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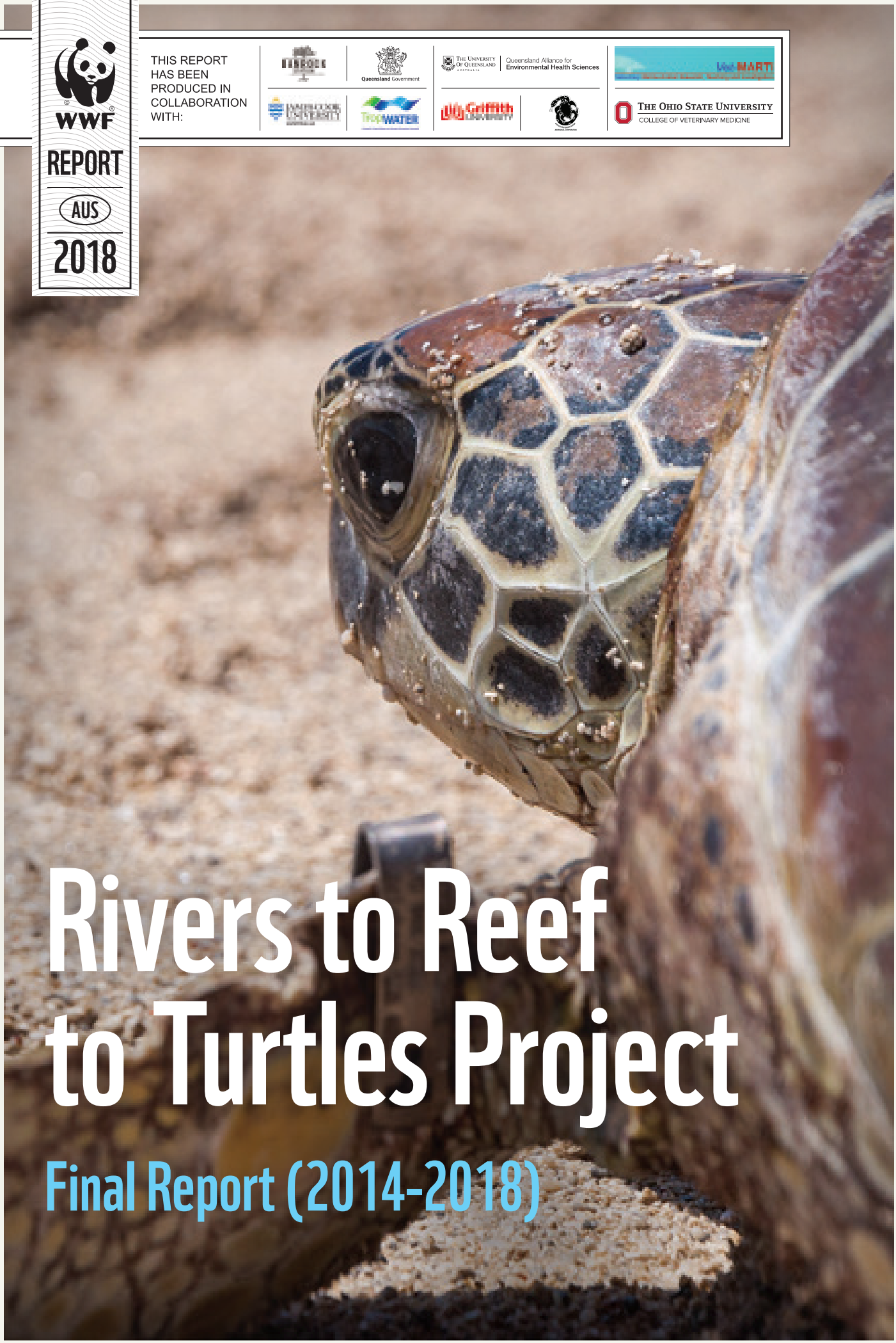

Rivers to Reef to Turtles Project

Final Report (2014-2018)

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Our mission is to create a world where people and wildlife can thrive together. To achieve this we work in partnership with organisations, communities and individuals to protect threatened species most in need.

This project would not have been possible without the passion and generous support of the Banrock Station Environment Trust. Since 2014, Banrock Station Environment Trust and WWF-Australia have been working together to bring the pleasure of fine Australian wine and the message of conservation to the world.

Central to Banrock Station's mission is its genuine commitment to the environment. Through re-investing profit to fund environmental projects, Banrock Station have contributed approximately $750,000 to the Rivers Reef Turtles project and helped raise awareness of the beauty and importance of the Great Barrier Reef and our Australian marine turtles.

Thank you, Banrock Station Environment Trust.

Editor
Christine Hof (WWF-Australia)

Independent Reviewer
Dr Fred Leusch (Griffith University)

Writers and Contributors
All Project Partners and Glen Holmes (contractor to WWF-Australia)

Design
Fresco Creative | www.frescocreative.com.au

Communication and Engagement team
Julie Chaise, Monique Isenheim, Mark Symons, Ron Newton, Josi Heyerdahl, Abigail Sexton (WWF-Australia)

Executive Summary

Background

Green turtles are among the many iconic and vulnerable species found on the Great Barrier Reef (GBR). They forage in coastal areas where they are exposed to complex mixtures of coastal pollutants. We currently know very little about the effects of these contaminants on the long-term health of turtles, and more generally on marine wildlife of the GBR. Understanding the impact that chemical contaminants have on turtles cannot only inform turtle conservation but can also, as they are sensitive and long-lived bioindicators of environmental health, guide efforts to protect, conserve and restore marine ecosystems such as the GBR.

The Rivers to Reef to Turtles (RRT) project has spent the past four years establishing a framework for studying and determining the chemical profile and health impacts of contaminants found in coastal waters, sediment, food (forage) and green turtles to understand how coastal pollutants affect marine wildlife on the GBR. Instigated by an unusual mortality event (UME) (or as publicised 'mass stranding') of green turtles in 2012 in the Upstart Bay area, the project was established to address knowledge gaps on the impacts of chemicals to the Reef outside a focussed program around sediment, nutrients and pesticides, and add to the existing body of knowledge about catchment pollution. The project developed tools to screen for a wide range of contaminants and detect chemical-induced changes and provides evidence that mixtures of coastal pollutants have led to negative health impacts for exposed green turtles. Many of the chemicals found are not included in routine monitoring programs, suggesting that despite routine monitoring we may be underestimating risks chemicals have on Reef ecosystem health.

The World Heritage-listed GBR marine park covers an area of 344,400 km^2 along the coast of northeast Australia. It receives freshwater run-off from 35 river catchments from 424,000 km^2 of land, largely modified from its natural state. These catchments transport sediments, nutrients, natural and anthropogenic chemical contaminants from agricultural, industrial, mining and urban activities, resulting in elevated contaminant loads and subsequent decreased water quality in the GBR lagoon. Degraded water quality from catchment run-off has long been known to be an issue for the GBR with both state and federal governments committing hundreds of millions of dollars to address it over the last 15 years with projects to date largely focussing on sediments, nutrients, herbicides and some other agricultural chemicals.

There are currently more than 40,000 chemicals registered for commercial use in Australia, including more than 3,000 pharmaceuticals. Thousands of new chemicals are registered for use worldwide each year, often with limited toxicological and environmental impact assessment. In addition, environmental processes such as photo- and bio-degradation can produce numerous transformation products for each one of those chemicals, exponentially multiplying the number of potential pollutants. This makes maintaining up-to-date environmental contaminant databases incredibly challenging but highlights the need to reassess existing monitoring programs and the methods used to detect and assess chemical pollution. The latest UNESCO report[1] recognises the role of water pollution as a key threat to the Reef ecosystem, but while the northeast Australian waterways are monitored using traditional water quality, coral and seagrass cover assessments, there is currently no routine monitoring of the impacts of new or emerging chemical contaminants on the environment, nor on any wildlife in the GBR. In 2012, 93 green turtles washed ashore dead or dying in the Upstart Bay area over a relatively short time period from June to July. Live turtles presented signs of neurological symptoms, appeared to have been well nourished and were mostly adult females. One theory proposed at the time was that the turtles had been exposed to a toxic compound resulting from catchment run-off. To test the theory of potential risks coastal contaminants pose to one of the iconic species of the GBR, the RRT project was initiated.

1 UNESCO (2017) State of Conservation Report 2017, available at https://whc.unesco.org/en/soc/3658

Green turtles are among the many threatened species found on the GBR exposed to complex mixtures of land-based contaminants. Although green turtles are a useful bioindicator of environmental health for their resident habitats in the GBR, correlating environmental monitoring and biological samples from turtles is a major challenge. The relationships between external contaminant doses (e.g., water/sediment/seagrass), internal exposure (e.g., blood concentrations) and subsequent toxicological and health effects in green turtles are poorly understood, yet establishing these links is critical to effectively inform future GBR monitoring and management. The RRT project was a four-year collaboration among several university and research partners, led by WWF-Australia, with in-kind support from various government agencies and philanthropic support from Banrock Station Environmental Trust.

The Rivers to Reef to Turtles (RRT) study

Three sites were selected as the focus for the investigation (Figure 1): Upstart Bay (UPB), which was the site of UME in between 2012 and 2014 and is influenced by the mouth of the Burdekin River, a large river in north Queensland contaminated by agricultural and legacy mining activities; Cleveland Bay (CLV), a site roughly 100 km north of UPB exposed to urban and industrial activity from the city of Townsville; and the Howick Group of Islands (HWK), a comparatively pristine site free from known anthropogenic pollution sources, roughly 500 km north of Townsville.

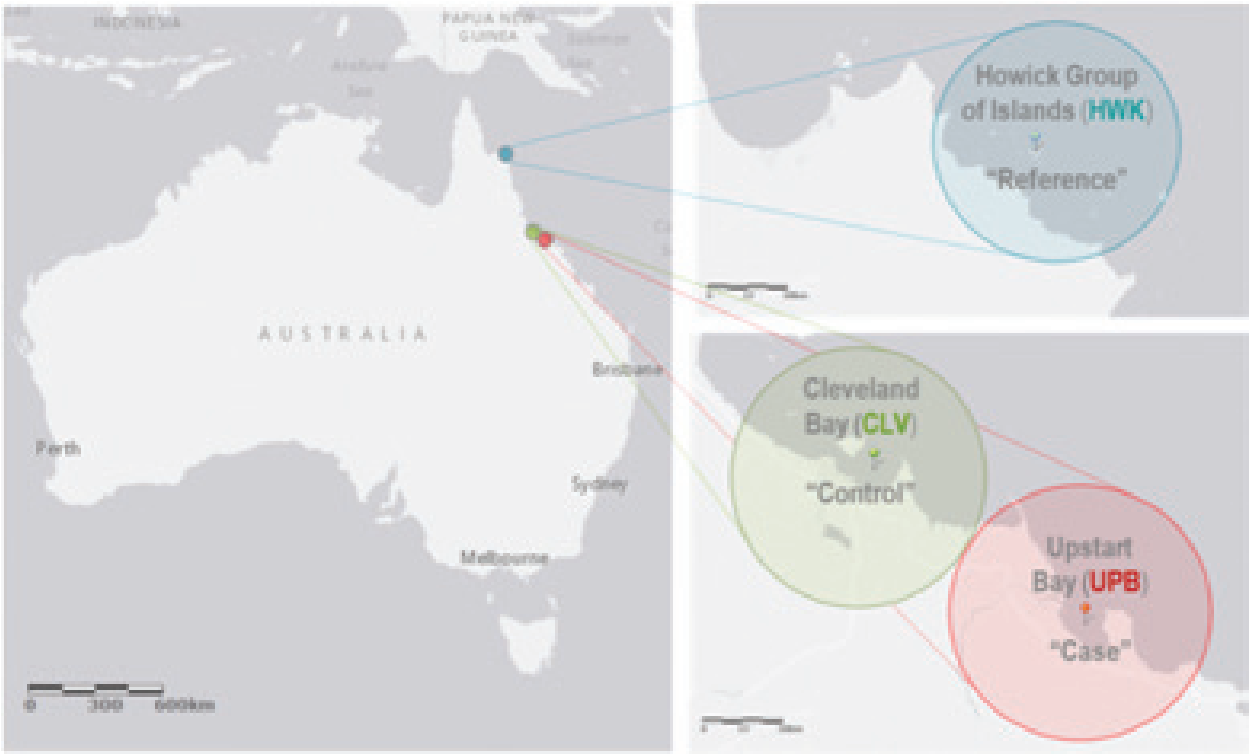


Figure 1: Location of the three study sites: Upstart Bay (UPB), Cleveland Bay (CLV) and the Howick Group of Islands (HWK).

The RRT study set out to develop non-target screening approaches combining environmental monitoring, turtle health and toxicology to understand the types of chemical contaminants present and their effects on green sea turtles (Figure 2). Turtles living in pristine offshore reefs served as a baseline for optimal turtle health.

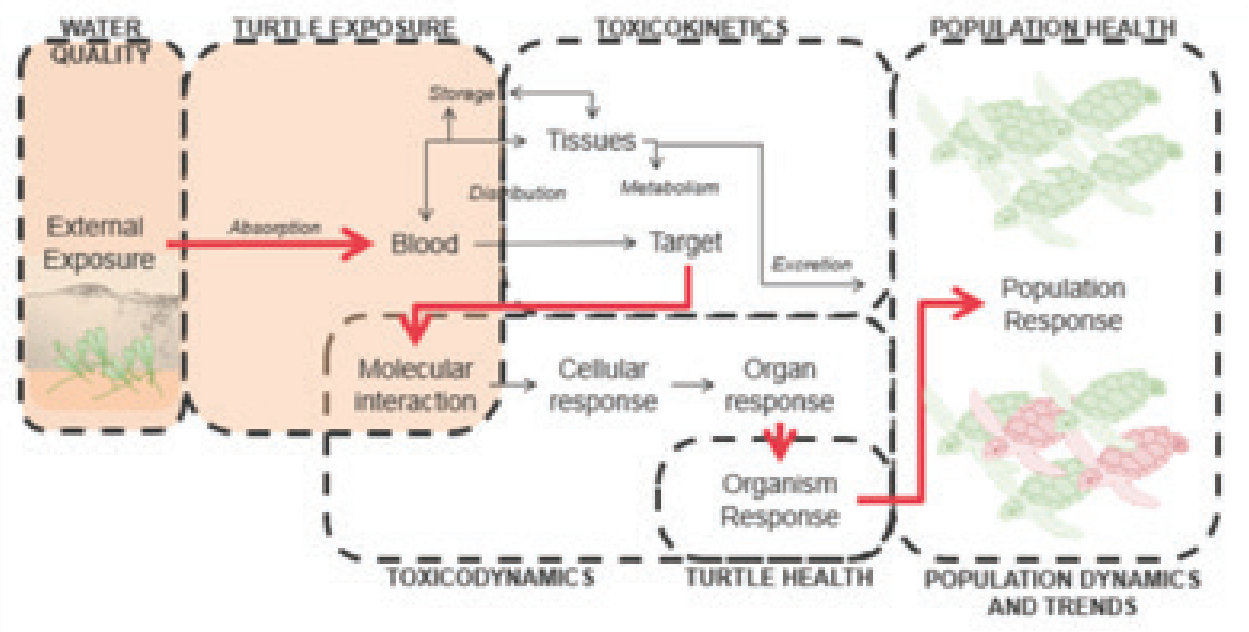


Figure 2: Conceptualised framework of the RRT program water quality and turtle exposure (pink highlight) and its links (red arrows) with other RRT program components. ***Note, the program components toxicokinetics and toxicodynamics are part of a future Phase II of the RRT.***

A study (Bell et al 2018, Chapter 1 p. 16) was initiated to better understand the population structure, dynamics, response to environmental disturbances, and recovery from major losses or environmental events (*e.g.*, mass stranding, coral bleaching). In addition, the population study aimed to provide more information on diet, turtle condition and genetic composition and diversity to better understand the origin and relationship of the turtle population to known genetic stocks and investigate morphometrics and biometrics as an indicator of health or environmental change. The health of individual turtles (Flint et al 2018, Chapter 2 p. 38) was also monitored during the three-year study through physical examinations, blood biochemistry and haematology.

Novel and exhaustive target and non-target chemical and bioassay methods were developed and applied to quantify inorganic elements (specifically metals and metalloids) and organic compounds in the turtle's environment (water, sediment and forage (food)) (Thomas et al 2018 and Gallen et al 2018, Chapters 3 p. 59 and 4 p. 131) and within the turtles (blood and scute) (Gaus et al 2018 Chapter 5 p. 176; Heffernan et al 2017 Chapter 6 p. 223; Dogruer et al 2017 Chapter 7 p. 224; Vijayasarathy et al 2018 Chapter 8 p. 225).

Reference intervals were established for trace elements in turtle blood and used to determine the extent of contamination at the coastal sites (Villa et al 2017 Chapter 9 p. 240). In addition, blood/scute ratio was shown to be useful in identifying turtles that have had elevated exposure to some contaminants. Recaptured turtles provided initial validation steps for this novel tool to examine temporal trends of contaminant concentration in green turtles (Villa et al 2018 Chapter 10 p. 241).

Finally, inform our understanding of toxicity of some of the elements commonly detected in this study in water, sediment and turtles, the toxicity of six frequently detected inorganic elements were tested in a newly developed turtle cell bioassay for acute toxicity (van de Merwe and Finlayson 2018, Chapter 11 p. 286).

Findings

Population and turtle health:

The population and health studies (Bell et al 2018 and Flint et al 2018, Chapters 1 p. 16 and 2 p. 38) indicated signs of poorer health in turtles from the two coastal areas compared to HWK. Signs include elevated total white cell counts and elevated creatinine kinase suggestive of inflammation and liver dysfunction, a higher prevalence of fibropapilloma (33-58× higher at UPB and CLV, respectively,

compared to HWK), and a significantly higher barnacle count (38-53× higher at UPB and CLV, respectively, compared to HWK). This was mainly apparent in the first two years of the study, most likely indicative of a chronic stressor of anthropogenic origin. Interestingly the effect was not evident in the third year of the study, possibly indicating that the stressor was no longer present at concentrations that negatively impact green turtle health. A previously unrecorded disease syndrome, including eye lesions, was identified in 10-23% of turtles from the coastal sites, persisting into the last year of the study. Despite extensive analyses, the cause of these lesions is currently unknown.

Chemicals in the environment (water, sediment and forage):

Comprehensive analysis of inorganic elements in water, sediment and forage (Thomas et al 2018, Chapter 3 p. 59) using both grab and passive samples indicated clearly elevated concentrations of several inorganic compounds at the coastal sites relative to offshore, sometimes exceeding available environmental protection guideline values. In marine water samples, the average cobalt (Co) concentration was above marine water quality guidelines (ANZECC, 99%ile) at all sites (3.6-16× higher than guideline value), and highest at the two coastal sites; copper (Cu) exceeded the marine water quality guidelines (ANZECC, 99%ile) at the two coastal sites only (1.6× higher than the guideline value at both sites); and while there is no ANZECC guideline for aluminium (Al), the concentration at all sites was 7.4-58× higher than an interim Al guideline.

Analysis of river water samples indicated high concentrations of aluminium (Al), copper (Cu) and zinc (Zn) (1.1-3.0× above ANZECC 95%ile freshwater guideline value), as well as iron (Fe) and silver (Ag), occasionally above guideline value.

Sediment metal concentrations were all below the relevant sediment quality guidelines; however, it was noted that cobalt (Co) in particular (65-88× higher) but also iron (Fe), copper (Cu), aluminium (Al), lead (Pb), nickel (Ni), selenium (Se), manganese (Mn) and vanadium (V) were present at higher (2.0-14.3× higher) concentrations in UPB and CLV sediment compared to sediment from the HWK site.

Likewise, the concentration of several metals was significantly elevated in above ground forage at the coastal sites compared to the HWK site, with many of the same metals as reported for sediment: manganese (Mn, 18-19× higher) and cobalt (Co, 8.7-9.4× higher) in particular, but also iron (Fe), silver (Ag), lead (Pb), zinc (Zn), molybdenum (Mo) and aluminium (Al) (2.5-4.6× higher). Inorganic elements occur both from natural and anthropogenic sources, and it is thus unclear whether the elevated concentrations of metals in water, sediment and above ground forage from the coastal sites are due to naturally high concentrations or to human activity in the catchment.

Organic chemical pollutants were also analysed in water and sediments (Gallen et al 2018, Chapter 4 p. 131). Targeted analysis of the water samples identified low concentrations of commonly detected anthropogenic pollutants at the coastal sites, including herbicides, active pharmaceutical ingredients and polycyclic aromatic hydrocarbons (PAHs). The herbicides atrazine, diuron and hexazinone are indicative of both agricultural use and domestic wastewater; the active pharmaceutical ingredients are indicative of wastewater inputs; while the PAHs indicative of both natural fires and human industrial activity. Non-target analysis for unknown organic chemicals found that the urban/industrial coastal site (CLV) differed significantly from the other two. A limited number of organic compounds were detectable in sediment samples, including PAHs, some polychlorinated biphenyls (PCBs) and two pesticides at the coastal sites, but not at the HWK site. Testing of sediment samples using bioanalytical tools, which include unknown pollutants and incorporate some measure of mixture toxicity, reported higher oxidative stress (generally associated with metals), aryl hydrocarbon (generally associated with dioxins and PAHs), estrogenic (generally associated with endocrine disrupting compounds) and inflammatory activity at one or both coastal sites higher than at the HWK reference site.

Although no organic chemicals were detected at concentrations dramatically above detection limits, these results demonstrate exposure to anthropogenic organic chemicals. Little is known about the sensitivity of green turtles to many of these compounds, and even low concentrations may produce adverse effects in exposed animals. In addition, it is well known that mixtures of chemicals can produce adverse effects

in exposed wildlife even when individual chemicals pose no hazard due to toxic mixture interactions. This mixture effect can be seen in the *in vitro* bioassay results. At the very least, the metal and organic chemical results indicate that turtles at the coastal sites are exposed to higher concentrations of complex mixtures of chemical contaminants compared to those at the relatively pristine HKW site.

