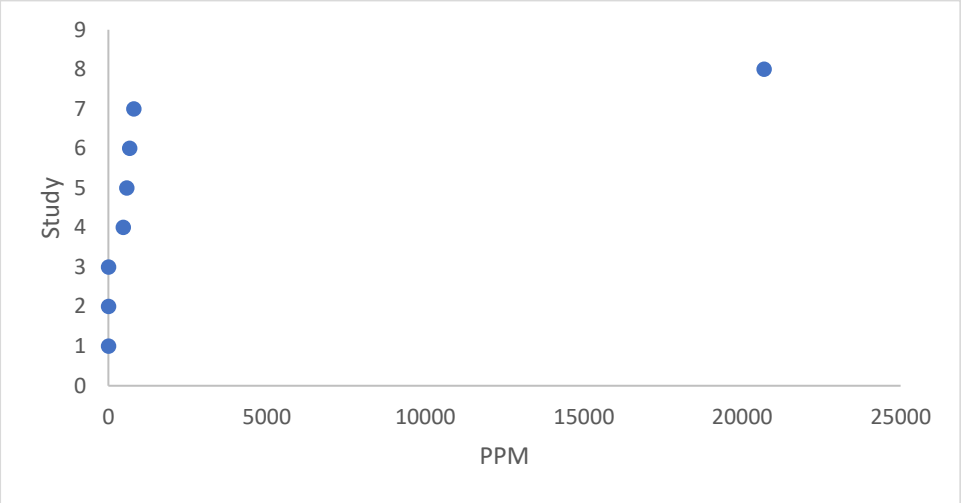
Appendix 38 – Ba Seagrass International magnitude range scatter/dotplot



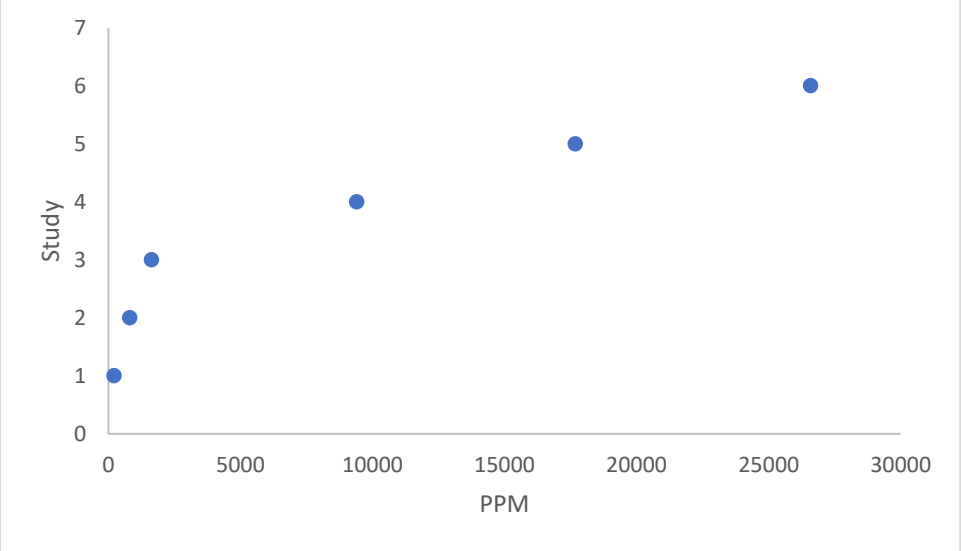
Appendix 39 – Bi Seagrass International magnitude range scatter/dotplot



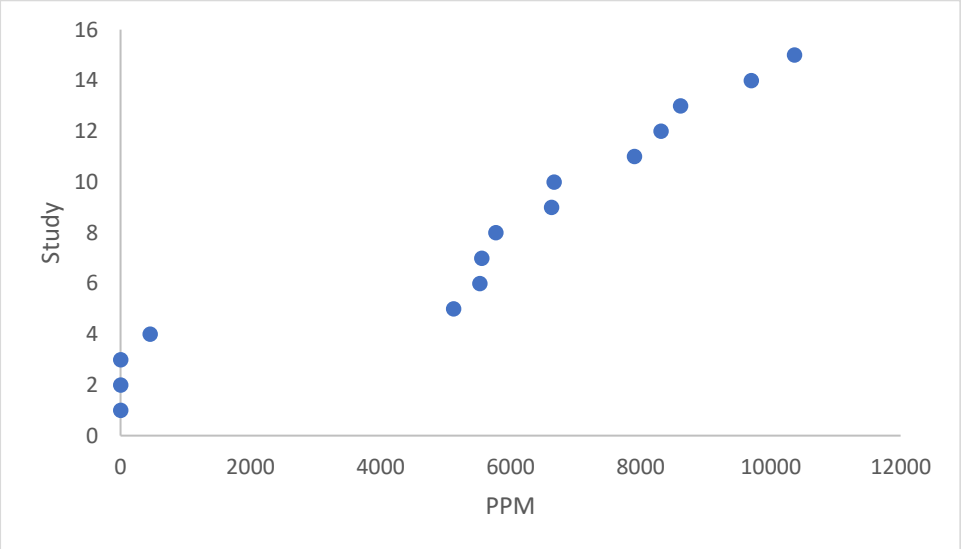
Appendix 40 – Ca Seagrass International magnitude range scatter/dotplot



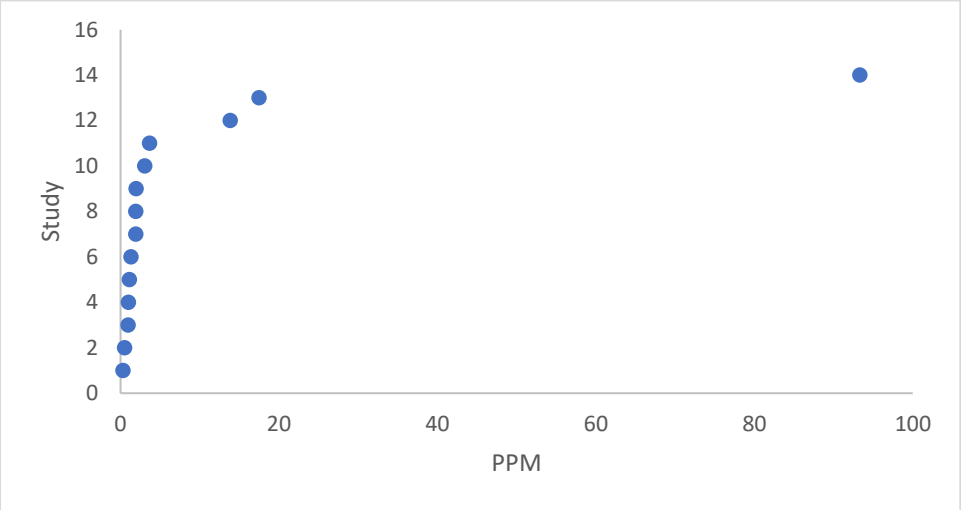
Appendix 41 – K Seagrass International magnitude range scatter/dotplot



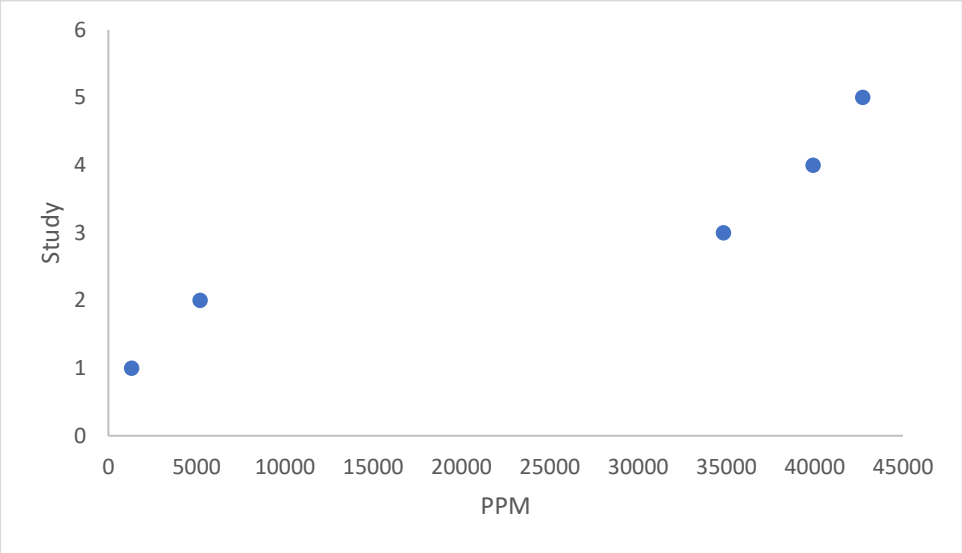
Appendix 42 – Mg Seagrass International magnitude range scatter/dotplot



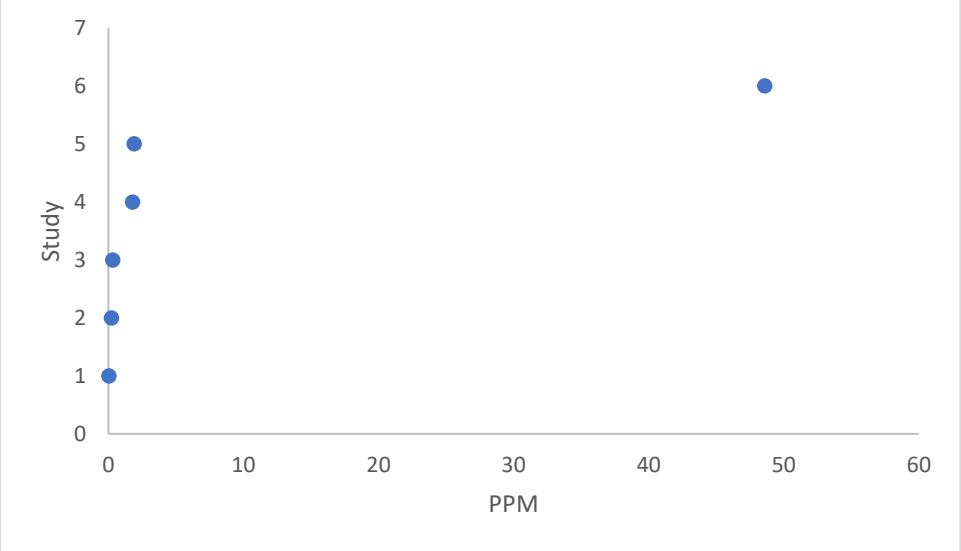
Appendix 43 – Mo Seagrass International magnitude range scatter/dotplot



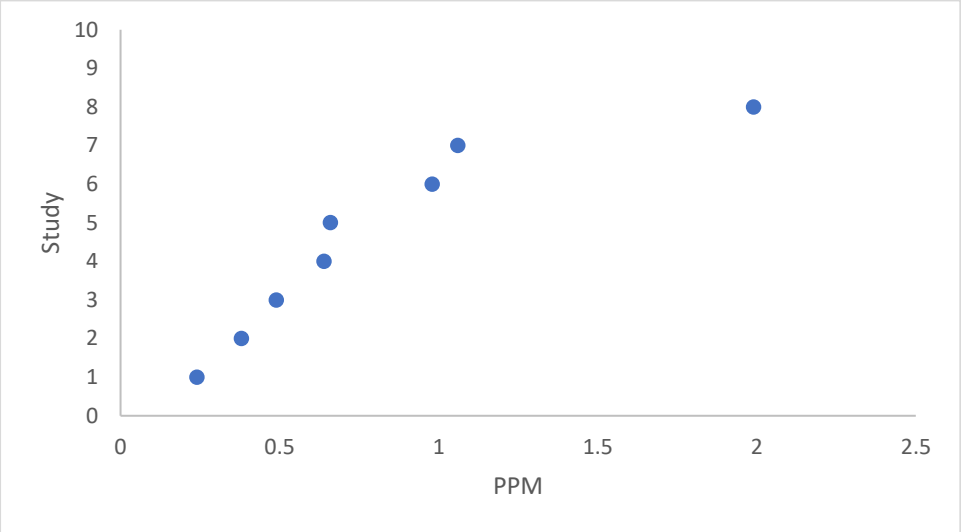
Appendix 44 – Na Seagrass International magnitude range scatter/dotplot



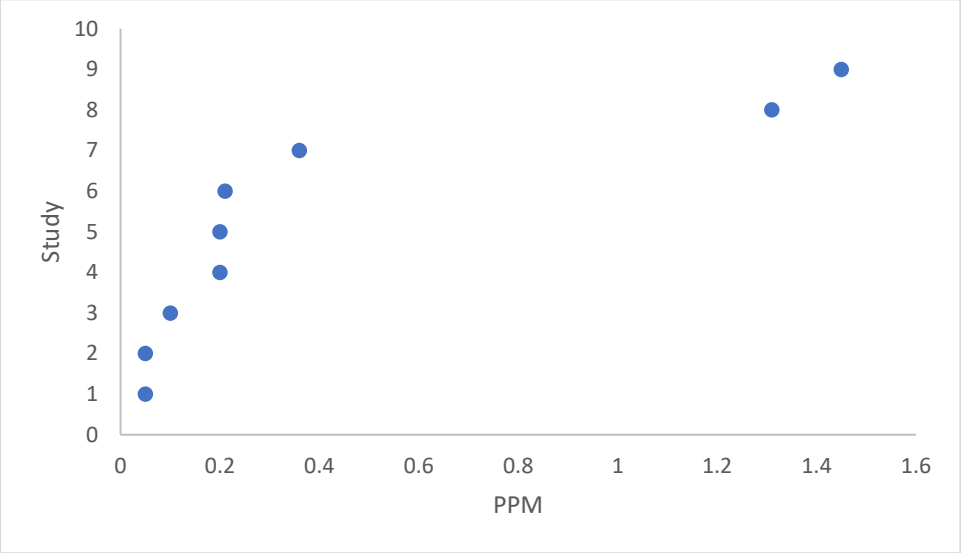
Appendix 45 – Sb Seagrass International magnitude range scatter/dotplot



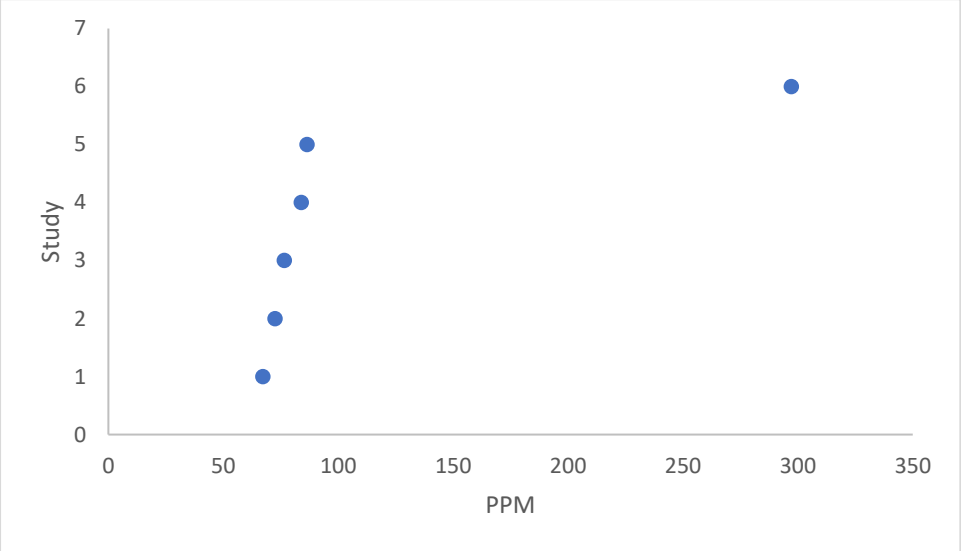
Appendix 46 – Se Seagrass International magnitude range scatter/dotplot



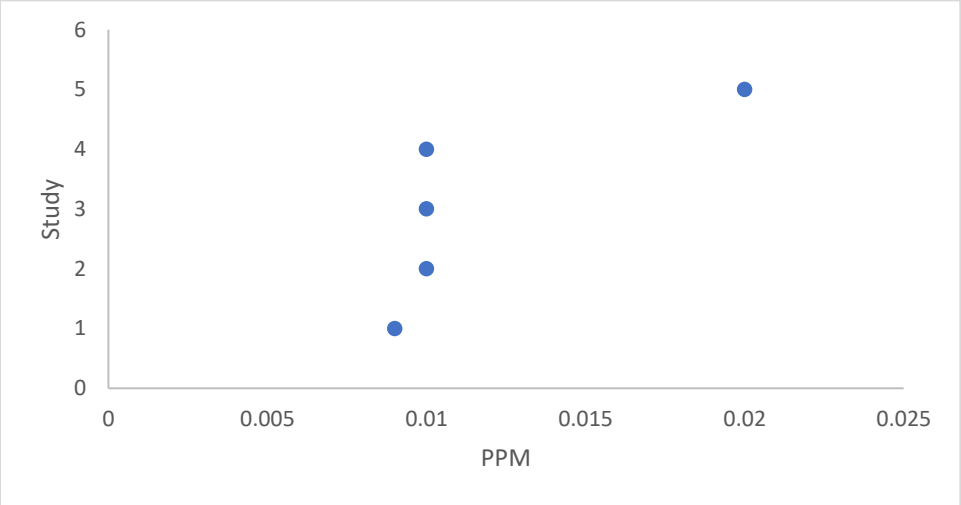
Appendix 47 – Sn Seagrass International magnitude range scatter/dotplot



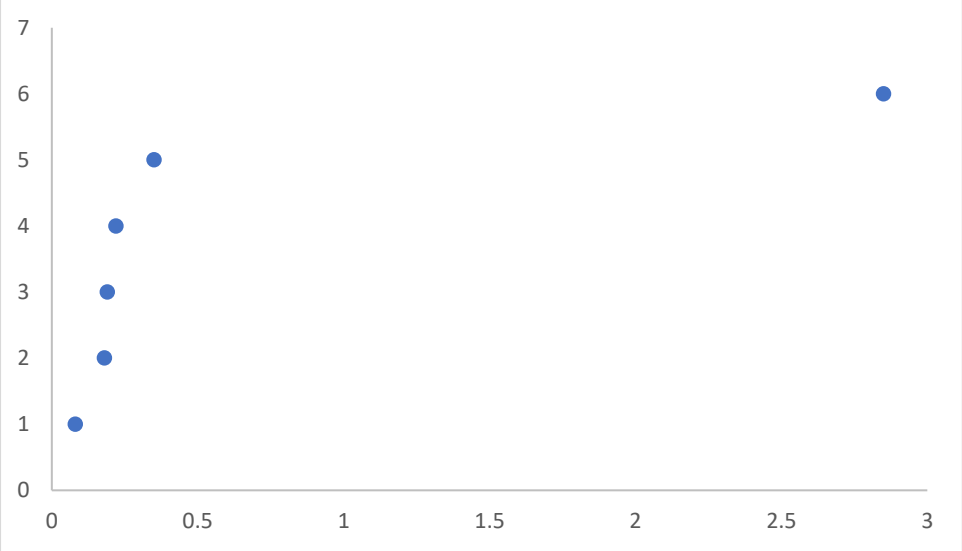
Appendix 48 – Sr Seagrass Intentional magnitude range scatter/dotplot



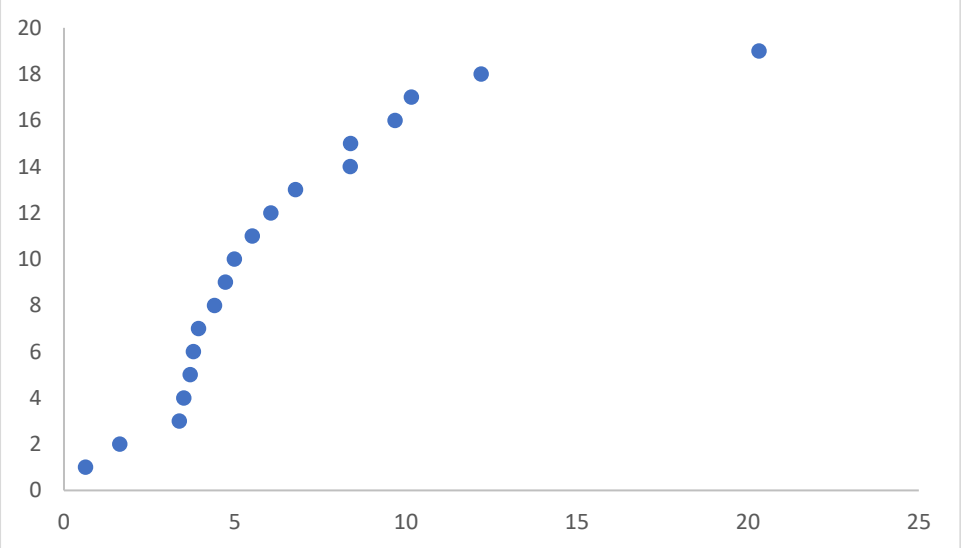
Appendix 49 – Tl Seagrass International magnitude range scatter/dotplot



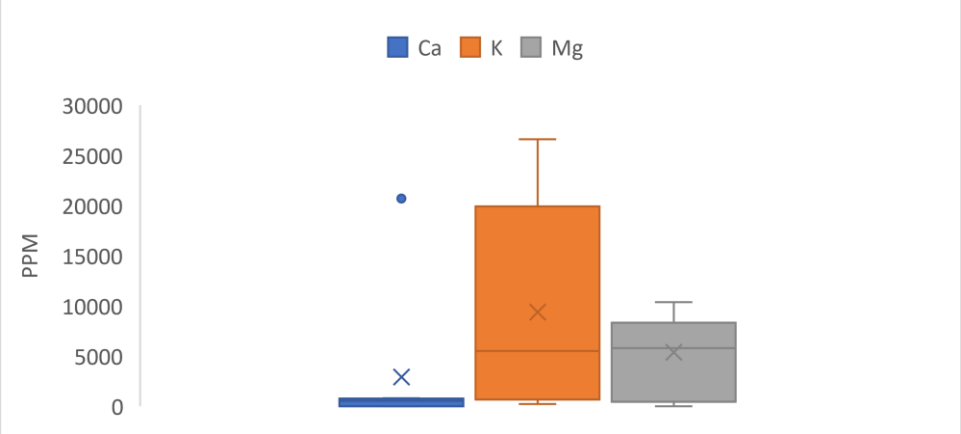
Appendix 50 – U Seagrass International magnitude range scatter/dotplot



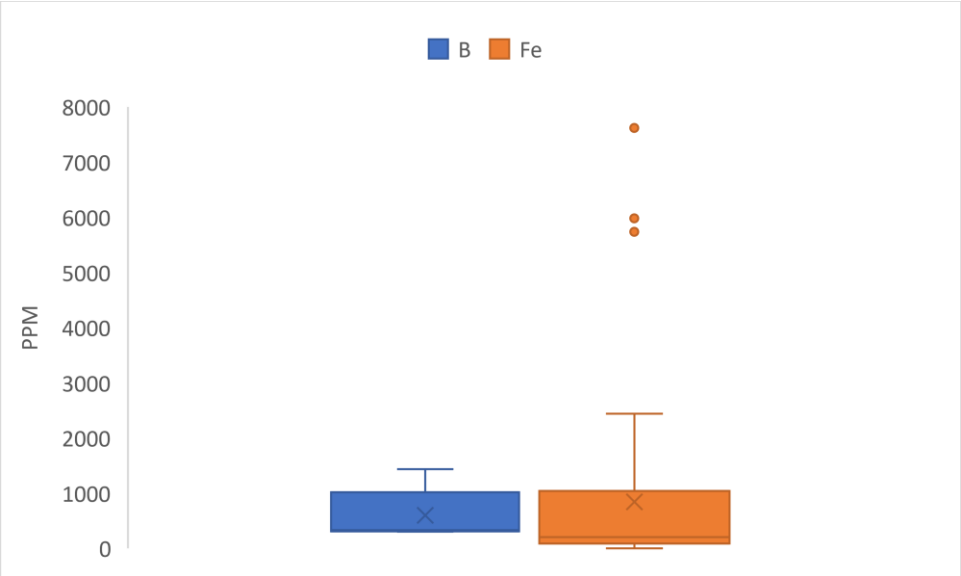
Appendix 51 – V Seagrass International magnitude scatter/dotplot



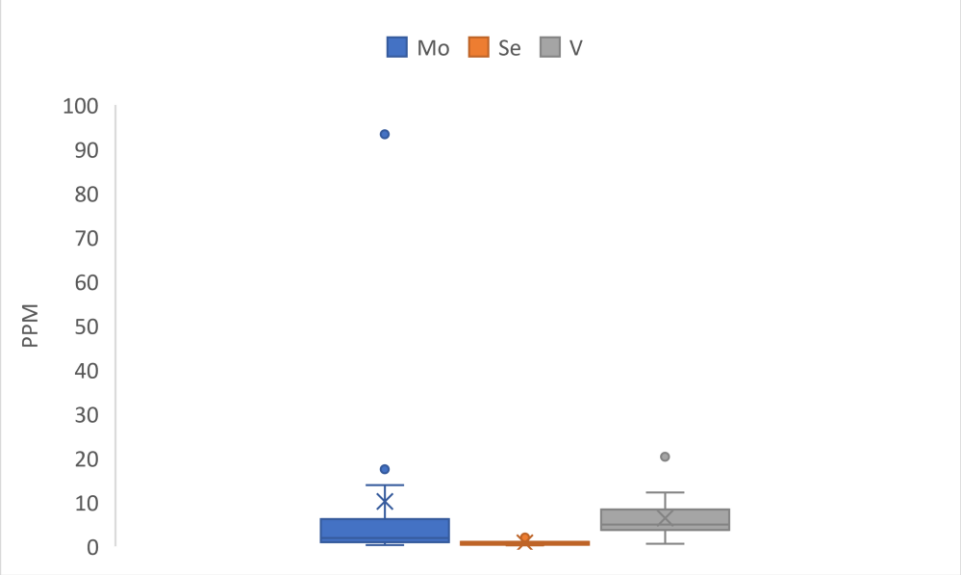
Appendix 52 – Boxplots of Essential seagrass macronutrients (Ca, K and Mg) magnitude ranges units in PPM



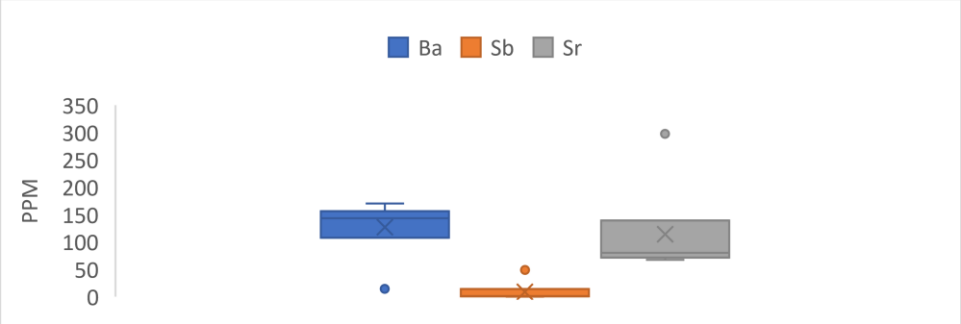
Appendix 53 – Boxplots of Essential seagrass micronutrients magnitude ranges (B and Fe) units in PPM



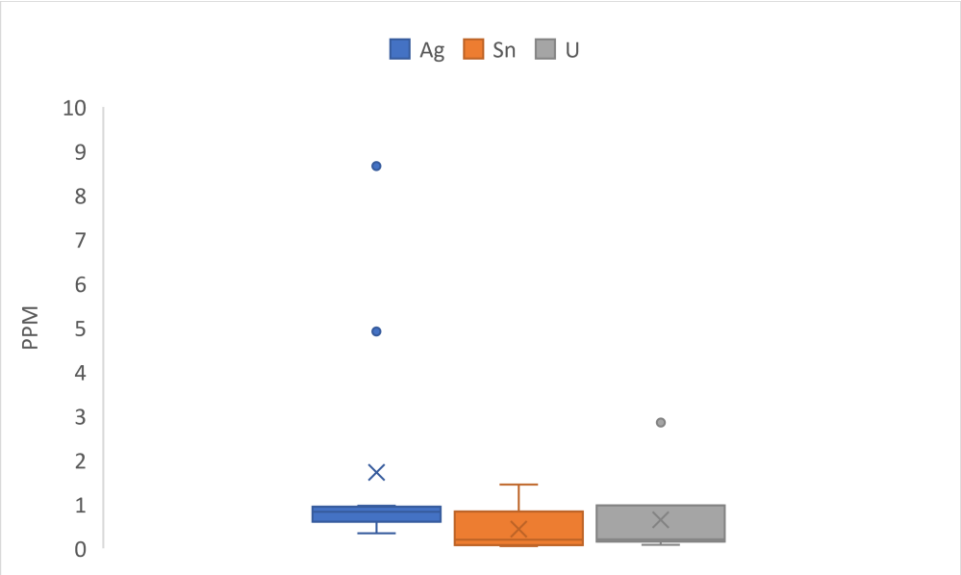
Appendix 54 – Boxplots of Essential seagrass micronutrients magnitude ranges (Mo, Se and V) units in PPM



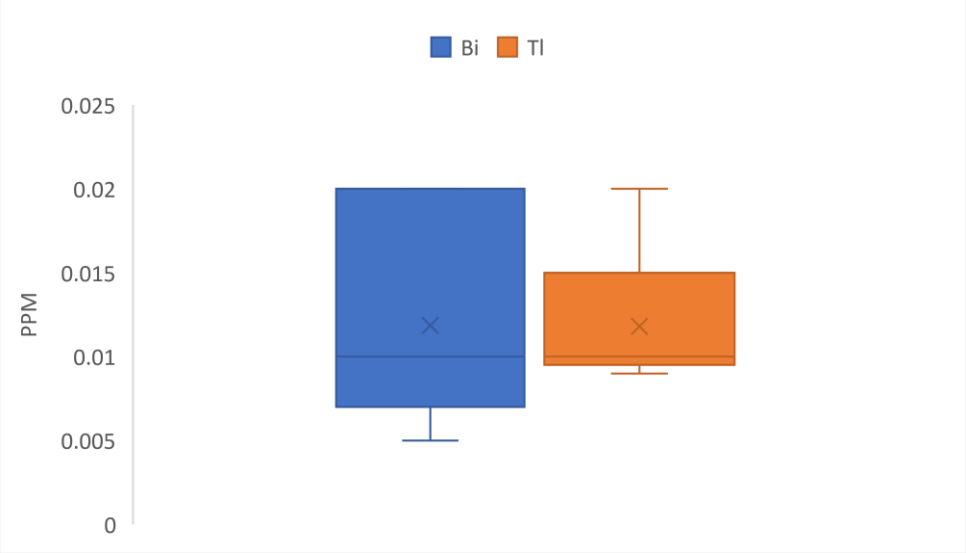
Appendix 55 – Boxplots of Non-essential seagrass element magnitude ranges (Ba, Sb and Sr) units in PPM