Chemicals within green turtles (blood and scute):

Turtle blood was first screened for a wide range of organic and inorganic compounds using a variety of novel non-target or multi-chemical and bioanalytical methods. Screening for polar (Heffernan et al 2017, Chapter 6 p. 223) and nonpolar compounds (Vijayasarathy et al 2018, Chapter 8 p. 225) was mostly unable to identify high concentrations of anthropogenic pollutant (although some metabolites of industrial chemicals were detected in blood from turtles from the urban/industrial site CLV and low concentrations of PAHs were detected in all turtle blood samples). However, various endogenous biomarkers indicative of neuroinflammation and oxidative stress were identified in blood from turtles from both coastal sites (Heffernan et al 2017). The bioanalytical screening of organic extracts of blood samples indicated the presence of baseline toxicants (compounds that affect biological organisms via non-specific modes of action), compounds that induce the oxidative stress pathway, and compounds that bind to the aryl hydrocarbon receptor (such as dioxins, PAHs and PCBs). All were present at higher concentrations in blood from turtles from the coastal sites (especially UPB) compared to HWK (Dogruer et al 2017, Chapter 7 p. 224).

In addition, several inorganic elements were detected at elevated concentrations in blood and scutes from turtles from the coastal sites (especially UPB), particularly cobalt (Co), but also molybdenum (Mo), manganese (Mn), magnesium (Mg), sodium (Na), arsenic (As) and antimony (Sb) (Gaus et al 2018, Chapter 5 p. 176). These were elevated when compared to reference intervals established based on data from HWK turtles used as a reference population (Villa et al 2017; Chapter 9 p. 240). Many of these metals are potent inducers of oxidative stress, and the blood metal and metalloid results (particularly Co) were well correlated with oxidative stress biomarkers and individual turtle health observations (reported in Flint et al 2018, Chapter 2 p. 38).

While hampered by the limited number of recaptures and thus to be taken as still preliminary, a comparison of inorganic blood concentrations (indicative of recent exposure) to scute concentrations (indicative of historical exposure) (Gaus et al 2018 and Villa et al 2018, Chapters 5 p. 176 and 10 p. 241) suggests that some of the metals frequently detected both within turtles and in their environment had decreased in concentration from 2012 to 2016, particularly cobalt (Co) and cadmium (Cd). In other words, green turtles at UPB were exposed to higher Co and Cd concentrations in 2012, with concentrations decreasing by 2016. It is worth noting that this observation fits both the timeline of the UME and the observations of gradual improvement in haematological and clinical indicators of systemic stress reported in the individual turtle health study.

The toxicity of some of the frequently detected elements was tested in a newly developed turtle cell line. Copper (Cu), cobalt (Co), arsenic (As) and molybdenum (Mo) were toxic to green turtle cells at micromolar (µM) to millimolar (mM) concentrations (van de Merwe and Finlayson 2018, Chapter 11 p. 286). While this confirms that these metals and metalloids pose a potential hazard to green turtles, none of the tested metals was cytotoxic at concentrations detected in (healthy) UPB or CLV turtles. Future research focussing on the development of *in vitro* bioassays that measure additional endpoints (such as neurotoxicity, endocrine disruption, genotoxicity), the inclusion of toxicokinetic modelling, and assessments of contaminant mixtures will provide a more comprehensive set of tools for assessing the effects of these elements (and other priority contaminants) in sea turtles.

Major conclusions and lessons learned

Water quality has long been known to be an issue for the health of the Great Barrier Reef. Despite efforts by various state and federal governments to improve water quality, the health effects observed in coastal

turtles have, for the first time, provided a (tentative) link between adverse water quality and the health of one of the iconic animals of the GBR ecosystem.

This study has shown that green turtle populations from the two coastal sites were in poorer health than those from the reference site. Chemical analyses show high concentrations of several inorganic elements in water, sediments, forage (food) and green turtles, in particular, cobalt (Co), manganese (Mn), molybdenum (Mo) and copper (Cu). Many of these inorganic elements were present at concentrations above environmental protection guidelines. Anthropogenic organic compounds such as pesticides, herbicides, pharmaceutical, PAHs and PCBs were also detected in the coastal environment, clearly demonstrating the impact of human activities on coastal environment and ecosystem quality. *In vitro* bioassay testing detected biological activity commonly associated with these types of contaminants, raising concerns about potential mixture toxicity.

The concentrations of cobalt (Co) in particular, which was detected in turtles and the environment above guideline levels, were well correlated with several of the adverse effects reported in this study and likely even more elevated in 2012. Together, these findings are suggestive of an influence of cobalt on the UME of 2012-2014. While ascertaining the role of contaminants in the 2012 UME is not possible due to the lack of adequate baseline data, the results highlight the importance that metals and metalloids play as pollutants in the GBR and for turtle health.

The following findings and specific recommendations for action emerge from the RRT study:

Finding 1:

Turtles in coastal areas were in poorer health than those in offshore islands of the GBR. While there is a wide range of differences between coastal and offshore turtle populations, turtles live and forage in coastal areas where they are exposed to complex mixtures of land-based contaminants. Their sensitivity to contaminants and long life spans means **that turtles could be used as an effective indicator of broader ecosystem health** and environmental degradation in the GBR, a sort of "canary of the Reef". The current GBR quality monitoring program does not routinely include monitoring of contaminants in biota. The current reluctance to monitor biota may have been due to a lack of baseline or reference values to interpret the results, particularly in the case of naturally occurring elements. The RRT project has addressed this barrier head-on by developing and validating reference values (*e.g.,* reference indices established for blood and scute inorganic element concentrations, blood biochemistry and haematology measures, barnacle count and BMI) to facilitate interpretation of the data, which can be a powerful tool to understand potential drivers of toxicity in marine wildlife such as green turtles.

Recommendation 1:

Expand monitoring of turtle population health on the GBR as an indicator of the health of the Reef itself. The monitoring should include health parameters (such as blood biochemistry and haematology measures, barnacle count, BMI, blood and scute elemental analysis) for comparison with the reference intervals (some of which were developed in this project) to benchmark the health of specific turtle populations within the GBR healthy reference range. At a minimum, barnacle count and BMI should be recorded. Importantly, blood and scute samples should be collected for inorganic and organic contaminant analyses (even if analysed at a later date).

Finding 2:

This study has shown that **there is a wide range of organic and inorganic pollutants in coastal areas of the GBR and in the turtles.** New techniques such as non-target chemical analyses and effect-based monitoring provide a more representative measure of organic chemical pollution, including mixture effects and emerging pollutants, and revealed the presence of thousands of chemicals within the GBR environment. **The current marine environmental monitoring of the GBR, focussed on a**

targeted and limited suite of chemicals[2,3], provides limited insight into the actual range of pollutants present in the environment; a wider range of techniques should be included in future monitoring to produce a more relevant assessment and management response. Inorganic elements and metals, in particular, appear to pose a risk to coastal marine life, including green turtles. Many metals are not routinely monitored, and there is very limited historical data to fully appreciate this risk. The findings of this study strongly support the inclusion of a wider range of inorganic elements (especially metals and metalloids) in routine monitoring programs of water, sediment and biota.

Recommendation 2:

A wider range of contaminants should be included in an updated routine monitoring of environmental quality of the GBR, including any water, sediment and seagrass monitoring programs. As a minimum, this should include a wide range of trace elements, prioritised for each location through initial screening tests. Preferably, the analysis should also include target and non-target organic chemical analysis. Ideally, it would also include effect-based monitoring (e.g., using in vitro bioassays) to measure overall toxicity and integrate possible mixture effects.

Finding 3:

Overall, the data provide a strong argument for the notion that trace element exposure may be impacting the health of coastal sea turtle populations. Understanding the impact of chemical contaminants on marine turtles is paramount to effective species conservation, reef catchment restoration, and the continued health of the GBR. This is especially relevant now as coastal development including urban and industrial land use, ports, and expansion of agricultural practices are expected to increase the sources and diversity of contaminants released into the sea.

Recommendation 3:

Studies should be carried out to better understand the hazard, exposure and ultimately the risk posed by trace elements (particularly cobalt, cadmium, manganese, molybdenum and copper) on turtle health, both individually and as complex mixtures with other inorganic and organic contaminants. This will require the development of alternative and ethical toxicity testing techniques (such as turtle cell bioassays), environmental fate and toxicokinetic models.

Finding 4:

Following on from the above findings, there are gaps in our current understanding of sources of pollution to coastal environments. Bays and estuaries are focal points of land-based pollution, but also feeding habitats for many turtle populations. A campaign to measure organic and inorganic chemical contaminants in water, sediment and turtles in some of the major bays and estuaries of the GBR would provide some much-needed data to prioritise investment. Solutions include restoration of critical catchment services in the GBR, such as remediation of gullies to mitigate agricultural impacts and wetlands to increase retention and removal of pollutants and sediments.

Recommendation 4a:

Chemical contaminants in water, sediment and turtles should be assessed in all major bays and estuaries of the GBR, using a comprehensive suite of analytes to assess a wide range of anthropogenic pollution sources. This should include both target and non-target organic chemical analysis, elemental analysis and in vitro effect-based assessment.

2 Huggins et al. 2017. Total suspended solids, nutrients and pesticide loads (2015-2016) for rivers that discharge to the Great Barrier Reef – Great Barrier Reef Catchment Loads Monitoring Program. Department of Environment and Science, Brisbane.
3 Waterhouse et al. 2017. Marine Monitoring Program: Annual Report for inshore water quality monitoring 2015-2016. Report for the Great Barrier Reef Marine Park Authority. Great Barrier Reef Marine Park Authority, Townsville, 227pp.

Recommendation 4b:

The data collected above would improve our understanding of pollution sources and help identify new sources of pollution so that effective strategies can be developed to lessen human impacts to this iconic and productive ecosystem, either through source control or implementation of additional treatment. In addition, a better understanding of pollution sources and impacted areas would inform investment in restoration work in bays and estuaries identified as high priority.

Finding 5:

The lack of water, sediment and/or biota archived samples prior to, during, and immediately after the UME have made the undertaking of the current investigation very difficult to conclusively identify a causative agent for earlier mass strandings. One of the important deliverables of this project is the development of a sample archive, which will be available to future researchers. In the event of a future UME, this will be invaluable to establish historical baselines and thus guide future rehabilitation and restoration efforts. But the future of the sample archive is in doubt without ongoing support to house it and make samples available.

Recommendation 5:

Maintain an archive of water, sediment and biota samples for future analysis, and to make those samples available to future scientists attempting to establish temporal contaminant trends.

Finding 6:

The project has resulted in the **development of new procedures for sampling and responding to turtle stranding.**

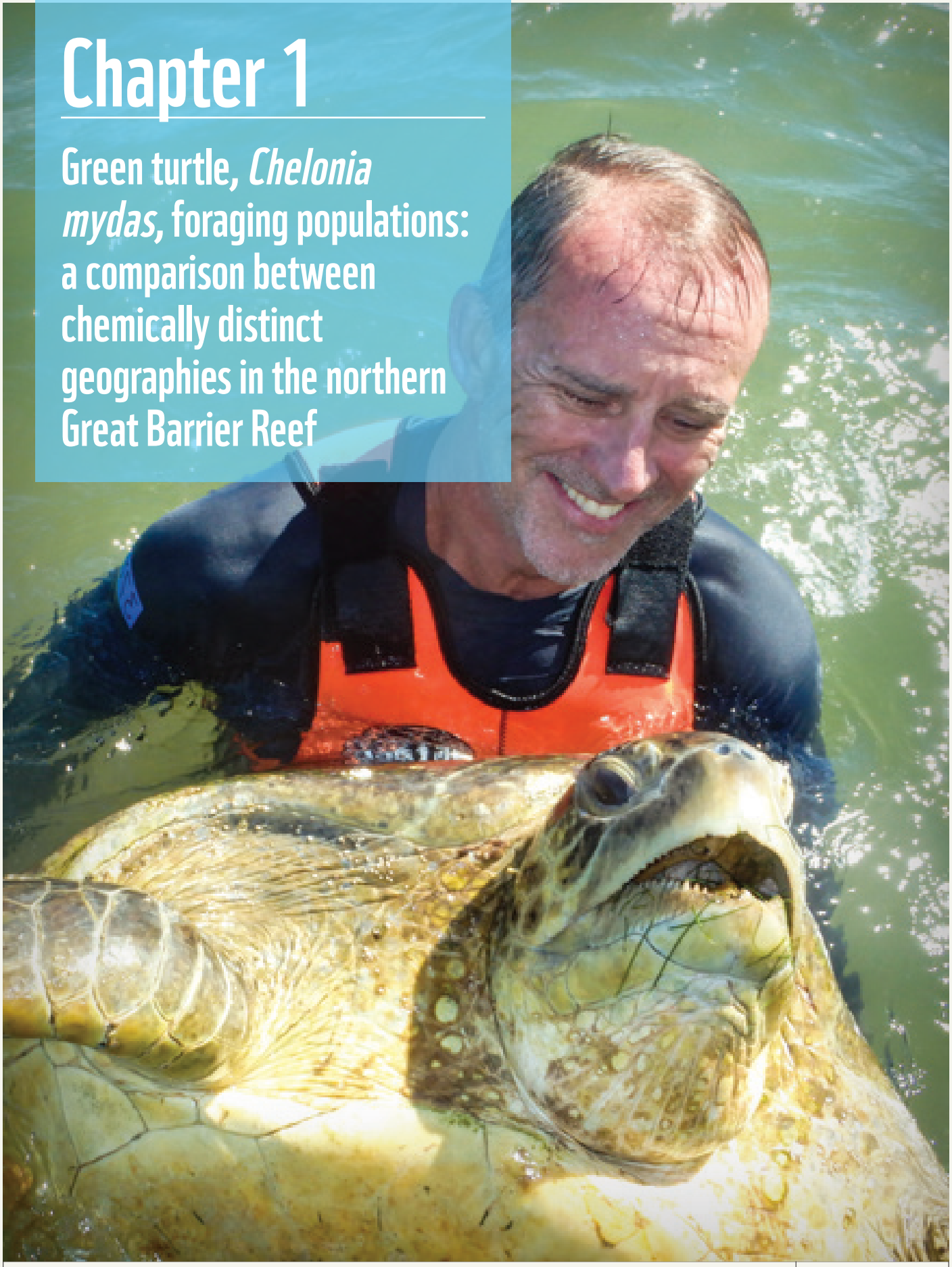
Recommendation 6:

Existing practice and standard operating procedures in response to UMEs or mass strandings should be updated. *The new procedures developed during this project need to be maintained to ensure that any future UMEs can be interpreted in context and that proper samples are taken to facilitate subsequent investigations into its causes. These include the provision of sampling kits and the development of capacity of local volunteer organisations and Queensland Government staff to respond to potential future stranding events, as well as tapping into various water quality monitoring programs in the region to ensure that environmental (water and sediment) samples are taken and preserved during key events (such as flooding) for future analysis. It would be wise to ensure that these operational measures are maintained and do not disappear over the next few years.*

Contents

Chapter 1

Green turtle, *Chelonia mydas*, foraging populations: a comparison between chemically distinct geographies in the northern Great Barrier Reef



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1. Green turtle, *Chelonia mydas*, foraging populations: a comparison between chemically distinct geographies in the northern Great Barrier Reef

Ian P. Bell[1], Justin Meager[2], Jason P. van de Merwe[3], Christine A. Madden Hof[4*]

[1] Aquatic Species Program, Queensland Department of Environment and Science, Townsville, Queensland 4810, Australia
[2] Aquatic Species Program, Queensland Department of Environment and Science, Brisbane, Queensland 4810, Australia
[3] Griffith Sciences and Australian Rivers Institute, Griffith University, Gold Coast, Queensland 4222, Australia
[4] WWF-Australia, Brisbane, Queensland 4000, Australia
[*] Corresponding author: chof@wwf.org.au

Abstract

The catchments of the Great Barrier Reef (GBR) have experienced significant modifications in recent decades, potentially leading to increases in sources of pollutants and declines in coastal water quality. As coastal waters of the GBR support the highest density green turtle (*Chelonia mydas*) foraging populations in the western Pacific Ocean, understanding the effects of contaminants on GBR green turtle populations is a priority. In 2012, elevated strandings of green turtles in the Upstart Bay region instigated the WWF's collaborative Rivers to Reef to Turtles (RRT) project to investigate if coastal pollutants are compromising green turtle health. Important to interpreting these investigations into toxicology and health is understanding the demographics of the green turtle populations being investigated. In three green turtle foraging grounds, Cleveland Bay (CLV), Upstart Bay (UPB) and the Howick Group of Reefs (HWK), this study explored population size, age class structure, sex ratio, growth rates, body condition and diet, as well as indices of turtle health, such as plastron barnacle loads and eye lesions. The three foraging populations had similar age class structure and sex ratios to other green turtle foraging populations in the GBR. Somatic growth rate was nonlinear, peaking in immature turtles, and was much slower in turtles foraging at HWK compared to the other two sites. This may have been due to differences in diet, which was supported by the mixture of seagrass and algae observed in gastric lavage samples of HWK turtles, compared to predominantly seagrass in CLV and UPB. There were also small differences in body condition between sites, as well as differences in barnacle loads, eye lesions and occurrence of fibropapilloma tumors. This study provides important context for the other chapters in this report investigating the toxicology and health of green turtles foraging in the GBR.

Keywords:

Population biology, body condition, demographics, somatic growth rate, barnacles

Introduction

Green turtle (*Chelonia mydas*) stocks are listed in 'threatened' categories at international, national and state levels (Seminoff, 2004; Environmental Protection and Biodiversity Conservation Act 1999; Nature Conservation Act 1994). Given their broad distribution in various life history phases, coupled with high site fidelity when inhabiting coastal areas, green turtle populations are exposed to many anthropogenic impacts globally, including along the Queensland coast and within the Great Barrier Reef (GBR) World Heritage Area. These impacts include bycatch in recreational and commercial fisheries, egg predation by feral (e.g. foxes and pigs) and native animals (e.g. goannas), harvest of adult female turtles and their eggs by indigenous groups, and extensive urban and industrial development encroaching on nesting and foraging habitats (Donlan et al., 2010).

The GBR catchments have experienced extensive degradation since the mid-19th century (Great Barrier Reef Marine Park Authority, 2014). Widespread native vegetation land clearing, predominantly for broad-acre farming and industrialised agriculture practices, combined with urban and industrial development

has led to substantial increases in the amount of sediment, nutrients and pollutants such as pesticides, heavy metals and other contaminants entering coastal waters (Waterhouse et al., 2017). Exacerbated by floods, cyclones and storms, there has been a dramatic decline in coastal water quality, subsequent degradation of seagrass foraging meadows and flow-on negative effects to green turtle populations in some years (Great Barrier Reef Marine Park Authority, 2014). As coastal waters of the northern GBR support some of the highest density green turtle foraging populations in the western Pacific Ocean, understanding the effects of increased pollutant or contaminant loads on GBR green turtle populations is a priority. In fact, understanding the effects of chemical pollution on sea turtles and their habitats has recently been identified as a global research priority (Hamann et al., 2010; Rees et al., 2016).

In June to July 2012, an unusual mortality event of green turtles was recorded in the Upstart Bay region, with a cluster of elevated strandings in the area from the tip of Cape Upstart to Cape Bowling Green (J. Meager, unpublished analysis of strandings data). This instigated the WWF's collaborative *Rivers to Reef to Turtles* (RRT) project to investigate if coastal pollutants are present at elevated levels, and if they are compromising green turtle health. Studies completed within this project to date have identified poor health (systemic disease, acute inflammation, oxidative stress, and liver dysfunction) and toxicological responses in green turtles in Upstart and Cleveland Bays, associated with elevated levels of trace elements and organic contaminants (Dogruer et al., 2018; Heffernan et al., 2017; Villa et al., 2017). Specifically, turtles from Upstart Bay have cobalt levels up to 25 times higher than the healthy reference population, and within the range expected to cause acute toxicity in other species (Villa et al., 2017). In addition, a wide range of exogenous compounds were detected in turtle blood from these sites (Heffernan et al., 2017), thousands of which have not yet been identified.

To better understand the effects that pollutant exposure and other threatening processes may be having on green turtles, it is important to assess the demographic structure of foraging populations (Chaloupka and Limpus, 2001; Hof et al., 2017). Capture-mark-recapture (CMR) is one of the most effective approaches to this, but requires long-term sampling of high intensity to adequately detect meaningful trends and demographic signals. Where CMR modelling is not possible, overall demographic composition (age class structure, sex ratios, genetic composition, etc.) can provide important insight into local and regional turtle population dynamics (e.g. Hamann et al., 2006; Limpus and Read, 1985; Limpus et al., 1994; Limpus et al., 2005). As these studies illustrate, the demographic composition of marine turtle foraging populations can vary significantly through time and habitat. In addition, because of a complex life history which depends on environmental conditions, diet quality and habitat availability, recruitment variability and haplotype diversity can also affect sea turtle foraging ground demographic composition and population dynamics.

As part of the RRT project, this study aimed to assess the demographics of the green turtle foraging populations at the three project locations: Upstart Bay, Cleveland Bay and the Howick Group of Reefs. The demographics of these three green turtle populations were assessed over a three year period by assessing: 1) age class structure, 2) sex ratios, 3) growth rates, 4) body condition, 5) diet, and 6) turtle health indices, such as barnacle loads, fibropapilloma tumors and eye lesions. Green turtles are the most abundant mega-herbivore found in the GBR, and are assumed to be year-round residents of foraging grounds, feeding in a range of tidal and subtidal habitats including coral and rocky reefs, seagrass meadows, algal turfs and mangrove forests (Limpus, 2009). Given their strong site fidelity, coupled with long-term residencies in these shallow coastal areas (Limpus et al., 1992; Schofield et al., 2010; Shimada et al., 2016), green turtles are considered good proxy indicators of exposure to anthropogenic impacts. Understanding foraging population demographics is therefore important to supporting investigations into the impacts of chemical pollutants on green turtles in the GBR.

Material and methods

Study sites

Green turtles were sampled from three foraging grounds in the northern GBR, categorised by their distinct anthropogenic influences of: 1) agricultural/legacy mining activities (Upstart Bay = UPB); 2)

urban/industrial (Cleveland Bay = CLV), and 3) remote from anthropogenic point sources (the Howick Group of Reefs = HWK) (Figure 1).

The Howick Group of Reefs (HWK) is a remote group of mid-shelf, uninhabited islands and reefs that lie within the northern Great Barrier Reef Marine Park. They are located approximately 130 km north of the nearest population centre (Cooktown) and 30 km offshore from the Cape York region catchment, a relatively undeveloped catchment with low pressures from nutrient, sediment, and pesticide loads, or water regime changes and habitat alterations. The green turtle foraging habitat in HWK is dominated by rock reef flats supporting coral outcrops, as well as macro-algae and seagrass meadows and some mangroves.

Upstart Bay (UPB) is an embayment, adjacent to a coastal area dominated by agricultural and legacy mining activities (Bartley et al., 2014). UPB receives waters from the Burdekin River, which runs through extensive agricultural land before discharging into the bay. It is also the main site of the green turtle mass mortality event of 2012 (Figure 2). The bay lies in a north-south orientation and is protected from the often strong prevailing south-easterly trade winds. Green turtle foraging areas of UPB are dominated by intertidal and subtidal seagrass and algae meadows occurring on a sand, rock and mud substrate that extend seaward from a predominately mangrove fringed shoreline along the Bay's southern and western side.

Cleveland Bay (CLV) is an embayment, adjacent of the city of Townsville. CLV is located 100 km north of UPB, and is geographically similar (i.e. it is influenced by similar environmental factors, and is a north-south facing bay), and influenced by urban/industrial activities adjacent to and within the city of Townsville. Like UPB, green turtle foraging areas of CLV are dominated by intertidal and subtidal seagrass and algae meadows.

Turtle capture, tagging and sampling

Green turtles were captured on algae and seagrass meadows at UPB and CLV, and on coral reef flats and among mangrove forests at HWK, between August 2014 and August 2017, using the standard turtle rodeo (boat or beach jumping) capture method (Limpus, 1978). To reduce bias in which turtles were selected for capture, when groups of turtles were encountered, the first turtle seen was pursued until it was captured or lost into deep or turbid water.

All turtles were captured between May and October each year, a period outside of the nesting season to ensure that all turtles associated with these foraging areas were available for capture (i.e. had not migrated for breeding). Following their capture, turtles were landed on a nearby beach for tagging, collection of morphormetric data and tissue sampling for parallel studies. They were typically released within 2 hours of being brought ashore. Turtles were tagged with self-locking standard titanium turtle tags (Stockbrands Company, Pty. Ltd., Western Australia). The tags were applied adjacent to the enlarged scale closest to the body on the posterior edge of the left and right front flippers (Limpus, 1992). If the animal was already carrying tags, the condition of the tag(s) was assessed and if less than two thirds of the tag was attached another tag(s) was applied. Recaptured turtles were released with a minimum of two securely attached titanium tags, one in each front flipper.

Prior to turtle release, a number of morphometric measurements, sex and health observations were taken. The curved carapace length (CCL) was measured (± 0.1 cm) along the midline from the junction of the skin and carapace above the neck to the posterior edge of the junction of the supracaudal scute (Limpus et al., 1994). Calibration of fibreglass tape was undertaken regularly against steel rules. Any large barnacles on the carapace that were likely to interfere with CCL measurement were removed. Turtle mass was measured (± 1 kg) using calibrated scales. Turtle sex was assigned to adults based on tail length past the junction of the supracaudal scutes (TLC), with males assigned if TLC >25 cm (Limpus, 2015).

A food (lavage) sample of the most recent feeding event of a subset of turtles from each site was obtained from the mouth, oesophagus and crop using the stomach flush technique described in (Forbes and Limpus, 1993). Visual observations and taxonomic identifications were made of the lavage samples at the time of sampling. A number of external indicators of health were also recorded for each turtle captured,

including barnacle (>1 cm diameter) number on the plastron, eye lesions and fibropapilloma tumors. All tagging, capture and observational data for the turtles were recorded in the Queensland Government's Turtle Research Program's database. All other data is stored on a database at WWF-Australia.

A range of other samples were also collected from sub-samples of all turtles captured, which were subsequently used in parallel studies. Skin samples (<1 cm^3) were taken from the front or rear edge of the flippers and stored in 70% ethanol for analysis of genetic diversity and composition (Jensen et al., 2018). Blood and scute (edge of carapace) samples were taken for analysis of chemical contaminants, toxicology (Dogruer et al., 2018; Heffernan et al., 2017; Villa et al., 2017), and testosterone levels for sex determination (Jensen et al., 2018). All sampling was undertaken under Queensland Government's G12/35326.1 permit and Animal Ethics permit number SA 2015/11/531.

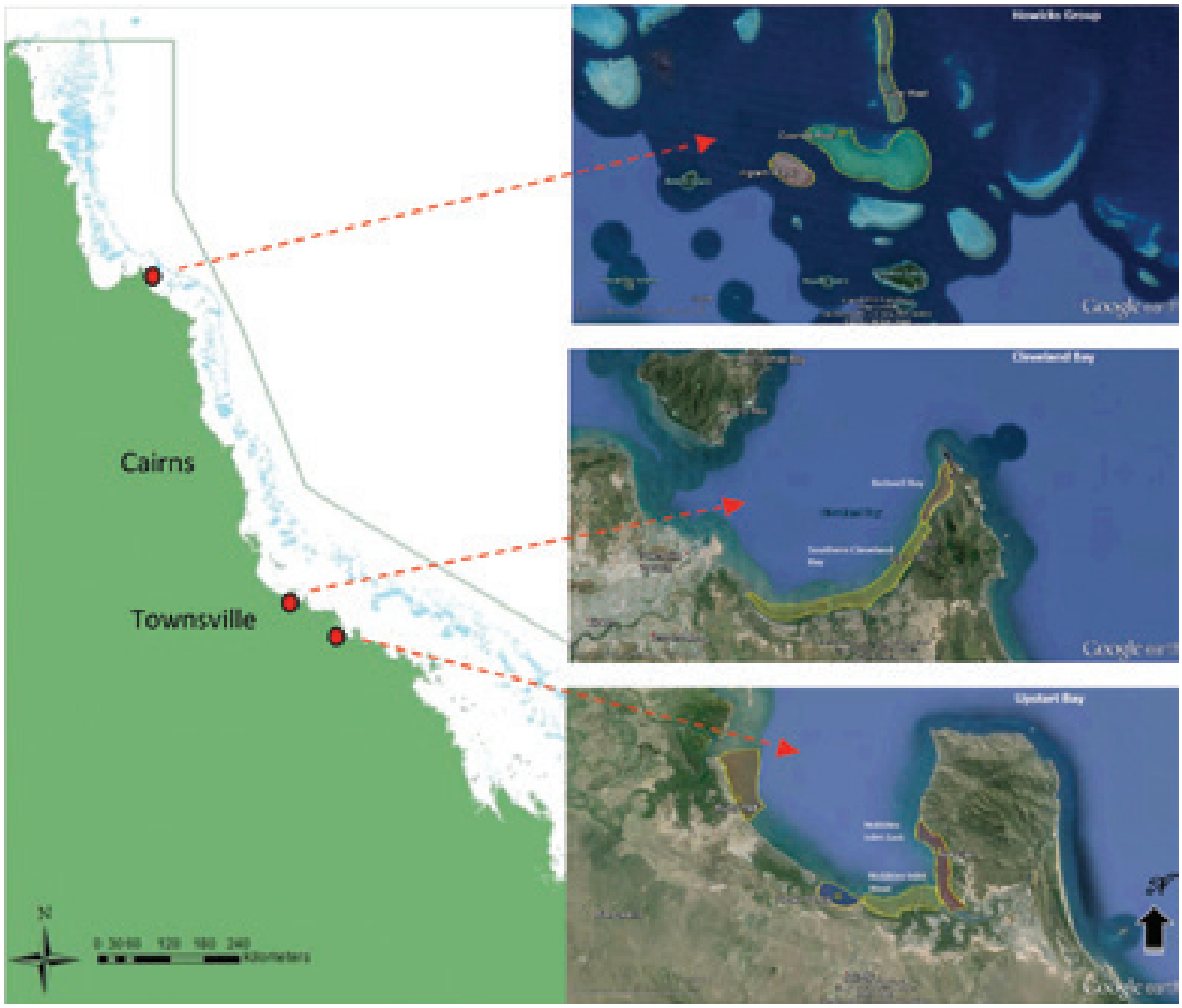

Figure 1: Location of the three study sites (Howick Group of Reefs, Cleveland and Upstart Bays), including the foraging grounds (enclosed in yellow boundaries) in which green turtles were captured.

Data analysis

Population structure

The age structure for each population was described as the percentage of juveniles, sub-adults and adults. The sex ratio at each site was only assessed for adults, as the sex of juveniles and sub-adults cannot generally be assessed without internal examination of the gonads. However, in a parallel study (at the HWK site only), the sex of a 247 immature turtles was assessed using a testosterone assay (Jensen et al., 2018), with 139 of these confirmed visually using laparoscopic examination following methods described in Limpus and Read (1985).

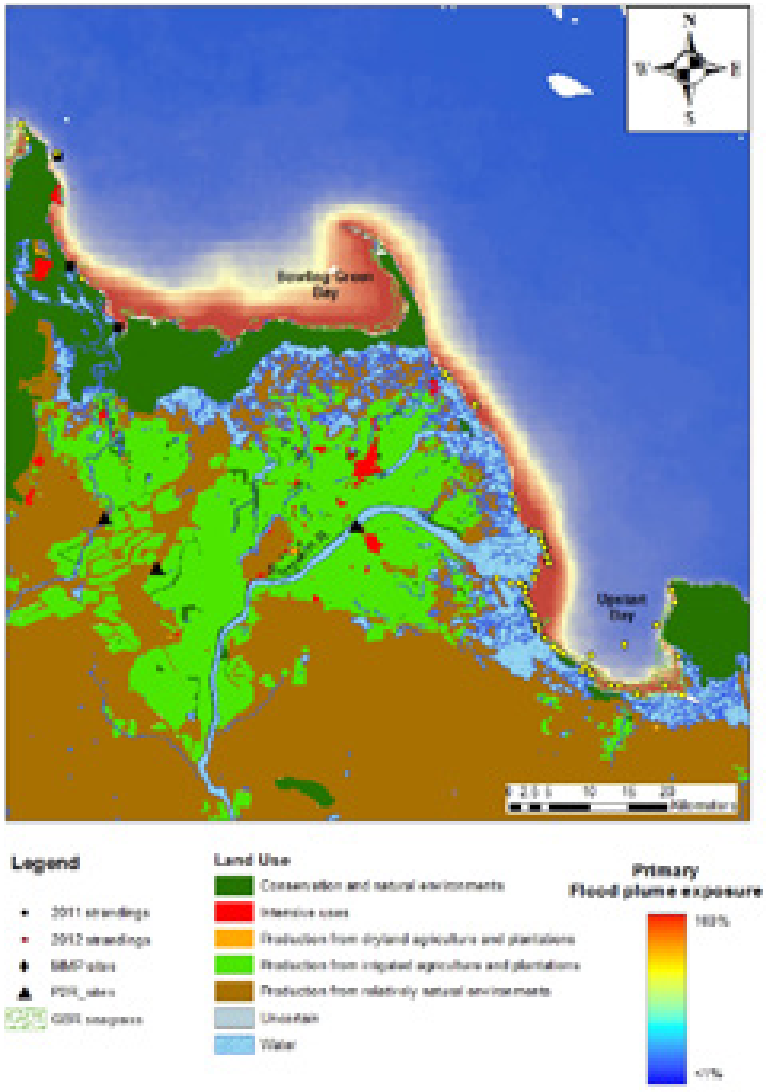

Figure 2: Land use and 2012 green turtle stranding locations in Upstart Bay.

It was assumed that the majority (>85%) of green turtles at UB and CB were from the southern GBR and Coral Sea genetic stock, as has been illustrated by previous genetic stock assessment at nearby Edgecumbe Bay (Jensen et al., 2016). In a parallel study conducted at HWK in 2014 and 2015, the genetic stock of 337 turtles captured were assessed using methods described in Jensen et al. (2018).

Population size estimates

One of the most reliable methods for estimating population parameters such as abundance, survivorship and recruitment of wildlife is the capture-mark-recapture (CMR) approach (Williams et al., 2002). However, obtaining accurate estimates depends on a sampling program that is both intensive enough to ensure high capture and recapture probabilities, and long enough to estimate annual survival (Pilcher and Chaloupka, 2013).

The low recapture rates of green turtles at UPB and CLV (see Table 1) precluded reliable CMR estimates of population abundance or survival. A more complete dataset for population modelling and survival estimates is available for HWK from historical datasets held by the Queensland Government (1997 – 2008). Within this study, only the 2014 recaptures of juveniles at HWK were analysed as a preliminary estimate of abundance. These turtles were sampled using a beach-jump or boat jump method from the reef flat and mangroves adjacent to Ingram Island. For the two-week period of sampling the population was considered closed to losses or gains (i.e., no deaths, immigration or emigration). The following four closed CMR models from Williams et al. (2002) were fitted: (1) capture probability is constant over

time (M0), (2) capture probability is a function of time (Mt), (3) consecutive captures are affected by behavioral responses of turtles (trap-happy or trap-shy; Mb), and (4) capture probabilities are affected by their response to captures and time (Mtb). The time varied (Mt) model provided the best fit to the data according to Akaike Information Criterion (AIC) (M0 = 4067.3, Mb = 3094.3, Mtb = 3076.9, Mt = 2989.5).

Growth rates

Growth rate data were collected from turtles that had been captured, tagged and recaptured during the study period and added to historical data (where available) to increase sample sizes. This resulted in a 2013-2017 dataset for UPB, 2014-2017 for CLV, and 1999-2017 for HWK. Data were divided into size-classes based on CCL: juvenile (<65cm), sub-adult (65-85cm) and presumed adult (>85cm).

Somatic growth rates were modelled using generalised additive models (GAMs), using the 'mgcv' (v 1.8-15; Wood and Wood, 2018), and 'gamm4' packages of R (Wood et al., 2017), following methods described in Bjorndal et al. (2016) and Limpus and Chaloupka (1997). The response variable was the absolute growth rate ($\Delta CCL/\Delta t$), where ΔCCL and Δt were the differences in CCL and time (t, in days) between captures and recaptures, respectively (Limpus and Chaloupka, 1997). Both negative and zero growth rates were included, but growth rates > 5 cm yr^{-1} and < -1 cm yr^{-1} were excluded because they were considered biologically implausible (and likely due to measurement and/or recording errors). Growth rates were standardised by the inclusion of smooth functions of the covariates 'mean CCL', 'year of capture' and the 'recapture interval' (in days, log transformed). 'Mean CCL' was considered to be a proxy of age, whilst the latter two variables were included to explain some of the variance in growth rates associated with cohort and environmental effects (Limpus and Chaloupka, 1997).

The first stage of the modelling process compared the residual plots of Gaussian, lognormal, scaled t and Tweedie error distributions and indicated that a Gaussian model was the most appropriate. We then explored the influence of recapture interval on the model by examining residual plots and by comparing models with different thresholds for inclusion. On this basis, recapture intervals of more than 13 years were excluded as clear outliers and recapture intervals of > 100 days were included. This resulted in recapture intervals that ranged from 114 to 6,218 days (0.3 to 13 years). As an additional step to reduce the influence of outliers on covariate smooth functions, we then set the gamma parameter to 1.4 (Wood, 2006). Next, we included a random intercept (turtle tag ID) to model longitudinal measurements using a mixed-model GAM (GAMM). This was necessary because 12% of the turtles were recaptured on more than one occasion. Finally, we tested for differences between sites and sexes using log-likelihood ratio tests (Wood, 2006). Here, both variables were included as categorical fixed effects. The final model was used to standardise growth rates to help interpreting differences between sites, and to compare growth rates with earlier studies.

Body condition

There has been much debate in the ecological literature on the appropriate method for comparing body condition between populations using length-weight data (Green, 2001; Jackson et al., 1990; Peig and Green, 2010; Schulte-Hostedde et al., 2005). However, all are based on the reasonable assumption that animals in better body condition have more fat and energy reserves, and are thus heavier for a given size than animals in poor condition. The common objective has therefore been to determine the weight of the animal relative to body size (Schulte-Hostedde et al., 2005), which for marine turtles has mostly been undertaken using ratio indices (e.g. Fulton's condition factor: Bjorndal et al., 2000; logarithmic transformation: Flint et al., 2014), or the Analysis of Covariance (ANCOVA) approach (e.g. Limpus et al., 2012). A more recent method that has been applied to amphibians, fish, birds and mammals is the scaled mass index (SMI), which standardizes body mass based on the scaling relationship between mass and length (Peig and Green, 2009; Peig and Green, 2010). Here we compared the condition of turtles between sites using the ANCOVA and SMI methods. We also calculated a ratio index [$\log_{10}$(mass, kg)/$\log_{10}$(CCL, cm)] for comparison with Flint et al. (2014).

For the ANCOVA, weight and CCL were first log transformed to normalise the response variable and make the relationship linear. The assumption of homogeneity of slopes was then tested (Neter, 1996). The ANCOVA model was then used to estimate a slope-adjusted condition index for each site and size class, by dividing the predicted log weight by the log CCL for three size classes (50, 70 and 90 cm CCL).

The scaled mass index was estimated following Peig and Green (2009), i.e.:

$$\text{Scaled mass index (SMI)} = W_i \left[\frac{L_0}{L_i}\right]^{b_{SMA}}$$

where Wi and Li are the weight (kg) and CCL (cm) for each turtle, respectively; L_0 is the CCL to which the index is standardised (here the overall mean, 61.9 cm) and the scaling exponent is b_SMA. The scaling exponent was estimated by standardised major axis regression (SMA) of log weight versus log CCL using Huber's M estimation because it is more robust to outliers (Venables and Ripley, 2002). SMI was then compared between sites using a Kruskal Wallis test.

Diet

Differences in diet preferences between sites and years were assessed qualitatively in 2014-2016 by visual examination of the gastric lavage samples as they were taken. These samples have been stored for more detailed analysis in the future.

Indicators of health

Epibiotic load has been considered an indicator of physically compromised turtles (Deem et al., 2009). In two coastal sites in Queensland, small immature green turtles with high plastron counts of the barnacle *Chelonibia testudinaria* were more likely to be unhealthy than those with no barnacles (Flint et al., 2010). We therefore compared plastron barnacle counts between sites using general linear models (glmmTMB package of R; Magnusson et al., 2017). An initial comparison of Poisson, quasipoison, negative binomial and zero-inflated Poisson models (ZIP) indicated better goodness of fit of the ZIP model because a large number of turtles had no barnacles.

The percent (%) of turtles with fibropapilloma tumors was calculated for each site, and in a parallel study, the percentage of turtles with eye lesions was also compared between sites.

Results and discussion

Population structure

There was a total of 4,124 captures of 3,643 individual green turtles within the three study locations over the three-year study (Table 1). Primary (P) turtles, those caught and tagged for the first time in this study, accounted for the majority (79%) of all turtles captured (Table 1). The ISR turtles included turtles that were either initially caught and tagged at this site prior to this study and captured again in this study (pre-2014), or captured and tagged for the first time in this study and caught again in a subsequent year of this study (this study). Of the 37 ISX NEST turtles at HWK (turtles that were initially tagged at a nesting beach), 23 (62%) were from nesting beaches in the sGBR (mainly the Capricorn Bunker) and 12 (32%) from the nGBR (mainly Raine Island). Of the remaining two, one was from an unknown nesting location, and the other from Fraser Island, south of the GBR.

Of the 3,643 green turtles captured in this study, 1,735 (48%) were identified (using secondary characteristics) as adults (1,370 females, 337 males, 28 indeterminate), 613 (17%) sub-adults, and 1,295 (35%) juveniles (Table 2). Turtles captured in this study encompassed all size classes from small immatures (CCL = 40 cm) to large adults (CCL = 122 cm). The coastal sites of CLV and UPB showed similar age class distributions, dominated by juveniles (>60%), while the offshore HWK site was

dominated by adult turtles (58%). The HWK age class distribution was similar to that observed in a 2001-2002 survey of foraging green turtles in the Gulf of Carpentaria (Hamann et al., 2006). The age class structures of the CLV and UPB populations are more similar green turtles captured in Moreton Bay in 1990-1992 (Limpus et al., 1994) and 2002-2005 (Arthur et al., 2008), and Edgecumbe Bay in 2003-2014 (Hof et al., 2017). However, there are also some dissimilarities of the age class distributions observed in this study to other previously published studies. For example, approximately equal proportions of adults, sub-adults and juveniles have been observed in green turtle populations foraging in Shoalwater Bay in 2000-2004 (Limpus et al., 2005), and the Heron/Wistari Reefs in 1984-1992 (Chaloupka and Limpus, 2001).