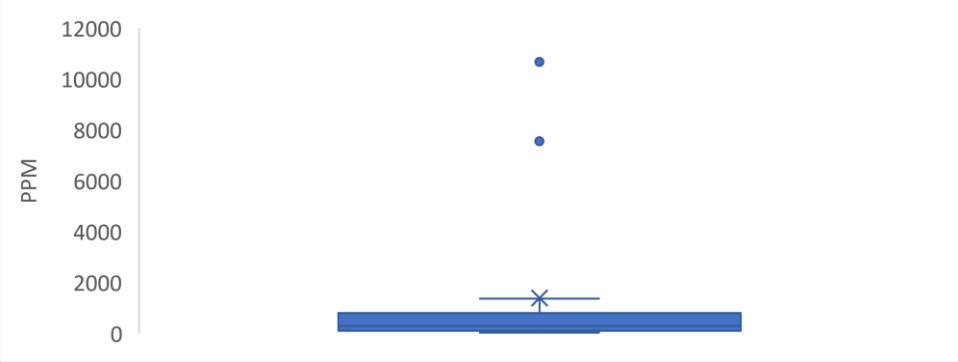
Appendix 56 – Boxplots of Non-essential seagrass element magnitude ranges (Ag, Sn and U) units in PPM



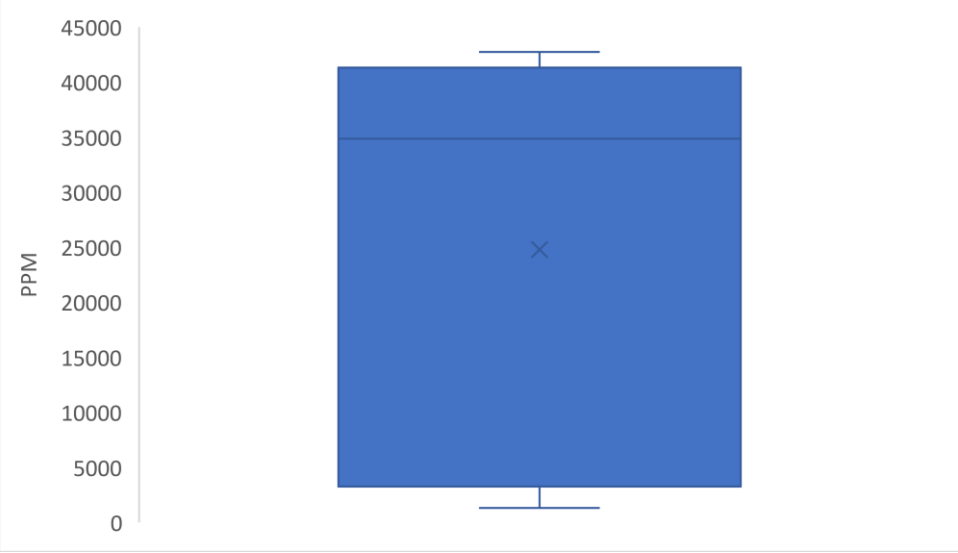
Appendix 57 – Boxplots of Non-essential seagrass element magnitude ranges (Bi and Tl) units in PPM



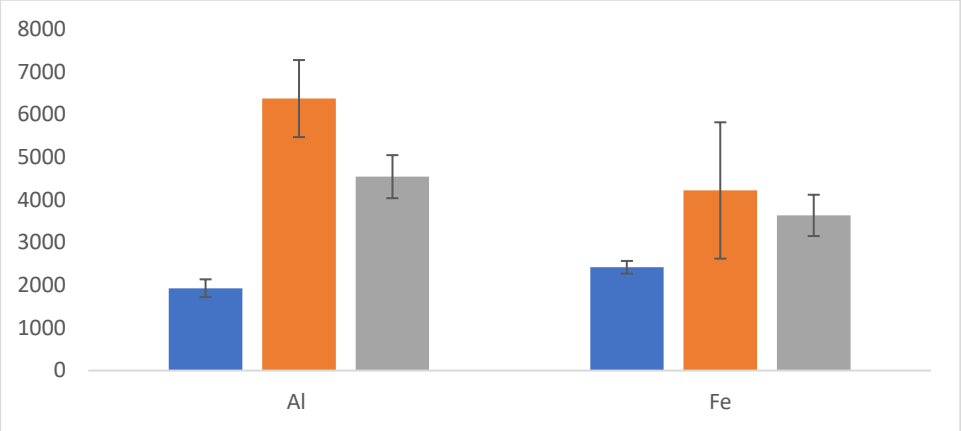
Appendix 58 – Boxplot of Al seagrass magnitude range units in PPM



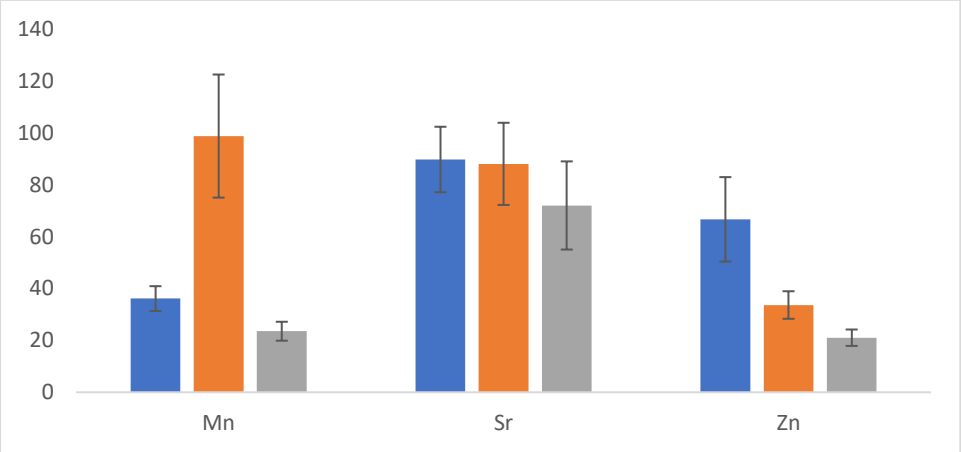
Appendix 59 – Boxplot of Na seagrass magnitude range units in PPM



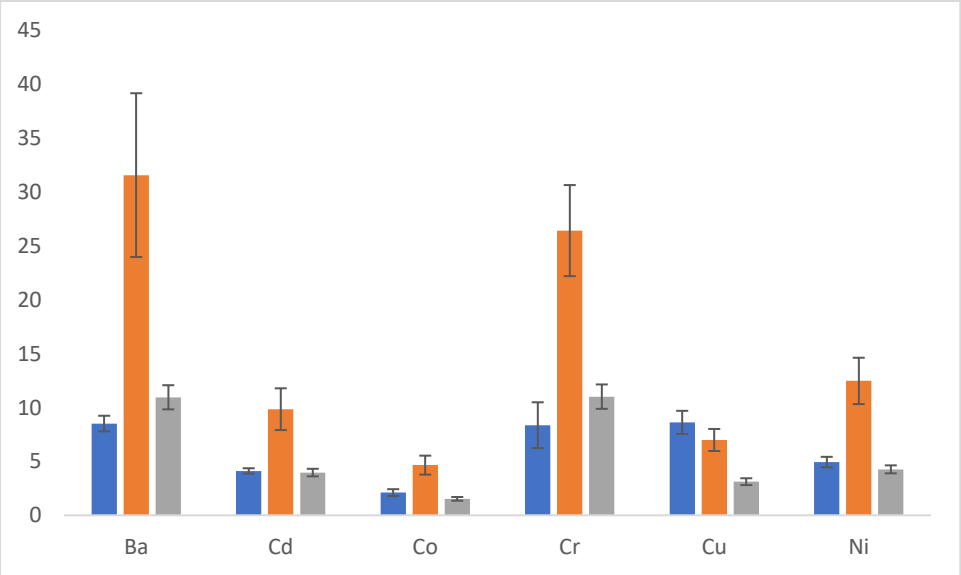
Appendix 60 – Al and Fe mean concentrations with standard errors in Sediment units are in mg/kg (PPM) Summer 2015/16



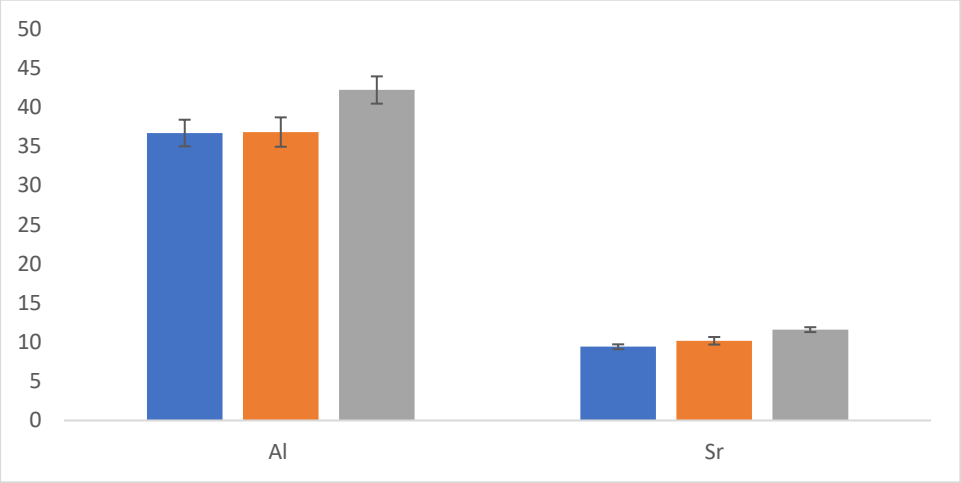
Appendix 61 – Mn, Sr and Zn mean concentrations with standard errors in Sediment units are in mg/kg (PPM) Summer 2015/16



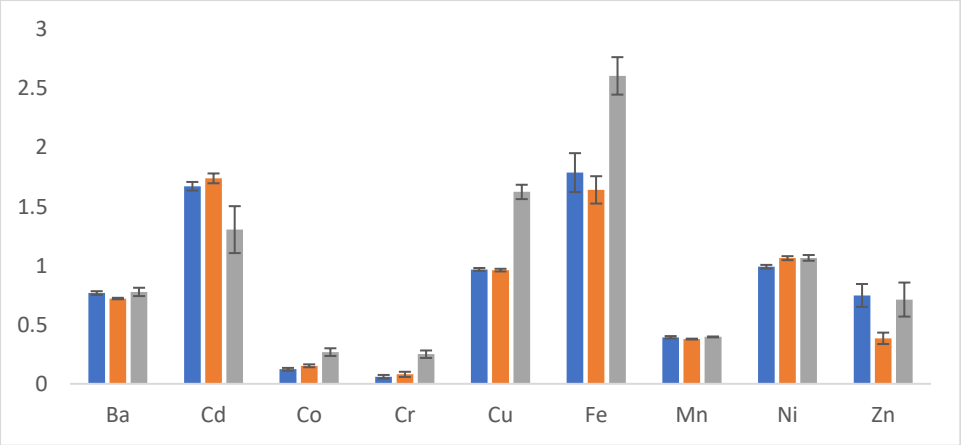
Appendix 62 – Ba, Cd, Co, Cr, Cu, and Ni mean concentrations with standard errors in Sediment units are in mg/kg (PPM) (Summer 2015/16



Appendix 63 – Al and Sr mean concentrations with standard errors in Water units are in mg/kg (PPM) –Summer 2015/16



Appendix 64 – Ba, Cd, Co, Cr, Cu, Fe, Mn, Ni and Zn mean concentrations with standard errors in Water units are in mg/kg (PPM) – Summer 2015/16



Appendix 65 – Seagrass raw data

See excel files labelled Seagrass.

Appendix 66 – Sediment raw data

See excel files labelled Sediment.

Appendix 67 – Water raw data

See excel files labelled Water.

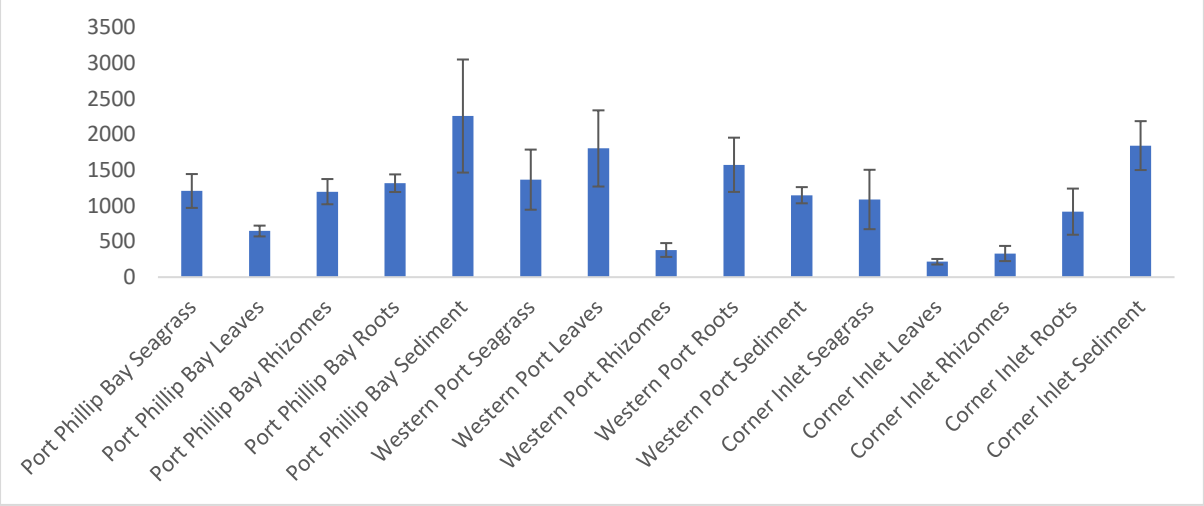
Appendix 68 – *Amphibolis antarctica*

This species of seagrass is found in the family Cymodoceaceae (Plants of the World Online 2020). It is also known as sea nymph, and it is found in coastal waters of southern and western Australia. It occurs primarily in the sublittoral zone, where it forms extensive meadows. It can occur as deep as 27 meters but does not often form meadows below 13 meters. It can also grow in extremely shallow waters, with its leaves floating on the surface, although this often results in leaf damage and loss. The species tolerates a range of habitats. It has been found growing on a variety of

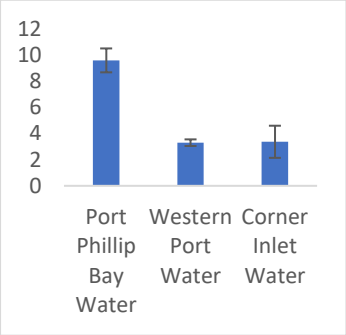
substrates, including sand covered rock, gravel, sand and clay. It grows in areas of both high and low water flow and occurs in areas of very high salinity (Edgar 2012).

Amphibolis antarctica – Source - https://collections.museumsvictoria.com.au/species/15191

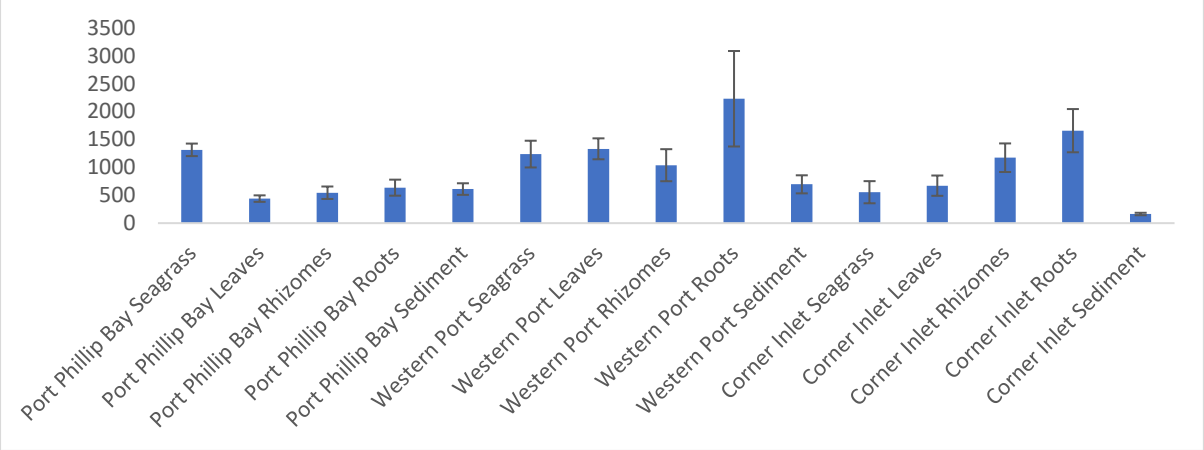
Appendix 69 – 2018 Mean Winter Al concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



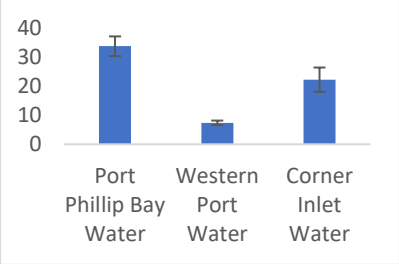
Appendix 70 – 2018 Mean Winter Al concentrations with standard errors in Water units in mg/kg (PPM)



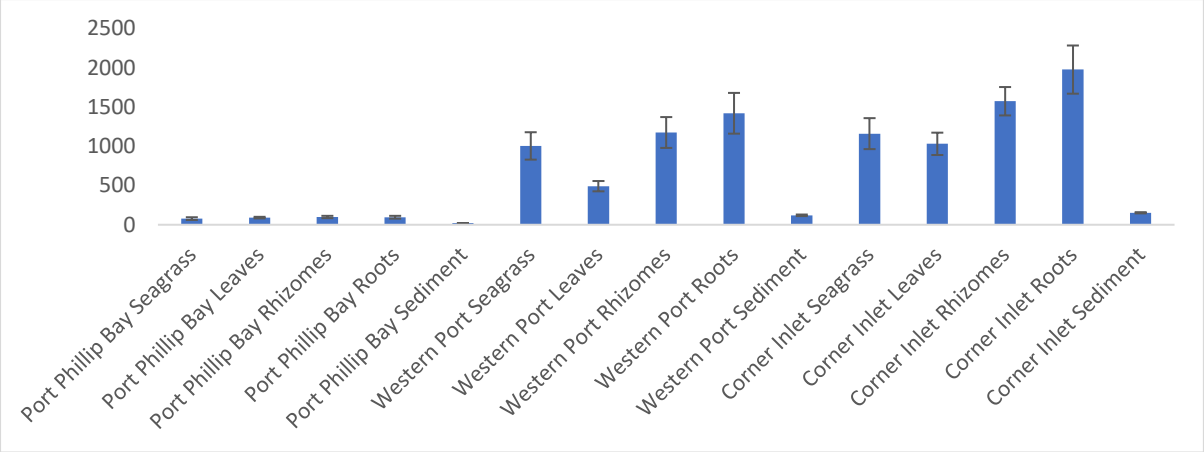
Appendix 71 – 2018 Mean Winter As concentrations with standard errors in Seagrass whole plants and associated organs and Sediment units in mg/kg (PPM)



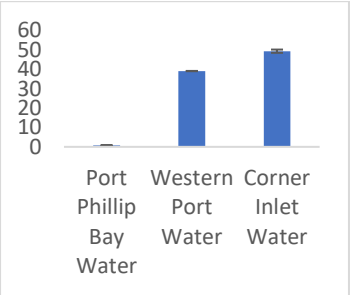
Appendix 72 – 2018 Mean Winter As concentrations with standard errors in Water units in mg/kg (PPM)



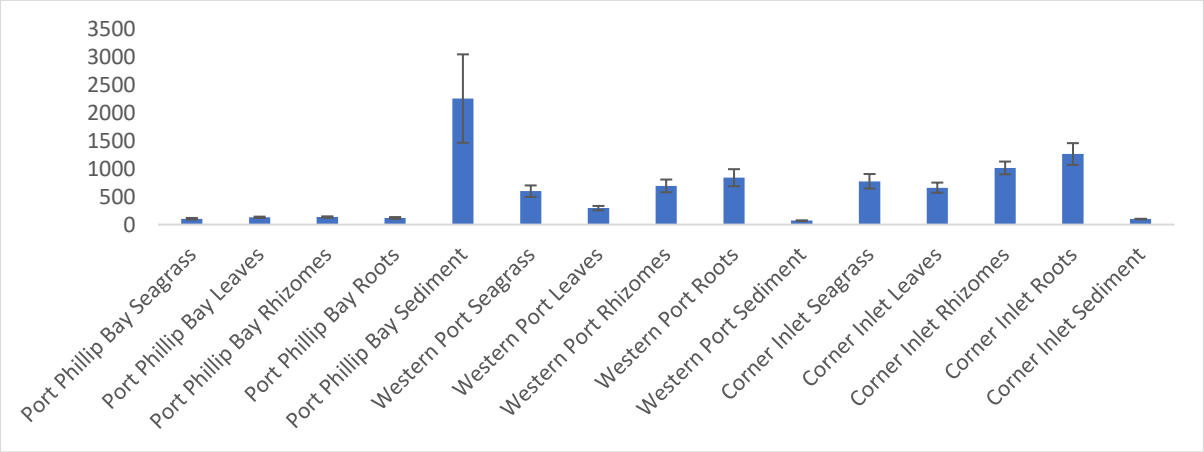
Appendix 73 – 2018 Mean Winter Cd concentrations with standard errors in Seagrass whole plants and associated organs and Sediment units in mg/kg (PPM)



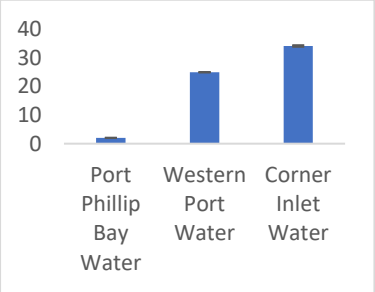
Appendix 74 – 2018 Mean Winter Cd concentrations with standard errors in Water units in mg/kg (PPM)



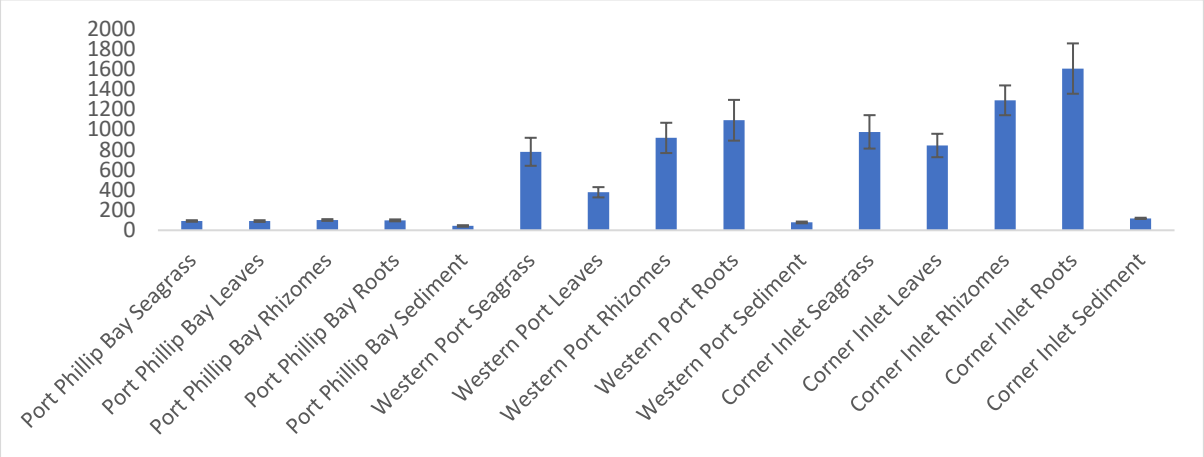
Appendix 75 – 2018 Mean Winter Co concentrations with standard errors in Seagrass whole plants and associated organs and Sediment units in mg/kg (PPM)



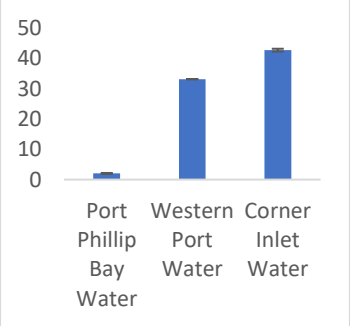
Appendix 76 – 2018 Mean Winter Co concentrations with standard errors in Water units in mg/kg (PPM)



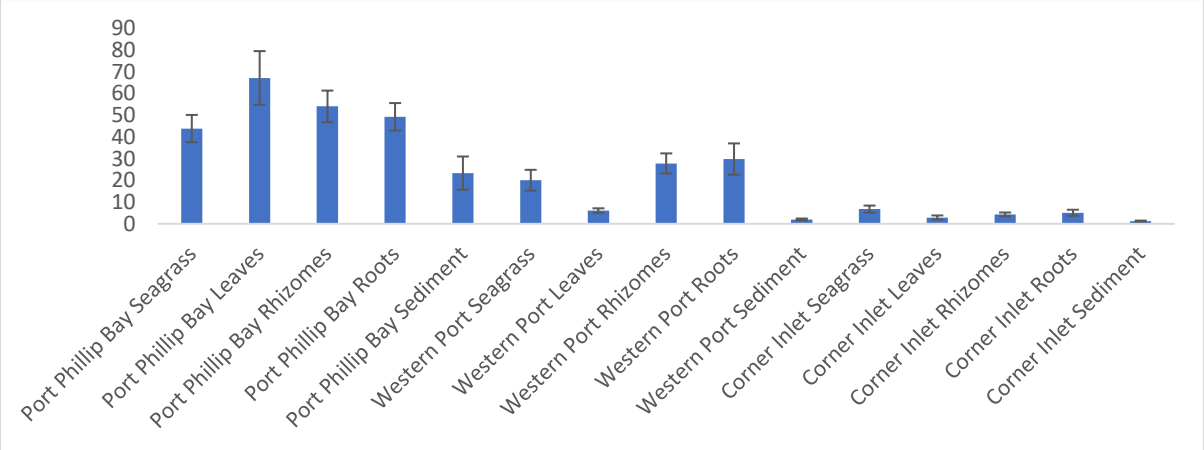
Appendix 77 – 2018 Mean Winter Cr concentrations with standard errors in Seagrass whole plants and associated organs and Sediment units in mg/kg (PPM)



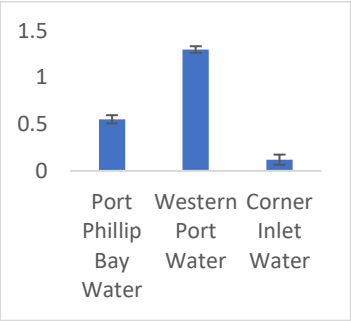
Appendix 78 – 2018 Mean Winter Cr concentrations with standard errors in Water units in mg/kg (PPM)



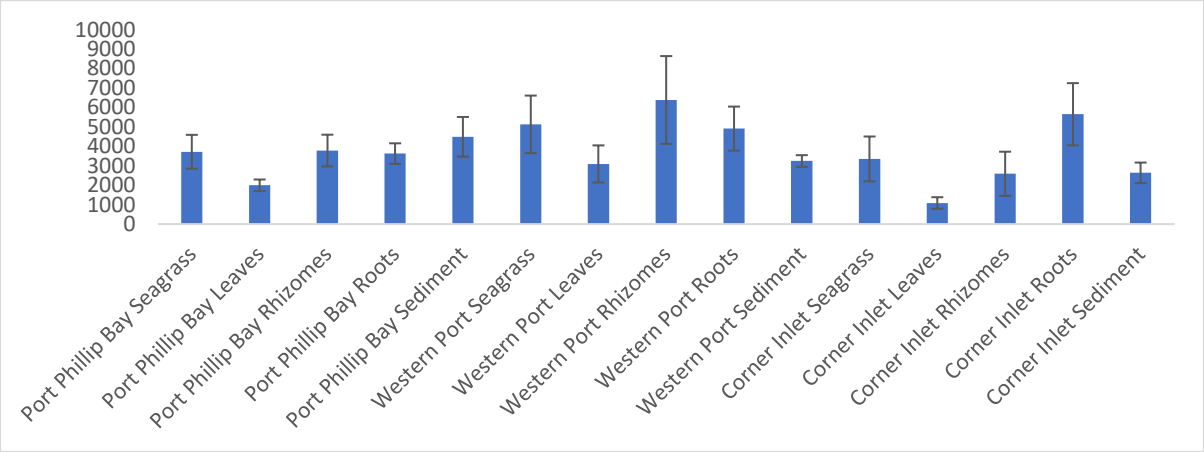
Appendix 79 – 2018 Mean Winter Cu concentrations with standard errors in Seagrass whole plants and associated organs and Sediment units in mg/kg (PPM)



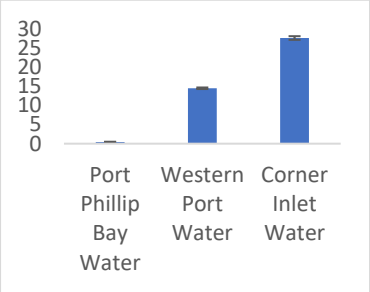
Appendix 80 – 2018 Mean Winter Cu concentrations with standard errors in Water units in mg/kg (PPM)