	Primary	ISR RTA	XFG	ISX NEST	ISR		WSR	Total
Study site					pre-2014	this study		
Howick Group	2175	27	1	37	274	218	154	**2886**
Cleveland Bay	365	6	0	0	3	26	20	**420**
Upstart Bay	719	13	0	0	23	42	21	**818**
Total	**3259**	**46**	**1**	**37**	**300**	**286**	**195**	**4124**

Table 1: Total turtle captures by tag status across three study sites from 2014 to 2017. ***P = primary turtle (first time caught/tagged); ISR = inter-season recapture; either initially caught and tagged prior to this study and then captured again in this study (pre-2014), or captured and tagged for the first time in this study and caught again in a subsequent year of this study (this study); WSR = within season capture (caught within the same sampling season); ISR RTA = inter-season recapture re-tagged animal; XFG = X feeding ground (originally tagged at a different feeding location, migratory or developmental); ISX NEST = inter-season nester (originally tagged at a nesting beach). The total number of individual turtles captured in this study (3643) can be calculated by subtracting WSR and ISR (this study) from the total.***

The differences in the age class structure of foraging green turtle populations within Queensland are likely due to sampling biases, as opposed to ecological factors. It is known that different age classes utilize different habitats, with juveniles generally using higher (shallower) intertidal habitats (including among mangrove forests), while larger turtles will tend to forage more in the mid intertidal and sub-tidal habitats (Limpus et al., 2005). Most catching in UPB and CLV was limited to the shallow intertidal areas due to poor visibility, which could account for the high proportion of juveniles captured at these sites.

The female:male (F:M) ratios of adult green turtles captured within this study are summarised in Table 3. The population was biased to females at all sites, and different between sites, ranging from 5:1 at UPB to 2:1 at CLV. For the parallel study that investigated the sex ratio (and genetic stock) of juveniles and sub-adult turtles captured at HWK (Jensen et al., 2018), F:M was also skewed towards females for juveniles (3.4:1) and sub-adults (3.1:1). The female biased sex ratios observed in this study are comparable to other green turtle populations within Queensland. For example, F:M ratios of green turtle foraging populations in Queensland ranged from 3.3:1 (sub-adults) to 1.7:1 (juveniles) at Shoalwater Bay (Limpus et al., 2005), from 0.65:1 (adults) to 1.7:1 (juveniles) at Heron/Wistari Reefs (Chaloupka and Limpus, 2001), from 2.1:1 (adults) to 4.2:1 (juveniles) at Clack Reef (Limpus et al., 2009), from 5:1 (adults) to 1.4:1 (sub-adults) at Moreton Bay (Limpus et al., 1994), and 1.6:1 (adults) at Edgecumbe Bay (Hof et al., 2017). While unsubstantiated, Chaloupka and Limpus (2001) suggest the possibility of sex-biased migratory behaviour and sex-biased recruitment to developmental habitats, to explain why certain foraging grounds appear

to favour a particular sex. In addition, as illustrated by Limpus and Read (1985), the use of secondary characteristics such as tail length to assign the sex of adult green turtles will bias the sex ratio towards females. A multi-site mark-recapture study on foraging grounds in the GBR using more reliable sex determination methods (e.g. laparoscopy, blood hormone concentrations) is needed to resolve many of these issues.

Study site	Adult	Sub-adult	Juvenile	Total
Howick Group	1468 (58%)	446 (18%)	598 (24%)	**2512**
Cleveland Bay	79 (21%)	55 (15%)	240 (64%)	**374**
Upstart Bay	188 (25%)	112 (15%)	457 (60%)	**757**
Total	**1735 (48%)**	**613 (17%)**	**1295 (35%)**	**3643**

Table 2: Age class (and percent contribution of each) of the green turtles captured between 2014 and 2017 at the Howick Group of Reefs, Upstart Bay and Cleveland Bay.

Study site	Female	Male	Ratio F:M	Indeterminate	Total
Howick Group	1163	280	4.2:1	25	1468
Cleveland Bay	52	26	2:1	1	79
Upstart Bay	155	31	5:1	2	188

Table 3: Sex ratio of adult green turtles captured between 2014 and 2017 at the Howick Group of Reefs, Upstart Bay and Cleveland Bay.

The sex ratios of foraging populations can provide important information on the sex ratios of nesting populations (or genetic stocks), especially if the genetic stock composition of the foraging population is known. While genetic stock analysis was not performed for CLV and UPB, a study in 2008 at a nearby foraging ground (Edgecumbe Bay) showed that nearly all green turtles were from the sGBR genetic stock (Jensen et al., 2016). It could therefore be expected that most turtles foraging at UPB and CLV are also from the sGBR stock. In addition, as described above, foraging green turtle populations south of UPB are generally female biased, with F:M ratios as high as 5:1. The sGBR breeding population is therefore likely to be female biased. Moderately female biased nesting populations such as that expected for the sGBR can likely be sustained over time. This is because adult male green turtles migrate for breeding more frequently than adult females (Limpus et al., 2005), leading to more balanced 'operational sex ratios' at breeding sites (Hays et al., 2010). In addition, due to the polygamous mating behavior of green turtles (Jessop et al., 1999), even female biased operational sex ratios may be able to sustain nesting populations, although the extent to how biased this can be toward females requires further investigation.

While the adult sex ratios assessed in this study at the HWK site were similar to UPB and CLV, the genetic composition of the HWK site is very different. In 2008, Jensen et al. (2016) showed that ~50% of the juveniles and ~70% of the sub-adults and adults foraging at the HWK site were from the nGBR stock, with the remainder coming from the sGBR/Coral Sea genetic stocks. By 2014/15 (the time of this study) this had shifted slightly to 39% of the juveniles and 77% of the sub-adults and adults at HWK coming from the nGBR genetic stock (Jensen et al., 2018). Interestingly, when the sex ratio of the different stocks was considered, Jensen et al. (2018) showed that 65-69% of the sGBR turtles foraging at HWK were females, whereas the sex ratio of the nGBR turtles was severely female biased (87-100%). This extreme female bias observed in the nGBR turtles foraging at HWK, particularly for the juveniles and sub-adults, may indicate a severe female bias in the nGBR nesting population, likely driven by excessive nesting beach sand temperatures at nGBR rookeries favouring the development of female hatchlings. However, this needs to be further investigated, as does whether this female bias observed at HWK is present in other foraging populations and breeding aggregations that support the nGBR nesting population.

Population size estimates

Age-and sex-specific survival rates, population abundance trends, and recruitment are best estimated using the CMR approach, but this requires intensive sampling and high recapture rates. In our study, low recapture rates at CLV and UPB did not allow meaningful CMR models to be developed at these sites. Robust survival estimates should also be based on a longer time series data. Nevertheless, recapture rates of juvenile turtles on the Ingram Island foraging grounds at HWK during the 2014 sampling season were adequate to estimate abundance. This yielded an estimate of between 356 and 598 juveniles (using the Mt model). While population growth and turtle survival rates were not modelled here, other green turtle foraging populations containing mixtures of sGBR and nGBR genetic stocks are increasing in abundance and have high survival rates (Chaloupka and Limpus, 2001; Hof et al., 2017). This has been attributed to the recovery of the sGBR green turtle population, in particular, from continued efforts to reduce human impacts on marine turtles nationally and the cessation of the commercial green turtle harvest in 1950. With continued capture-recapture sampling at the foraging grounds featured in this study (HWK, CLV and UPB), population parameters could be modelled in the future.

Growth rates

Recapture interval ($F = 3.35$, $EDF = 2.77$, $p = 0.015$), mean CCL ($F = 3.87$, $EDF = 3.87$, $p < 0.001$) and capture year ($F = 3.79$, $EDF = 2.36$, $p = 0.03$) had significant influences on estimated growth rates (Table 3). The size-specific growth rate function was dome shaped (non-monotonic) and skewed to the left (Figure 3b), and growth rate reached an asymptote with increasing recapture interval (Figure 3a). Somatic growth also varied between turtles caught in different years, suggesting cohort and/or environmental effects (Figure 3c). There were no significant differences in standardised growth rate between sexes ($D = 0.96$, $p = 0.61$), but there were insufficient samples to test for sex-specific differences in growth rates between sites.

Somatic growth rate differed significantly between sites (Figure 4; $D= 46.82$, $p < 0.01$). Standardised growth rate at HWK was significantly slower than at CLV ($\beta = 1.12$, $se = 0.22$, $t = 5.05$, $p <0.01$) and UPB ($\beta = 1.19$, $se = 0.19$, $t = 6.35$, $p <0.01$). Size-specific growth rate was estimated from the final GAMM to be 1.9 cm yr^{-1} at HWK (se: 0.15), 3.0 cm yr^{-1} at CLV (se: 0.26) and 3.1 cm yr^{-1} (se: 0.21) at UPB (based on a 60 cm CCL turtle caught in 2016 and recaptured in 2017).

Green turtles at all three foraging sites displayed the typical non-monotonic growth pattern that we have come to expect from free ranging wild turtles within the GBR (Chaloupka et al., 2004; Limpus and Chaloupka, 1997) and elsewhere (Bjorndal et al., 2017). The smaller resident turtles grow slowly, typically growing <2 cm yr^{-1}, with annual growth rate increasing as they approach ~60 cm CCL. After this size, annual growth rate slows and approaches zero as they reach adult size. For most animals, the single most important factor to either promote or limit growth rate and reproductive periodicity is the availability of nitrogenous food (White, 1978). This holds true for marine turtles, whose life history strategy is one of

high fecundity, but lower survivorship of immature age-classes (Limpus, 2009). It is logical therefore that immature turtles grow rapidly, but this relies on access to high quality forage.

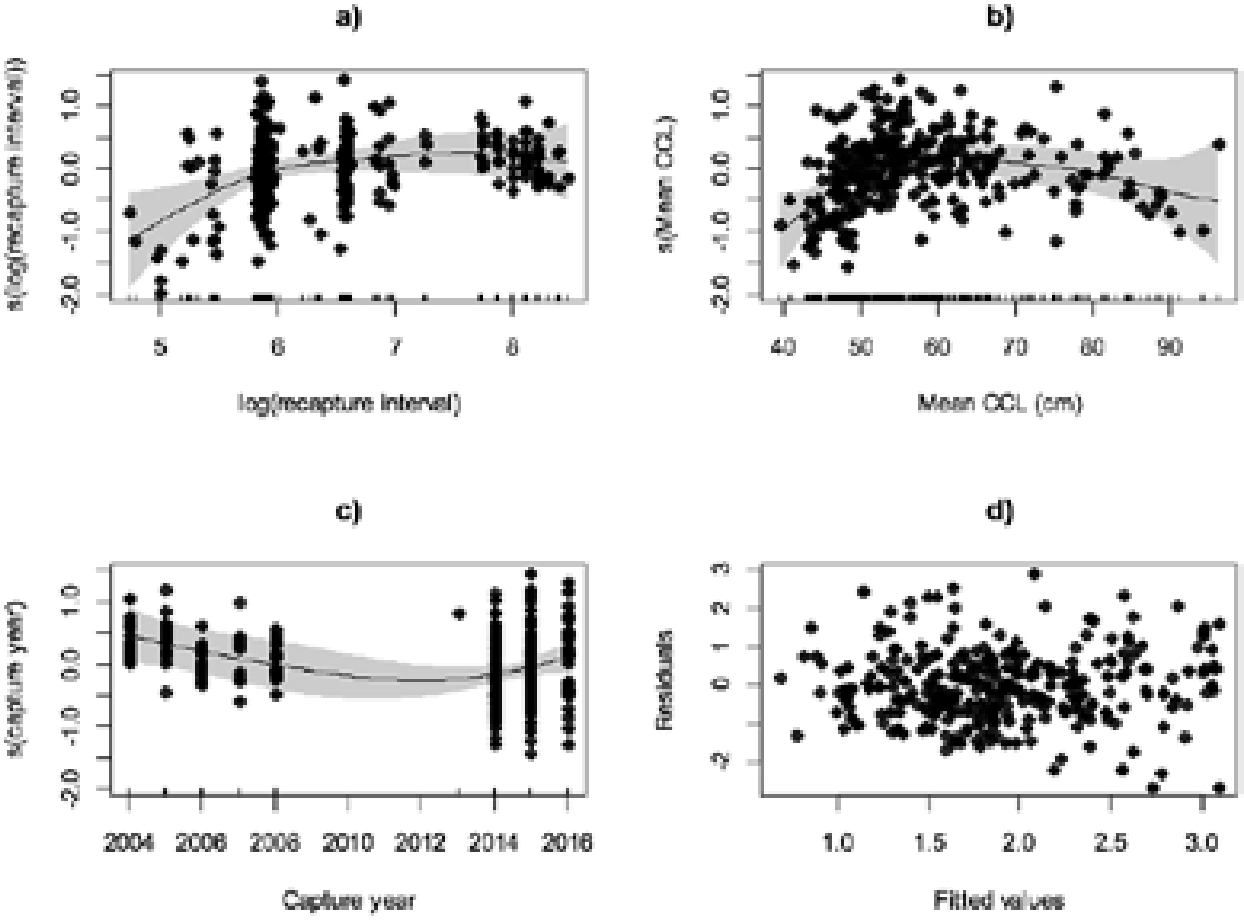

Figure 3: Smoothers from the final GAMM for (a) recapture interval (log transformed, days), (b) mean CCL (cm) and (c) capture year (the residuals are indicated by the solid circles). Figure (d) indicates the model residuals versus the fitted values.

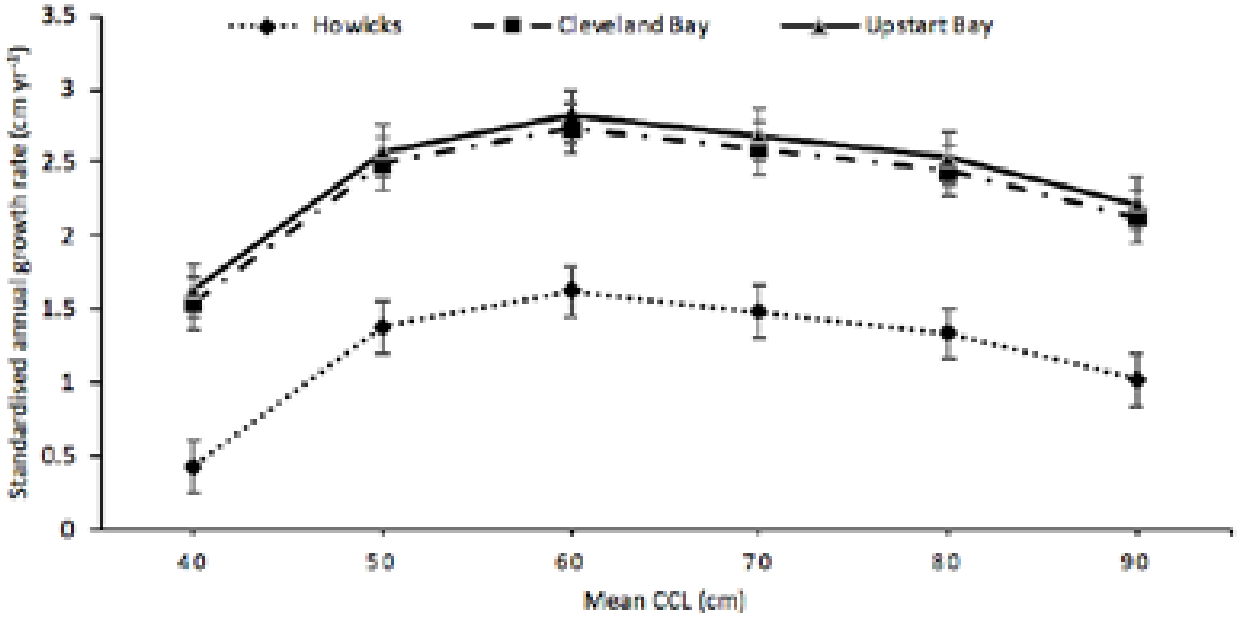

Figure 4: Standardised annual growth rates (±SE) of green turtles (*Chelonia mydas*) foraging in the Howick Group of Reefs, Cleveland Bay and Upstart Bay.

The size-specific growth rates observed in this study were comparable to other green turtle foraging populations in Queensland (Chaloupka et al., 2004; Hof et al., 2017; Limpus and Chaloupka, 1997) and globally (Bjorndal et al., 2017; Koch et al., 2007; López-Castro et al., 2010; Seminoff et al., 2002). The growth rate was considerably slower at HWK than CLV and UPB. This may be due to differences in diet between the sites, with HWK turtles often including large portions of algae in their diet, compared to predominantly seagrass at UPB and CLV (see below). This hypothesis is supported by Bjorndal et al. (2017), who recently illustrated that green turtles feeding on algae alone or a mixture of algae and seagrass displayed slower growth rates that those feeing on predominantly seagrass. However, contradictory to this, two foraging green turtle populations in the sGBR, Shoalwater Bay and Heron/ Wistari Reefs, had very similar growth rates (~2 cm yr^{-1}), despite predominantly feeding on seagrass and algae, respectively (Chaloupka et al., 2004). Density-dependent processes can also shape growth rates and thereby complicate comparisons between sites, time periods or studies (Bjorndal et al., 2000). Further, annual variability (see Fig 2c), presumably because of extrinsic environmental drivers (Chaloupka et al., 2004) or cohort effects, makes it problematic to compare growth rates between studies undertaken during different time periods. Future research into the impact of diet, and more specifically diet shifting, as well as the calorific value of algae compared to seagrass to green turtles may provide further insight into the differences in growth rate observed between sites in this study. There may also be other factors affecting the health and/or metabolism of green turtles foraging at HWK that account for their reduced growth rates observed in this study.

Body condition

The ANCOVA analysis indicated significant differences in the slope of the log(weight)-log(CCL) relationship between sites ($F = 9.09$; $p < 0.001$), with an estimated slope of 2.986 (se = 0.012) for CLV; 2.983 (se= 0.012) for UPB and 2.979 (se= 0.011) for HWK. The slope-adjusted condition indices suggested that overall, turtles were in similar body condition at CLV and UPB, and slightly poorer condition (~1% difference) at HWK (Figure 5, Table 4).

Site	CCL (cm)	Slope-adjusted CI	Lower 95%	Upper 95%
Howick Group	50	0.673047	0.610971	0.735122
	70	0.855633	0.798479	0.912787
	90	0.974195	0.920203	1.028186
Cleveland Bay	50	0.68033	0.618217	0.742444
	70	0.862917	0.805708	0.920126
	90	0.981478	0.92742	1.035537
Upstart Bay	50	0.67715	0.615069	0.739231
	70	0.859737	0.802562	0.916911
	90	0.978298	0.924275	1.032322

Table 4: Slope-adjusted condition indices (CI) for each site and for three turtle body sizes (mean ± 95% confidence intervals).

The SMI also indicated a statistically significant difference between sites (Kruskal Wallis chi square = 38.93; $p < 0.001$), although there was considerable overlap between sites and the difference between sites was only small (~3.4 % difference between CLV and HWK, Figure 5). Average SMI at CLV (mean = 27.341, SD = 3.708) and UPB were similar (mean = 27.004, SD = 3.575), and only slightly better than at HWK (mean = 26.411, SD = 3.381). The ratio indices (Table 5) suggested that on average body condition was good at each site, and similar to the ranges found by Flint et al. (2014) in green turtles foraging in Gladstone. However, there was considerable variation between individuals with some juveniles in very poor condition at all three sites.

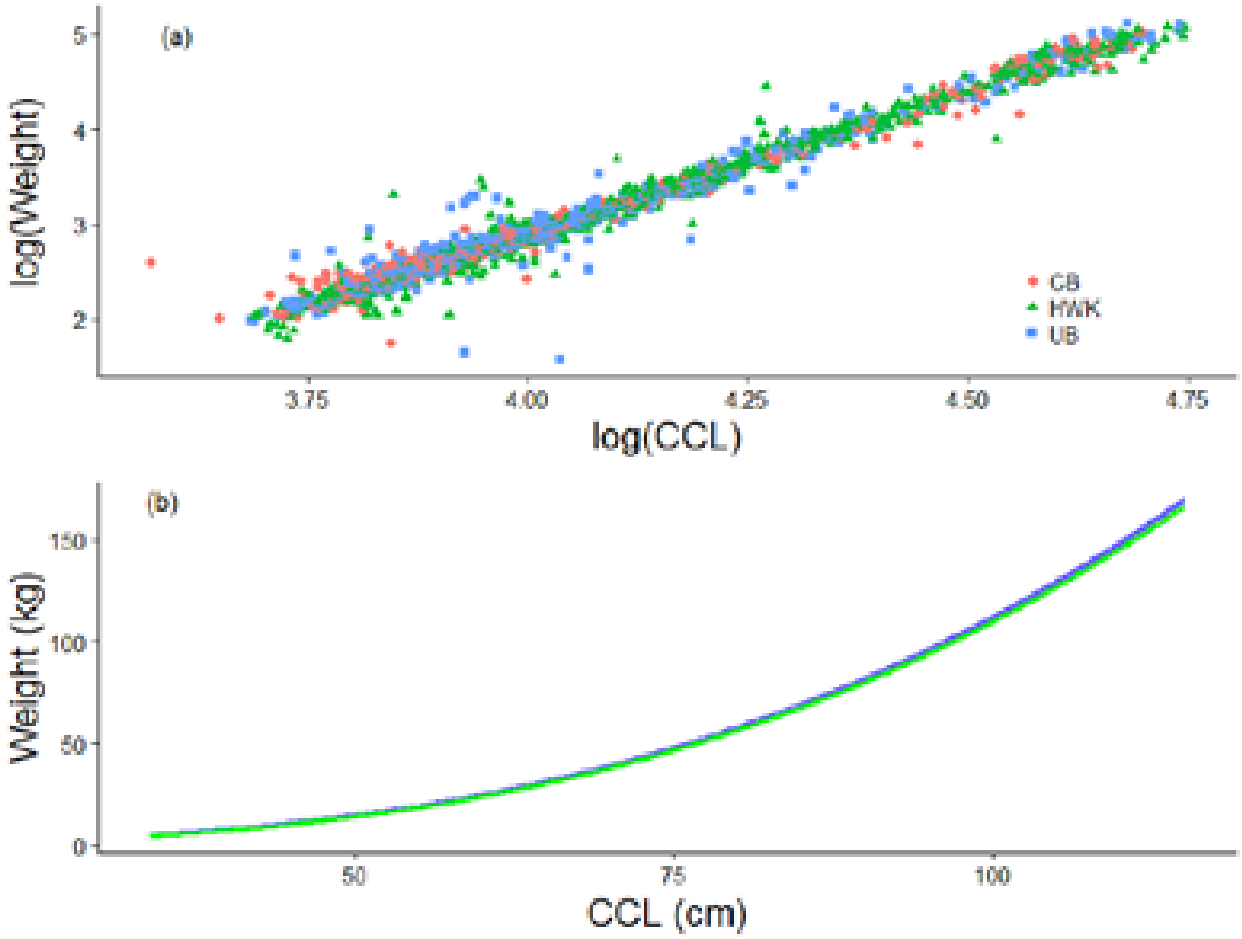

Figure 5: Green turtle body condition relationships. Linear relationship between log weight and log CCL (a); fitted mixed-slopes ANCOVA model back transformed to the original variable scales (b). Red: CLV; Green: HWK, Blue: UPB. Note, in (b) that the red line for CLV is behind the blue line for UPB because they had very similar fitted values.

The slightly poorer body condition of the HWK turtles is possibly related to the reduced growth rates and different diet compositions (see below) also observed at this site. However, it is important to note that the differences are very small, and unlikely to be ecologically significant. Regardless, the body condition indices presented here that encompass all age classes, provide important baseline values of green turtle condition, that are useful for comparisons with other foraging populations monitored in the future and/or turtles undergoing rehabilitation. The relationships between body condition and other health parameters (e.g. barnacle loads, blood biochemistry) are discussed in detail by Flint et al. in the following chapters.

Site		Juvenile	Sub-adult	Adult	Overall
Howick Group	Mean	0.687822	0.892079	1.025713	**0.785286**
	SD	0.077209	0.048762	0.030721	**0.14837**
	Min	0.48092	0.787804	0.858629	**0.48092**
	Max	0.89951	1.040167	1.075999	**1.075999**
	N	496	169	126	**791**
Cleveland Bay	Mean	0.673155	0.877405	1.02401	**0.751972**
	SD	0.057585	0.049195	0.033508	**0.143444**
	Min	0.45733	0.746148	0.913917	**0.45733**
	Max	0.830033	0.969275	1.072556	**1.072556**
	N	258	45	54	**357**
Upstart Bay	Mean	0.69526	0.867161	1.02906	**0.749741**
	SD	0.066137	0.049609	0.03706	**0.12074**
	Min	0.386061	0.676976	0.946018	**0.386061**
	Max	0.910211	0.996762	1.091808	**1.091808**
	N	453	45	48	**597**

Table 5: Observed ratio condition indices for comparisons with previous studies, with the mean, standard deviation (SD), range (max and min) and sample size (N) for each site and age class. Condition index is derived from log10(mass, kg)/log10(CCL, cm) for comparison with earlier studies. Note, that this index should not be used to compare condition between sites or age classes because of a number of statistical properties of ratio indices that have been well recognised in the literature (e.g. Jackson et al., 1990; Schulte-Hostedde et al., 2005).

Turtle diet

Qualitative observations of gastric lavage samples indicated that turtles had distinctly different diets in the two embayment habitats (CLV and UPB) compared to the offshore site (HWK). The major dietary components of turtles found foraging within CLV and UPB were seagrasses, dominated by *Cymodocea serrulata* and *Halodule uninervis*. There was no difference in diet between sub-adults and adults, or adult males or female turtles found feeding in these seagrass habitats. Conversely, turtles found foraging at HWK alternated between a predominantly seagrass and a red macro-algal diet (*Gelidiella* and *Laurencia* sp.). Gastric lavage sampling at HWK revealed that the majority of turtles were feeding on red macro-algae in 2014, primarily on seagrass in 2015, and an approximate equal split in the number of turtles feeding on macro-algae and seagrass in 2016.

While further investigation is warranted, observational estimates of seagrass density in Cleveland and Upstart Bays appeared substantially higher than the sparse biomass seen on the sandy reef flat at HWK. In addition, there is evidence of significant temporal and spatial variability in algal abundance around HWK reefs (Coles et al., 2007), and also for seagrass stocks along the Queensland coast (Lanyon and Marsh, 1995; Lee-Long et al., 1993). This supports more consistent consumption of seagrass by green turtles at CLV and UPB, and a need for green turtles at HWK to regularly shift diet preferences. This switch in diet between seagrass and algae at HWK between sampling years shows a clear diet preference dichotomy, which has also been observed in other studies (André et al., 2005; Brand et al., 1999). It has been proposed that geographic variations in food availability result from localised stochastic events influencing productivity (Garnett et al., 1985) and nutrient uptake rates (Bjorndal, 1997).

This study provides a contemporary baseline understanding of green turtle dietary breadth and therefore niche utilisation within embayments and reefal areas in the northern Great Barrier Reef, and is important for allowing predictions to be made on how populations may respond to, or cope with the ramifications of a changing climate. For example, an increase in mean sea level combined with an increase in oceanic acidification due to carbon dioxide uptake, may have profound, albeit unknown implications for global reef and neritic marine ecosystem diversity (Hoegh-Guldberg et al., 2007), with flow-on effects to the green turtles foraging in these habitats.

Turtle health indices

Barnacle counts

Overall, 84% of green turtles at UPB had plastron barnacles compared to 64% at CLV and only 13 % at HWK. There was a significant difference in plastron barnacle load (i.e. the number of barnacles per turtle) between sites ($D = 101.8$, $df = 2$, $p < 0.001$). On average, the likelihood of turtles having a high barnacle load was considerably lower at HWK than CLV (-4.11, $z= -7.9$, $p < 0.001$), and lower at UPB than CLV (-0.57, $z= -5.07$, $p < 0.001$). The average number of barnacles per turtle was 0.125, 4.77 and 6.58 for HWK, UPB and CLV, respectively. The barnacle load between sites was likely to have been influenced by environmental and habitat differences, for example, cleaning stations and reef fish species that forage on turtle epibionts (Losey et al., 1994) are more likely to occur at the coral reef habitat of HWK than at the inshore, seagrass dominated habitat of UPB and CLV. Plastron barnacle load is therefore likely to be more informative in comparing the health of individual turtles within sites. The links between barnacle loads and turtle health are discussed in more detail by Flint et al. in the following chapters.

Fibropapilloma tumors

During the study, 21 incidences of fibropapilloma (FP) tumors were observed, representing 0.5% of all turtles caught. Although prevalence of FP was low across all sites, turtles in CLV had the highest occurrence of FP (2.3%), followed by UPB (1.3%) and HWK (0.04%). The very low prevalence of FP observed in the present study is not unexpected as there is only very low incidences of FP reported in coastal green turtle populations of the GBR generally (Jones et al., 2016; Limpus et al., 2005).

Eye lesions

Over the three years of this study (2014-2017), eye abnormalities were observed in 23% of the turtles in Upstart Bay, of which six cases were sampled for closer examination. Similar lesions were seen in 9% of the turtles in Cleveland Bay. The Howick Group of Reefs turtles had a much lower prevalence at 0.2%. Prevalence across all three sites increased from 7% in 2014 and 3% in 2015 to 14% in 2016 and 25% in 2017. The eye lesions mostly affected small immature (<60 cm CCL) and adult turtles (both 42.2%), while large immature (60-85 cm CCL) turtles were the least affected age group (15.6%). The links between eye lesions and turtle health are discussed in detail by Flint et al. in the following chapters.

Conclusions

This study provides important demographic information about three green turtle foraging populations sampled throughout the Rivers to Reef to Turtles project from 2014 to 2017: Cleveland Bay (CLV), Upstart Bay (UPB) and the Howick Group of Reefs (HWK). Age class and sex ratio structures of the three populations differed considerably, although all were within the ranges of typical GBR green turtle foraging populations, and differences were likely driven by sample biases. The growth rates of green turtles captured in these foraging grounds were also within the range of those observed for green turtles previously, although the HWK turtles were growing approximately 1.6 times slower than CLV and UPB turtles, possibly driven by a diet dichotomy in this population. This was supported by observations of gastric lavage samples that indicated UPB and CLV green turtles fed predominantly on seagrasses, while HWK turtles regularly fed on either seagrasses or algae (or a mixture of the two), with the contribution of each varying over the four year study period. This may also be linked to the poorer body condition observed in turtles foraging at HWK compared to the other two sites, although these differences were

very small, and unlikely to be ecologically significant. A number of sea turtle health indices also varied between the three foraging populations. Barnacle loads, eye lesions and FP varied between sites (and were generally lowest at HWK), the implications of which are discussed in following chapters looking specifically into the health of these foraging populations.

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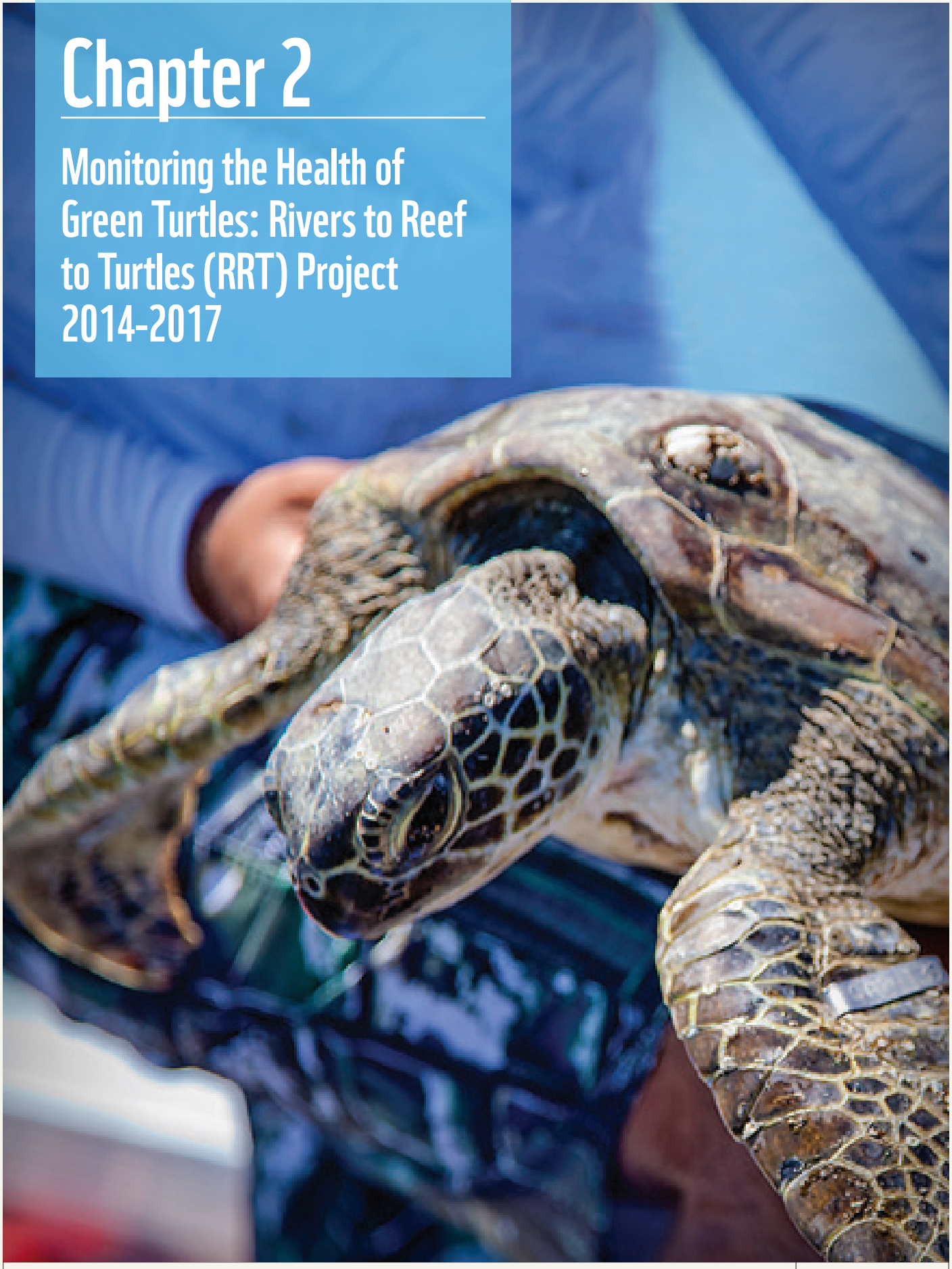
Chapter 2

Monitoring the Health of Green Turtles: Rivers to Reef to Turtles (RRT) Project 2014-2017



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2. Monitoring the Health of Green Turtles: Rivers to Reef to Turtles (RRT) Project 2014-2017

Mark Flint[1,2*], Anne-Fleur Brand[3,4], Ian P Bell[5] and Christine A. Madden Hof[6]

[1] *Veterinary* - Marine Animal Research, Teaching and Investigation unit, School of Veterinary Science, The University of Queensland, Gatton Campus, QLD 4343 Australia.
[2] Ecosystem Health Unit, Department of Veterinary Preventive Medicine, College of Veterinary Medicine, The Ohio State University, 1920 Coffey Rd, Columbus, OH 43210 USA.
* Corresponding author email: flint.71@osu.edu
[3] Institute for Risk Assessment Sciences, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 104-106, 3584 CM Utrecht, The Netherlands.
[4] Queensland Alliance for Environmental Health Sciences Faculty of Health and Behavioural Sciences, The University of Queensland, 39 Kessels Road, QLD 4108 Australia.
[5] Aquatic Species Program, Queensland Department of Environment and Heritage Protection, Townsville, Queensland 4810, Australia.
[6] WWF-Australia, Brisbane, Queensland 4000, Australia

Abstract

Over more than three years the Rivers to Reef to Turtles (RRT) project examined the health of sea turtles at two coastal sites impacted by urban and agricultural human activities (Cleveland and Upstart Bays) and one proposed pristine site (Howick Group of Islands) in Northern Queensland, Australia, through physical examinations and blood biochemistry and haematology; conducted necropsies when possible to determine causes of death; attempted to advance diagnostics through testing associations between toxins and turtle health; and investigated a previously unrecorded disease syndrome (ulcerative eye lesions) of the turtles of this region. No comprehensive health assessments had been conducted at these sites prior to this study.

The coastal Cleveland and Upstart Bays both demonstrated effects likely to be in response to stressors suspected to be anthropogenic in origin (elevated total white cell counts and creatinine kinase levels across the populations, respectively). This was associated with a suite of trace elements, in particular cobalt. While these indicators of stress resolved by the final year of the study, a chronic stressor was suspected to be persisting with ongoing low albumin:globulin. Necropsies did not elucidate any specific diseases, but eye lesions affecting up to 23% of the coastal study site turtles were examined and found to have mixed bacterial infections and active inflammatory processes.

Although body condition index did not closely correlate with site health, barnacle counts in juvenile turtles may prove a reliable indicator of site health. Based on previously established indicators of poor health, barnacle counts showed that 10% of the population was in poor health at Upstart Bay and nearly 20% of the population at Cleveland Bay. This is above what would be expected for a normal population.

Overall, the health component of this study suggested that the pristine turtle population was healthy and the coastal turtle populations were under active stressors, most likely caused by anthropogenic effectors such as chemical pollutants, when initially examined in 2014. These stressors resolved by the conclusion of the study in 2017; but chronic stressors remained absent in the pristine site and present within each of the studied coastal populations.

Keywords:

Green turtle, eye lesion, clinical pathology environmental stressor, health, Queensland

Introduction

Ecosystem health is a concept that holistically describes the condition of an environment, and the organisms within it. The health of an ecosystem can vary as a result of various stressors. A stressor is an event or a stimulus that causes stress to an organism and may manifest as a physical, chemical or behavioral response. Mapping the stressors in a specific area is essential to diagnose the area's health. This information can then be used to develop appropriate mitigation measures (Flint 2018).

To make this determination information is gathered through a variety of methods and disciplines, about a defined area (ecosystem) either before (baseline) or after (e.g. disease investigations) a defined event to make a "diagnosis" of the area's health. From here, mitigation measures can be developed.

Monitoring the health of sentinel species is commonly used as proxies of the health of their living environment. In the marine environment, sea turtles have been long proposed as sentinel indicators for several reasons. First, they are long-lived species, believed to live for a long time. Furthermore, they have a high site fidelity (after recruiting from the ocean, most sea turtle species will live in the same coastal region for the rest of their lives), and may inhabit waters close to human populations (thereby exposing themselves to anthropogenic stressors). Conversely, their populations are low in numbers when compared with abundant species such as fishes or corals and they undertake long-lasting (several months) migrations every three plus years to breed, making them less suitable as sentinels. Also, as reptiles, turtles are slow to respond to diseases and often the stressor (e.g. pathogen, contaminant) is long-gone before clinical signs appear (Flint 2018).

Sea turtles are a collection of species of global concern, with populations ranging from increasing to locally extinct. Understanding their health and the health of the environment in which they live is very important for the preservation of both and of heightened importance when an unusual mortality event (UME), such as the one that occurred in Upstart Bay in Northern Queensland in 2012 and 2013 where over a hundred sea turtles died over a short period of time and the cause went undetermined.

Among the sea turtle species, some green turtles (*Chelonia mydas*) inhabit nearshore waters close to human populations for prolonged periods of their life. This makes them particularly prone to exposure to chemicals that are used on land and wash onto the reef. Field observations of chronic disease prevalence and poor health in green turtles from such habitats (Aguirre and Lutz 2004) prompts the question whether chemical mixtures contribute to poor health status and population declines.

In Northern Queensland, multiple unusual mortality events (UME) of unknown cause occurred in the past decade, where hundreds of sea turtles died in short period of time. Events like this highlight that the understanding of turtle health and the health of their living environment is crucial for conservation.

Flint and collaborators investigated the health of green sea turtles that mass stranded in Gladstone, Australia in 2011 (Flint et al. 2015a). To determine if this mass stranding could be attributed to disease, they clinically examined live animals and necropsied the deceased turtles. Also, blood samples were taken from live animals for routine blood profiles. Gaus and colleagues analysed these same blood samples, together with pooled liver samples and pooled fat samples and found several contaminants to be at concentrations where acute adverse health effects are possible (Gaus et al. 2012). Unfortunately, the absence of exposure baselines and toxicological reference data for the green sea turtle presented limitations for the interpretation of the findings at the time.

Cleveland and Upstart Bays in Northern Queensland both suffered ongoing increased sea turtle mortalities due to suspected toxicoses in recent years, with an UME in Upstart Bay in 2012 and 2013. In the Rivers to Reefs to Turtles project conducted by WWF Australia, the health of green sea turtles from this area was examined during the recovering phases of this UME. By contrast, the offshore Howick Group of Islands have remained presumably unaffected, supporting resident populations of turtles, thus offering an ideal control site.

Over more than three years, the Rivers to Reefs to Turtles' project objective was to gather information on the health of sea turtles in these three areas and combine the health data with the other disciplines to investigate and report on the holistic impact of stressors originating in upstream rivers, flowing to our embayments, and on to our reefs.

This was achieved by through the following aims:

- Analyzing and interpreting the green turtle health reference ranges for Cleveland Bay, the Howick Group of Islands, and Upstart Bay for the duration of the project December 2013 -December 2017.
- Comparing green turtle health reference ranges within the study sites- Howick Group of Island (control site), Cleveland Bay (reference site), and Upstart Bay (study site)- using previously published reference ranges (biochemistry, haematology and plasma protein fractions) and visual indicators of health (plastron barnacle counts and body condition scores).
- Correlating green turtle health reference ranges with results of toxicological analysis (of blood and scutes samples) to help determine whether contaminant exposure adversely affected turtle health at individual and/or population levels.

Materials and methods

Study sites

Three study locations were selected. Upstart Bay, one of the sites of the UME in 2012 and 2013; Cleveland Bay, a similar embayment of moderately different environmental stressors; and the Howick Group of Island, a group of offshore reefs in the northern Great Barrier Reef as a 'control' location (see Brodie et al. 2018 for a full description).

Cleveland Bay (-19.767,554°S, 147.702,955°E), located 100km north of Upstart Bay, is geographically similar (i.e. it is influenced by similar environmental factors, and a north-south facing bay) but influenced by urban/industrial activities adjacent to the city of Townsville, heavy industry and a port. Detailed site descriptions are available (Villa et al. 2017). Townsville is both an urban and an industrial area. The Port of Townsville is frequently dredged and dredged material is placed at the northern side of the bay. A long-term marine water monitoring program is in place, which indicates chronic low levels of different groups of chemicals (PoTL 2015).

Upstart Bay (-19.235,428°S, 146.938,284°E), a rural coastal area, is dominated by agricultural and legacy mining activities and in part, receives waters from the Burdekin River, one of the largest polluted rivers in Queensland (see Brodie et al. 2018). It is the site of the green turtle mass strandings in 2012 and 2013. The bay lies in a north-south orientation and is protected from the often strong prevailing south-easterly trade winds. Intertidal seagrass and algae meadows occur on a sand, rock and mud substrate and extend seaward from a predominately mangrove fringed shore-line along the Bay's southern and western side. The bay receives input from the Burdekin River catchment, which is predominantly affected by agricultural land use, but also by historical and ongoing mining activities. Mass standing events of green turtles occurred in 2012 and 2013 of which the cause remains to be clarified.

The Howick Group of Islands is a remote collection of mid-shelf, uninhabited reefs which lie within the northern Great Barrier Reef Marine Park (-14.416,695°S, 144.880484°E), located approximately 30 km offshore from the Cape York region catchment, a relatively undeveloped catchment with low pressures from nutrient, sediment, and pesticide loads, or water regime changes and habitat alterations (Villa et al. 2017) The Howick represented the control site, distanced from anthropogenic contaminant point sources such as agricultural or riverine runoff. Sea turtles foraging at the Howick are considered clinically healthy (Villa et al. 2017).