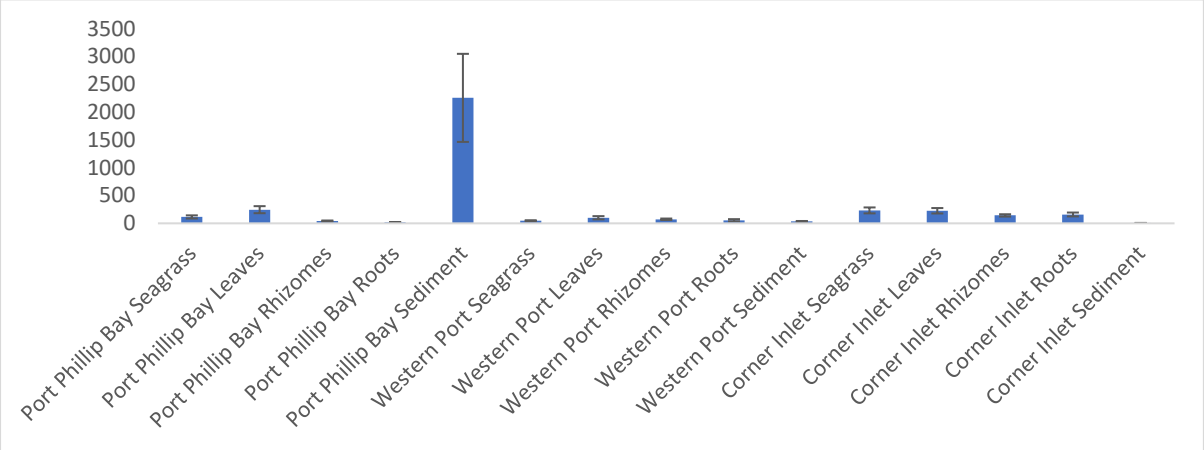
Appendix 81 – 2018 Mean Winter Fe concentrations in with standard errors Seagrass whole plants and associated organs and Sediment units in mg/kg (PPM)



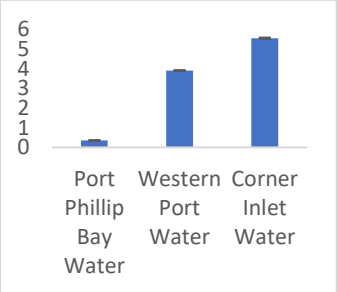
Appendix 82 – 2018 Mean Winter Fe concentrations with standard errors in Water units in mg/kg (PPM)



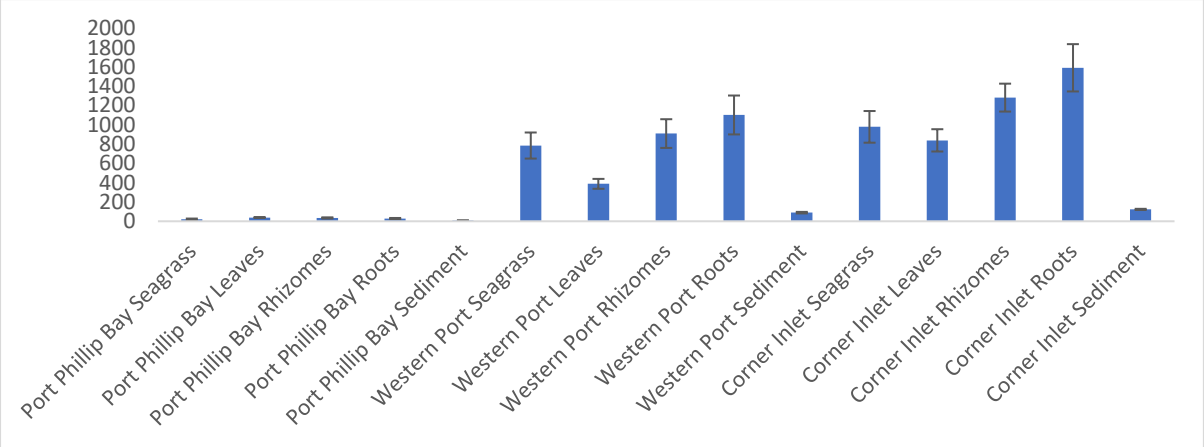
Appendix 83 – 2018 Mean Winter Mn concentrations with standard errors in Seagrass whole plants and associated organs and Sediment units in mg/kg (PPM)



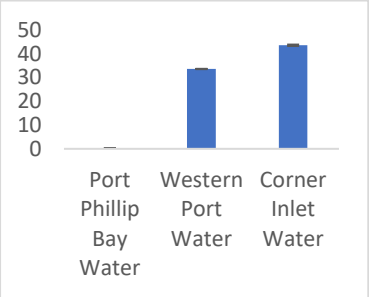
Appendix 84 - Mean 2018 Winter Mn concentrations with standard errors in Water units in mg/kg (PPM)



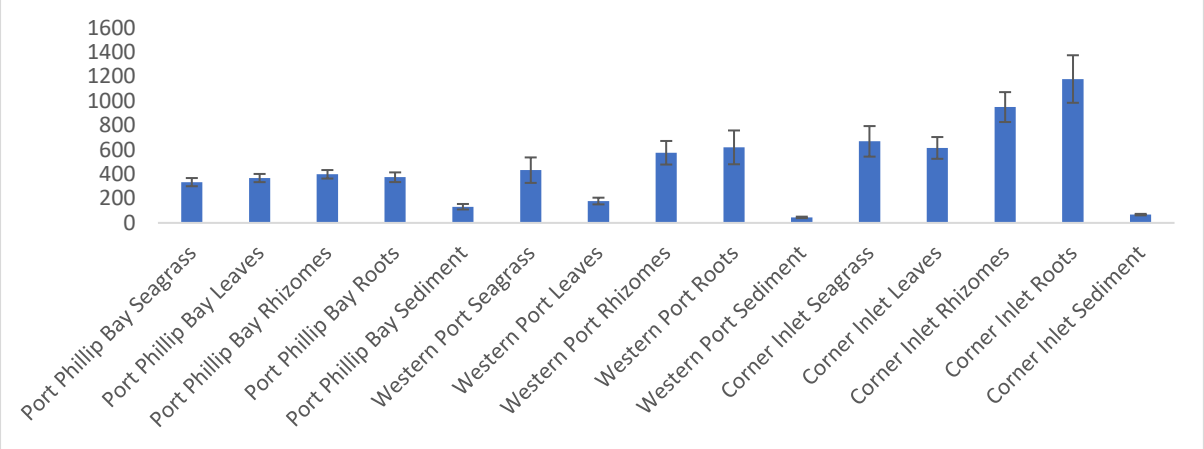
Appendix 85 – 2018 Mean Winter Ni concentrations with standard errors in Seagrass whole plants and associated organs and Sediment units in mg/kg (PPM)



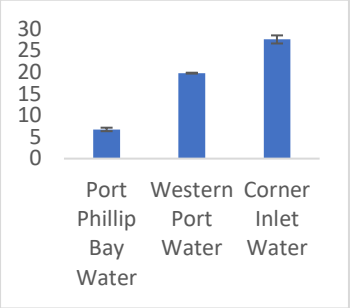
Appendix 86 – Mean 2018 Winter Ni concentrations with standard errors in Water units in mg/kg (PPM)



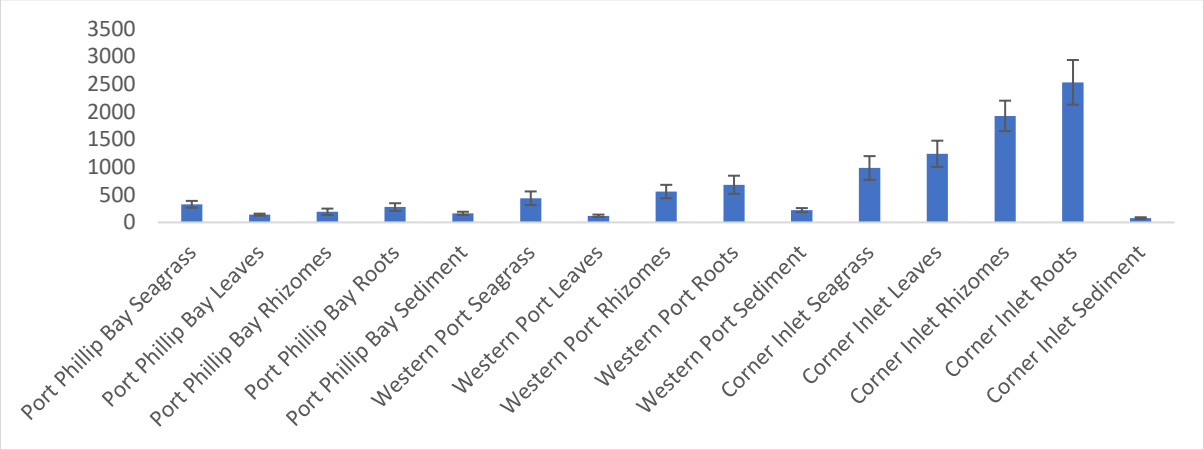
Appendix 87 – Mean 2018 Winter Pb concentrations with standard errors in Seagrass whole plants and associated organs and Sediment units in mg/kg (PPM)



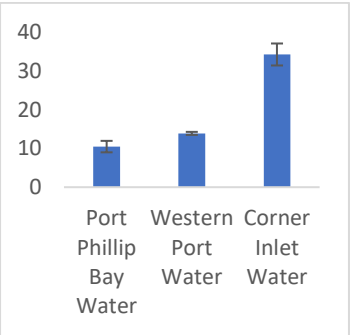
Appendix 88 – Mean 2018 Winter Pb concentrations with standard errors in Water units in mg/kg (PPM)



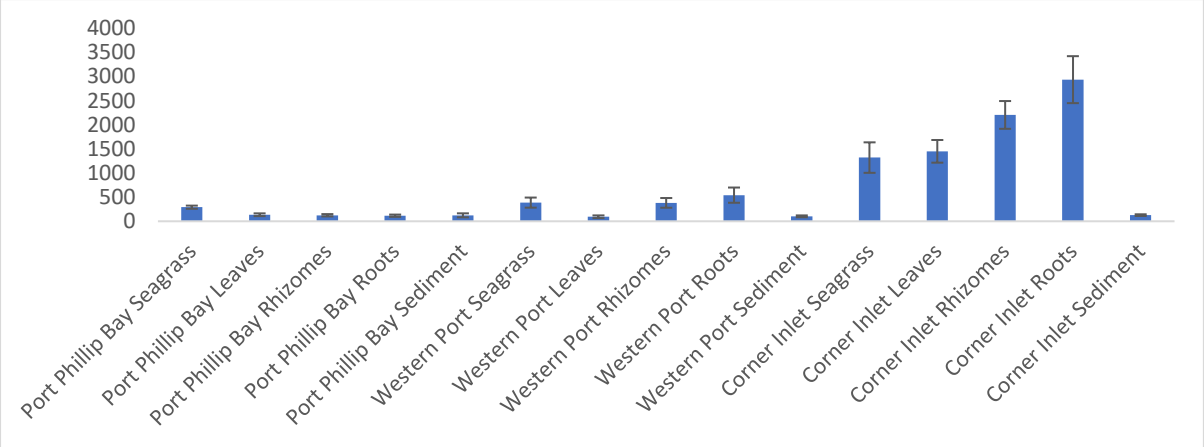
Appendix 89 – Mean 2018 Winter Se concentrations with standard errors in Seagrass whole plants and associated organs and Sediment units in mg/kg (PPM)



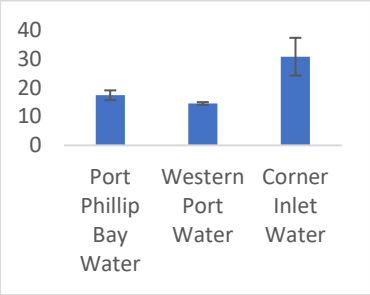
Appendix 90 – 2018 Mean Winter Se concentrations with standard errors in Water units in mg/kg



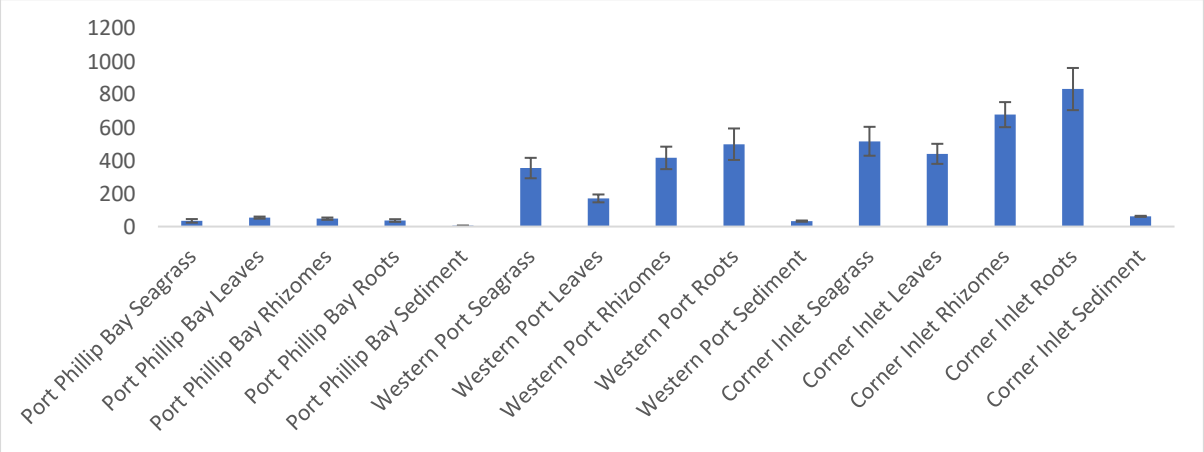
Appendix 91 – 2018 Mean Winter Sn concentrations with standard errors in Seagrass whole plants and associated organs and Sediment units in mg/kg (PPM)



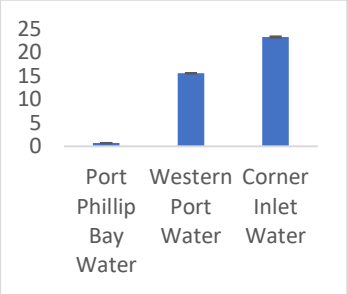
Appendix 92 – 2018 Mean Winter Sn concentrations with standard errors in Water units in mg/kg (PPM)



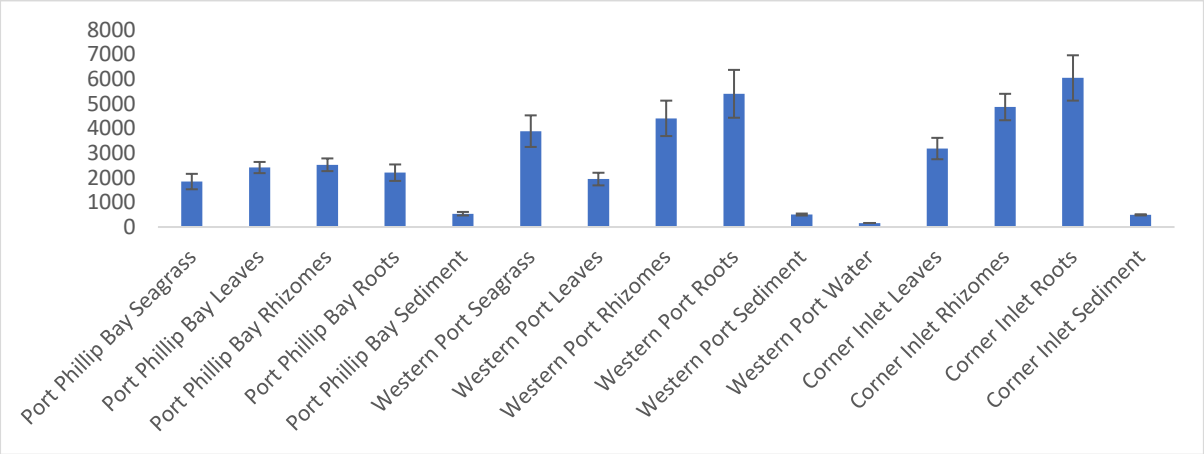
Appendix 93 – 2018 Mean Winter V concentrations with standard errors in Seagrass whole plants and associated organs and Sediment units in mg/kg (PPM)



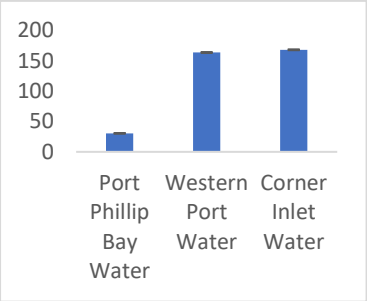
Appendix 94 – 2018 Mean Winter V concentrations with standard errors in Water units in mg/kg



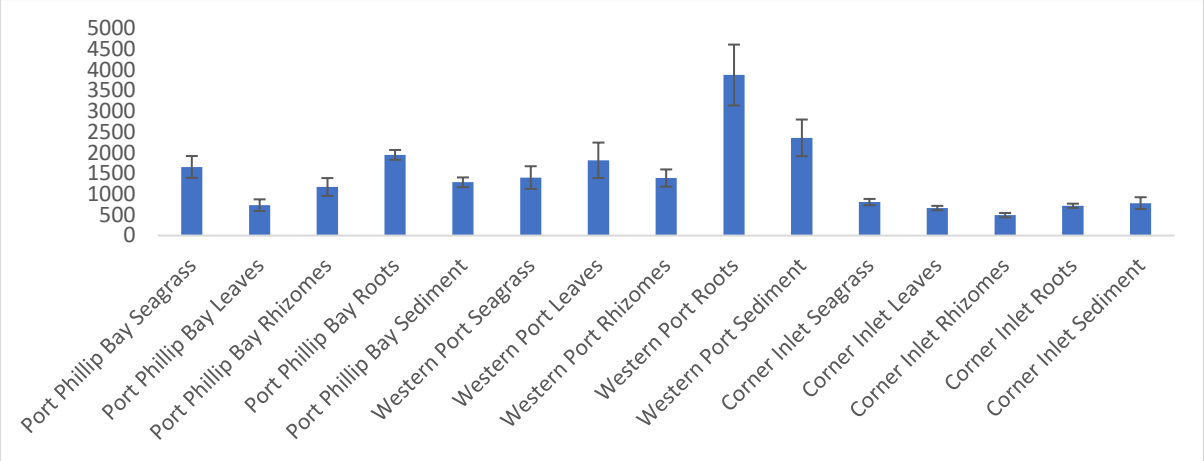
Appendix 95 – 2018 Mean Winter Zn concentrations with standard errors in Seagrass whole plants and associated organs and Sediment units in mg/kg (PPM)



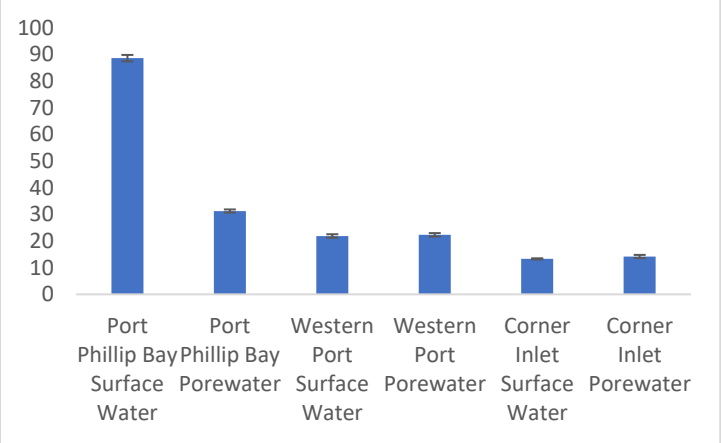
Appendix 96 - 2018 Mean Winter with standard errors Zn concentrations in Water units in mg/kg (PPM)



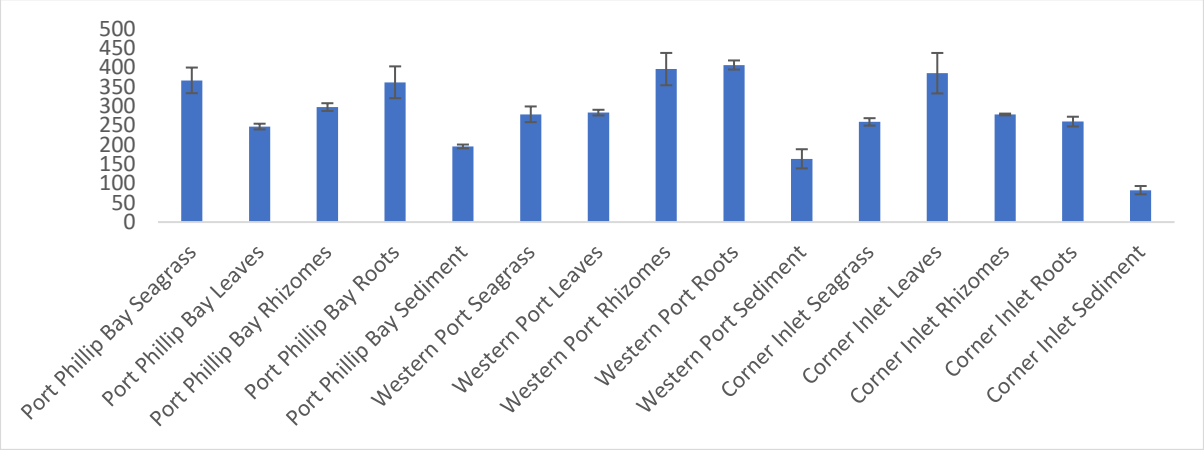
Appendix 97 – 2018 Mean Spring Al concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



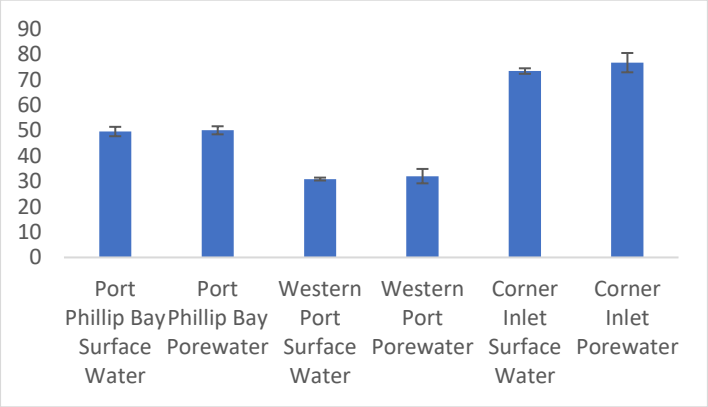
Appendix 98 – 2018 Mean Spring Al concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



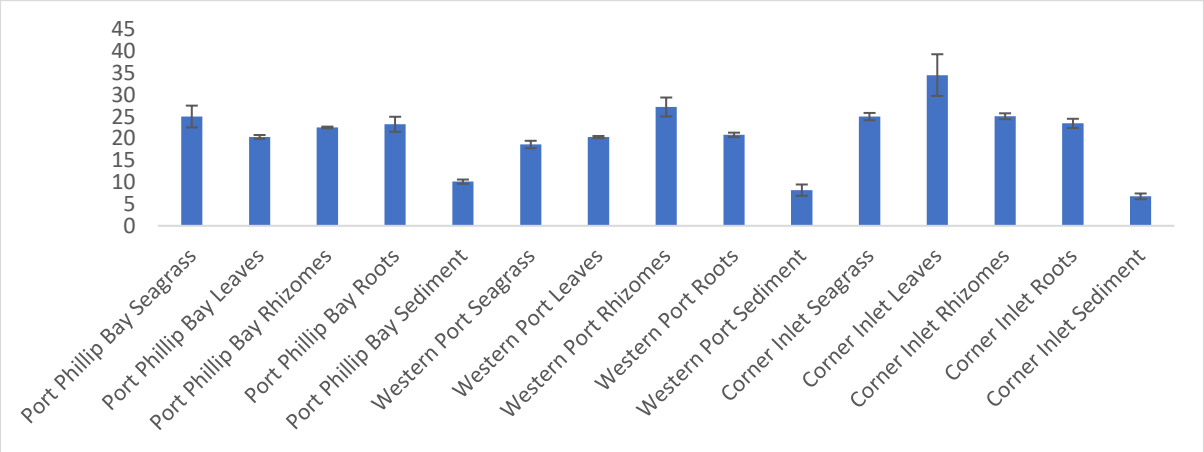
Appendix 99 – 2018 Mean Spring As concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



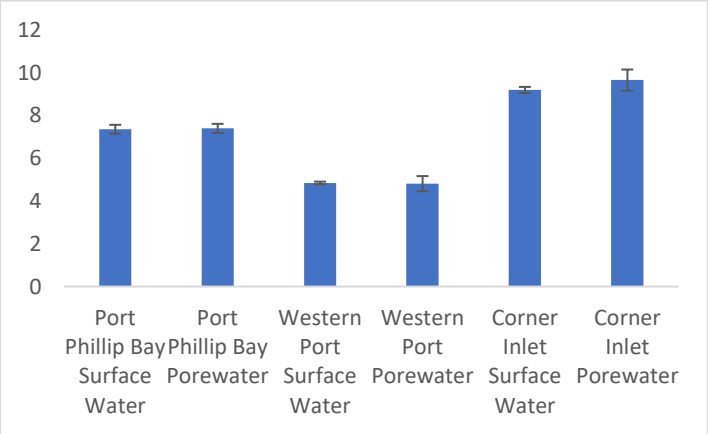
Appendix 100 – 2018 mean Spring As concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



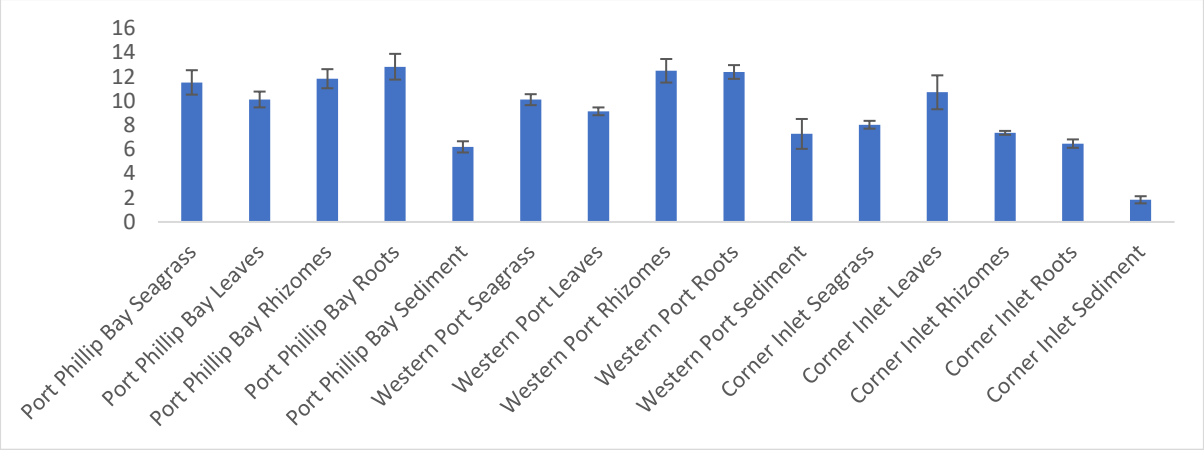
Appendix 101 – 2018 Mean Spring Cd concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



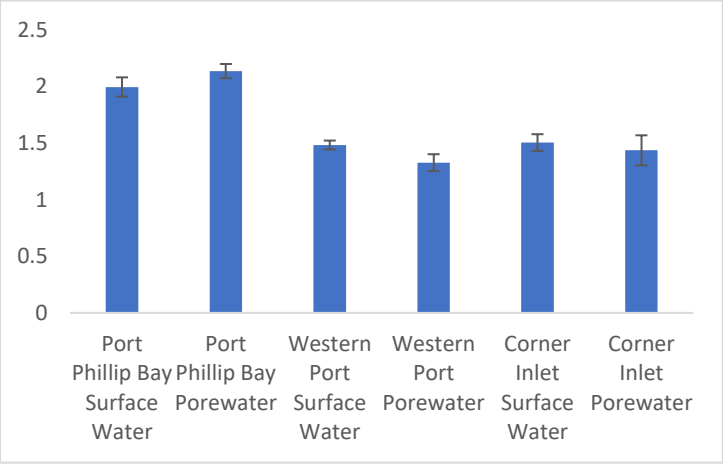
Appendix 102 – 2018 Mean Spring Cd concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



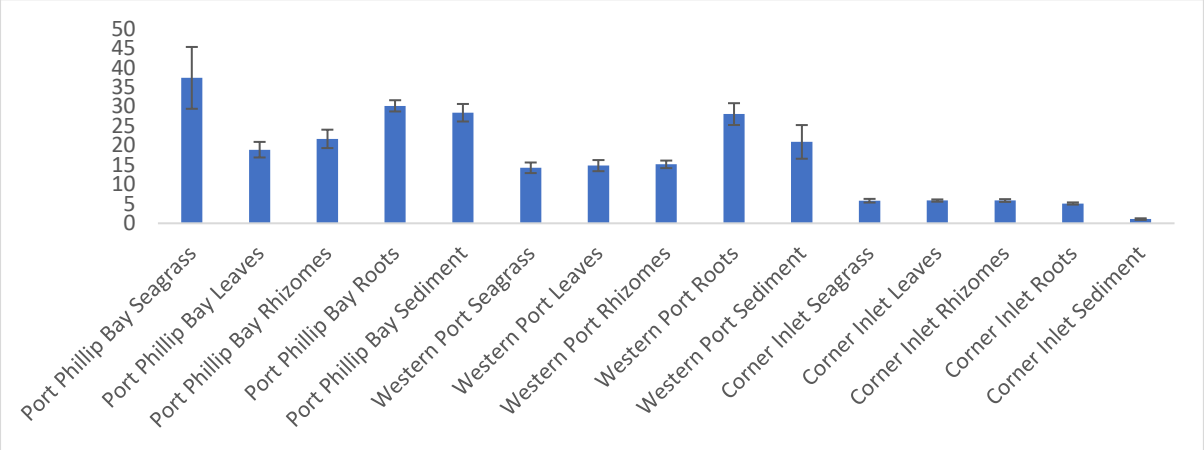
Appendix 103 – 2018 Mean Spring Co concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