Animal selection

Turtles were captured in the intertidal and sub-tidal waters using the turtle rodeo method and beach jumping. All turtles were released back to their foraging regions as soon as the sampling and collating information on biometrics was completed.

In Year 1 of the study, the focus was on collecting a representative sub sample (n = 40) of the population from each of three sites and priority was placed on obtaining carcasses for comprehensive necropsies. During the Year 2 sampling, eye lesions were observed in turtles from the coastal sites. In Year 3,

sampling focused on 42 recaptures to estimate any health changes in the study population over time. Furthermore, diagnosing the eye lesion was undertaken as at least two of the people involved in the study who had come in contact with these animals developed dermal lesions with secondary infections that required antimicrobial therapy to resolve. All suitable quality carcasses within the region had comprehensive post mortem examinations conducted.

Sampling

During the three years of sampling for health, physical health indicators, blood biochemical and haematological profiles were assessed to make a comparison between sites and to monitor changes in population health over time.

Furthermore, the application of plasma protein electrophoresis was advanced as a tool for the identification of specific changes in the plasma protein profile which may be indicative of responses to certain diseases and stressors. Available cases of mortality that were deemed to be in suitable carcass condition capable of delivering a diagnosis were subjected to comprehensive postmortem examination.

Clinical assessment

Body condition was initially determined based on visual assessment by experienced researchers and later confirmed by comparing with the index derived from the ratio of curved carapace length to weight. Turtles in good body condition, displaying no clinically apparent abnormalities (Herbst and Jacobson 2003), including neurological deficits (Chrisman et al. 1997) or lesions of the carapace or plastron (Flint et al. 2010a), were classified as 'clinically healthy'. Animals being of 'average body condition' were classed as healthy or poor depending on lesions and other recorded abnormalities. Turtles in 'poor body condition' and/ or exhibiting clinical abnormalities were classified as 'clinically unhealthy' and examined in detail to determine cause of disease. Barnacle counts were conducted on each turtle and the number of barnacles >1cm diameter on the plastron were recorded. Morphometrics (growth) were used to aid interpretation.

Blood collection, preparation and analyses.

Blood sampling was assessed for 120 animals (40 per site) in Year 1 and 42 selected animals (25 Upstart Bay, 15 Cleveland Bay and 7 recaptures from Howick) in Year 3. Year 1 compared results with established standards to determine any anomalies. Year 3 compared populations over time to see if their health status had changed.

Blood samples were collected via the external jugular in the cervical dorsal sinus, prepared and examined using previously described techniques (Owens and Ruiz 1980, Flint et al. 2010a), with minor modifications. Blood samples (>20 mL) were collected from turtles using an 18 G 38 mm needle attached to a 50 mL syringe. For the blood biochemistry, voided samples were transferred to a heparinised evacuated tube, and then placed in coolers (4°C), separated and serum frozen at -80°C until assessment of blood biochemistry, which occurred within 12 months of collection. For haematology, a blood smear was made with fresh blood prior to transfer to the heparinised evacuated tube by placing a small drop of blood on a clean glass slide and spreading the blood in a monolayer smear using the edge of another glass slide held at approximately 30° to the vertical. Slides were air dried and transported before being professionally stained with H&E and examined under a light microscope.

Both blood chemistry and haematology were performed at UQ Diagnostic Laboratories in 2014-2015 and QML Laboratories in 2016 and 2017 using standard protocols and test methodologies. A selection of comparable (between the two laboratories) biochemistry parameters measured included aspartate transaminase (AST), alkaline phosphatase (ALP), total protein, albumin, globulin, albumin: globulin ratio (A:G), phosphate, creatinine kinase and glucose. Haematological parameters measured were thrombocytes, erythrocytes cells and from the leukocytes, lymphocytes, heterophils, eosinophils, basophils and monocytes were calculated and used to estimate the presented total white cell counts (TWCC) (Work et al. 1998, Flint et al. 2010a). For a subset of samples in 2014-2015, we conducted Plasma Protein Electrophoresis analysis on the plasma of 24 blood samples to assess protein fractions in turtles known to be exhibiting clinical pathology tentatively associated with heavy metal stressors.

Ocular examination included the sampling of abnormal growths seen in animals' eyes of Upstart and Cleveland Bays. Presentations between the two Bays were suggested to differ. For a subset (n = 6) of the turtles with eye lesions, samples were harvested for secondary diagnostics. The growth was gently manipulated with forceps and if it could be lifted from the cornea without causing trauma, it was sampled by sharp dissecting the lesion from the cornea and placed immediately into a vial and frozen at -20°C and then -80°C on return to Brisbane. Samples were processed (slides made from smears and sample culturing) for microbial screening to search for potential aetiologies. Slides were processed as impression smears and by standard histopathology and stained with H&E. Cultures were grown in the UQ Microbiology Department in the School of Veterinary Science on sheep's blood agar for 7 days.

In the field, a subset of turtles with suspected active ocular lesions had their eyes stained using fluorescein to detect ulceration.

All turtles with suspected eye lesions had both the left and right eye photographed at close range for diagnosis. Lesions were classed as present, absent and undetermined. Present lesions were further identified as mild, moderate or severe and an estimation of active or resolved made.

Standard necropsy examination involved placing the turtle in sternal recumbency and removing the plastron using a circumferential incision around the suture line of the plastron with the soft tissue and carapace. Retracting and removing the plastron, the forelimbs were removed to expose the organs of the coelom. From here, each organ was systematically examined, removed, opened and findings reported. Samples from each organ were obtained and stored in 10% neutral buffered formalin for histology and/or samples were wrapped in an inert foil or glass jar and frozen for toxicological or microbial screening. Any appropriate smears or slides were made at the time of the dissection (Flint et al. 2009).

Sampling was undertaken under Queensland Government's G12/35326.1 permit and Animal Ethics permit number SA 2015/11/531.

Results and discussion

For Year 1, health assessments and serum biochemistry and haematology performed on 120 green sea turtles sampled from the Howick Group of Islands, Cleveland Bay and Upstart Bay suggested each of the latter two sites were having a different negative influence on its respective population of sea turtle. Urbanized Cleveland Bay with its industry dominated landscape showed low frequency signs of elevated creatinine kinase. Upstart Bay, with its agriculturally fed tributary, had a marked increase in total white cell counts across all age classes (Table 1).

By 2017, recaptured turtles showed a resolution of these elevated parameters. In Year 3, blood from recaptured turtles from Upstart Bay (n=25), Cleveland Bay (n=10) and Howick Group of Islands (n=7) were submitted to QML for serum biochemical and haematological analysis for comparison with Year 1 findings. Total white cell counts had decreased at the study sites. Creatinine kinase returned to within normal limits at Cleveland Bay. A:G ratio remained lower than normal limits between 2014-2015 and 2017 for all sites (Table 1).

No time shift was noted for any examined site or parameter other than the resolution of creatinine kinase and total white cell counts (Table 1).

	Upstart		Cleveland		Howick	
	2014-2015 (40)	*2017 (25)*	*2014-2015 (40)*	2017 (10)	*2014-2015 (40)*	2017 (7)
Albumin	12.8 (3.1)	12.5 (3.0)	11.5 (3.2)	8.5 (3.6)	12.1 (1.8)	10.8 (2.4)
Alkaline Phosphatase	23.6 (7.4)	29.2 (13.5)	23.7 (11.2)	19.8 (6.7)	15.0 (3.6)	12.4 (2.7)
Aspartate Transaminase	150.0 (31.9)	151.7 (35.0)	139.2 (30.8)	106.8 (28.2)	122.5 (24.0)	114.7 (35.7)
Calcium	1.1 (0.6)	1.5 (0.8)	1.1 (0.4)	1.3 (0.4)	1.7 (0.5)	1.8 (0.4)
Creatinine Kinase	803.0 (668.6)	1065.5 (944.2)	2147.4 (5489.4)	734.6 (626.7)	1109.9 (624.1)	1174.6 (548.8)
Globulins	29.9 (8.6)	29.5 (4.1)	29.1 (6.4)	24.3 (4.7)	32.1 (4.7)	30.6 (3.3)
A:G	0.4	0.4	0.4	0.3	0.4	0.3
Glucose	5.6 (1.7)	5.4 (1.4)	6.7 (1.7)	5.5 (1.9)	6.0 (1.5)	4.8 (1.0)
Phosphorous	2.0 (0.5)	2.3 (0.6)	2.1 (0.7)	1.9 (0.2)	1.4 (0.4)	1.3 (0.4)
Total Protein	42.4 (11.9)	42.1 (5.7)	40.7 (9.0)	32.8 (7.9)	44.2 (5.8)	40.7 (4.5)
Total White Cell Count	38.7 (13.4)	14.0 (3.9)	28.3 (11.6)	17.1 (5.8)	25.5 (8.3)	18.6 (4.1)

Table 1: Averages (±SD) of select biochemistry and haematology results for all turtles sampled in Upstart Bay, Cleveland Bay and the Howick Group of Islands in 2014-2015 compared with 2017 resampling.

The average, maximum and minimum values for presumable healthy turtles at each site in 2014-2015 were compared to the existing published reference ranges used for Australia (Flint et al. 2010a), and the wider reference ranges used globally. There was no difference between the healthy green sea turtles' normal blood parameters and those published, except for the values highlighted in blue (Table 2). In all cases, these values were only just outside the published reference ranges.

	Upstart (n = 40)			Cleveland (n = 40)			Howick (n = 40)		
	Average	*Max*	*Min*	*Average*	*Max*	*Min*	*Average*	*Max*	*Min*
Albumin	12.8	6.6	18.2	11.5	6.2	17.5	12.1	8.8	16.6
Alkaline Phosphatase	23.6	11.0	47.0	23.7	10.0	65.0	15.0	9.0	24.0
Aspartate Transaminase	150.0	86.7	249.9	139.2	80.0	224.6	122.5	69.2	165.2
Calcium	1.1	0.2	2.4	1.1	0.2	2.0	1.7	0.8	2.9
Creatinine Kinase	803.0	157.1	3176.2	2147.4	145.8	3051.9	1109.9	379.4	3014.1
Chloride	111.4	101.0	124.6	115.7	102.1	235.0	109.0	97.2	119.7
Creatinine	21.3	12.6	29.6	25.8	15.7	33.5	26.9	17.8	34.1
GGT	0.4	0.1	1.4	3.4	0.1	113.3	0.3	0.0	1.1
Globulins	30.7	17.7	45.5	29.1	14.8	41.9	32.1	24.7	43.1
Glucose	5.8	3.3	10.4	6.7	3.7	10.7	6.0	2.8	9.6
Potassium	4.3	3.3	5.2	4.7	2.6	10.6	4.1	3.2	5.4
LDH	118.5	41.1	348.4	183.1	0.5	2136.9	90.1	26.0	263.7
Magnesium	4.0	2.4	5.9	3.7	1.8	5.5	3.5	2.1	5.5
Sodium	153.1	142.3	167.5	153.6	142.2	168.3	153.6	143.6	162.2
Phosphorous	2.0	0.8	2.8	2.1	0.7	3.8	1.4	0.9	2.7
Total Bilirubin	3.1	1.7	8.9	2.7	1.4	4.2	2.5	2.0	3.3
Total Protein	43.5	24.3	62.7	40.7	24.8	57.3	44.2	36.2	56.2
Uric Acid	55.0	25.0	155.0	66.7	22.0	146.0	54.1	2.0	176.0
Urea	1.1	0.1	10.5	1.5	0.2	15.9	1.5	0.4	3.8

Table 2: Comparison of the maximum, minimum and average values for common parameters for presumable healthy turtles at each site in 2014-2015 with the published literature.

Toxicology

Analysis of water, sediment, seagrass and turtle blood showed clear distinctions between the study locations in the profiles of both organic and inorganic contaminants. Most notably, the concentrations of several heavy metals were elevated at both coastal foraging grounds and concentrations of cobalt at Upstart Bay were many times greater than considered safe in mammalian species. For tested metals and organic contaminants (e.g. polar and non-polar compounds), Upstart Bay > Cleveland Bay >> Howick Group of Islands and reefs.

Several elements were detected at elevated concentrations in blood from turtles from the coastal sites (especially UPB), particularly Co (ranging from 160-840 mg/L), but also Mo, Mn, Mg, Na, As and Sb (Chapter 5).

Plasma protein electrophoresis- Samples were selected based on Queensland Alliance for Environmental Health Sciences' screens indicating high levels of metals as well as total white cell counts showing some degree of inflammatory response. In addition, 6 controls of known no contaminant exposure and no high white cell counts were compared. Plasma protein electrophoresis did not prove to be a good biomarker of contaminant burden for alpha, beta and gamma-globulins or for albumin fractions. It did show depressed pre-albumin levels in 7 of the 24 samples with elevated metal loads. The single elevated pre-albumin level was found in the control (Table 3).

	α- Glob (%)	β- Glob (%)	γ- Glob (%)	Pre-Albumin (%)	Albumin (%)
Howick- Control (n = 6)	0 (0)	0 (0)	3 (50)	1 (17)	3 (50)
Cleveland- high TWCC and 2 metals (n = 5)	0 (0)	0 (0)	0 (0)	2 (40)	1 (20)
Cleveland - normal TWCC and 2 metals (n = 7)	1 (14)	1 (14)	3 (43)	3 (43)	3 (43)
Upstart- high TWCC and 2 metals (n = 12)	2 (17)	1 (8)	6 (50)	6 (50)	1 (8)
Cleveland - normal TWCC and 2 metals (n = 0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
High TWCC and 2 metals (n = 17)	2 (12)	1 (6)	6 (36)	3 (18)	1 (6)
Normal TWCC and 2 metals (n = 7)	1 (14)	1 (14)	3 (43)	3 (43)	3 (43)

Table 3: Fraction of protein (n (%)) for normal total white cell counts and metal loads, high total white cell counts and elevation in at least 10 important heavy metals, and combinations thereof for the three study sites.

Ocular lesions

Eye abnormalities were observed in 23% of the turtles in Upstart Bay, of which 6 cases were sampled for closer examination. Similar lesions were seen in 10% of the turtles in Cleveland Bay. The Howick Group of Island turtles had a much lower prevalence at 0.2% (Table 4). There was a wide range of reported lesions presentations. Within this range, turtles appeared to minimally have a thin film of hazy gelatinous substance across the cornea, to moderately healed white scar tissue to maximally active inflamed lesions. Physical examination of the eye and sampling indicated lesions occurred both unilaterally and bilaterally with a higher prevalence in adults. Lesions were often associated with ulceration when examined with fluorescein staining. The lesions appeared to originate from one aspect (outside) of the eye. Some involvement of the cornea was occurring as swelling of the cornea was occasionally noted with potential associated hyperplastic tissue. The conjunctiva and glandular tissue also presented as inflamed and markedly swollen (Figure 1). Grossly, the lesions included potential secondary involvement with Ozobranchus sp leeches and small fibropapilloma tumours; with evidence of suspect Ozobranchus sp leeches, mix bacterial infections and mixed inflammatory infiltrates being noted histologically. No bacteria were cultured on sheep's blood agar media from the harvested eye samples (n = 6). No viral inclusions were found. There was no strong data to allow the assessment of severity of lesions.

Although the proportion of positive cases increased each year, the proportion of negative cases remained approximately the same with a reduction in the proportion of inconclusive cases (Table 4).

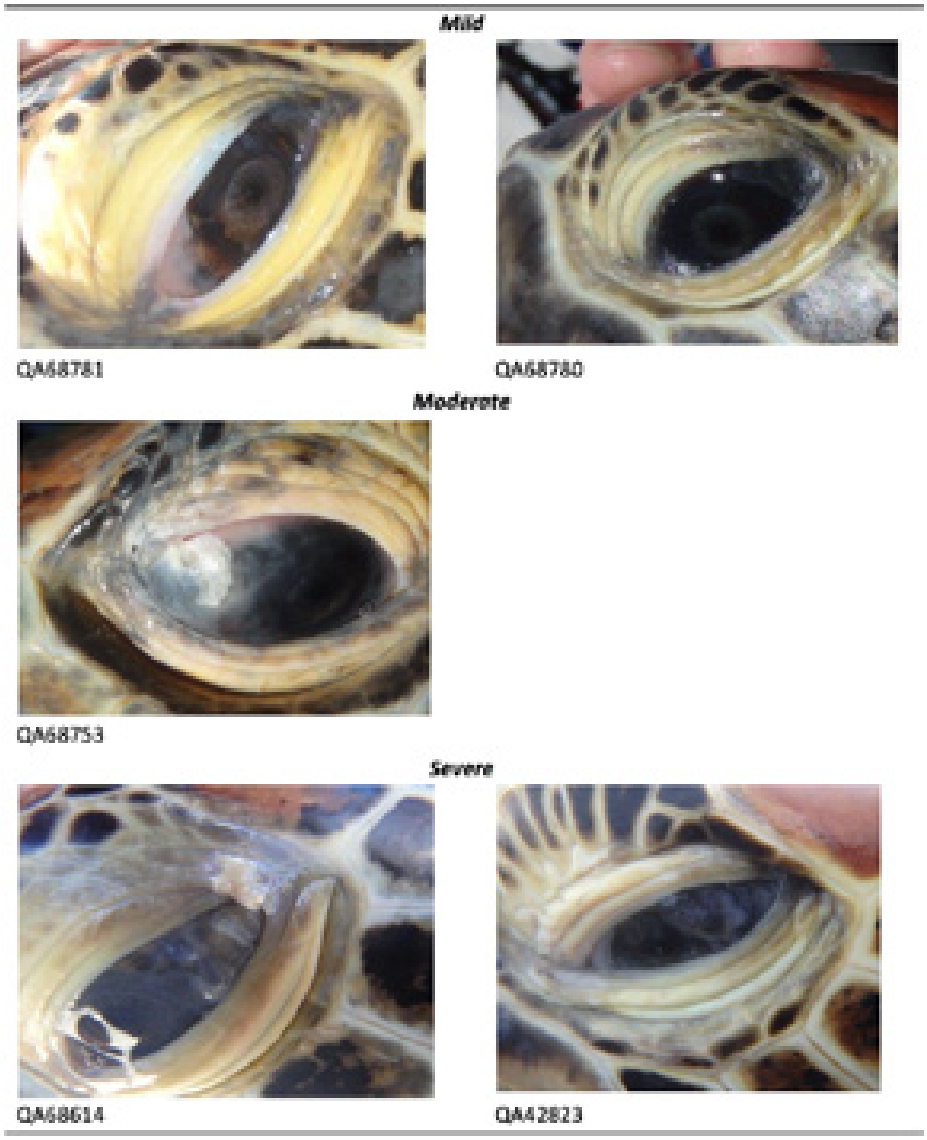

Figure 1: Ocular lesions from samples taken in Upstart Bay in May 2017 showing range of damage from mild alteration of the cornea to moderate corneal opacity to severe tissue change (minus signs of active infection).

Year by Site	I	N	Y	Total
2014	**63**	**259**	**26**	**348**
Cleveland Bay	14	42	13	69
Howick Group of Islands	40	152	1	193
Upstart Bay	9	65	12	86
2015	**35**	**409**	**14**	**458**
Cleveland Bay	1	35	2	38
Howick Group of Islands	28	284	0	312
Upstart Bay	6	90	12	108
2016	**56**	**205**	**41**	**302**
Cleveland Bay	13	30	1	44
Howick Group of Islands	11	132	0	143
Upstart Bay	32	43	40	115
2017	**1**	**81**	**28**	**110**
Howick Group of Islands	0	32	3	35
Cleveland Bay	0	0	0	0
Upstart Bay	1	49	25	75
Grand Total	**155**	**954**	**109**	**1218**

Table 4: Prevalence of eye lesions (positive- "Y", negative- "N" and inconclusive "I") for each year 2014-2017 for the three study sites.

When comparing eye infections with blood results for those available data, no bacterial growth was isolated from the eye lesions. The debris present on the eyes were indicative of bacteria or could be associated with a virus; but neither were possible to elucidate.

Parameter	Positive Lesions			Negative Lesions			Undetermined Lesions		
	Min	Max	Average	Min	Max	Average	Min	Max	Average
Alb/Glob Ratio	0.4	0.2	0.5	0.4	0.3	0.5	0.5	0.3	0.6
Albumin (g/L)	10.7	5.0	17.0	13.7	12.0	17.0	13.3	12.0	16.0
ALP (U/L)	24.4	15.0	58.0	38.3	22.0	67.0	31.3	14.0	41.0
Amylase (U/L)	648.9	510.0	960.0	716.7	670.0	760.0	625.7	480.0	950.0
AST (U/L)	149.3	99.0	220.0	162.7	142.0	179.0	158.3	96.0	225.0
Bile Acids (umol/L)	0.1	0.0	1.0	0.5	0.0	1.0	0.3	0.0	1.0
Calcium (mmol/L)	1.2	0.2	2.2	0.8	0.2	1.1	1.8	0.6	2.6
Cholesterol (mmol/L)	3.6	2.2	5.4	4.5	3.7	5.2	4.1	3.0	5.3
Creatinine Kinase (U/L)	1297.7	186.0	4496.0	759.0	419.0	1427.0	1134.7	506.0	1934.0
Globulin (g/L)	28.2	22.0	35.0	33.3	28.0	36.0	29.7	24.0	36.0
Glucose (mmol/L)	5.1	3.9	7.0	6.0	4.4	7.7	6.1	4.6	8.8
Glutamate Dehydrogenase (u/L)	15.0	5.0	24.0	17.0	7.0	29.0	21.2	6.0	52.0
Phosphate (mmol/L)	2.3	1.4	3.5	2.9	2.2	3.6	2.1	1.8	2.3
TP (g/L)	38.9	30.0	48.0	47.0	40.0	53.0	43.0	36.0	48.0
Urate (mmol/L)	0.1	0.0	0.2	0.1	0.1	0.1	0.1	0.0	0.2
Heterophils (x109/L)	5.0	3.0	8.6	4.3	3.9	4.8	7.2	2.8	11.0
Lymphocytes (x109/L)	5.2	2.5	8.5	7.9	5.3	10.5	5.8	0.5	11.3
Monophils (x109/L)	1.2	0.0	2.6	1.2	0.4	1.7	0.9	0.0	1.6
Eosinophils (x109/L)	2.0	0.2	3.8	1.7	1.1	2.1	1.1	0.2	2.4
Basophils (x109/L)	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.2
TWCC (x109/L)	13.4	10.0	16.0	15.0	12.0	19.0	14.9	3.5	24.0

Table 5: Effect of eye lesion (presence, absence or undetermined) on blood biochemistry and haematology for 2017.

For 2016-2017, blood results from Upstart Bay, where the eye lesions were most prevalent, there was no association of evidence of clinical pathology with active eye infections (n = 9). Further, there was no difference in any of the measured clinical analytes between positive for eye infection, negative for eye infection and indeterminable (n = 9, 4, and 7 respectively) (Table 5).

Necropsies

Due to low numbers (n = 2), there were no significant diagnoses derived from necropsies. The two cases, both in 2016, can be summarized as:

1. An adult green turtle from Wunjunga was missing its head and flippers with suspected teeth rakes on the soft tissue. Samples were collected and frozen for further analysis if indicated.
2. A prepubescent female green turtle from was found moribund washed up on a Wunjunga beach. She was taken to a local rehabilitation centre, but died two days later with suspected neurological deficits (head tilt). Gross necropsy (by Dr. Ian Bell) indicated mild serous atrophy of fat but otherwise good musculature and organ integrity. No obvious signs of death.

A third turtle that was tagged as part of the program was found stranded shortly after being recorded as caught and tagged by Rivers to Reef. Not cause of death was assigned.

There were no noted links between toxicology, health and presence of eye lesions. Health and toxicology were reported by Villa et al. 2017.

Morphometrics

Growth rates and body condition scores

Growth rates and body condition scores are discussed in Chapter 1- Bell et al. (2018).

In brief, somatic growth rate differed significantly between sites (D= 46.82, $p < 0.01$). Standardised growth rate at Howick was significantly slower than at Cleveland Bay ($\beta = 1.12$, se = 0.22, $t = 5.05$, $p < 0.01$) and Upstart Bay ($\beta = 1.19$, se = 0.19, $t = 6.35$, $p < 0.01$). The final model predicted that a 50 cm CCL green turtle caught in 2010 and recaptured again in 2011 grew at 1.38 cm year-1 at Howick (se: 0.23), 2.50 cm year-1 at Cleveland Bay (se: 0.31) and 2.58 cm year -1 (se: 0.29) at Upstart Bay.

For body condition scores for all age classes, a small but significance difference in slopes was evident between sites, with an estimate of 2.95 (2.91-2.30) for Cleveland Bay (intercept: -8.87), 3.02 (2.96-3.07) for Upstart Bay (intercept: -9.17) and 3.06 (3.02-3.09) for Howick (intercept: -9.34) (Bell et al. 2018- Figure 6).

Site	Age class	Average (SD)
Upstart Bay	Juvenile	5.9 (10)
	Sub adult	5.4 (9.6)
	Adult	2.2 (7.7)
Cleveland Bay	Juvenile	8.7 (12.6)
	Sub adult	14.9 (22.7)
	Adult	3.1 (8.1)

Table 6: Average number of barnacles for each age class at the coastal sites where the highest concentration of sick animals were present between 2014-2017.

Barnacle counts - Barnacle counts are reported in Chapter 1- Bell et al. (2018). In brief, there was a significant difference in barnacle counts between locations across all age classes ($D = 101.8$, $df = 2$, $p < 0.001$). On average, the likelihood of turtles having a high barnacle load was considerably lower at Howick

than Cleveland Bay (-4.11, z= -7.9, $p < 0.001$), and lower at Upstart Bay than Cleveland Bay (-0.57, z= -5.07, $p < 0.001$). The average number of barnacles per turtle was 0.125, 4.77 and 6.58 for Howick, Upstart Bay and Cleveland Bay, respectively.

Barnacle load differed significantly between sites for sub-adults ($D = 92.14$, $df = 2$, $p < 0.001$). Subadult green turtles at Cleveland Bay were the most likely to have high barnacle load, followed by Upstart Bay and then Howick. The model predicted an average of 7.53 barnacles per turtle at Cleveland Bay (se: 0.91), 4.22 barnacles per turtle at Upstart Bay (se: 0.54) and only 0.13 barnacles per turtle at Howick (se: 0.07).

Collating Upstart Bay and Cleveland data to compare age class for barnacle count of noted poor conditioned animals, yielded only 7 animals (4 Upstart, 3 Cleveland) recorded as emaciated between 2014-2017. All reported animals were juveniles. However, 6 out of these 7 fitted the >15 barnacles criteria for being likely to be in poor health.

Comparison of each age class at the compromised coastal sites demonstrated adults were the least impacted age class and sub adults were the most impacted age class (Table 6).

Site	Age class	No. turtles > 15 barnacles	Total # of turtles	%
Upstart Bay	Juvenile	47	455	10.3
	Sub adult	10	102	9.8
	Adult	3	136	2.2
	All	60	693	8.7
Cleveland Bay	Juvenile	51	259	19.7
	Sub adult	18	55	32.7
	Adult	5	62	8.1
	All	74	376	19.7

Table 7: Proportion of poor health turtles across age classes for the coastal sites recorded 2014-2017.

Site	Year	No. turtles > 15 barnacles	Total # of turtles	%
Upstart Bay	2014	10	84	11.9
	2015	15	145	10.3
	2016	15	114	13.2
	2017	-	-	-
Cleveland Bay	2014	12	103	11.7
	2015	17	54	31.5
	2016	15	63	23.8
	2017	7	39	17.9

Table 8: Proportion of poor health juvenile turtles for the coastal sites recorded each year between 2014 and 2017.

Assuming that >15 barnacles meant poor health (Flint et al. 2010a), the proportions for poor health turtles in each age class were divided by the total population for Cleveland Bay and Upstart Bay (excluding missing values recorded as "999" in counts) to express the percentage of turtles in poor health at the two coastal study sites. Across all age classes, Cleveland Bay was in poorer health than Upstart Bay. For juveniles, which have been shown to be in poor health if found with >15 barnacles are found on the plastron, 10% of the population was impacted at Upstart Bay and nearly 20% of the population at Cleveland Bay (Table 7).

For juveniles, comparison of the proportion of animals with more than 15 barnacles on their plastron for each site with each year the study recorded barnacle counts, indicated Upstart Bay maintained approximately equal numbers of poor health juvenile turtles each year while Cleveland Bay showed a rapid peak in 2015 of poor health individuals and a steady decline since that time (Table 8).

There was no noted "time shift" in normal biochemical or haematological values. Comparing the 2015 blood results to 2017 results for common analytes (Table 1), there was a resolution of active stressors, as reflected in the total white cell counts for the two coastal study sites. However the low A:G ratio indicates stressors may still be present, although this is present in Howick Group of Islands samples too, suggesting this parameter should be interpreted with caution. Normal (control- Howick Group of Islands) values did not shift when comparing 2015 with 2017. All were within the normal ranges (Flint et al. 2010a) and comparable to each other, with the caveat that n = 7 in 2017 for the Howick.

These reference indices compared favourably with the published known standards for Australian waters (Flint et al. 2010a) which is included among the wider internationally reported values (Stacy and Innis 2017). The average values for all turtles at each site was taken and an unadjusted maximum and minimum value was calculated to intentionally create the likelihood that anomalous values would skew data outside of the established reference index. By doing so, the only Howick Group of Islands' blood data parameters outside of the published reference indices were UA, Ca, CK and Na (Table 2- highlighted). All of these variations were very minor with no clinical significance and are likely the result of the all-inclusive max-min approach which would be treated as 95% exclusion in any of the commercial reference index calculators. The exception was sodium (Na), which was moderately elevated across all sites, suggesting an analysis error (possibly due to time between sampling and analysis as this analyte is volatile). This is not a physiological response as functional cell sodium concentration is very narrow.

All examined values fell within the middle of these reported ranges suggesting the northern Great Barrier Reef stock sampled population and the southern Great Barrier Reef sampled populations are comparable. This is further supported by the trace element reference indices that show recaptured Howick Group of Islands control sample population fall within the previously established ranges (Villa et al. 2017).

With confidence in the employed reference ranges, analysis of samples from the urbanized Cleveland Bay showed low frequency signs of an active systemic stressor that may have been caused by a myriad of reasons. This type of depressed health is consistent with findings from other catchment draining bays along with Queensland coastline (Flint et al. 2010b, Flint et al. 2015a). Upstart Bay had a marked increase in inflammatory response but no directly identified causative agent. This response was present in a relatively high proportion of animals and was indicative of an active process warranting closer examination. A recheck of blood parameters in these study sites in 2017 to determine the persistence of these stressors indicated both active stressors had resolved with CK levels in Cleveland Bay returning to within normal limits and total white cell counts in Upstart Bay dropping to below those of the control site. For both sites, the total white cell counts were markedly lower between 2014-2015 and 2017. This may have been a result of laboratory error as laboratories changed between Years 1 and 3 of the project; however, the smears were validated by MF and the relationship between the sites (higher in coastal, lower in control) was comparable. The coastal environments showed a normalization of values when compared with the control site, suggesting no active inflammatory process persisted from 2014-2015 to 2017 (Table 1).

da Silva found evidence for a possible relationship between metal contamination with copper, iron and lead and STFP through oxidative stress generation (da Silva et al. 2016). Unfortunately, linking observed contaminant concentrations to their biological significance remains challenging and can be further confounded by other natural events that occurred in these regions such as wide spread flooding which has linked increased strandings to freshwater influxes (Flint et al. 2017). This study attempted to elucidate the effects environmental stressors were having on sea turtles by characterising the inflammatory responses through the use of plasma protein electrophoresis as a potential bio-indicator that could offer a more sensitive indicator to specific toxins (Table 3); i.e. a signature profile that could help pin-point a toxin and its impact (Flint et al. 2015b). All samples were selected based on their known blood levels of heavy metals as per Queensland Alliance for Environmental Health Sciences' data and previous known biochemical and haematological profile. Only one of the seven animals with depressed pre-albumin had elevated enzymes associated with liver pathology (QA42616) that could be associated with heavy metal toxicoses; resulting in a failure of the PPE technique to pinpoint specific stressors or an association of heavy metal intoxication. Plasma protein electrophoresis did not prove to be a good indicator of contaminant burden for alpha, beta and gamma-globulins or for albumin fractions in the examined samples.

However, in the same year, Villa and colleagues did demonstrate turtles from Upstart Bay had elevated heavy metal loads, including cobalt at levels greater than those recorded previously for any vertebrate species and correlated these with serum biochemical indicators that indicated systemic stressors (Villa et al. 2017). This suggests that serum biochemistry is still a reliable and currently best available parameter that can be associated with heavy metal burdens in sea turtles.

The emergence of a new potential pathology among sea turtles within the affected Bays causing eye lesions was identified. Its across study site prevalence was only 7.5% in 2014 and 3% in 2015, before elevating to 14% in 2016 and 23% in 2017 (Table 4). However, despite this marked increase in positive cases, the proportion of negative cases remained approximately equal over time and the proportion of inconclusive cases decreased. This may be due, in part at least, to increased vigilance recording and photographing eye lesions at the same rate throughout the whole study as opposed to disease outbreak. Regardless, these eye lesions warranted investigation and causes determined.

Eye lesion presentation ranged from mild to severe (Figure 1) and included cellular debris, rafts of bacteria and evidence of ulceration. No specific aetiology of the lesions was identified through histology or microbial culturing. There was no indication of viral involvement. However, this disease syndrome cannot be discounted as a potentially serious issue among coastal sea turtles of Northern Australia. Two people working with these animals during the time of escalating positive cases developed coincidental drug sensitive Staphylococcus aureus infections immediately post field work with these animals. This bacterium was not isolated from any of the examined turtle eye lesions and cannot be linked to the human counterpart outbreak, but caution should be used when dealing with animals diagnosed with these lesions. Further, for turtles with, without and inconclusively with eye lesions, we examined biochemistry and haematological parameters (Table 5). There was no relationship between the presence or absence of lesions and clinical health; suggesting the eye lesions were either non-infectious, viral (and not detected on clinical pathology) or chronic resulting in no noted clinical changes.

From Chapter 1, it was demonstrated there was an inverse growth relationship with Howick Group of Island turtle growing slower than the two coastal site turtles. Reasons for this could include dietary differences or compensatory growth from the flooding events of 2010 as well as many other reasons, but this could not be correlated with our measured health parameters taken between 2014/15-2017.

Also from Chapter 1, there was no detectible relationship between health status and body conditions score. Data implied Howick Group of Island turtles had a consistent body condition whereas Upstart Bay had poor conditioned juveniles and good conditioned adults and Cleveland Bay having the opposite. These trends were non-significant and minor. No site demonstrated the population was in peril. For a more finite examination of proxies of health status, we examined barnacle counts.

Previous studies have demonstrated the likelihood of being in poor health is greater when more than 15 barnacles are present on the plastron of juvenile green sea turtles as opposed to less than 15 barnacles. This serves as a quick non-invasive proxy for health. The low values seen in this study cannot be aligned clinically when looking at the sites as they are currently presented. However, the barnacle count was developed to be a proxy of the health status of individuals, not an entire population. This is because usually only a small percentage of any population is sick and group level results can hide disease outbreak patterns. However, by breaking down the population into age class to determine which cohorts may be impacted, some rudimentary patterns develop (Table 6). As a population, nobody is clinically sick, but adults were the least impacted age class and sub adults were the most impacted age class. The caveat to this was barnacle count scales were developed only for juvenile green turtle age class from two inshore embayment populations (Flint et al. 2010a). Sub adult and adult interpretations are assuming the same relationship holds true.

To compare age classes, this study looked at age against each site (Table 7). Across all age classes, Cleveland Bay turtles were in poorer health than Upstart Bay turtles based on barnacle count alone. As this is not validated, it cannot be over-interpreted, but for juvenile green sea turtles, which have demonstrated this >15 barnacles relationship, this approach showed that 10% of the population was impacted in Upstart Bay and nearly 20% of the population at Cleveland Bay study sites. Although there are no set levels as to what constitutes a population disease outbreak rate, this is above what would be expected for a "normal" population (e.g. in herd health management of cattle and sheep the aim is less than 5% of the herd being ill) and suggested a potential population of sick animals.

Taking this analysis one step further, this study compared only juvenile turtles (as they composed the bulk of the data and it has been demonstrated that more than 15 barnacles is a strong proxy of poor health) for each year of the study and found that the populations were not getting sicker or totally recovered (Table 8). It appeared Cleveland Bay had the highest proportion of sick juvenile green sea turtles in 2015 and returned to above ideal (compared with an arbitrary 5-10% of population) but more "normal" values and Upstart Bay remained mildly elevated.

Conclusions

Overall, the health component of this study suggested that coastal turtle populations were under active stressors, most likely caused by anthropogenic effectors such as chemical pollutants, when initially examined in 2014. Cleveland Bay was greatly impacted (by tentatively shown toxic insult) and biomarkers were elevated above what we would consider a normal ratio of unhealthy: healthy population. This impact lasted 2015-2016 but there was evidence by the end of 2017 the population was returning to normal, albeit still above that which we would expect for a normal functional wildlife population. This was based on blood results from this period which showed elevated CK in Cleveland Bay, which was indicative of an acute insult/ catabolic state and mildly elevated total white cell counts in Upstart Bay which was consistent with a persistent potentially subclinical stressor. These conclusions were further supported by barnacle counts indicating the health status of juveniles to be poorer than would be expected for an arbitrary normal population. In these turtle populations, a clear link between turtle health and heavy metals was demonstrated when examining serum biochemistry.

Furthermore, closer investigation suggested these chronic stressors may have been manifesting as ocular lesions. Such manifestations may impact the ability to forage (reduced sight) and reproduce (poor health leading to poor condition resulting to reduced fecundity). During the trip where escalating positive cases of eye lesions were recorded, at least two people working with these infected animals had skin infections caused by S. aureus. The zoonotic potential of this syndrome should be considered.

We are now looking at a recovering population (Cleveland Bay) that warrants monitoring and an ongoing stressed population (Upstart Bay) that warrants elucidating.

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Chapter 3

Metal screening in turtle forage, sediment, sea water and river water of the Great Barrier Reef

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3. Metal screening in turtle forage, sediment, sea water and river water of the Great Barrier Reef

Colette R. Thomas[1,2,*], Will Bennett[3], Andrew Simmonds[4], Ryan Turner[5], Carol Honchin[6], Christine A. Madden Hof[7], Ian Bell[8], Jon E. Brodie[9,10,11]

[1] Centre for Tropical Water and Aquatic Ecosystem Research (TropWATER), James Cook University, Townsville 4811
[2] SEED Science, Townsville 4810
[*] Corresponding author: colette@seedscience.com.au
[3] Environmental Futures Research Institute, School of Environment and Science, Griffith University, Gold Coast, 422
[4] Torres Strait Regional Authority, Thursday Island, 4875
[5] Department of Environment and Science, Queensland Government, Dutton Park, 4102
[6] Great Barrier Reef Marine Park Authority, Townsville, 4805
[7] World Wide Fund for Nature-Australia, Brisbane, 4000, Australia
[8] Aquatic Species Program, Queensland Department of Environment and Heritage Protection, Townsville, Queensland 4810, Australia
[9] ARC Centre of Excellence for Coral Reef Studies, James Cook University, Townsville, 4811, Australia
[10] C2O Consulting, Townsville, 4810
[11] formerly TropWATER, Townsville

Abstract

Green turtles are iconic residents of the north Australian coastline. Estuarine and coastal systems in Australia, as elsewhere, are in a state of continuing deterioration from a range of complex coastal pressures. The exposure to and impact of pressures such as metal loads on turtle health is poorly understood, and metal concentration data for Australian tropical coastal and offshore waters frequented by turtles are limited. Turtle forage is a critical exposure pathway of chemicals to green turtles. Environmental concentrations of 24 metals in above-ground forage were assessed at three green turtle foraging locations in the Great Barrier Reef and Burdekin River water over three consecutive years from 2014-17. Metal concentrations in sea water and surficial sediment were also measured in 2014. Most metals were present at significantly higher concentrations in coastal compared to offshore forage, reflecting terrigenous inputs. Co and Al exceeded marine water quality guidelines at all locations, and mean dissolved Al, Cu, and Zn exceeded freshwater guidelines for river water. Marine sediment guidelines were not exceeded but As and Fe were enriched at coastal sites relative to background. Mean forage concentrations of Al, As, Fe, Mn, U, and V were higher than reported in recent literature reviews for similar forage species. The contribution of anthropogenic activity to current forage metal loads and the potential impact of observed loads to forage ecosystems cannot be clearly understood without first identifying local metal baseline reference values for forage species. Metals may require routine monitoring to establish reliable estimates which allow trends in contamination to be confidently detected and promptly addressed.

Keywords:

Integrated assessment, Seagrass, Rhodophyta, Biomonitoring , Bioaccumulation

Introduction

Environmental pressures such as physical disturbance, resource exploitation, and the release of materials such as nutrients, sediment, and other organic and inorganic substances has led to widespread deterioration of estuarine and coastal systems at multiple scales (Lotze et al. 2006; Duarte et al. 2015). These pressures can occur separately or simultaneously, and their impacts can be cumulative (Bevilacqua et al. 2018). Despite its World Heritage status, the Great Barrier Reef (GBR) has not escaped this trend (Brodie et al. 2012). A raft of policy instruments is in place which aims to improve, directly or indirectly, the status and condition of the GBR's interconnected estuarine, coastal, and reef ecosystems (Brodie and Pearson 2016). However, multiple interacting stressors including a dynamically changing climate and near-daily emergence of new contaminants (Gwenzi et al. 2018, Kroon et al. 2015) make ecological events (whether ongoing, rare, idiosyncratic, or catastrophic) hard to prevent or diagnose.

The degradation of coastal habitats is increasingly associated with the increased delivery of organic and inorganic material (Sobek et al. 2016; de Souza Machado et al. 2016), however traditional measurements of these materials are confined to a targeted suite which rarely includes metals and metalloids (hereafter 'metals'), except when required for permitting purposes or special research projects. The most recent data for metal concentrations in tropical Australian coastal and offshore surface waters were collected almost two decades ago for a handful of metals (Munksgaard and Parry 2001, Esslemont 2000). Metals provided one focal target within a broader research project into the effects of coastal pollutants on green turtle health.

Metals are natural and ubiquitous components of the environment. Neither created nor destroyed in the environment, they undergo continuous (uneven) redistribution throughout both the Earth's crust and near-surface (surficial) rocks, soils, and sediments. 'Geoavailability' refers to the ease with which metals are remobilised in surficial material and depends on total metal content and access to weathering agents and processes (Smith and Huyck 1999). Land runoff and atmospheric deposition are the primary natural inputs of metals to the marine environment (Pacyna et al. 1995; Pacyna and Pacyna 2001). In addition, dust from catchments and volcanic activity can release metals into the atmosphere, which later deposit onto the land or sea. Historical and current climate, land use, volcanic activity, and parent rock geochemistry combine to determine the unique natural geochemical profile of a given region.

Anthropogenic activities such as mining, smelting, and discharge of industrial waste can alter the natural distribution and abundance of metals in surficial environments and can alter patterns of surficial metal geoavailability and bioavailability. For example, drainage and reclamation of intertidal sediments and acid mine drainage can enable development of acid sulphate soils, which mobilise metals such as Al, As, Ni, Mn, and Zn and increase their bioavailability (Johnston et al. 2010). Similarly, dredging and resuspension of fine-grained sediment can release dissolved (bioavailable) metals even beyond the dredging period (Hedge et al. 2009; Kalnejais et al. 2010). 'Bioavailability' broadly refers to the degree to which a substance is free to move into or onto biological organisms (Smith and Huyck 1999). A metal's bioavailability is determined by its geoavailability and its capacity to be physically dispersed and chemically mobilised (Smith and Huyck 1999).