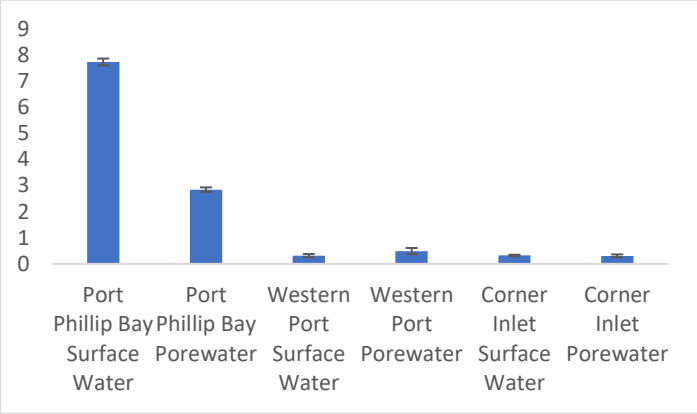
Appendix 104 – Mean 2018 Spring Co concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



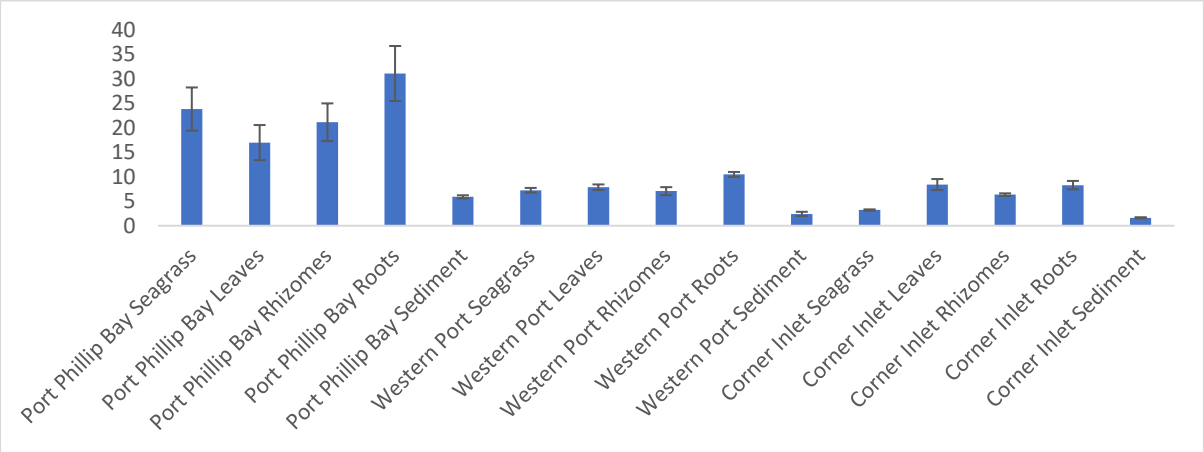
Appendix 105 – 2018 Mean Spring Cr concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



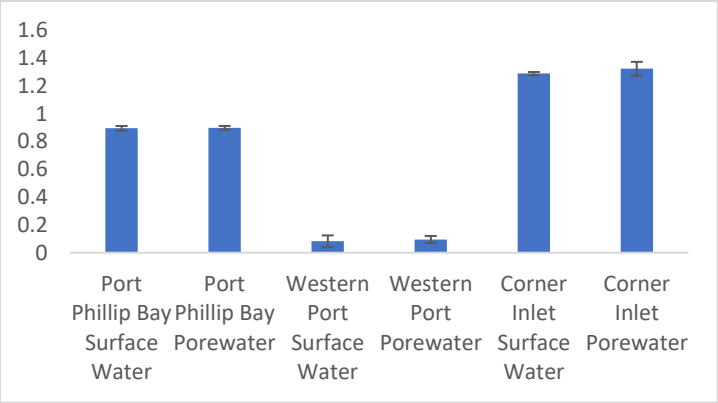
Appendix 106 – 2018 Mean Spring Cr concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



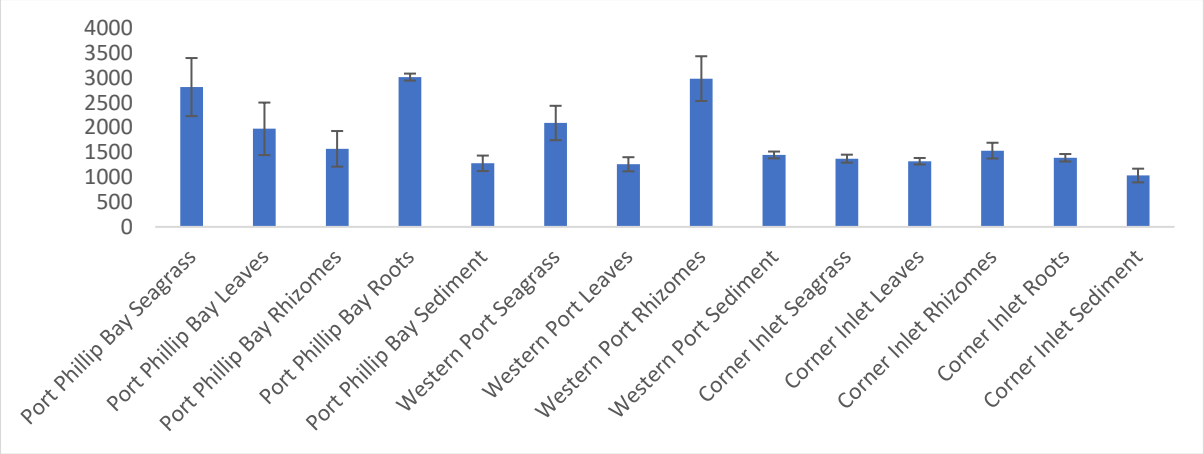
Appendix 107 – 2018 Mean Spring Cu concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



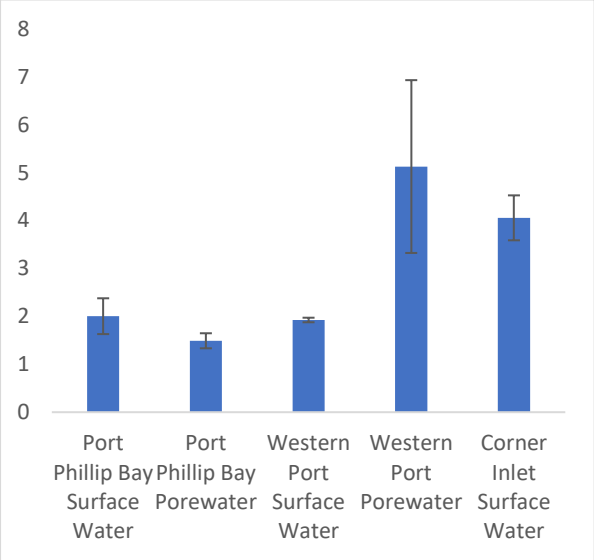
Appendix 108 – 2018 Mean Spring Cu concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



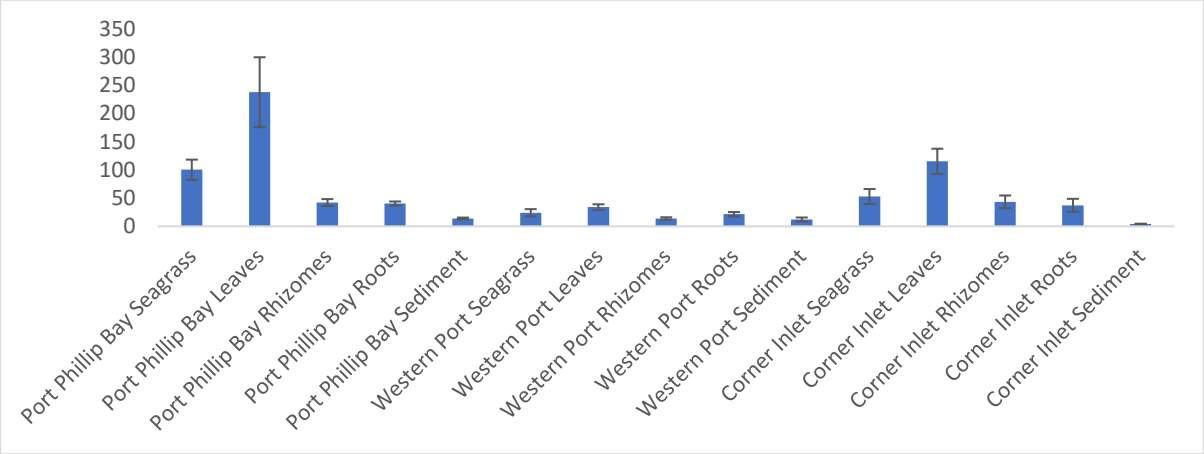
Appendix 109 – 2018 Mean Spring Fe concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



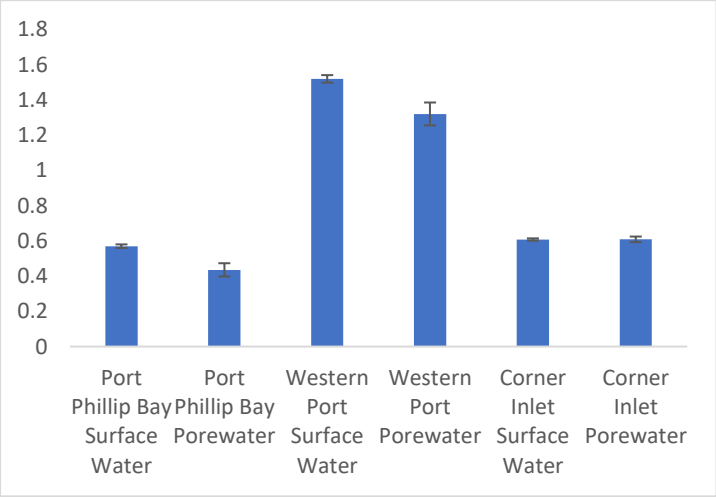
Appendix 110 – 2018 Mean Spring Fe concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



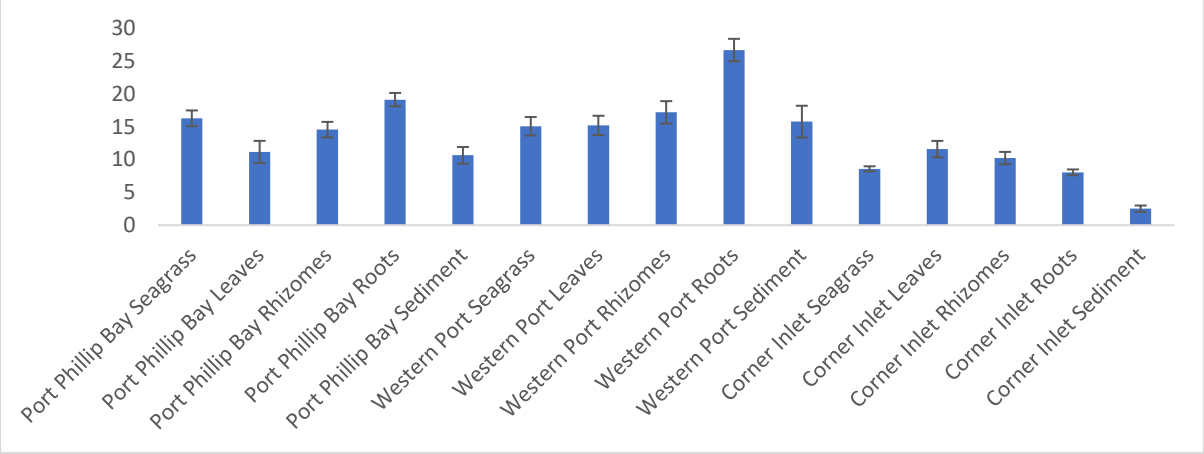
Appendix 111 – 2018 Mean Spring Mn concentrations in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



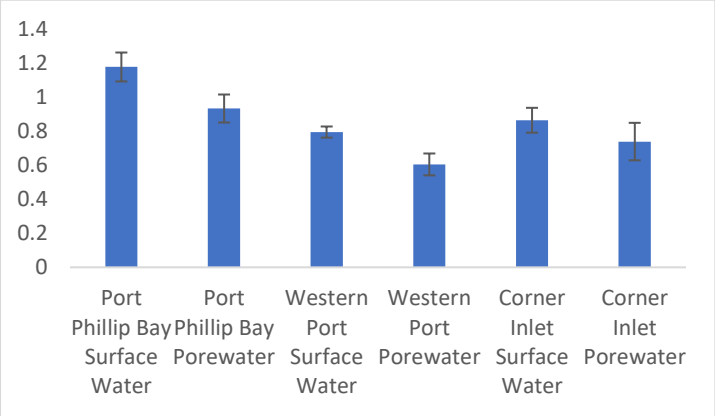
Appendix 112 – 2018 Mean Spring Mn concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



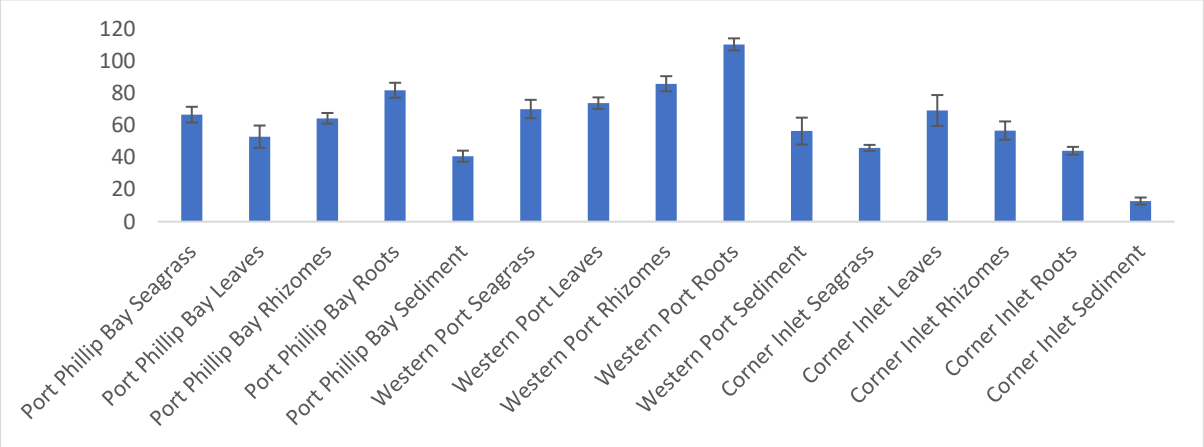
Appendix 113 – 2018 Mean Spring Ni concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



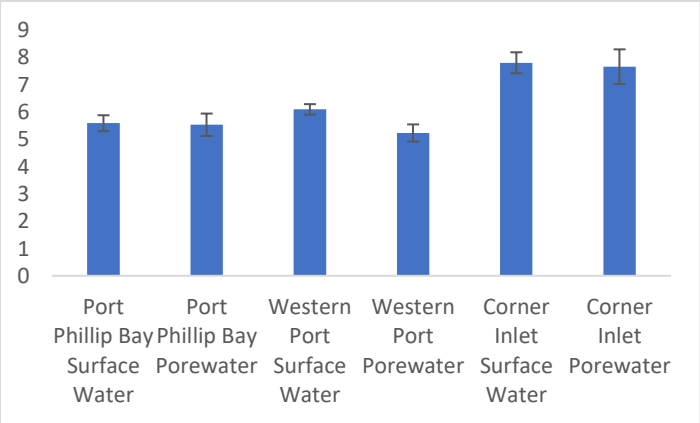
Appendix 114 – 2018 Mean Spring Ni concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



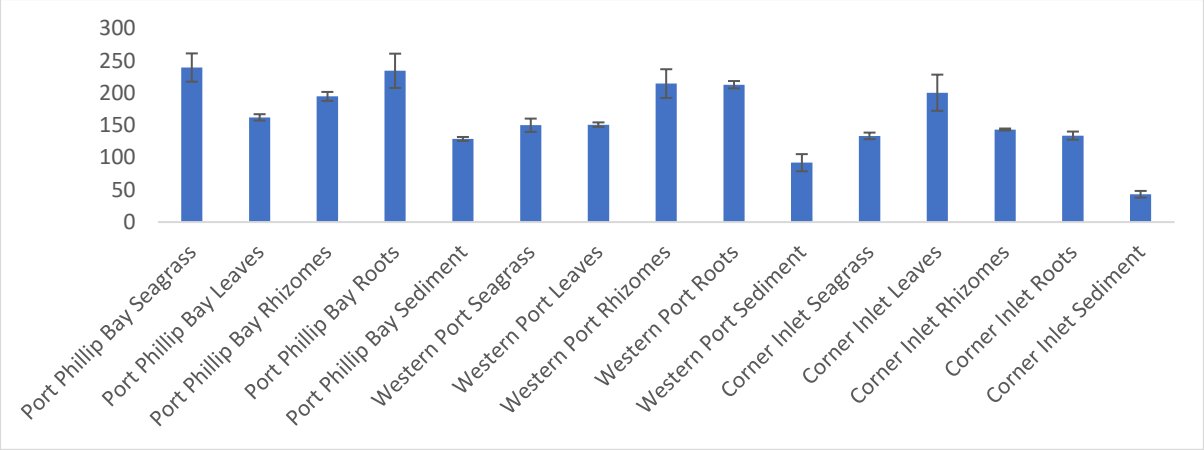
Appendix 115 – 2018 Mean Spring Pb concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



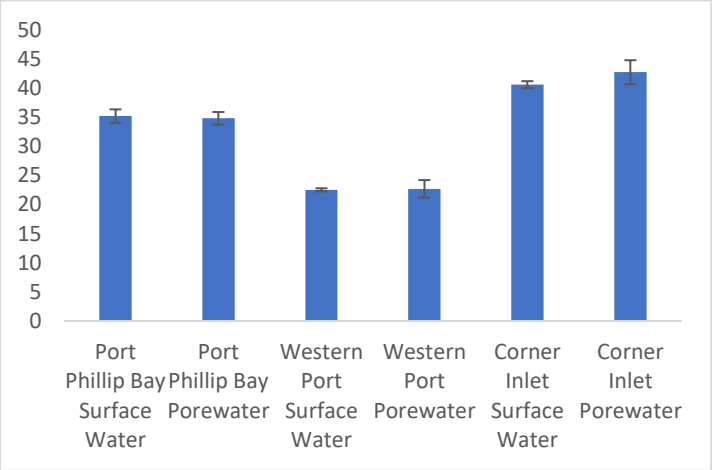
Appendix 116 – Mean 2018 Spring Pb concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



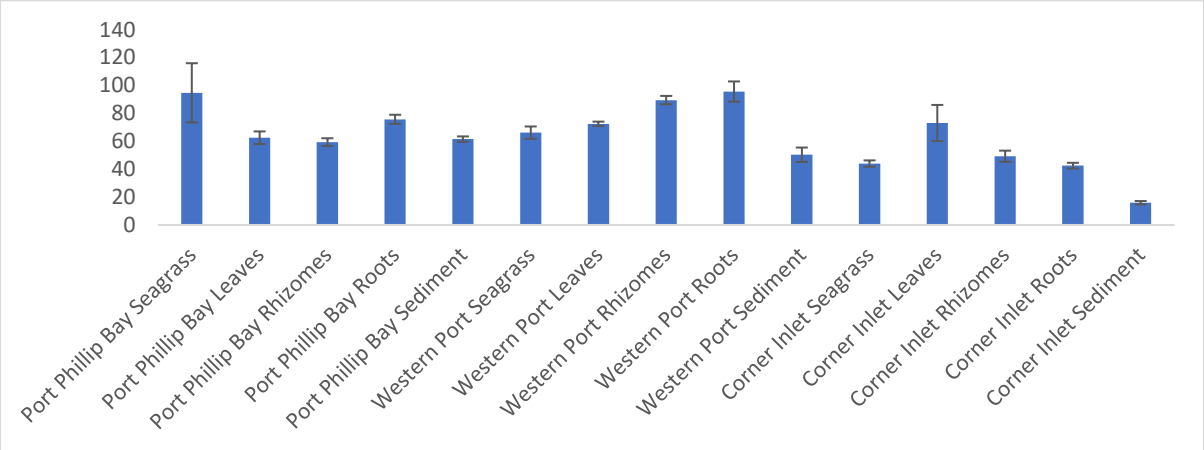
Appendix 117 – Mean 2018 Spring Se concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



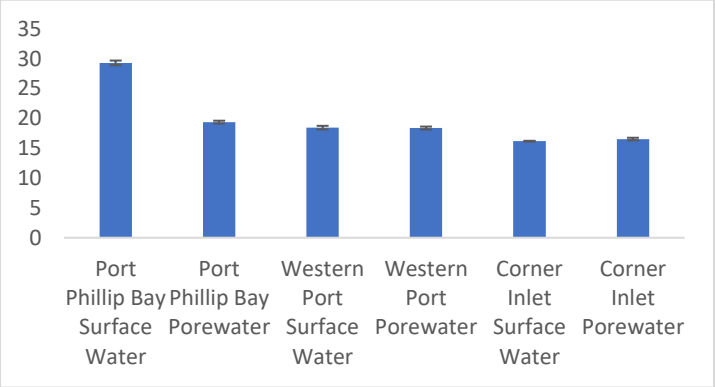
Appendix 118 – Mean 2018 Spring Se concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



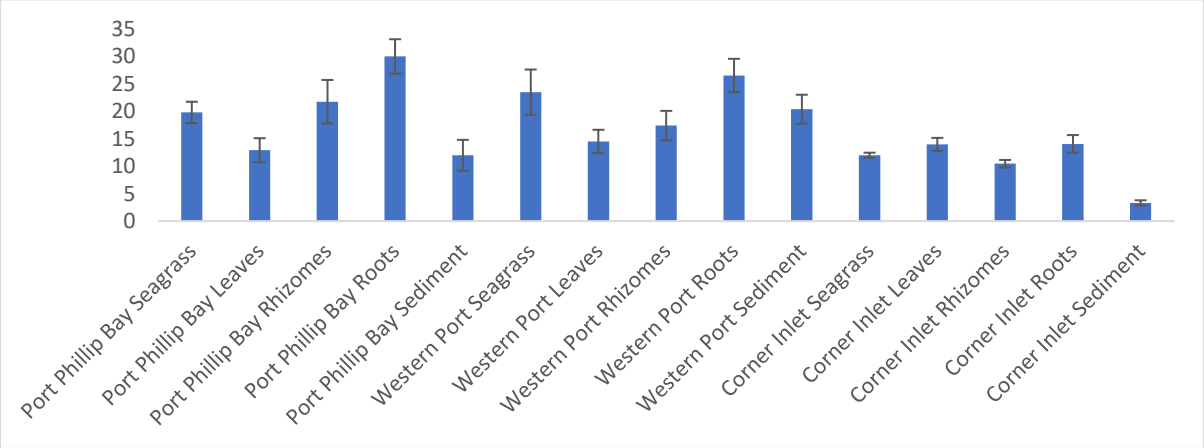
Appendix 119 – 2018 Mean Spring Sn concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



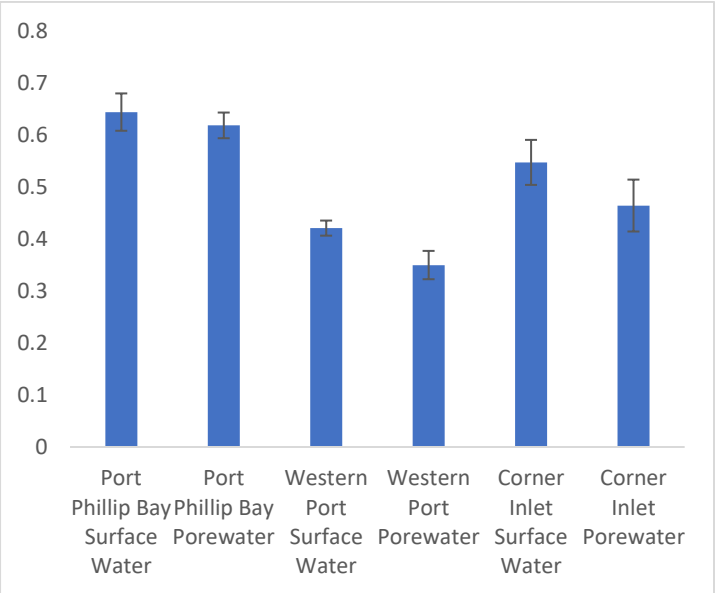
Appendix 120 – 2018 Mean Spring Sn concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



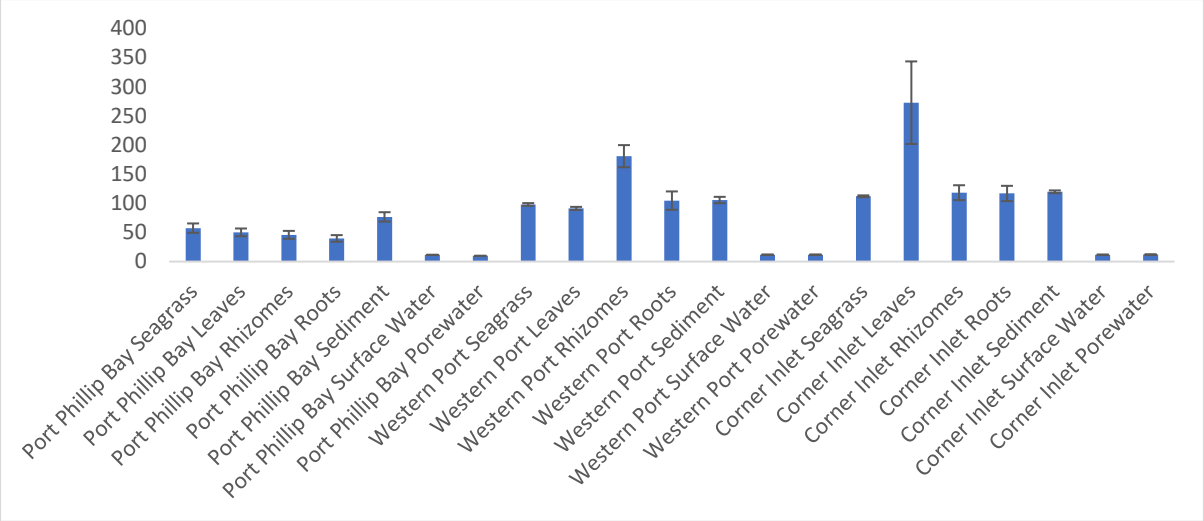
Appendix 121 – 2018 Mean Spring V with standard errors concentrations in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



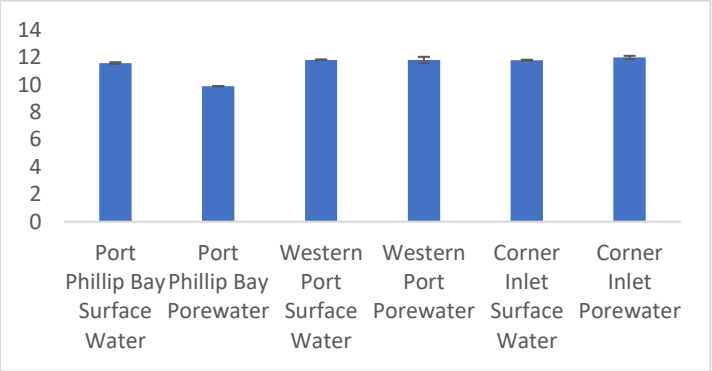
Appendix 122 – Mean 2018 Spring V concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



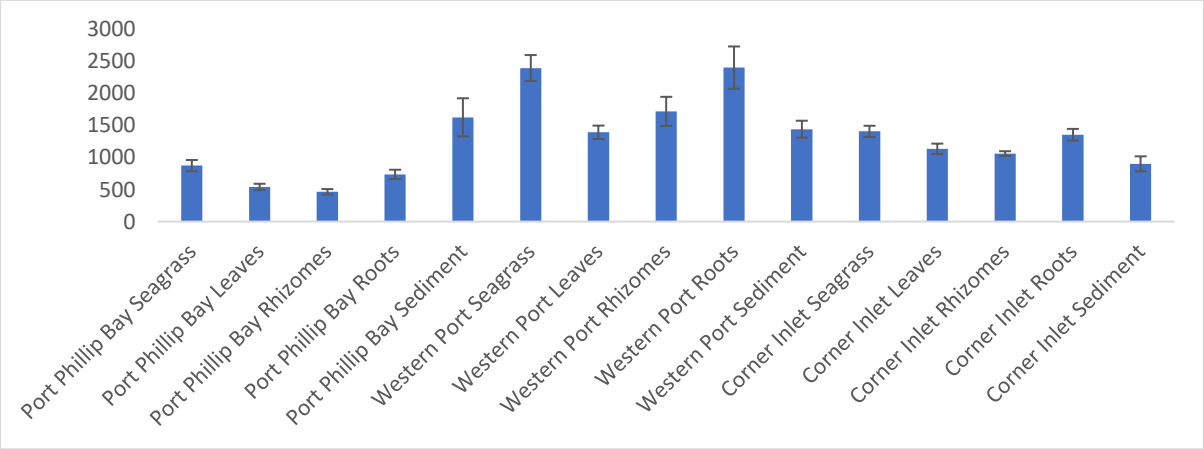
Appendix 123 – 2018 Mean Spring Zn concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



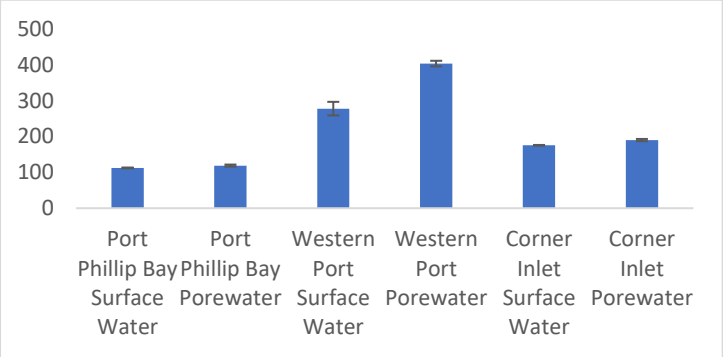
Appendix 124 – 2018 Mean Spring Zn concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



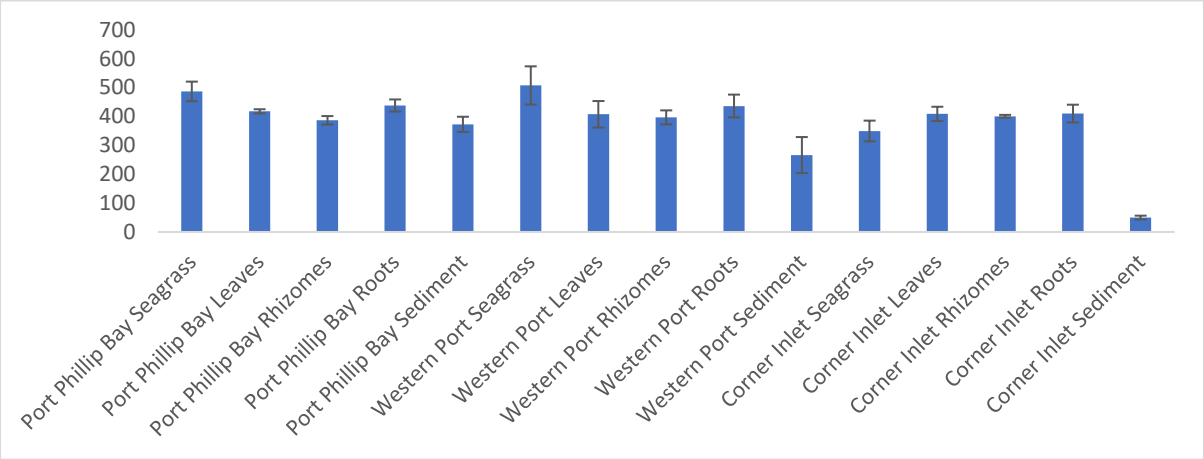
Appendix 125 – 2018/19 Mean Summer Al concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



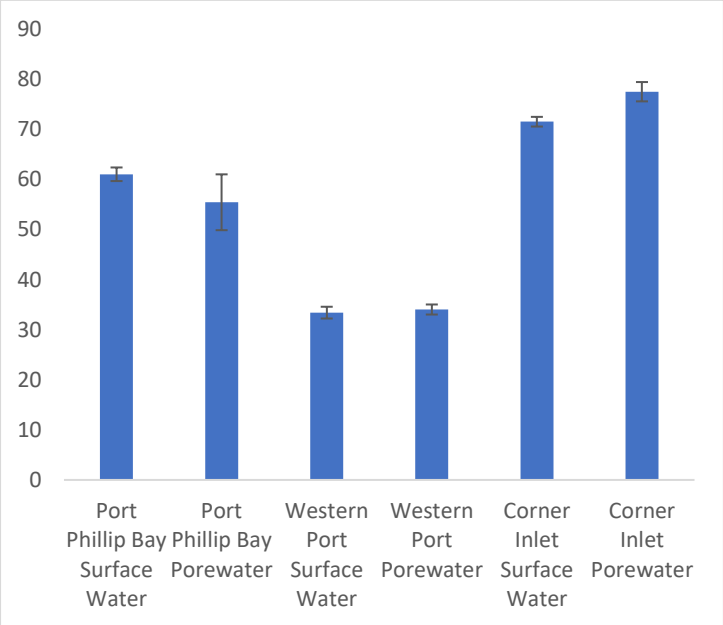
Appendix 126 – 2018/19 Mean Summer Al concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



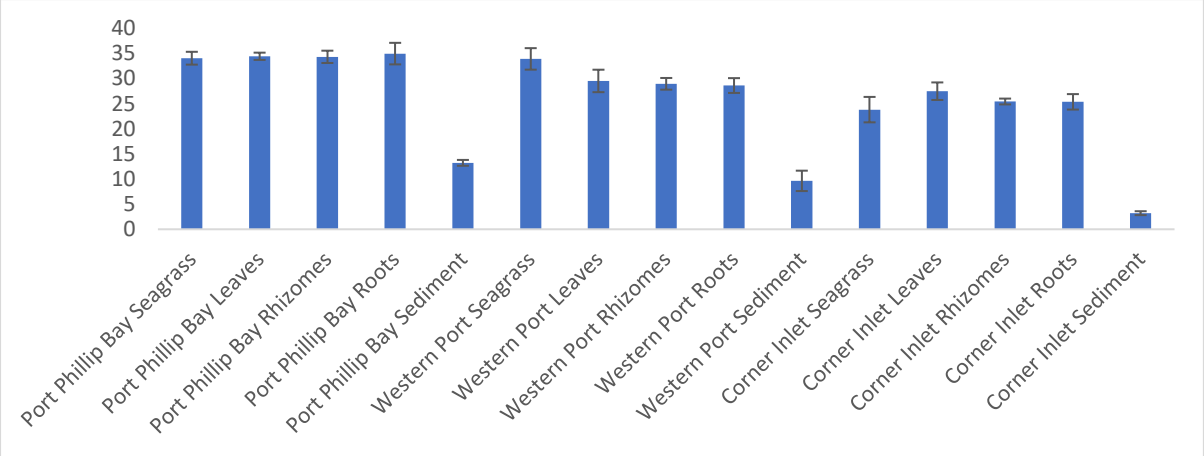
Appendix 127 – 2018/19 Mean Summer As concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



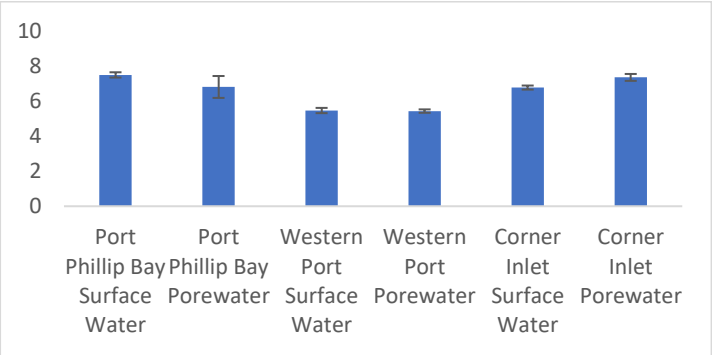
Appendix 128 – 2018/19 Mean Summer As concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



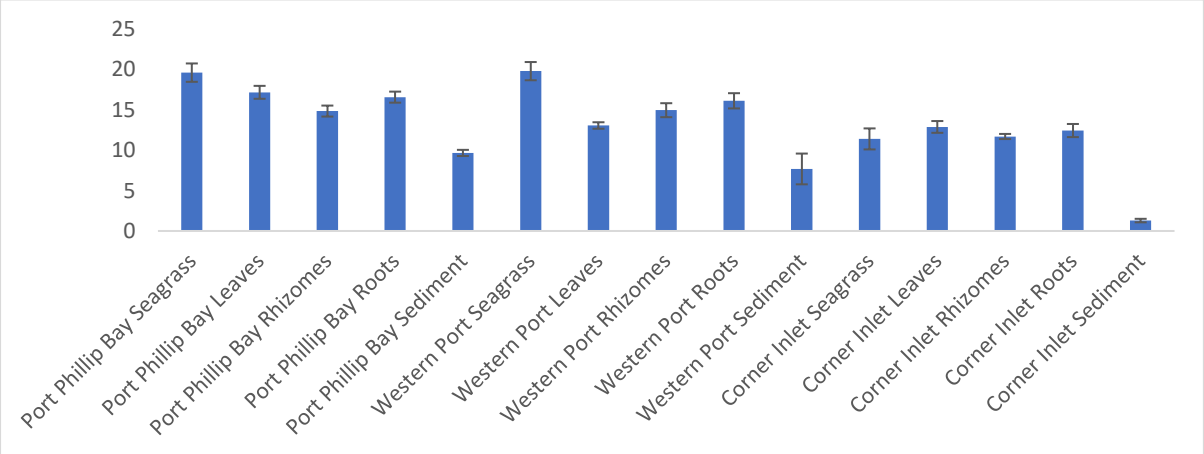
Appendix 129 – 2018/19 Mean Summer Cd concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



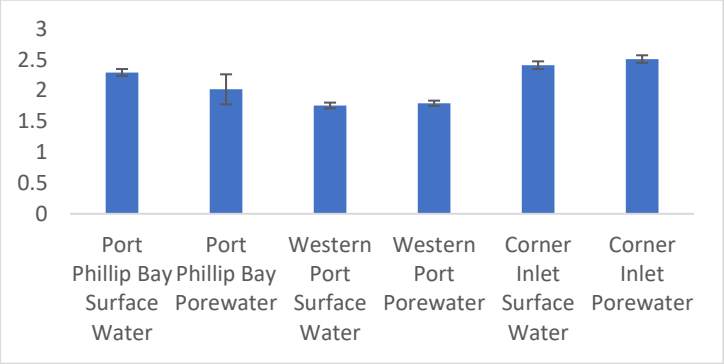
Appendix 130 – 2018/19 Mean Summer Cd concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



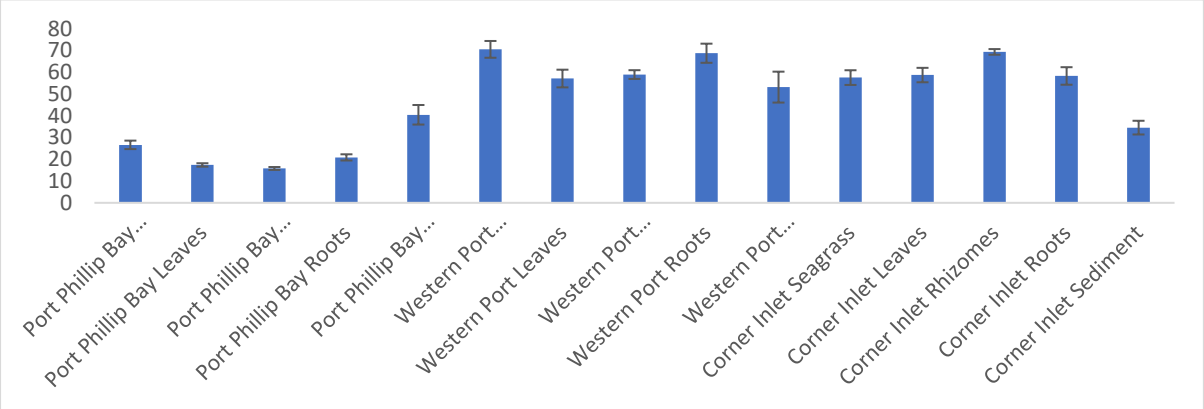
Appendix 131 – 2018/19 Mean Summer Co concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



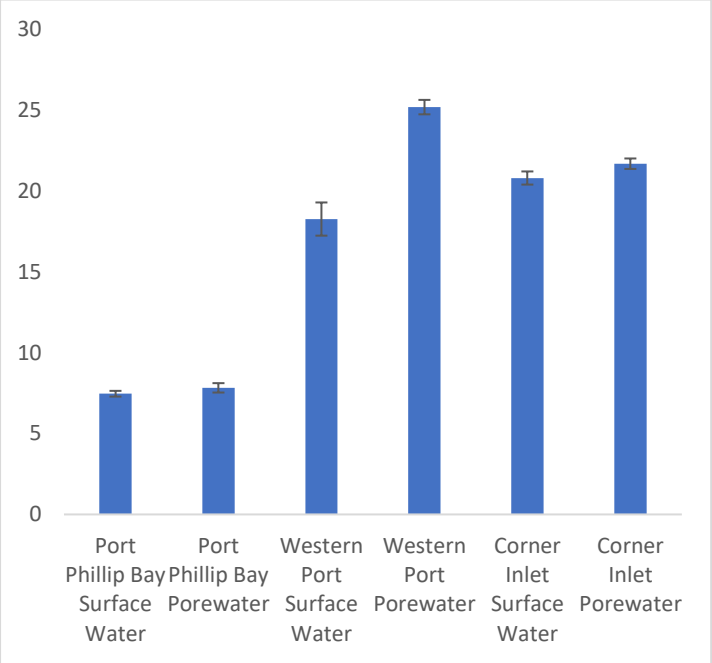
Appendix 132 – 2018/19 Mean Summer Co concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



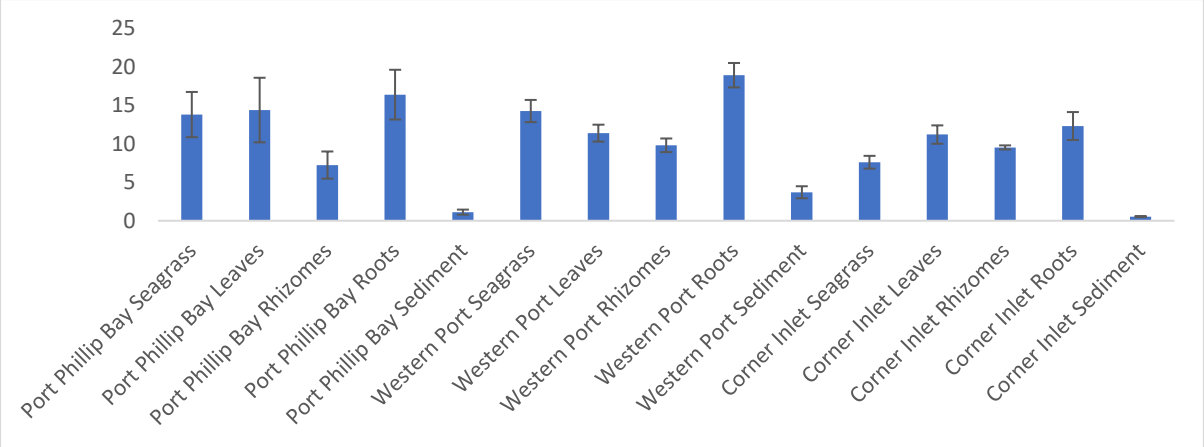
Appendix 133 – 2018/19 Mean Summer Cr concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



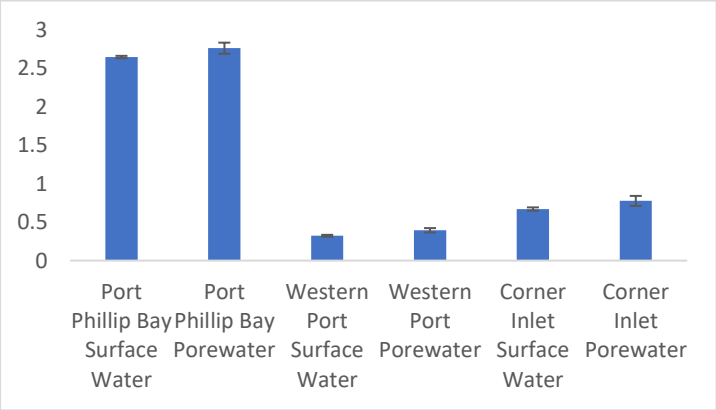
Appendix 134 – 2018/19 Mean Summer Cr concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



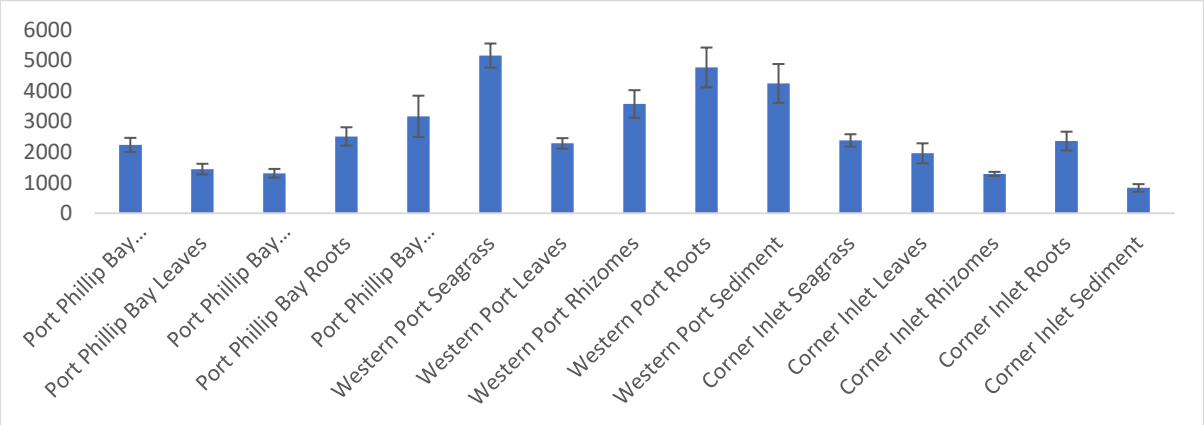
Appendix 135 – 2018/19 Mean Summer Cu concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



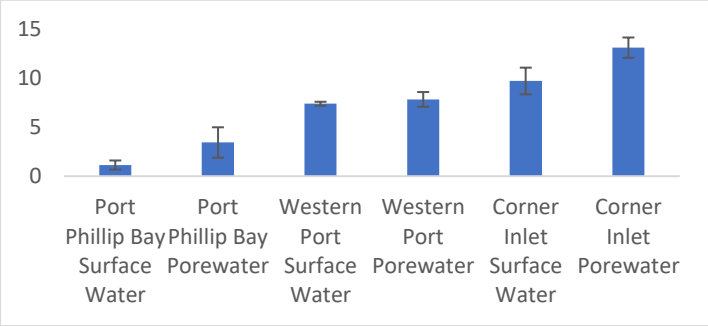
Appendix 136 – 2018/19 Mean Summer Cu concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



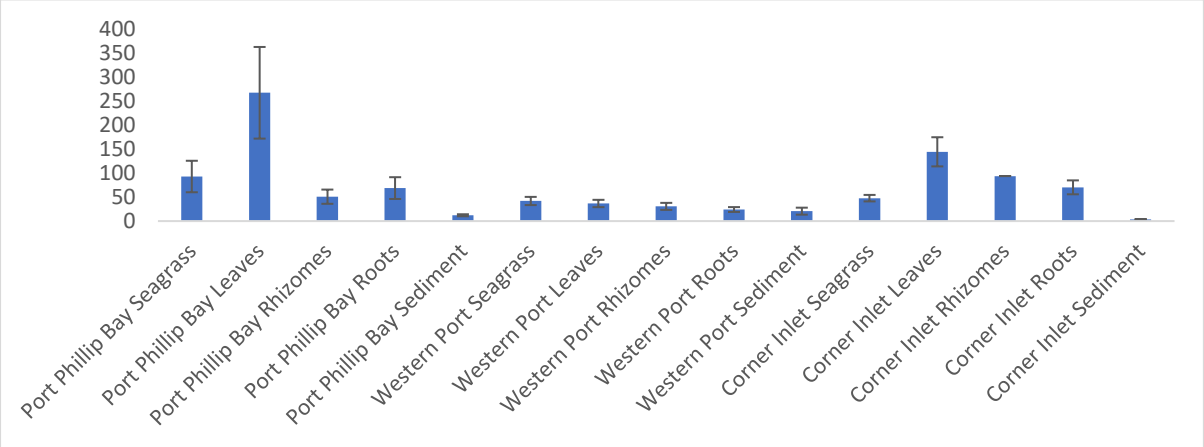
Appendix 137 – 2018/19 Mean Summer Fe concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



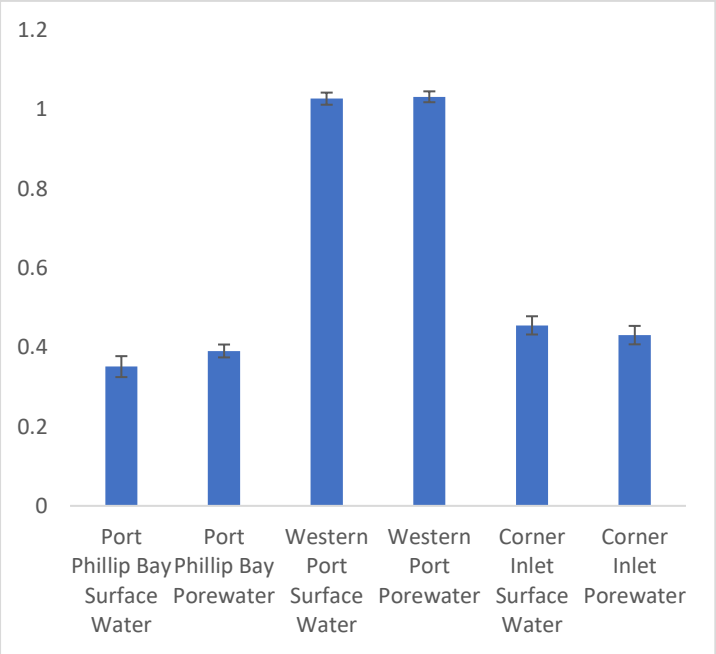
Appendix 138 – 2018/19 Mean Summer Fe concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



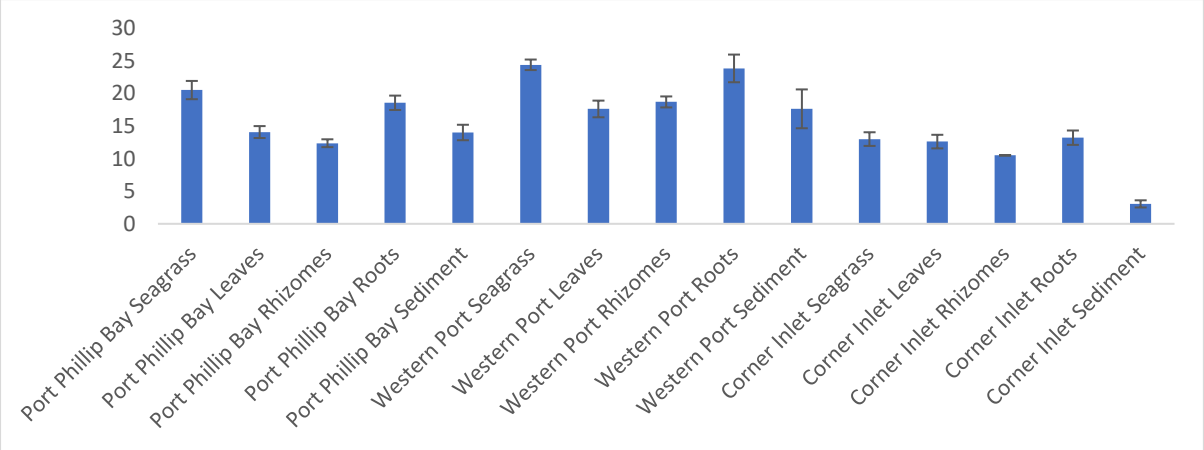
Appendix 139 – 2018/19 Mean Summer Mn concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



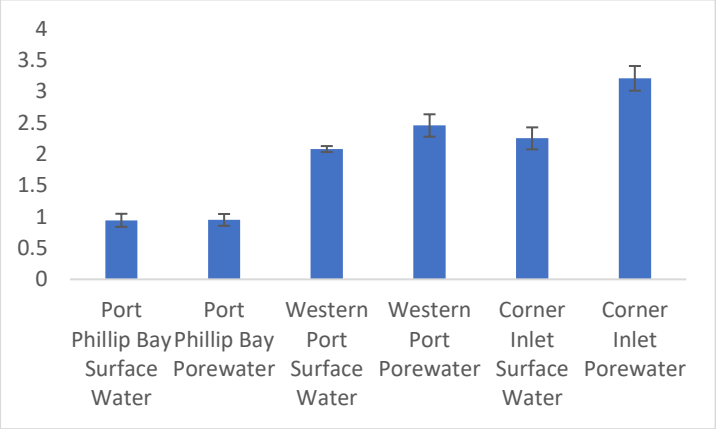
Appendix 140 – 2018/19 Mean Summer Mn concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



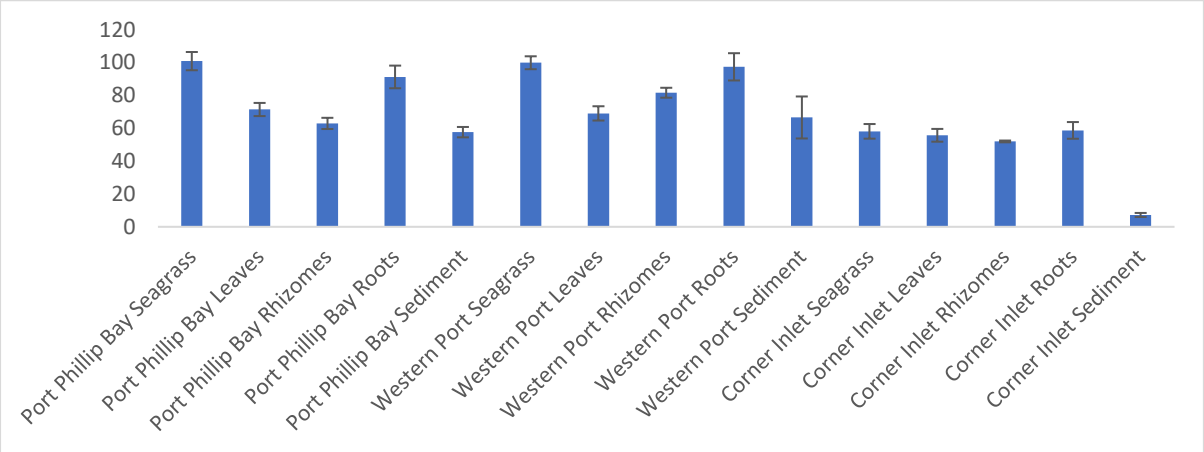
Appendix 141 – 2018/19 Mean Summer Ni concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



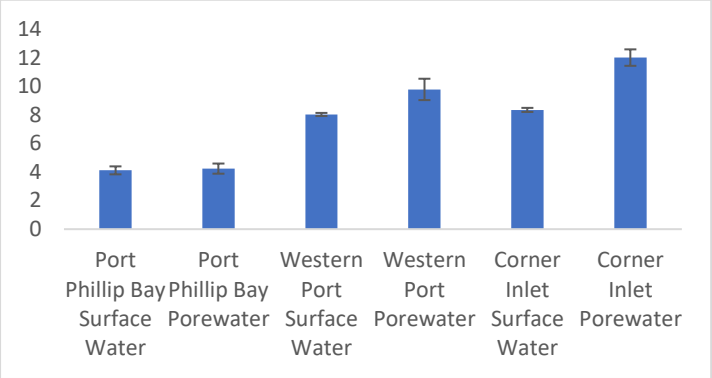
Appendix 142 – 2018/19 Mean Summer Ni concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



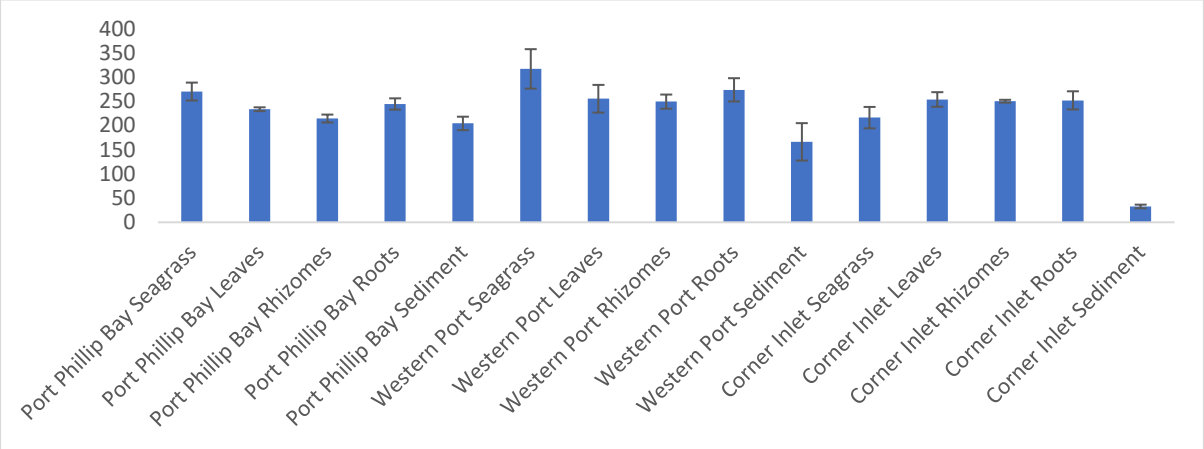
Appendix 143 – 2018/19 Mean Summer Pb concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



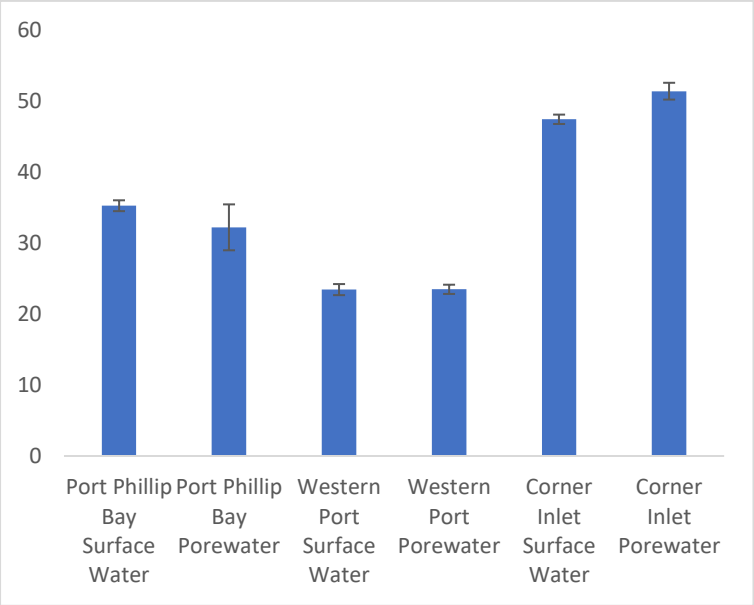
Appendix 144 – 2018/19 Mean Summer Pb concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



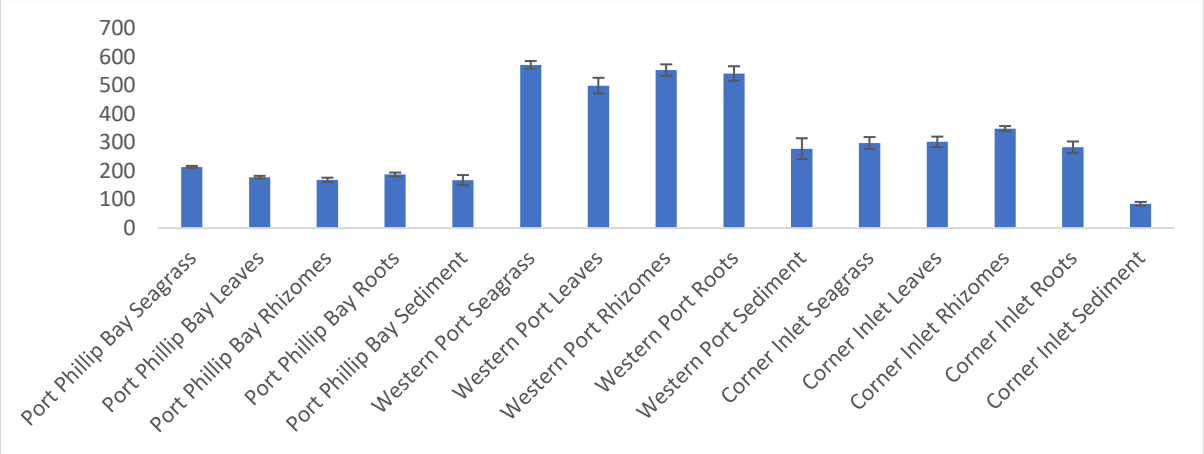
Appendix 145 – 2018/19 Mean Summer Se concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



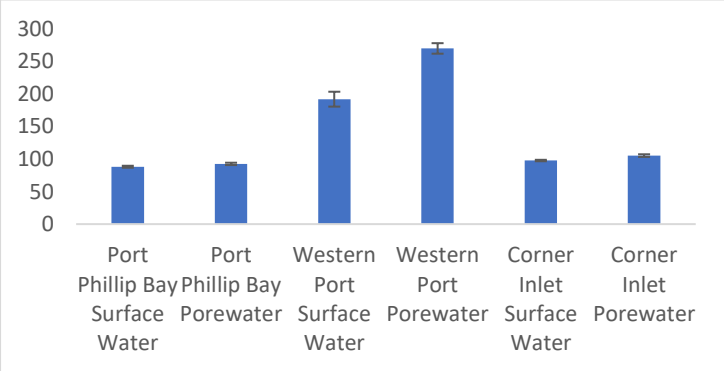
Appendix 146 – 2018/19 Mean Summer Se concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



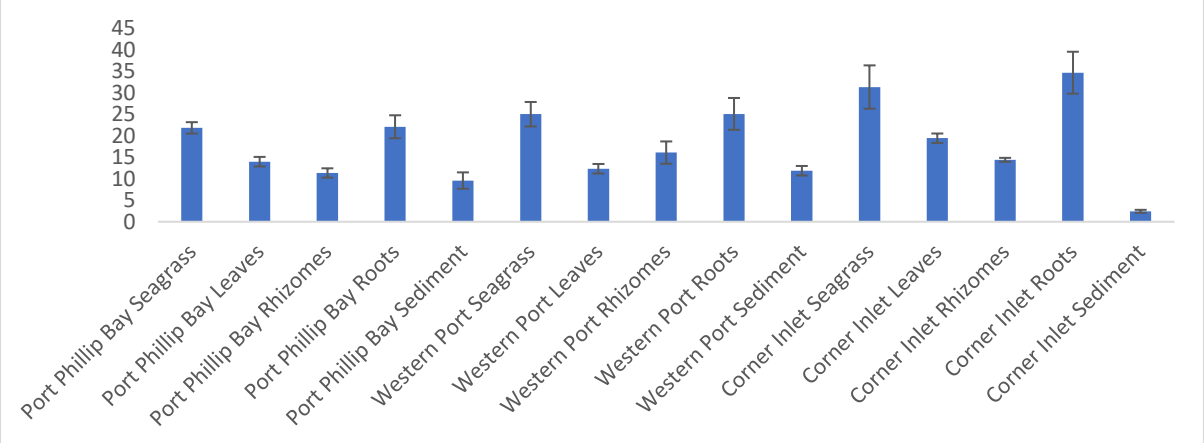
Appendix 147 – 2018/19 Mean Summer Sn concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



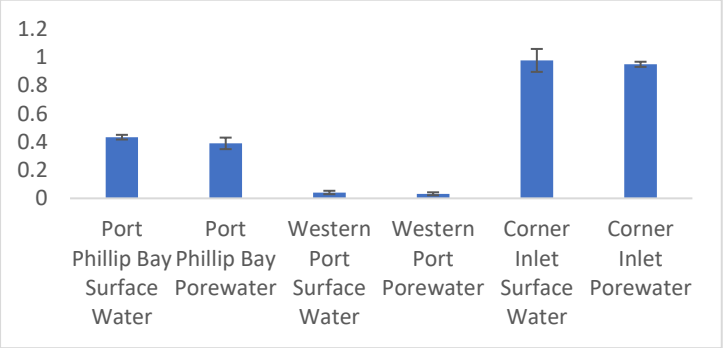
Appendix 148 – 2018/19 Mean Summer Sn concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



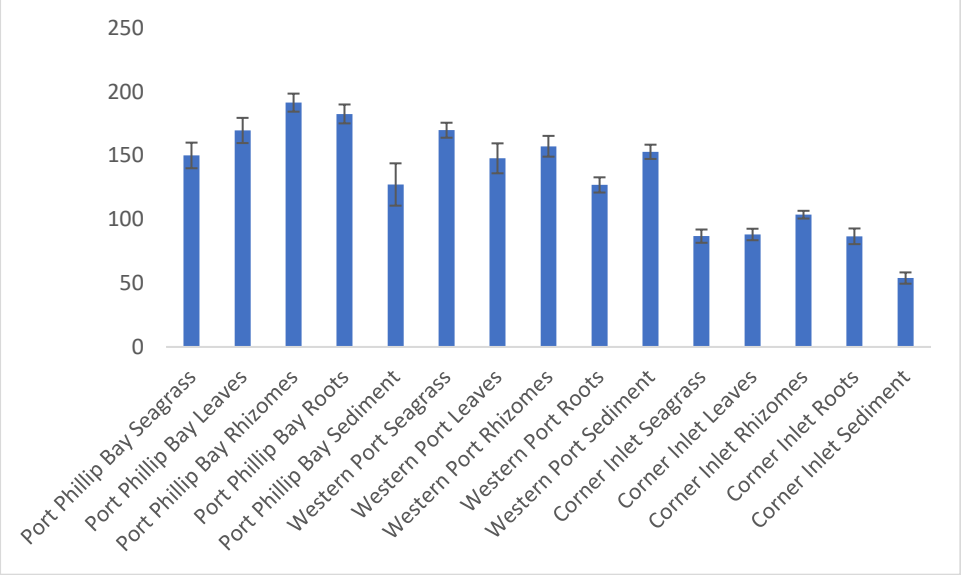
Appendix 149 – 2018/19 Mean Summer V concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



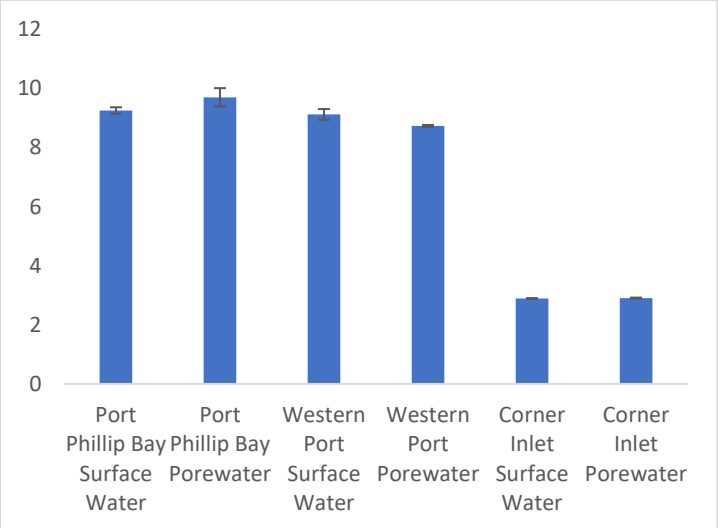
Appendix 150 – 2018/19 Mean Summer V concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



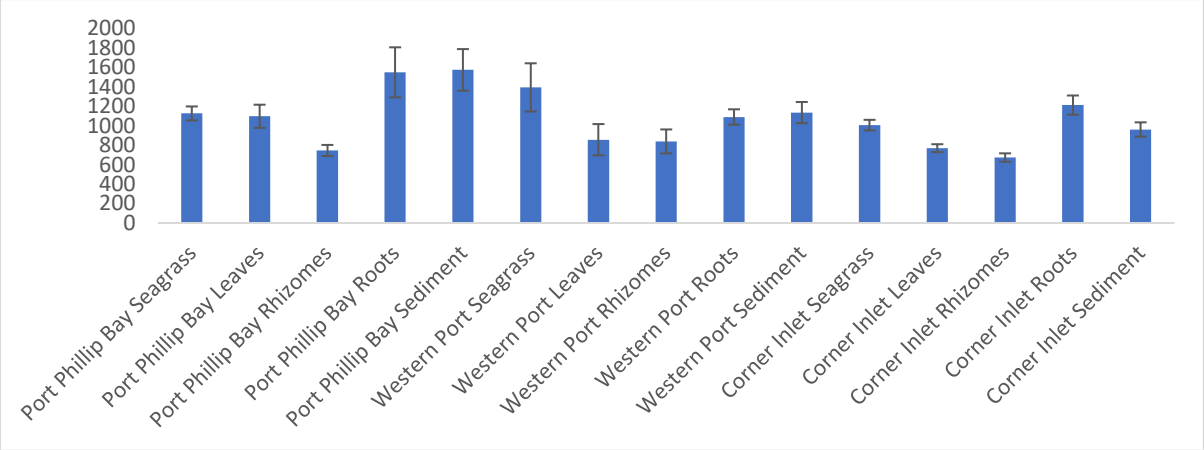
Appendix 151 – 2018/19 Mean Summer Zn concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



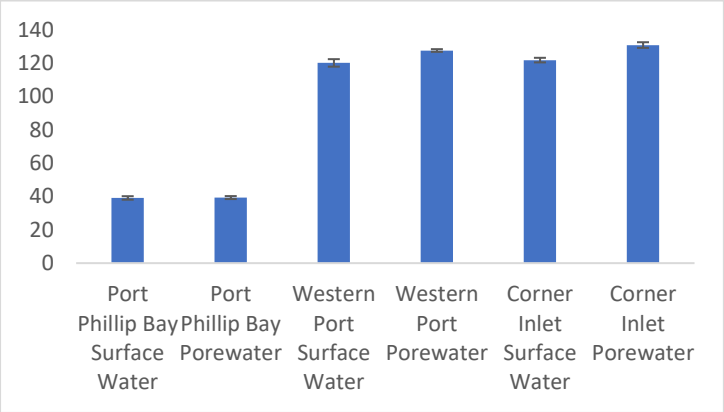
Appendix 152 – 2018/19 Mean Summer Zn concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



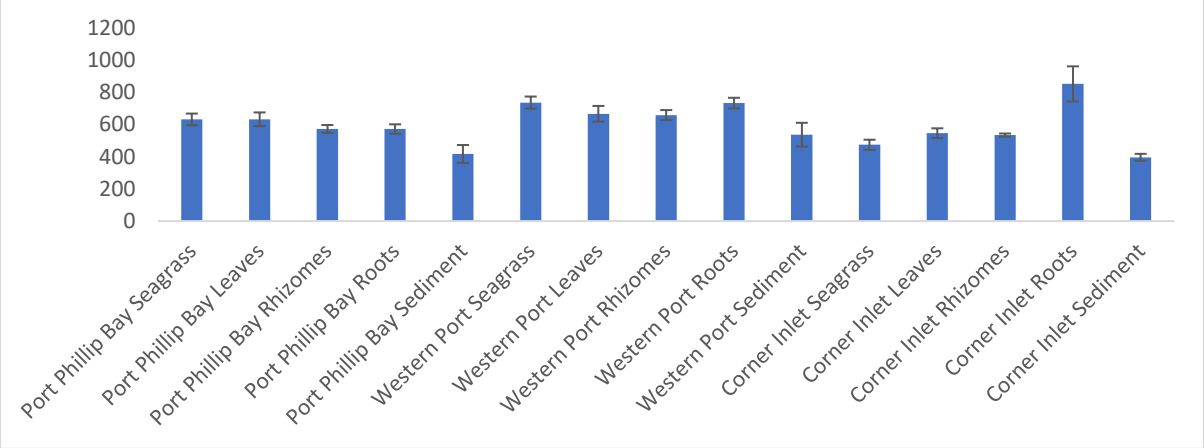
Appendix 153 – 2019 Mean Autumn Al concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



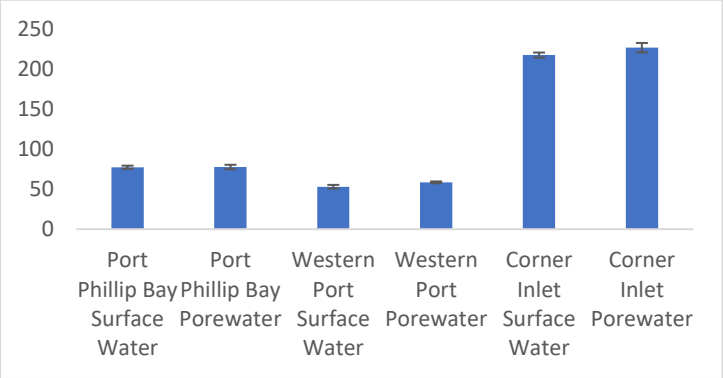
Appendix 154 – 2019 Mean Autumn Al concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



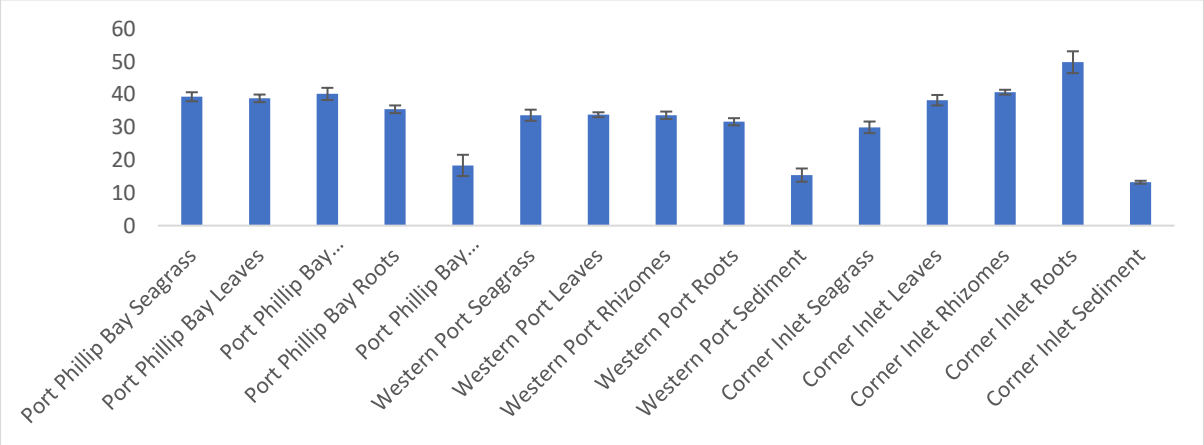
Appendix 155 – 2019 Mean Autumn As concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



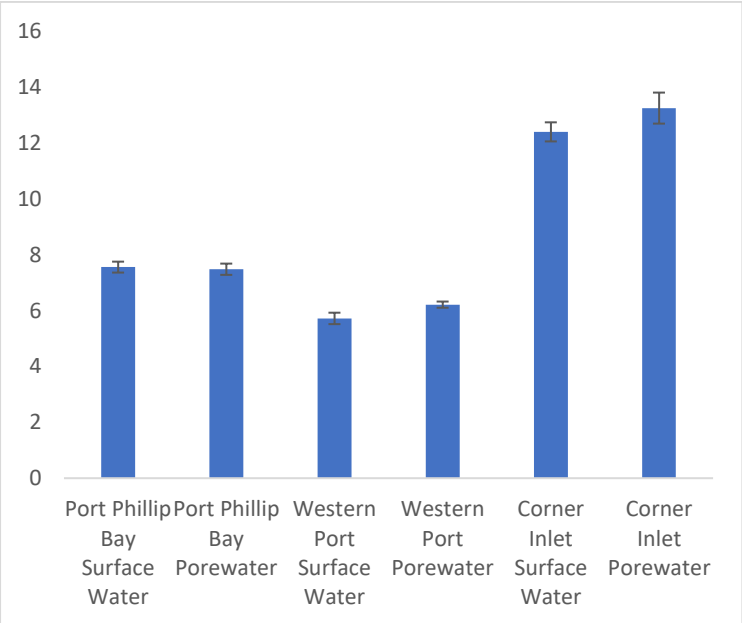
Appendix 156 – 2019 Mean Autumn As concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



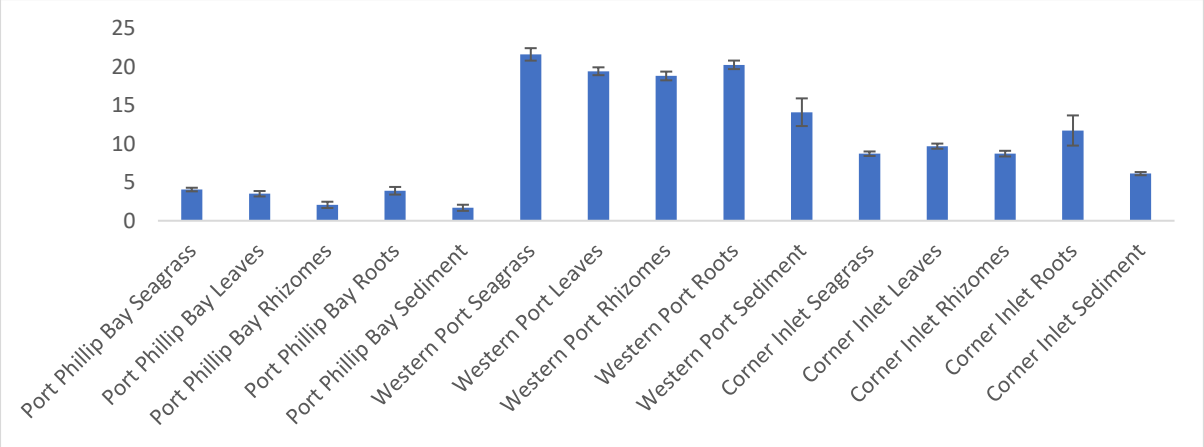
Appendix 157 – 2019 Mean Autumn Cd concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



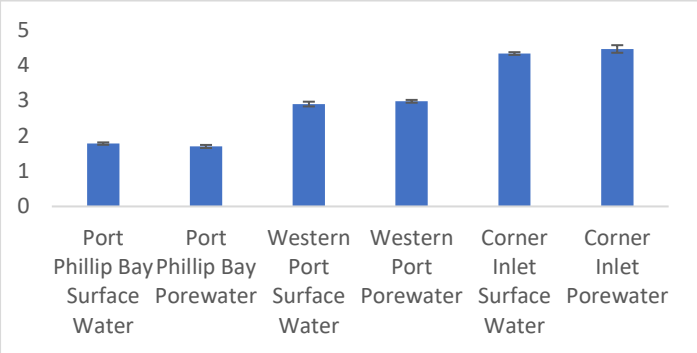
Appendix 158 – 2019 Mean Autumn Cd concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



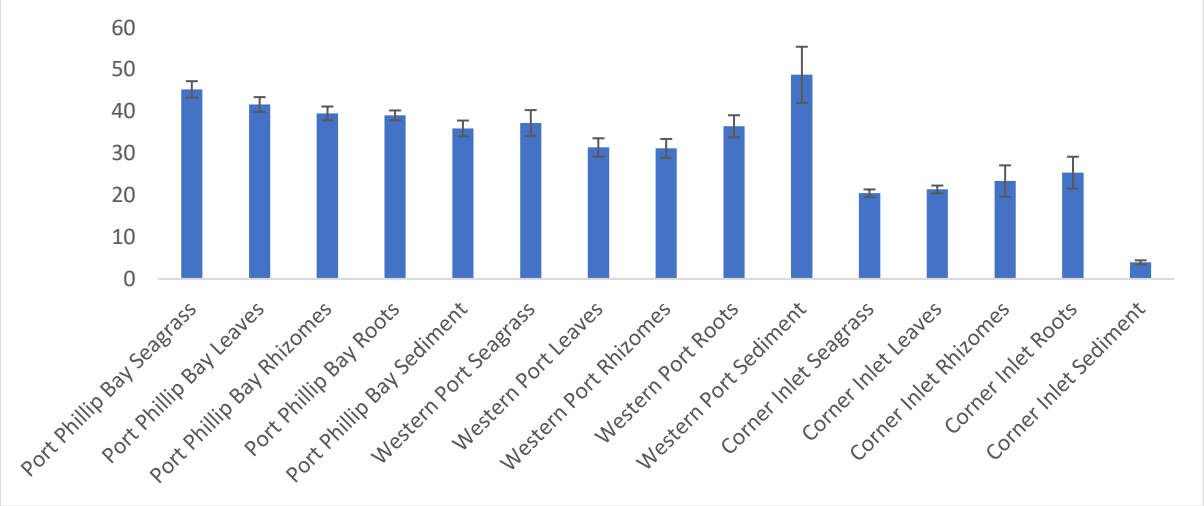
Appendix 159 – 2019 Mean Autumn Co concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



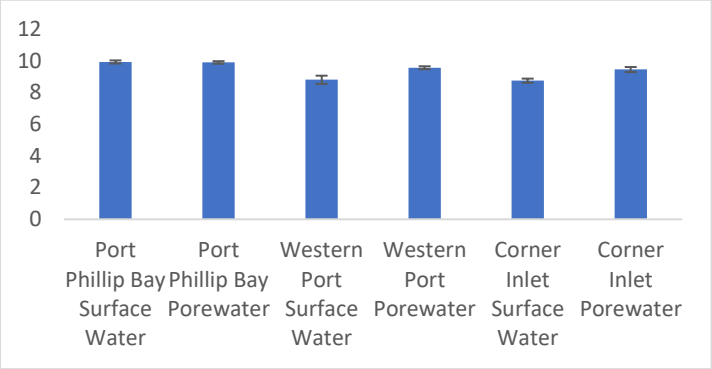
Appendix 160 – 2019 Mean Autumn Co concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



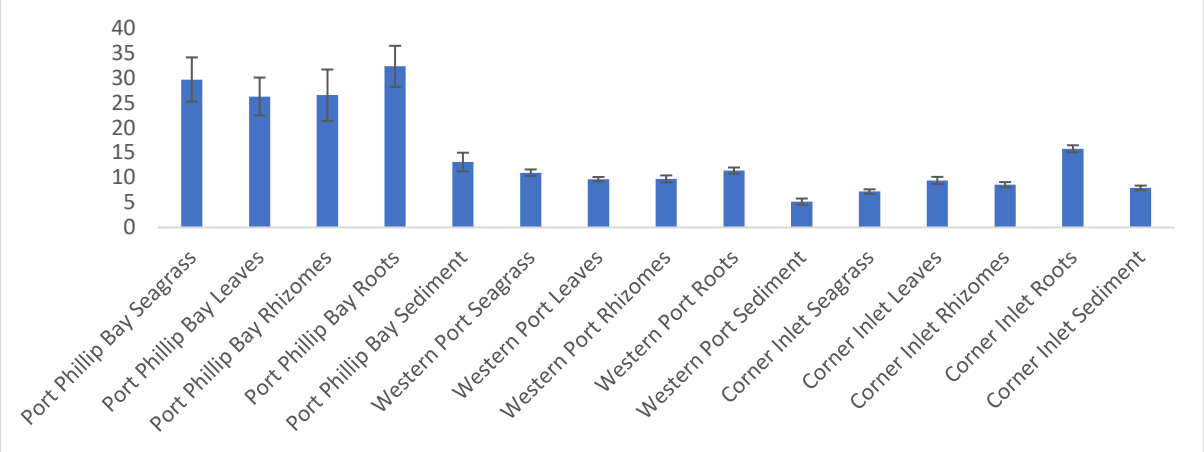
Appendix 161 – 2019 Mean Autumn Cr concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



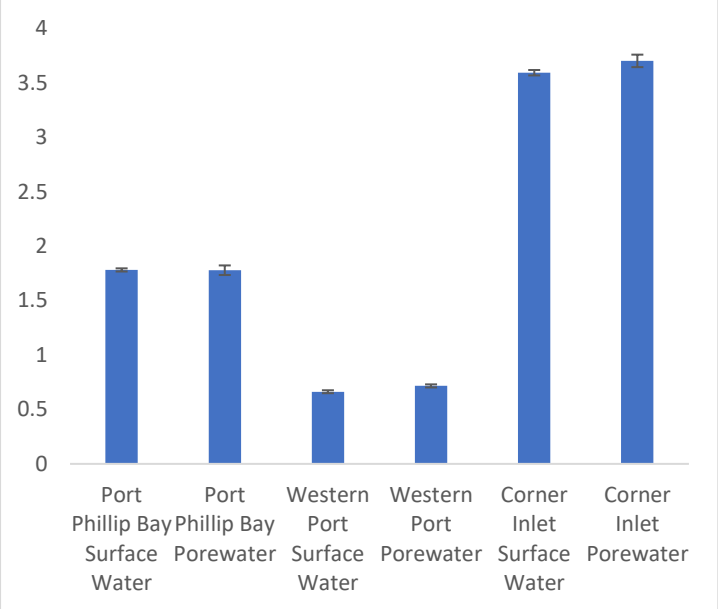
Appendix 162 – 2019 Mean Autumn Cr concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



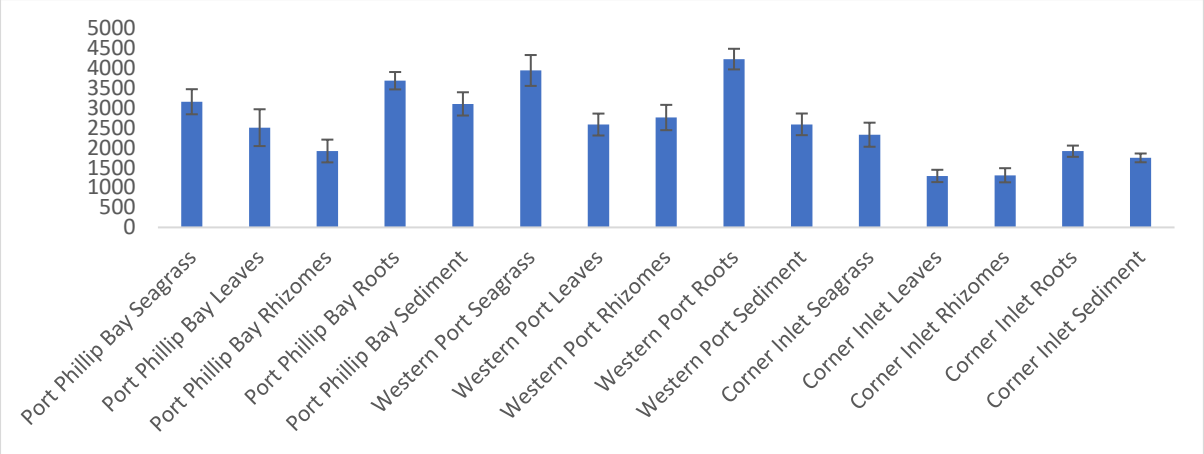
Appendix 163 – 2019 Mean Autumn Cu concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



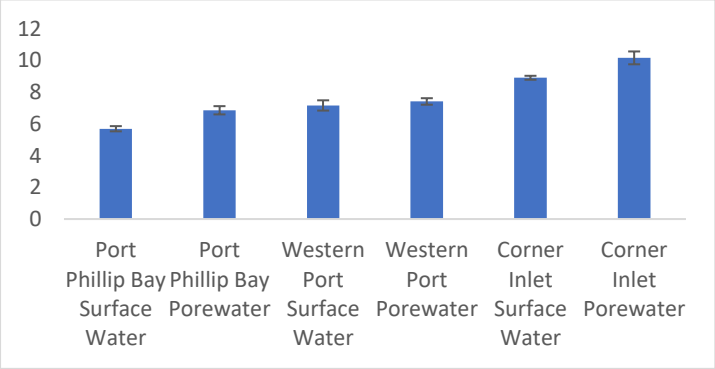
Appendix 164 – 2019 Mean Autumn Cu concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



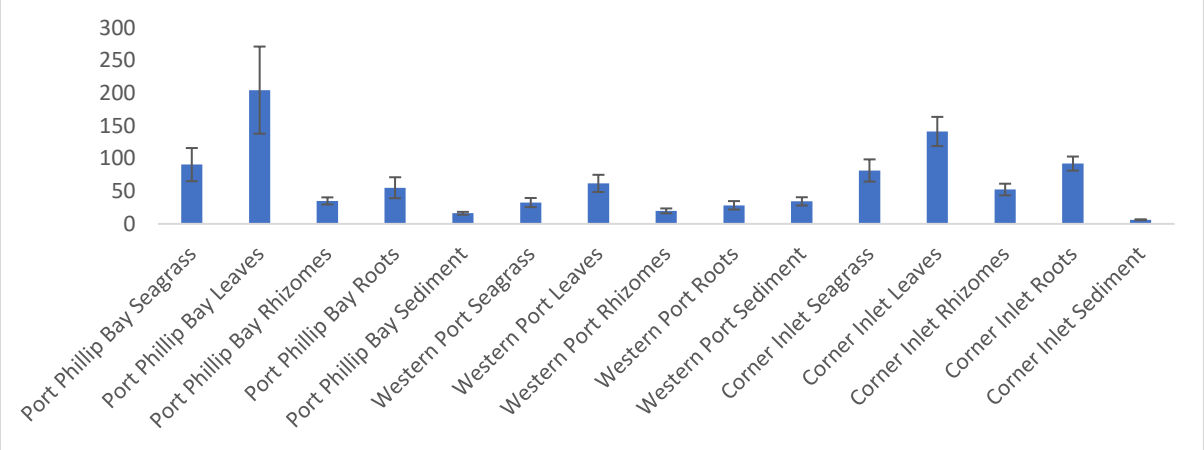
Appendix 165 – 2019 Mean Autumn Fe concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



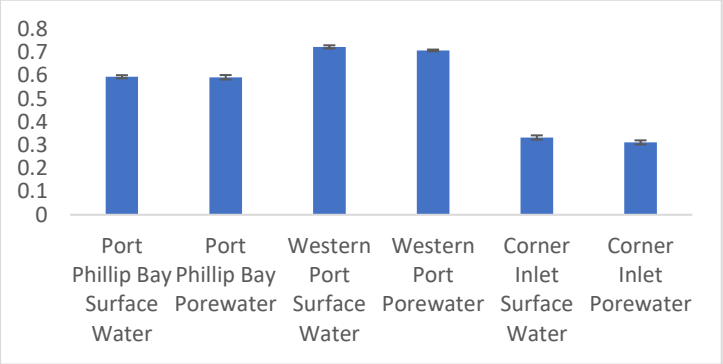
Appendix 166 – 2019 Mean Autumn Fe concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



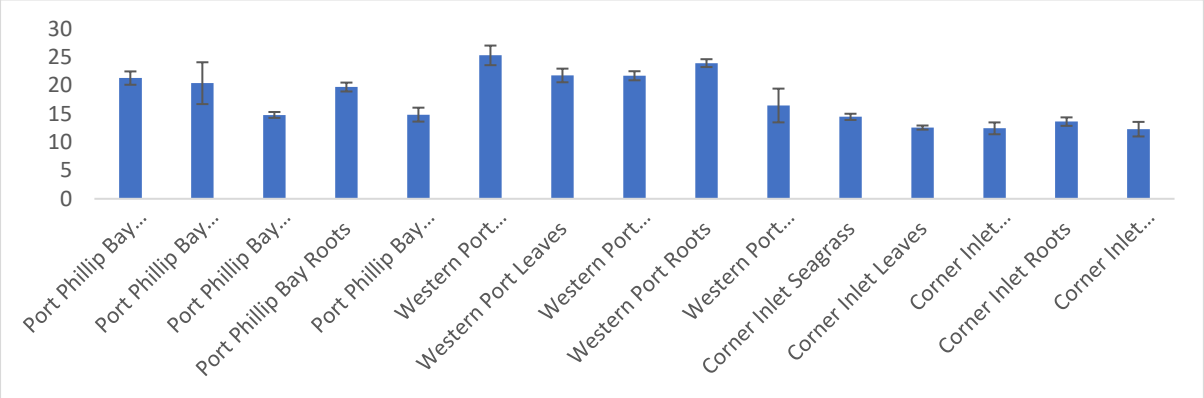
Appendix 167 – 2019 Mean Autumn Mn concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



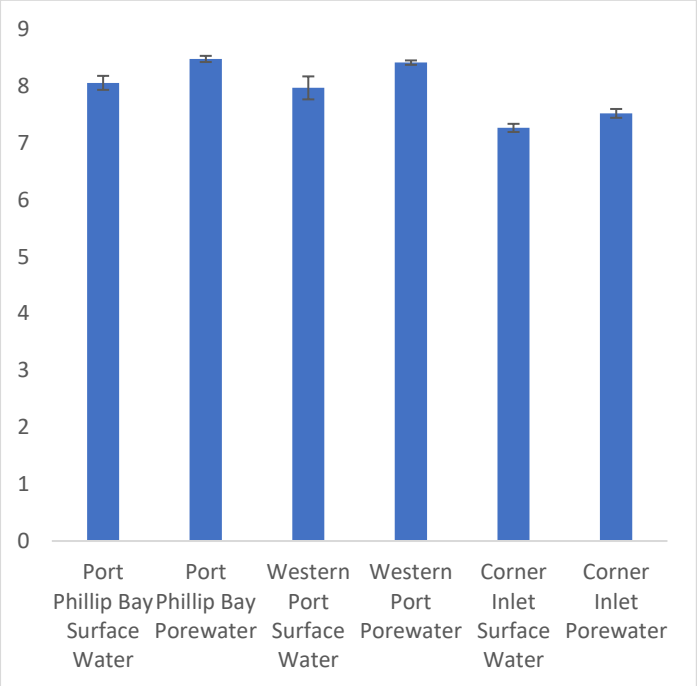
Appendix 168 – 2019 Mean Autumn Mn concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



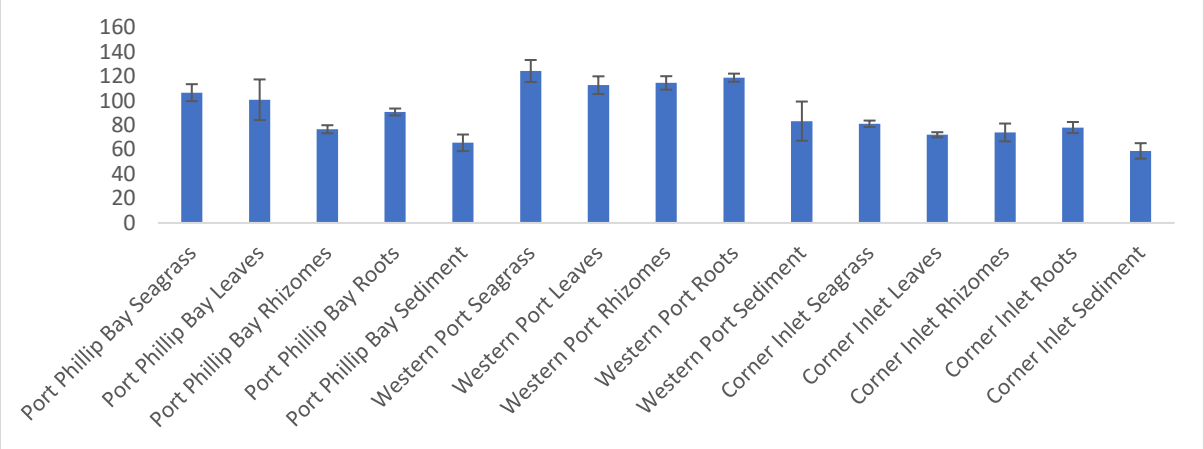
Appendix 169 – 2019 Mean Autumn Ni concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



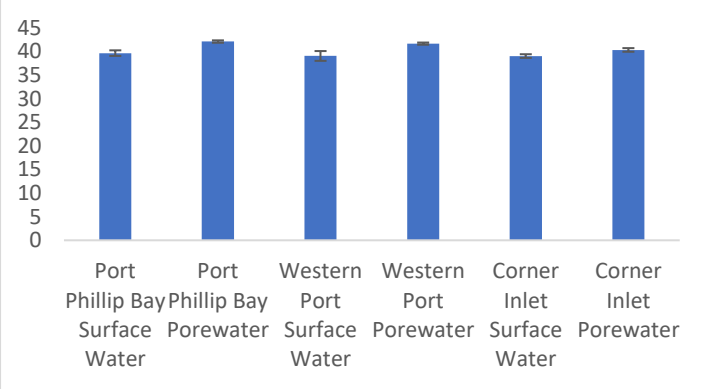
Appendix 170 – 2019 Mean Autumn Ni concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



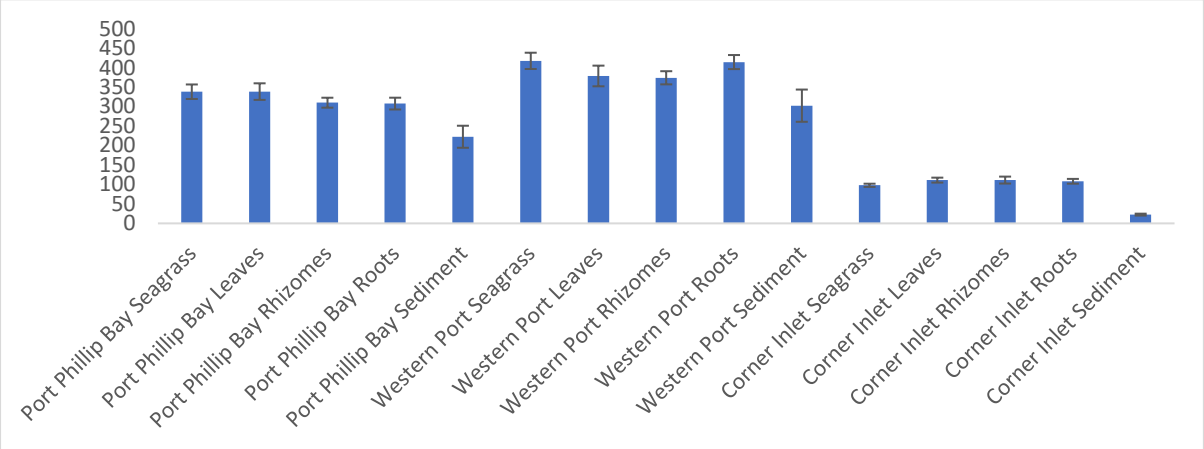
Appendix 171 – 2019 Mean Autumn Pb concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



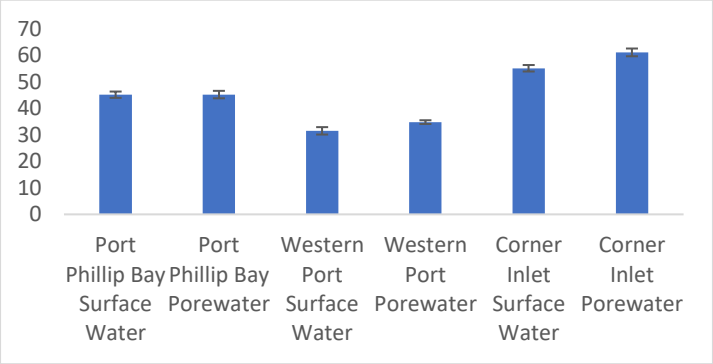
Appendix 172 – 2019 Mean Autumn Pb concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



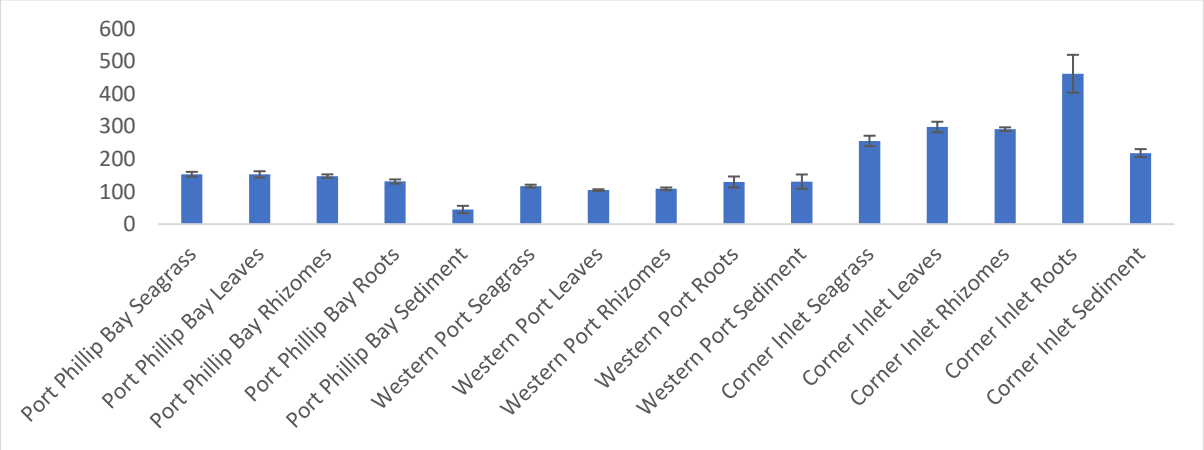
Appendix 173 – 2019 Mean Autumn Se concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



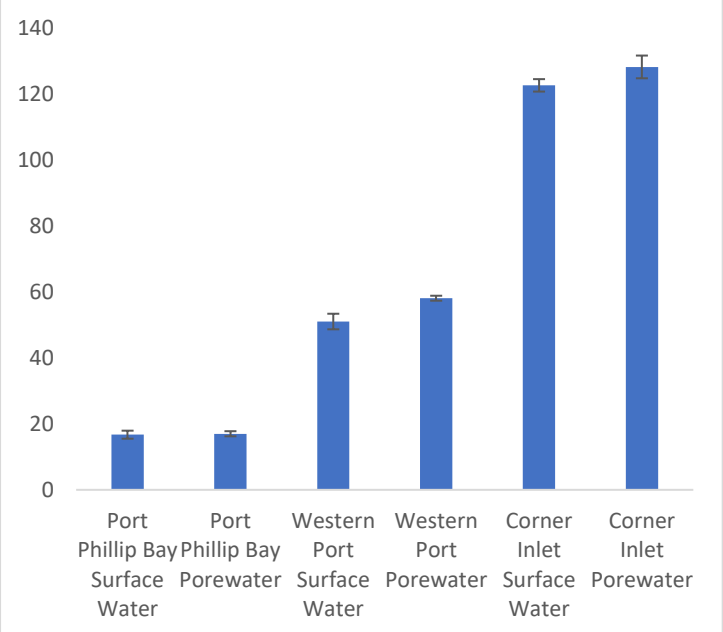
Appendix 174 – 2019 Mean Autumn Se concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



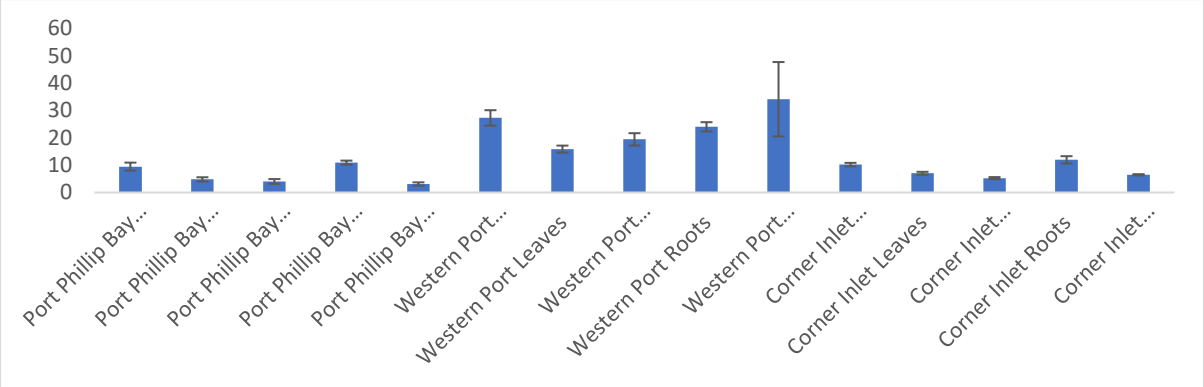
Appendix 175 – 2019 Mean Autumn Sn concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



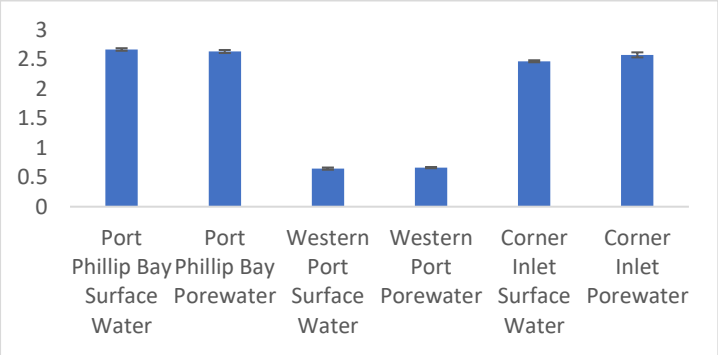
Appendix 176 – 2019 Mean Autumn Sn concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



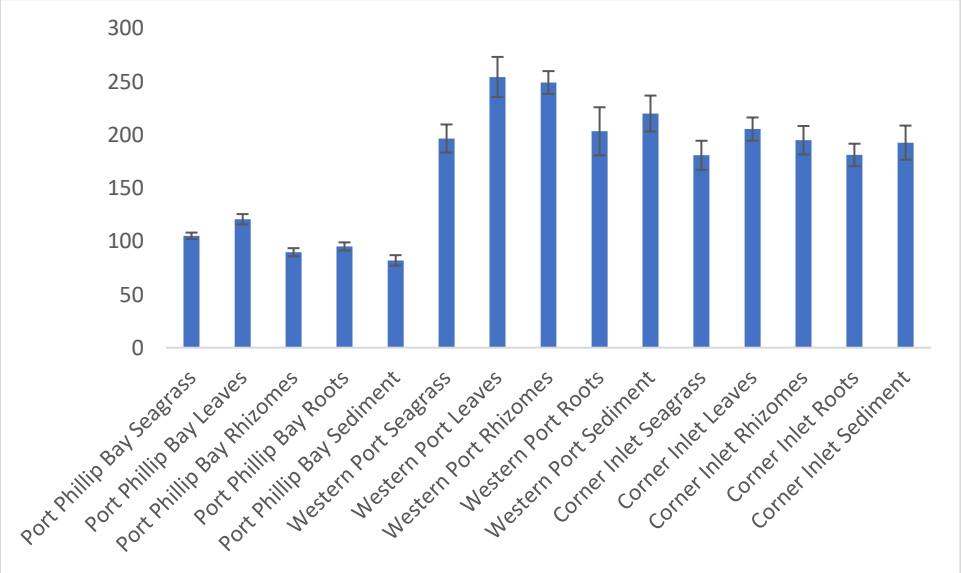
Appendix 177 – 2019 Mean Autumn V concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



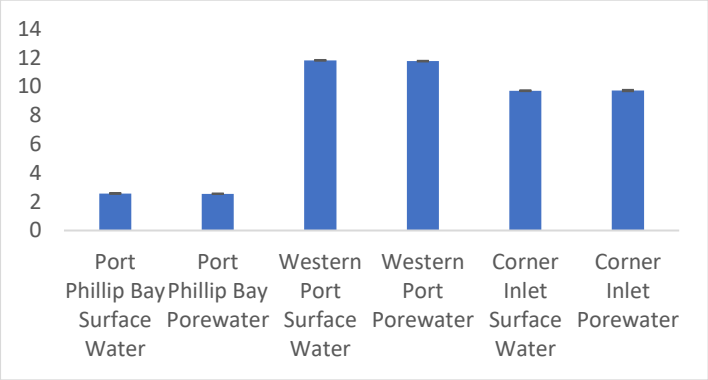
Appendix 178 – 2019 Mean Autumn V concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



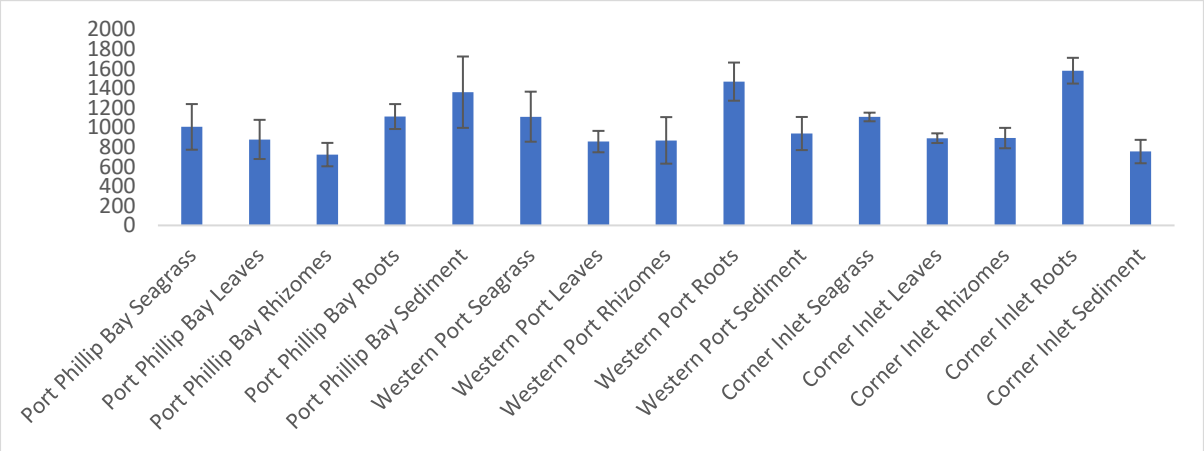
Appendix 179 – 2019 Mean Autumn Zn concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



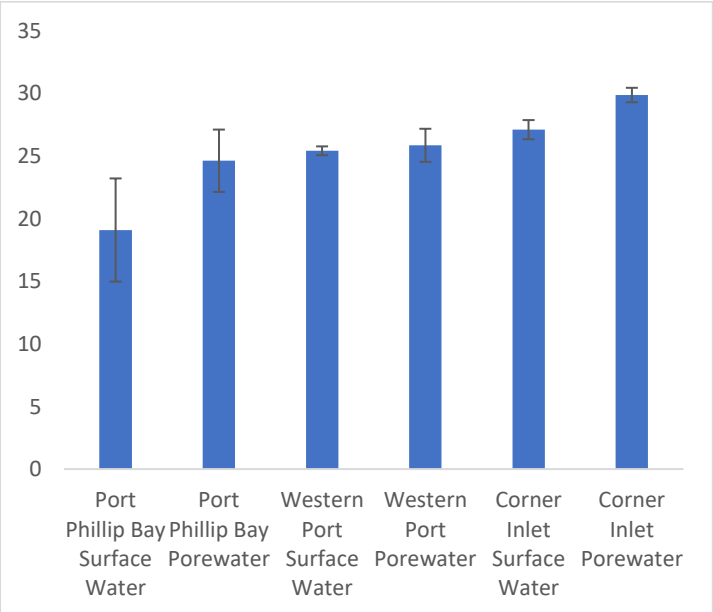
Appendix 180 - 2019 Mean Autumn Zn concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



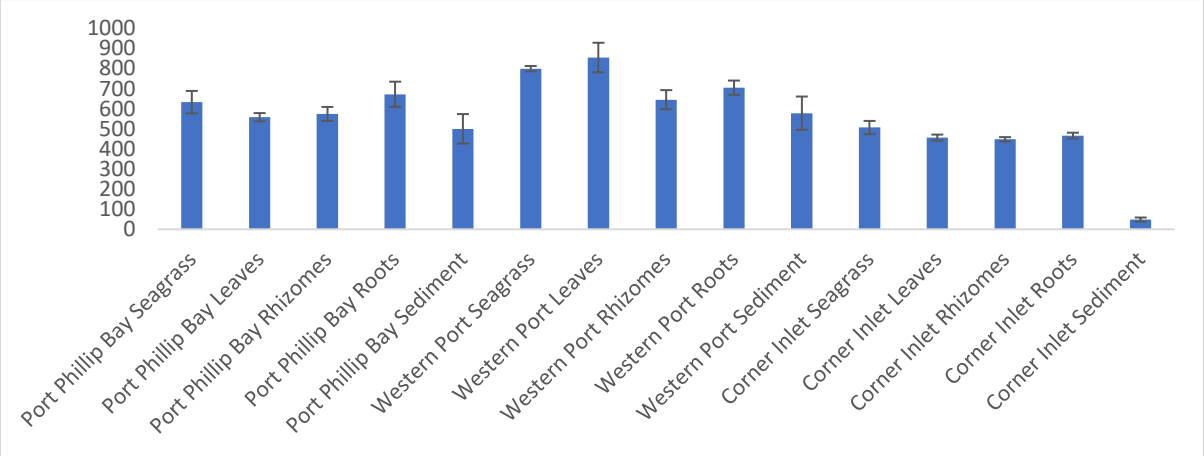
Appendix 181 – Mean Winter Al concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



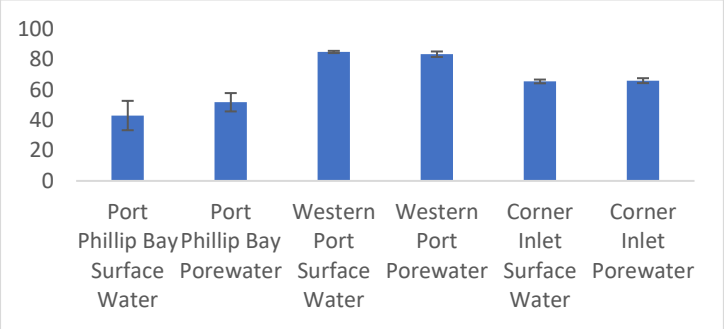
Appendix 182 – 2019 Mean Winter Al concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



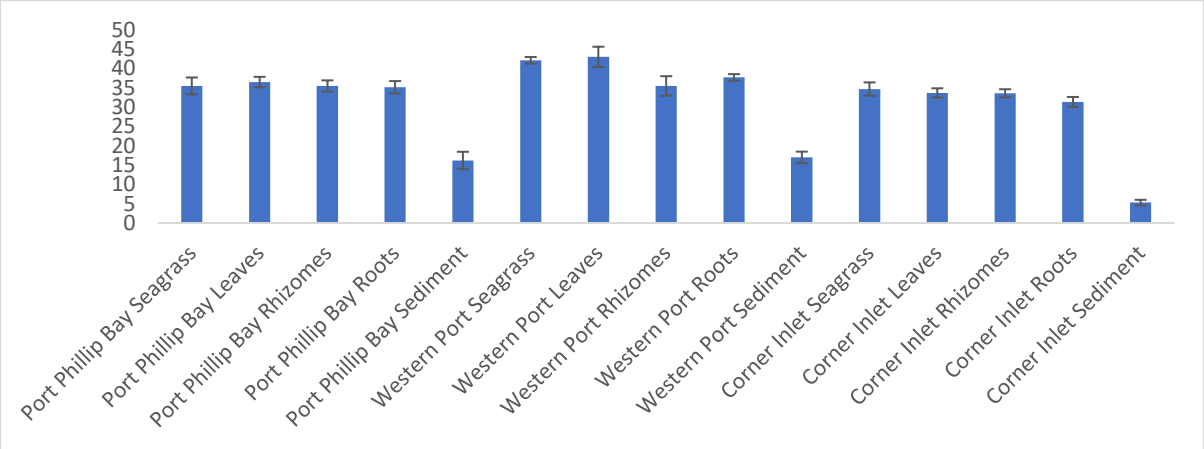
Appendix 183 – 2019 Mean Winter As concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



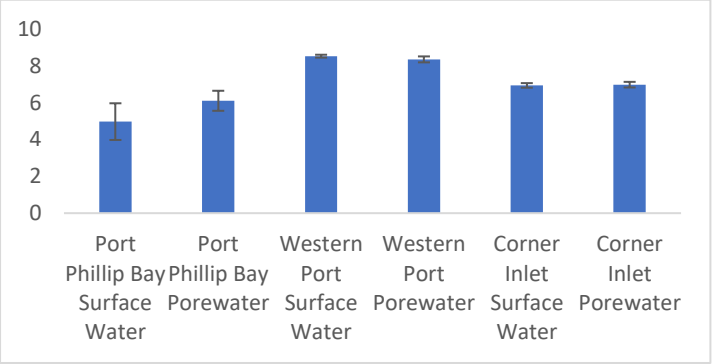
Appendix 184 – 2019 Mean Winter As concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



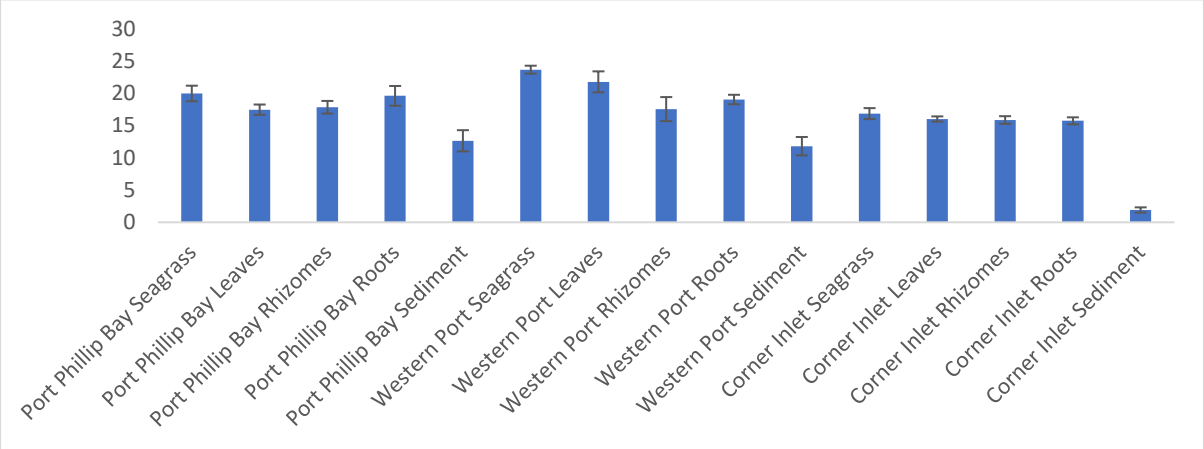
Appendix 185 – 2019 Mean Winter Cd concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



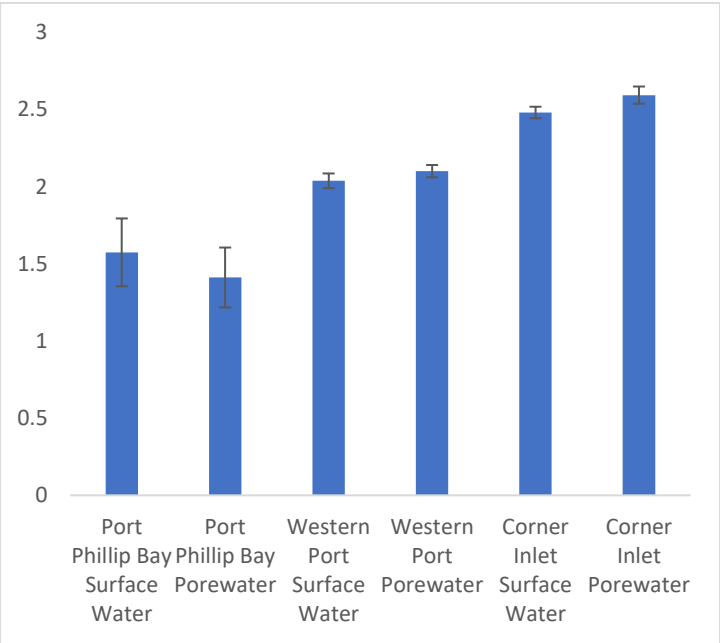
Appendix 186 – 2019 Mean Winter Cd concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



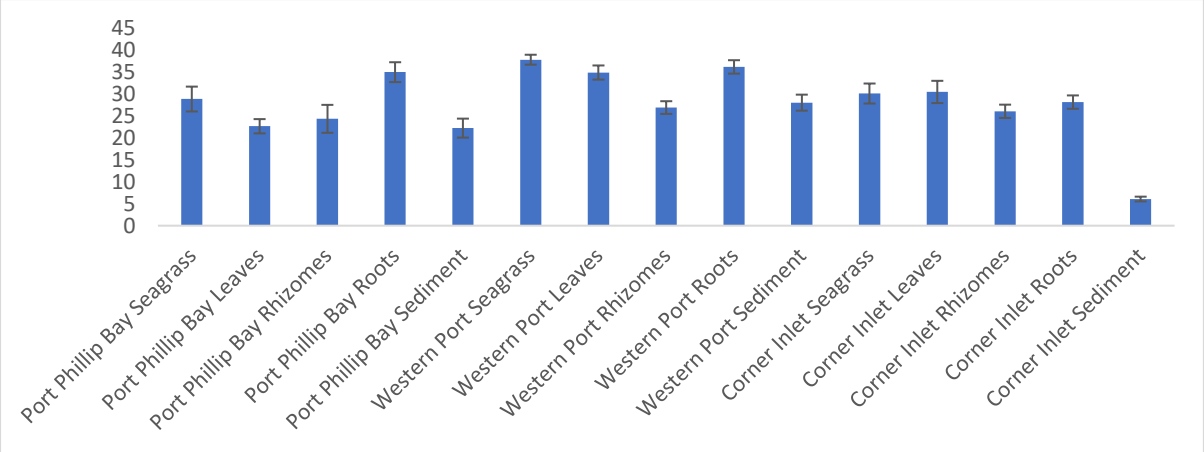
Appendix 187 – 2019 Mean Winter Co concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



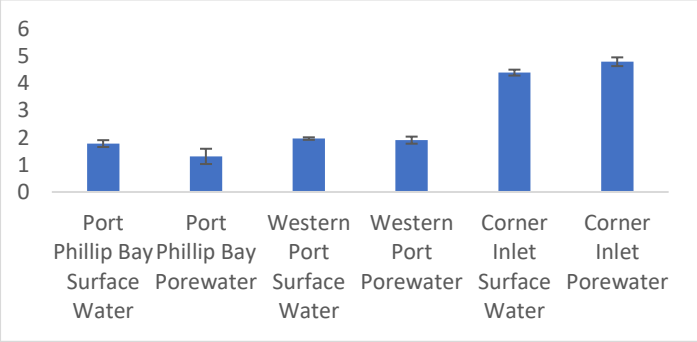
Appendix 188 – 2019 Mean Winter Co concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



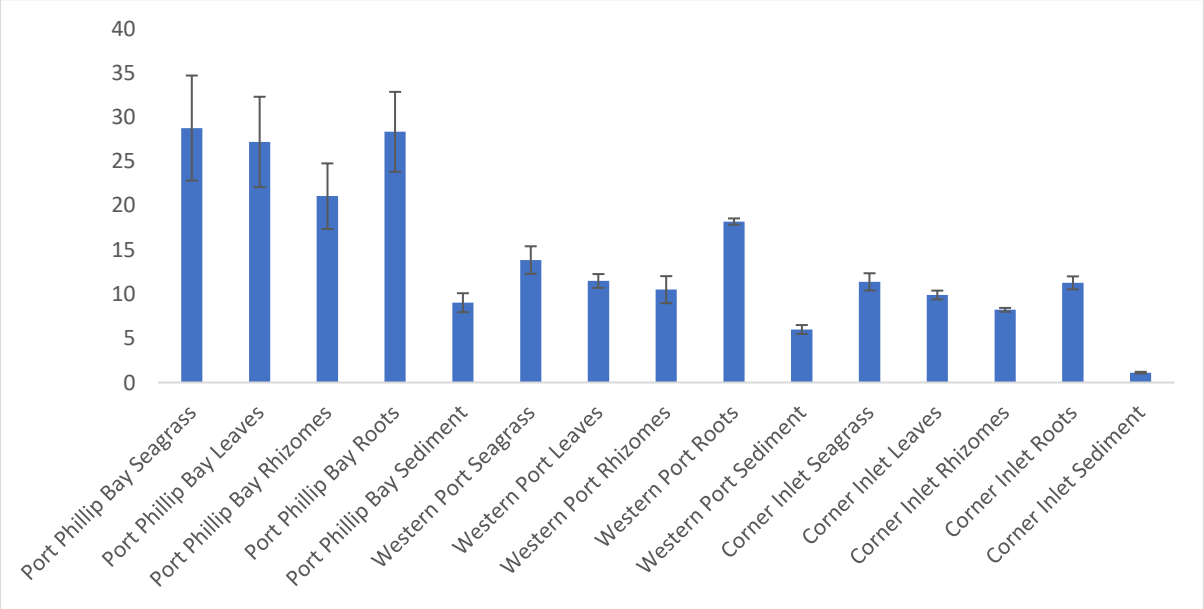
Appendix 189 – 2019 Mean Winter Cr concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



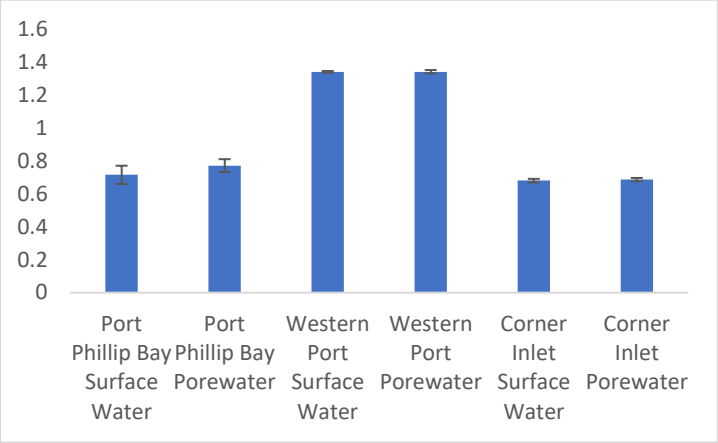
Appendix 190 – 2019 Mean Winter Cr concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



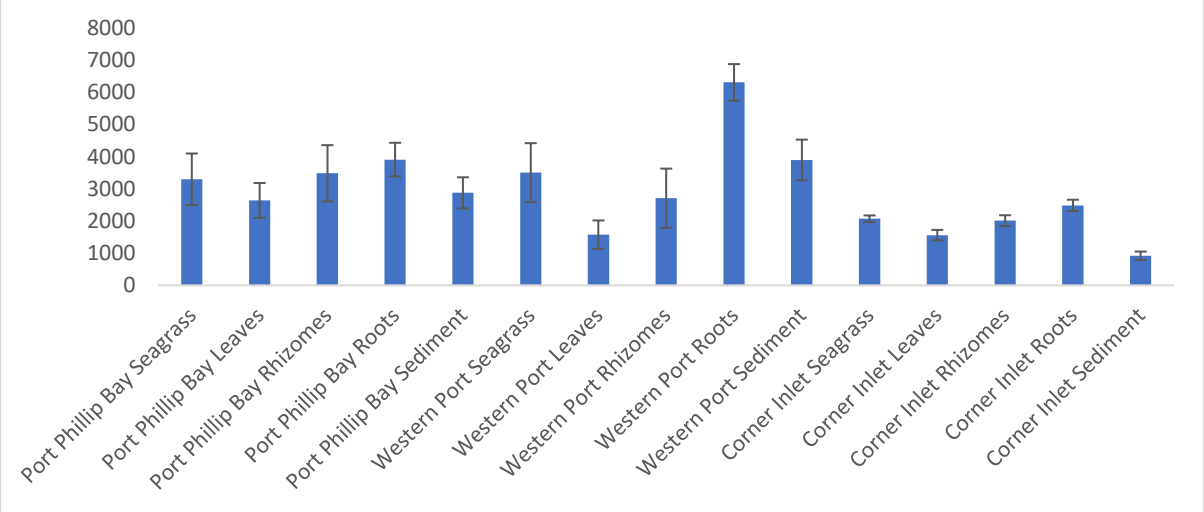
Appendix 191 – 2019 Mean Winter Cu concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



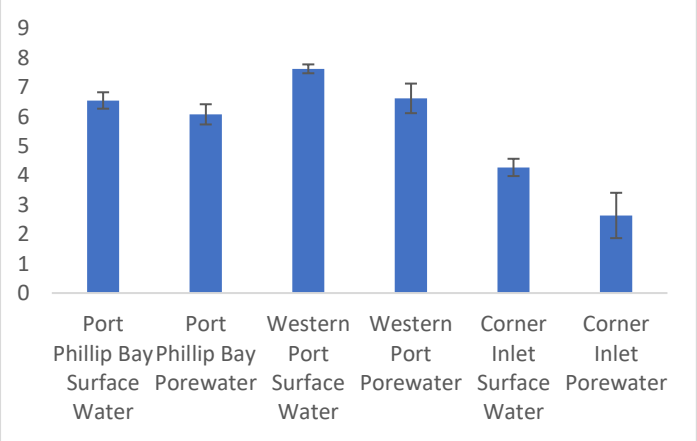
Appendix 192 – 2019 Mean Winter Cu concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



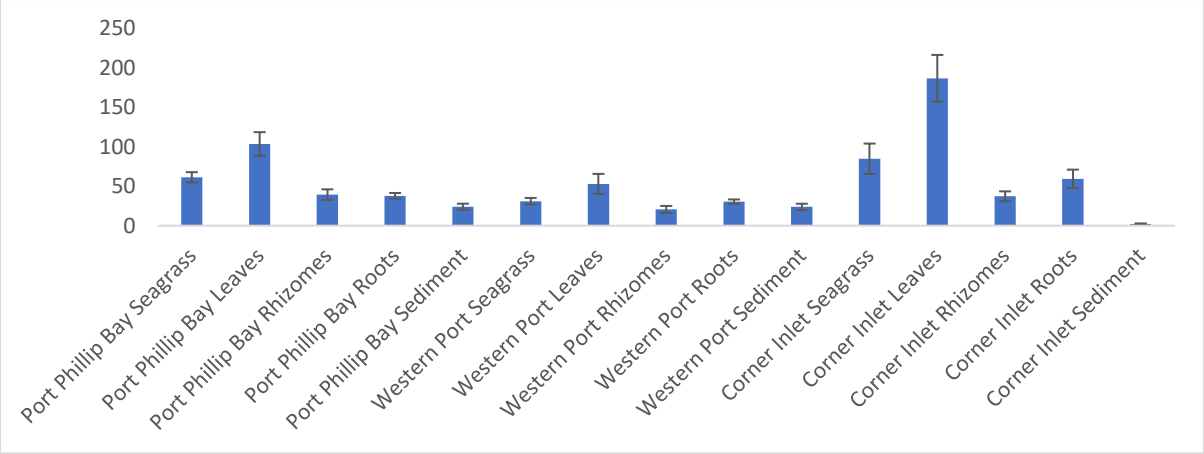
Appendix 193 – 2019 Mean Winter Fe concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



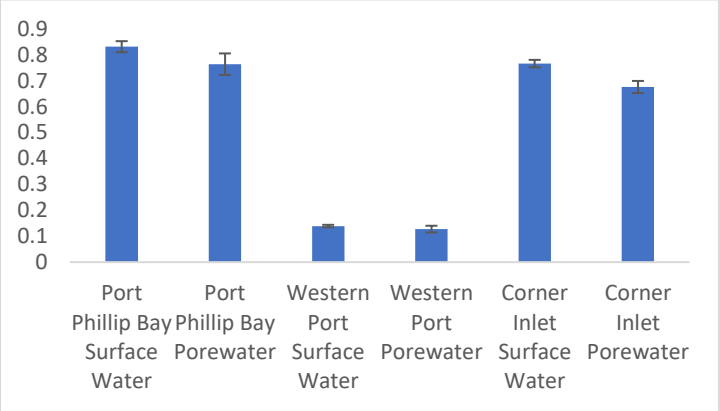
Appendix 194 – 2019 Mean Winter Fe concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



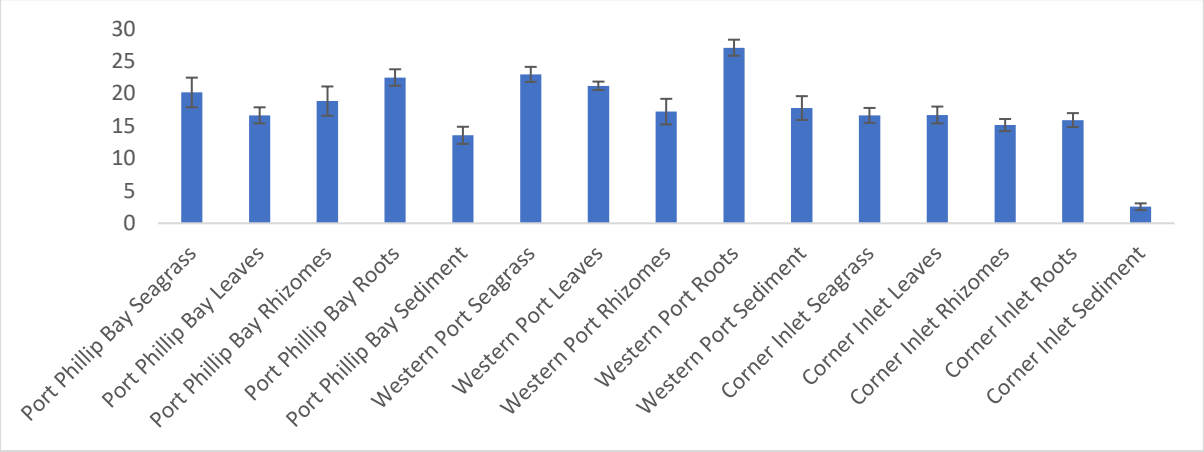
Appendix 195 – 2019 Mean Winter Mn concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



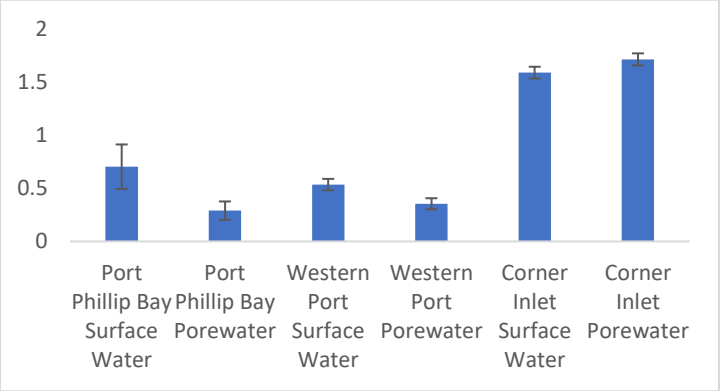
Appendix 196 – 2019 Mean Winter Mn concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



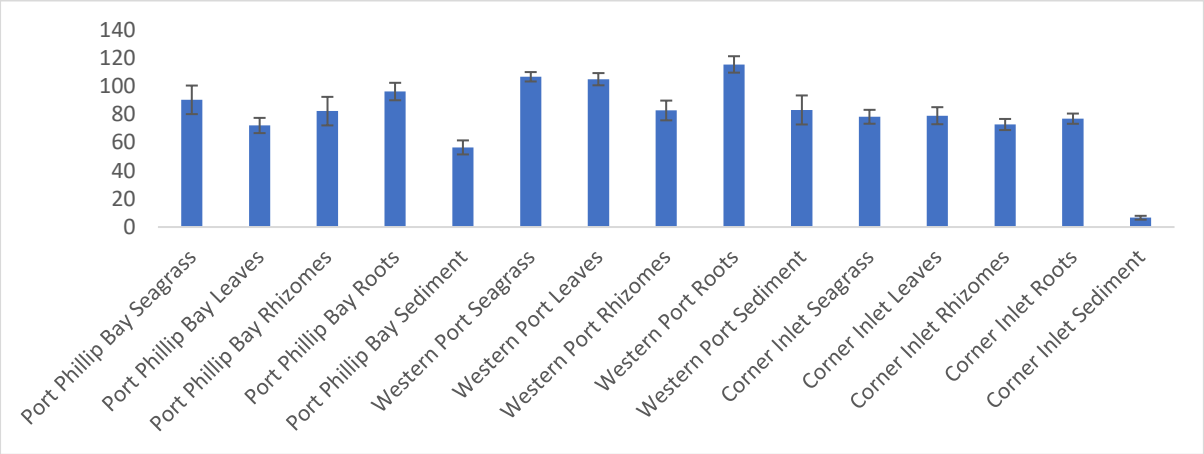
Appendix 197 – 2019 Mean Winter Ni concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



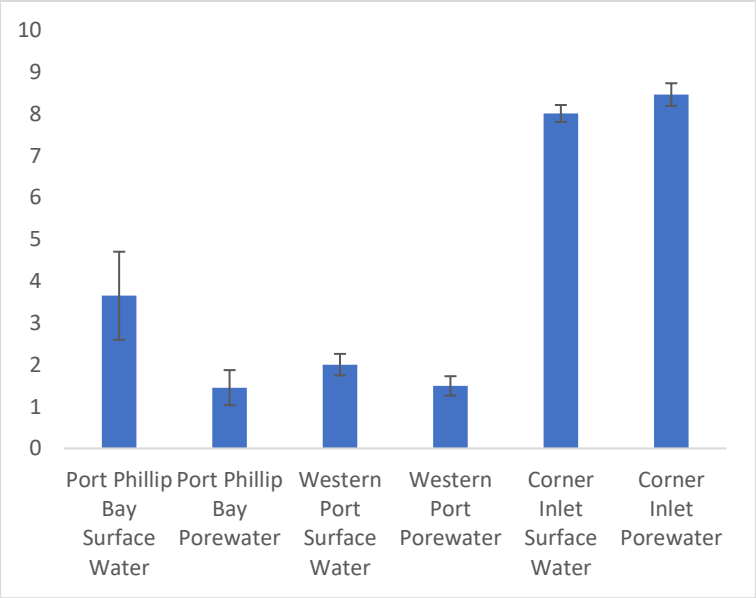
Appendix 198 – 2019 Mean Winter Ni concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



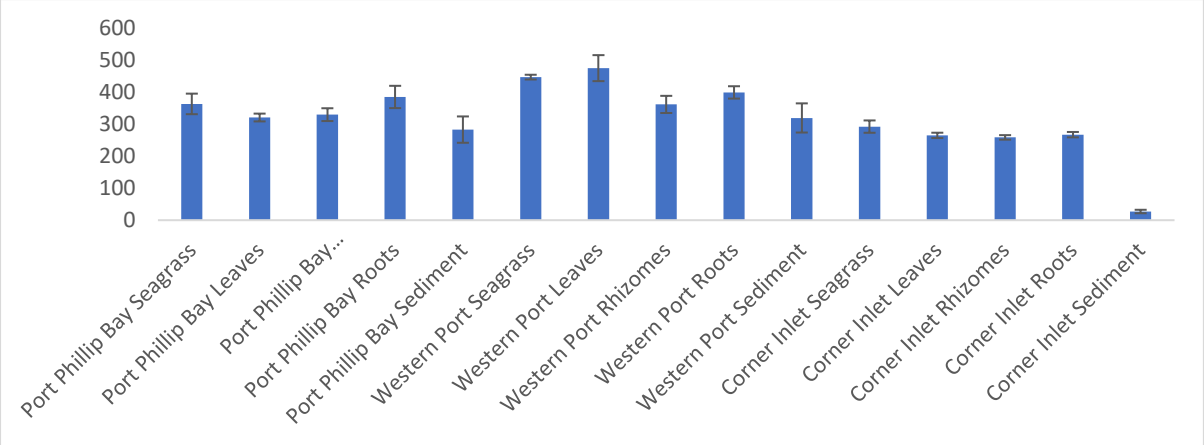
Appendix 199 – 2019 Mean Winter Pb concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



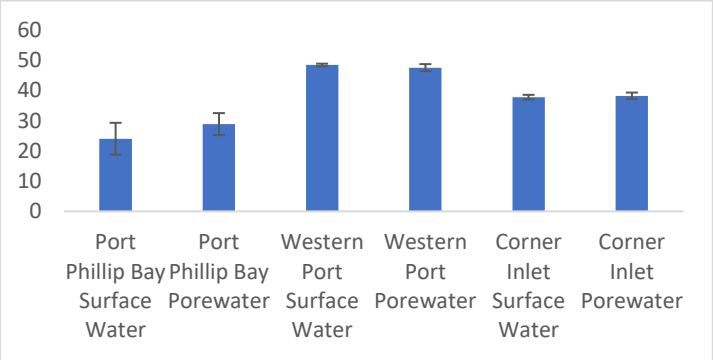
Appendix 200 – 2019 Mean Winter Pb concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



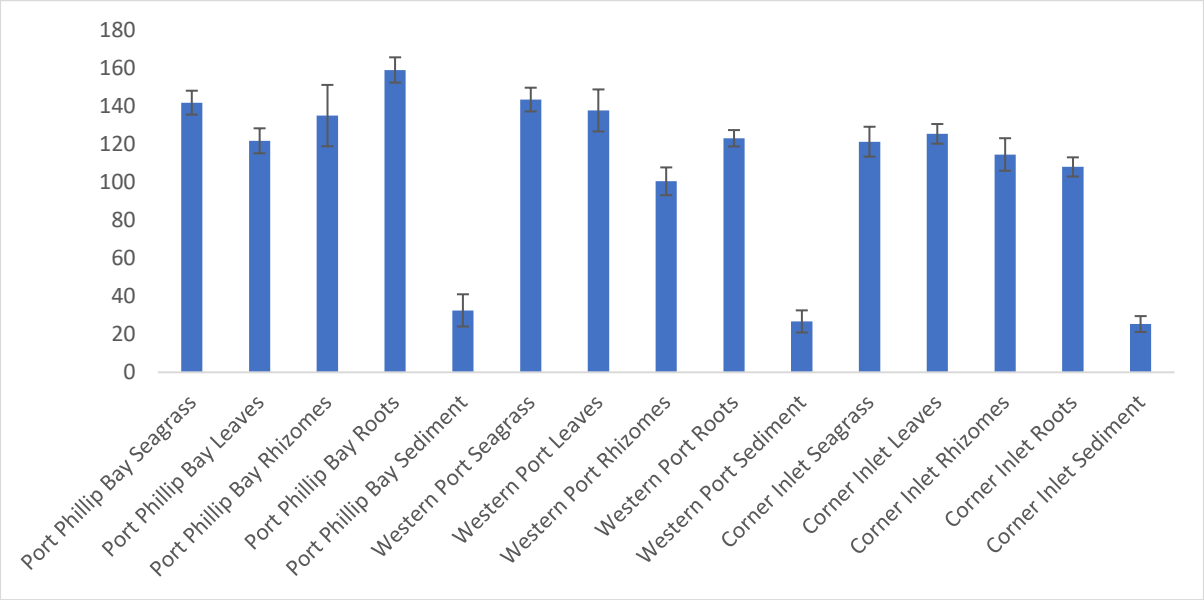
Appendix 201 – 2019 Mean Winter Se concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



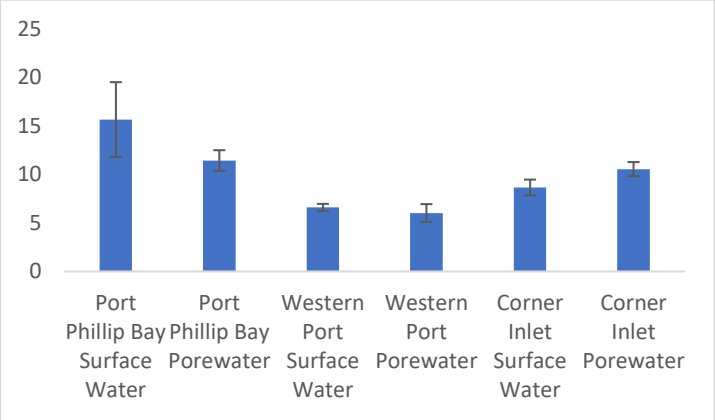
Appendix 202 – 2019 Mean Winter Se concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



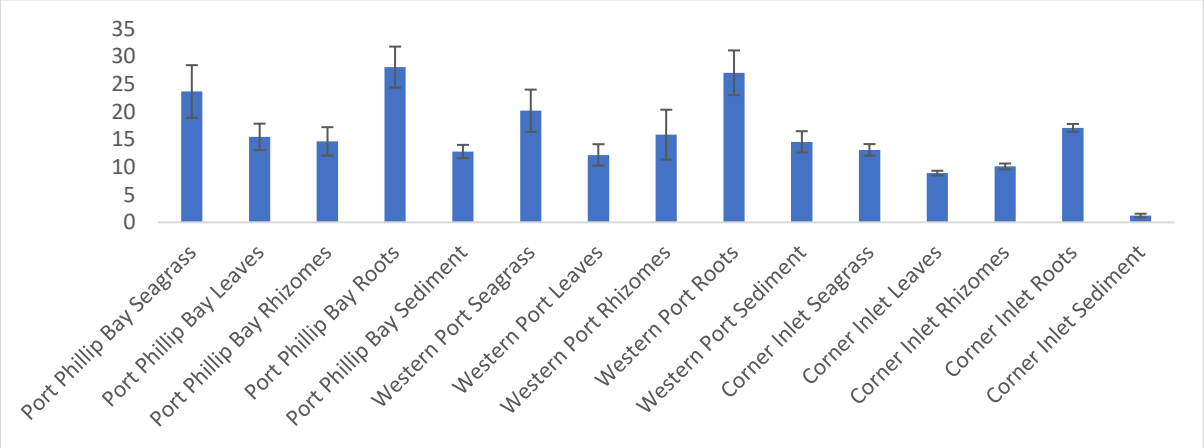
Appendix 203 – 2019 Mean Winter Sn concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



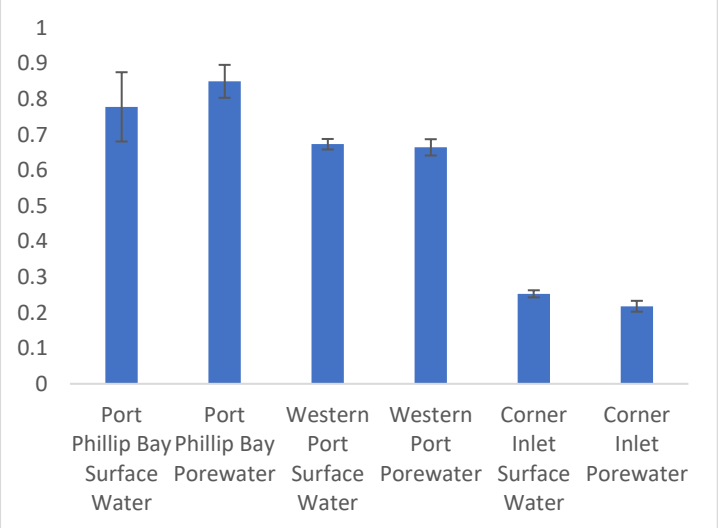
Appendix 204 – 2019 Mean Winter Sn concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



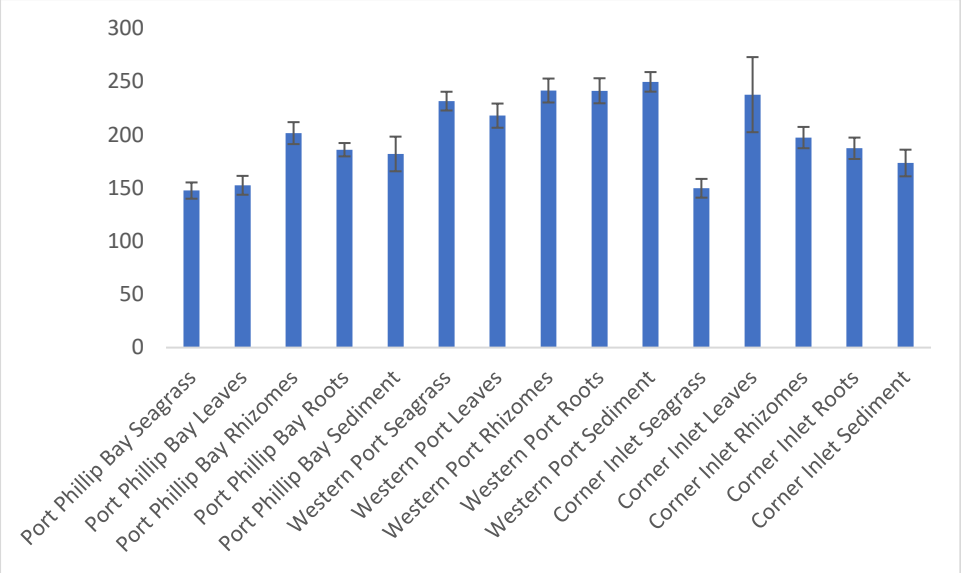
Appendix 205 – 2019 Mean Winter V concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



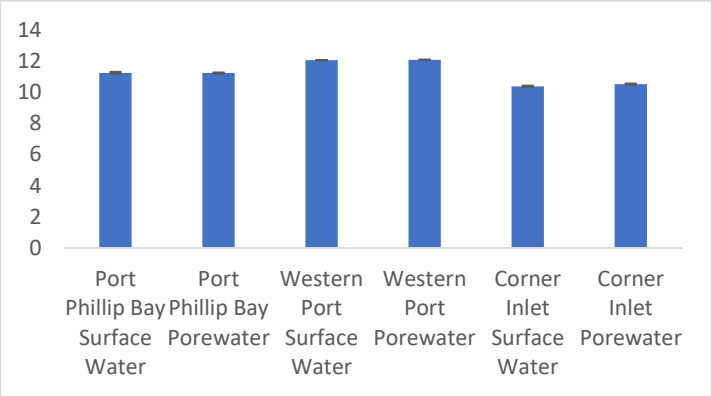
Appendix 206 – 2019 Mean Winter V concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



Appendix 207 – 2019 Mean Winter Zn concentrations with standard errors in Seagrass whole and plants and associated organs and Sediment units in mg/kg (PPM)



Appendix 208 – 2019 Mean Winter Zn concentrations with standard errors in Surface and Porewater units in mg/kg (PPM)



Appendix 209 – PCA analysis of Seagrass, Sediment and Water

Please see attach Excel spreadsheet labelled PCA.