Seagrasses and macroalgae are capable of accumulating metals at several orders of magnitude higher than their surrounding environments and are often used as bioindicators of metal pollution (Bonanno and Orlando-Bonaca 2018). Turnover rates can influence availability and impact. Compared with turtle forage (primarily seagrass and macroalgae) and sea water, surficial sediment is expected to have a relatively high uptake and binding affinity for metals, and relatively low turnover and dispersal rate. Sediment levels of metals are expected to vary over annual-decadal timeframes. Green turtles feed on seagrass, macroalgae, mangrove fruits, jellies, bluebottles, dead fish and small crustaceans. Seagrass and macroalgae forage species (hereafter 'forage') are sessile and have variable but considerable capacity for metal uptake and binding (Bonanno and Di Martino 2016; Bonanno and Orlando-Bonaca 2018). For them, metals are expected to vary at moderate time scales (e.g., monthly). Sea water levels of metals are dynamic and expected to fluctuate with daily changes in currents and tides. The rapidly changing conditions and lower contact exposure of sea water is expected to present a lower contamination load to green turtles than surficial sediment or forage.

Turtles ingest large volumes of forage, which can accumulate metals. In coastal environments the capacity of marine phytobenthic species such as seagrasses and macroalgae are increasingly being considered as bioindicators of trace elements in sediment and sea water (Bonnano and Orlando-Bonaca 2017). In phytobenthos and other organisms, some metals are essential for growth and homeostasis, but all metals are potentially toxic at high concentrations (de Souza Machado et al. 2016; Babula et al. 2008). Bioaccumulation in forage creates a critical exposure pathway for metals to green turtles, and forage metal loads comprise the focus of this work.

During the winter of 2012 and into 2013 in Upstart Bay, north-central Great Barrier Reef, a mass stranding and mortality event was recorded for green turtles (*Chelonia mydas*; DEHP 2012; Brodie et al. 2014). The source and potential recurrence of the stranding was unknown and remains unlikely to

be identified with certainty. We investigated the prevalence of as many metals and metalloids (hereafter 'metals') as possible from turtle habitat, both at the site of the stranding and at two other locations that appeared to be unconnected to the stranding event. Investigations into environmental concentrations of organic contaminants are reported elsewhere (Gallen et al. in prep), as are the investigations into metal and organic concentrations in turtle tissue (Heffernan et al. 2017, Vijayasarathy et al (in prep), Villa et al. 2017).

The objective of this study is primarily focussed on screening green turtle habitat matrices (i.e. sea water, sediment, forage) for as wide a range of metals as possible to; 1) integrate with data on metal loads in turtle tissues reported by Heffernan et al. (2017), Vijayasarathy et al (in prep), and Villa et al. (2017) so that potential relationships between internal (turtle) and external (turtle habitat) loads can be identified, and 2) as a screening assessment of metal prevalence in selected GBR coastal habitats. We also screened metal concentrations in river water from the primary river discharging to these coastal locations. While we do present results within the context of selected local and global data, the study does not investigate the toxicity or potential source of any metal observed in any matrix.

Materials and methods

Three marine study locations were selected (refer Executive Summary, Figure 1). Upstart Bay (UPB) was the site of the green turtle mass stranding event in 2012 and 2013 and was the main investigation location for the turtle toxicity studies. Cleveland Bay (CLV) is two bays to the north of UPB; both bays are influenced by the northward-flowing material that is routinely discharged from the Burdekin River and thus share similar geochemical source environments (Table A1), albeit CLV to a lesser degree (Bainbridge et al. 2012). A group of offshore reefs, cays, and islands in the northern Great Barrier Reef provided a near-pristine comparison location (Howick Group of Islands; HWK).

The Great Barrier Reef is a mixed siliciclastic-carbonate system, in which northward-facing bays and intertidal zones protect fine sediments from wave action and dispersal by trade winds (Doherty et al. 2000, Orpin et al. 2004). From the coast to the offshore shelf, zones of sedimentation exist along a gradient from terrigenous siliciclastic inner shelf sediments to carbonate reefal sands on the outer shelf (Orpin et al. 2004). Inner shelf sediments are significantly higher in clay and quartz and lower in carbonate than outer-shelf sediments (Bannister et al. 2012). UPB and CLV are inner-shelf embayments dominated by mud and clay (Doherty et al. 2000, Orpin et al. 2004), whereas HWK is an outer-shelf group of reefs and islands dominated by carbonates. These sedimentary environments are expected to influence the geochemical profile of sediments, as well as forage and sea water.

The Burdekin catchment has several major sub-catchments (Table A2) with varying size, discharge characteristics, land-uses, and geology (Table A4). The coastal bays receive drainage and runoff water from catchments characterised by different land-uses: UPB primarily from agricultural activities on the Burdekin Catchment and CLV from Burdekin agriculture as well as inputs from urban and industrial sources (Figure A3, Table A1). HWK is offshore and remote from human land-use activities. Land use in the Burdekin Region is dominated by cattle grazing which extends over 90% of the region (Figure A3). A nickel/cobalt mine operated at Greenvale until recently, two coal mines operate in the eastern part of the catchment (in the Bowen sub-catchment near Collinsville), and a gold mine operates at Ravenswood. Extensive areas of both ancient and recent basalts are present in the Burdekin catchment (Figure A4), with old basalts in the eastern sub-catchments (e.g. Bowen) and recent basalts in the north and north-west. Soils derived from basaltic ricks are enriched in minerals including cobalt (Turekian and Carr 1961). Areas enriched in cobalt and nickel include the Greenvale area (Figure A3).

During the first year (Aug 2014 – Aug 2015) a pilot study was conducted to assess metal levels in sediment, above-ground forage, and water at all locations. A screening approach was adopted, in which 24 metals were assessed in sediment and forage samples and 12 metals were assessed in sea water using passive samplers (Diffusive Gradients in Thin Films; DGT). Sampling and analytical efforts in the following two years focused on forage, however river water samples are also reported for the full term.

Sample collection

Within each location, the primary feeding habitat for green turtles (seagrass meadows) was chosen as the sampling area. Samples were collected over a period of three consecutive years (late 2014 to early 2017). A combination of sediment, forage (seagrass and macroalgae), and river water grab sampling, and passive sea water sampling techniques were used.

Forage samples were collected at three points along a 50m intertidal transect at three sites per location. Samples were collected in July-August 2014-2016 at HWK, and at UPB and CLV in September/October 2014-2016 and May/June 2015-2017. At each location five representative samples of forage were collected from three sites 50m apart. Seagrass and macroalgae leaves (if present) were torn by hand from roots/rhizomes using gloves and rinsed in sea water to remove loose sediment. Epiphytes were not separated from seagrass or macroalgae, but visible faunal species, shells, and detritus were removed prior to storage and analysis. Samples collected in the first round were pooled in glass jars in the field. Subsequent samples were stored individually, and two replicates were pooled for analysis. In some years there was no seagrass at reef-flat sites (HWK), but turtles were observed feeding on macroalgae (predominantly red macroalgae *Laurencia* and *Graciliaria* species). Macroalgae samples were collected and analysed following the same analytical protocol used for forage collected in seagrass meadows. Samples were stored on ice during transport and frozen within 12 hours of collection.

Sediment was collected adjacent to seagrass sampling points in the late 2014 sampling round. Samples were collected with gloved hands from the top 5 cm of sediment and pooled in glass jars. Visible plant and animal material was removed prior to analysis. Samples were stored on ice during transport and frozen within 12 hours of collection. Samples were taken from archived vibrocore USB-1CO, collected in 2012 from northern Upstart Bay offshore from the mouth of the Burdekin River by Lewis et al. (2014). The core had been stored, sealed and unopened, at the JCU Earth Sciences Laboratories at 4°C. The core was halved using a circular saw dedicated to this purpose. Care was taken to immediately remove any shards of the aluminium core tube during sample collection. Aside from the circular saw, plastic tools were used throughout the sample collection process. This core displayed the same two distinct layers as described by Lewis et al. (2014) for the original USB-2CO core; comprising an upper ~1.5m section characterised by a 'coarsening upward sand unit containing lenses of clay and organic material (wood and plant) and varying amounts of muscovite mica', and the lower ~1m section characterised by 'stiff organic-rich silty clay with occasional lenses of muscovite-rich fine to medium sand'. Slices were collected at four stratigraphic locations matching those at which radiocarbon and Optically Stimulated Luminescence (OSL) ages have previously been determined (original core USB-2CO, refer to Lewis et al. 2014 for detail).

Three DGT samplers were deployed at each site in the first (late 2014) sampling round. DGT samplers with a Chelex 100 - Metsorb mixed binding layer were prepared as described by Panther et al. (2013). DGT samplers remained in their plastic containers at 0-4°C until just prior to deployment. To deploy DGT samplers, the sampler holder was removed from the plastic container using a clean pair of latex gloves and attached to a rope, buoy or fixed structure using plastic cable ties. The surface of the DGT samplers was not touched or allowed to contact anything that could cause contamination. DGT samplers were submerged at least 20 cm below the surface of the water, at a depth that ensured they would remain submerged during the entire tidal cycle. Temperature loggers were deployed alongside samplers to record water temperature. DGT samplers were deployed no longer than five days to prevent biofouling of the sampler surface, which can influence the accuracy of the measurement. Samplers were retrieved by cutting cable ties and removing the sample holder from the water using a clean pair of latex gloves, without touching the sampler surface. The samplers were immediately rinsed (in their sample holder) with a fast stream of Milli-Q deionized water to remove debris and returned to their containers for transport to the lab. Trays were stored on ice during transport to the lab then frozen until analysis.

River water was sampled once per year between 2014 and 2016, during the traditional Burdekin River flood season (December to March). Samples were collected from Home Hill, close to the Clare gauging station (Figure A3), which is the station closest to the mouth of the Burdekin River that can be routinely sampled during flood and where regular sampling is carried out for suspended sediment, nutrients, and

pesticides (e.g. Turner et al. 2013). The only significant river flow to occur during the project period at this station occurred in January 2017, and samples were collected at that time (Figure A5).

Sample Analysis

Above-ground Forage

Forage samples were processed for total metals using microwave assisted digestion in a CEM MARS 6 system, as per EPA Method 3052. Briefly; forage was first washed in deionised water to remove loosely-adhered particulate matter then oven-dried at 60°C. Dried samples were ground in an agate mortar and pestle and homogenised. A 0.25 g sample of dried and ground forage was directly weighed into a TFM PTFE digestion vessel, followed by the addition of 9 mL concentrated nitric acid, 2 mL of concentrated hydrochloric acid, and 1 mL of concentrated hydrogen peroxide. Vessels were placed into the microwave system and heated to 180°C, maintained at 180°C for at least 9.5 min, and then allowed to cool. Visual inspection of the digestion solution confirmed complete dissolution of the sample, with no solid material remaining. An aliquot of each sample was diluted 50-fold with Milli-Q water prior to analysis by ICP-MS, as described in the previous section.

Surficial Sediment

Sediments collected in 2014 were processed for both total recoverable metals and dilute-acid extractable metals. Metals extracted with weak acids correlate with the bioavailable fraction, and these methods are recommended for sediment metals data intended for application in anthropogenic or contaminant enrichment factor calculations (Birch 2017). Total recoverable metals were obtained by microwave-assisted extraction in a CEM MARS 6 system, as per EPA Method 3051A. Briefly, up to 0.5 g of homogenised wet sediment was directly weighed into a TFM PTFE digestion vessel, followed by the addition of 9 mL concentrated nitric acid and 3 mL concentrated hydrochloric acid. Vessels were placed into the microwave system and heated to 175°C, maintained at 175°C for at least 4.5 min, and then allowed to cool. Extraction solutions were transferred to polypropylene tubes, centrifuged, and an aliquot of the supernatant transferred to a polypropylene tube and diluted 50-fold with Milli-Q water. Dilute-acid extractable metals were obtained by extraction of wet sediment with 1 mol/L HCl (Instrument Quality, Seastar) in a sediment : acid ratio of 1:50, for 1 h at room temperature (Simpson et al. 2016). Diluted extract solutions were analysed for metals with an Agilent 7900 ICP-MS operated in collision mode with He as the cell gas for kinetic energy discrimination (KED) interference removal. Concentrations were converted from wet weight to dry weight using the measured moisture content of each sample obtained by weighing a sub-sample of wet sediment, oven-drying at 105°C to a constant weight, and reweighing. Independent quality control solutions (10 µg/L) were analysed regularly throughout each analytical run. A certified reference material of stream sediment (NCS DC 73309) was analysed (n=6) to verify the efficiency of the total recoverable metals procedure and the accuracy of the ICP-MS analysis. Recoveries were between 84 – 119% of certified values which are within acceptable limits. Core samples were analysed for weak-acid extractable metals (AEM) as described for surficial sediment. Slices were also analysed for total recoverable metals (TRM), loss on ignition (LOI), and percent organic carbon and carbonate content. (Heiri et al. 2001).

Sea Water

DGT samplers collected in 2014 were disassembled inside a Class A laminar flow hood, located in a Class B clean room, to minimise trace metal contamination. The binding gel from each DGT sampler was eluted first in 1 mL of 1 mol/L ultra-pure HNO_3, followed by 1 mL of 1 mol/L NaOH. Eluents were combined, diluted 10-fold with 2 % ultra-pure nitric acid, and analysed by ICP-MS as described previously (Panther et al. 2013). Time-integrated concentrations were determined using the DGT equation, as described previously (Zhang and Davison, 1995).

River Water

Each sample was sampled according to the Australian Standard: AS/NZS 5667.1:1998 (Standards Australia, 1998a, 1998b). All samples were passed through a 0.45 µm cellulose acetate filter (Sartorius)

to obtain an estimate of the dissolved metal concentrations. Samples were preserved with ultrapure nitric acid to a final sample concentration of 1% (Eaton et al. 2005) and then refrigerated at 4°C till analysed. All samples were sent to a laboratory accredited by the National Association of Testing Authorities (NATA) for chemical analysis, and therefore, complied with ISO 9001 and ISO17025 as a minimum.

Ambient water quality sampling occurred monthly with an increased frequency of sampling during high flow conditions. Samples were collected between July 2014 and June 2016 and samples were analysed using a Thermo-Fisher, x-series, inductively coupled plasma-mass spectrometer (ICP-MS) (Thermo Electron Corporation, 2004; Eaton et al. 2005) for Ag, Al, As, Ba, Cd, Co, Cr, Cu, Fe, Mn, Mo, Ni, Pb, Sb, Se, Tl, U, V, and Zn. Surface water metal concentration data were compared to the ANZECC and ARMCANZ (2000) surface water quality trigger values using the 95% level of ecosystem protection for slightly-to-moderately disturbed ecosystems.

Statistical Analysis

Metal concentration data in forage and sediment typically were not normally distributed, nor were variances equal for any of the three locations. We used Welch's ANOVA (one-way) with a homogeneity test prior to the analysis with the Games-Howell post-hoc test for multiple comparisons. All statistical tests were performed in IBM SPSS Statistics 24.0. Censored data were always statistically analysed as 50% of the LOD.

Enrichment Factors

These background sediment metal concentrations were also used to calculate Enrichment Factors. Enrichment Factors (EFs) simplify the comparison of sample and source material Me / Re ratios by normalising the sample ratio to the source ratio, as shown in Equation (1), where Me = any metal of interest and Re = the reference metal, usually Al or Fe. Each background sample was assessed to identify the most abundant metal for application as the reference metal in Enrichment Factor and MPI calculations (described further in Results).

$$EF = \frac{\left(\frac{Me}{Ref}\right)_{sample}}{\left(\frac{Me}{Ref}\right)_{background}}$$

Normalisation also reduces the confounding that arises from variable grain size, which is not otherwise accommodated in the calculation (Brady et al. 2015). Using Al as an independent variable also normalises for differences in mineralogy by preferentially leaching mineral phases such as clays and oxides that have a natural affinity for metals (Ward and Larcombe 1996). Metals for which EFs are greater than five are considered indicative of potential pollution (Brady et al. 2015). Implementation of the EF approach requires valid background geochemistry values. Censored data (below the limit of detection; LOD) were converted to half the LOD value prior to use.

Enrichment Factors (Equation 1(1)) are used to calculate the Modified Pollution Index (MPI; Birch et al. 2017, Brady et al. 2015). The MPI allows for non-conservative behaviour of sediments by elementally normalising the data to account for the impact of one high-concentration metal from the tested suite (Equation 2).

$$MPI = \sqrt{\frac{(EF)^2 + (EF_{max})^2}{2}}$$

Bioaccumulation Factors

Bioaccumulation factors (BAFs) were calculated for forage relative to sediment at each location, and forage metal concentration values were compared to the literature. Bioaccumulation Factors (BAFs) were calculated for forage from sediment using mean values, as shown in Equation 3:

$$BAF = \frac{C_{forage}\ (\mu g.g^{-1}\ DW)}{C_{site\ sediment}\ (\mu g.g^{-1} DW)}$$

Results and discussion

Metal concentrations were compared to available data or guidelines for all matrices tested. National water quality guidelines were used to identify exceedances in river water, sea water, and sediment samples. Sediment quality guidelines are not available for most metals, so site-specific guideline values were also determined. In the absence of guideline values, metals concentrations that are ≥2-fold higher than the accepted median natural background (reference) value can be treated as site-specific exceedances of sediment quality guidelines (Simpson et al. 2013).

Above-ground forage

Of the 22 metals tested in above-ground forage in 2014, Hg was below analytical limits of detection at all locations (data not shown). Five metals (Mn, Mo, Sb, Sr, and Zn) detected in UPB were within 20% of CLV forage values. All other metals detected in Upstart Bay forage were at least 20% higher than CLV forage. The highest values were observed for Cd, Co, and Fe. Based on the 2014 results, the following years of the project focussed on developing a better understanding of metal levels in turtle forage at all locations.

Given the environmental persistence and bioaccumulation of Hg, and the consistent non-detection of this metal in sediment or forage at any location, Hg was dropped from the analytical screening suite. A further five metals (Cs, Sn, Ti, W, and Zr) were added to broaden the scope of the screening suite to 26 metals. Acceptable recoveries for CRMs are typically 80-120% of the certified value (Table 4). For Al and Ti, this is not achievable without including hydrofluoric acid in the digest solution, which is not necessary for most metals and is hazardous to handle. Given these constraints, reported recoveries for these elements are within expected ranges. In this study W and Zr had certified values below the LOD and since recovery is not reliable for these metals they are not reported further (see Table A2).

Total metal concentrations were obtained for 24 metals in above-ground forage in the three years between late 2014 to early 2017 (Table 1, Figure 1). Summary statistics are provided in . Significantly higher metal concentrations were detected at coastal locations (CLV or UPB) compared to the offshore location (HWK) for Ag, Al, As, Ba, Co, Cs, Cu, Fe, Mn, Mo, Ni, Pb, Se, Sn, Th, Ti, V, and Zn. Arsenic (As) was significantly ($p < 0.05$) higher in CLV forage compared to UPB and HWK. Metals Sr and U were significantly ($p < 0.001$) higher at HWK compared to UPB and CLV, which is not unexpected for marine metals. U was also higher ($p < 0.001$) in UPB compared to CLV (Table 1, Figure 1). U enrichment can result from mineral sands (Brady et al. 2015), indicating that UPB samples may have higher marine sediment content.

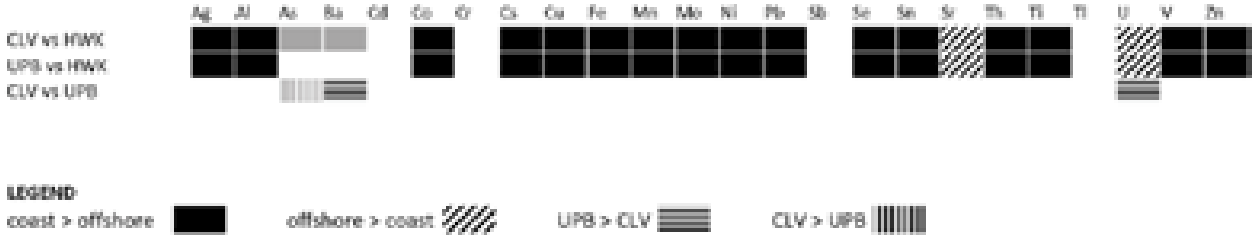

Figure 1: Map of significant differences between metal concentrations in forage collected from each location, using data from Table 1. Black cells are significant at $p < 0.001$ and grey cells are significant at $p < 0.05$

Comparison of forage metal concentrations with other global data provides an indication of whether turtles feeding in the study site are likely to be exposed to higher levels of metals than other turtle populations. Two recent comprehensive reviews of metals in seagrass and macroalgae were used to compare our results (Table 2) and these show variations of several orders of magnitude for some metals (e.g., Al, Fe). We consider only red macroalgae data, since turtles were directly observed feeding on red macroalgae. We report maximum concentrations of As, Fe, Mn, U, and V which are 3.5, 9.0, 2.3, 13, and 2.0 times higher respectively than maxima reported in the literature reviews (Table 2). The selected reviews contained no information on Cs, Th, Ti, or Tl, thus concentrations of these metals in GBR forage relative to other regions remains unknown. All other metals tested in this study were within ranges reported in Table 8, which cover a variety of exposure contexts.

	Ag	Al	As^	Ba	Cd	Co*	Cr^	Cs	Cu*
Howick	0.017 ± 0.013^{x,y}	3,100 ± 1,600^{x,y}	6.2 ± 5.8^{a}	7.6 ± 2.2^{a,x}	0.22 ± 0.083	0.54 ± 0.22^{x,y}	5.4 ± 1.8	0.29 ± 0.15^{x,y}	2.4 ± 3.1^{x,y}
Cleveland	0.057 ± 0.027^{x}	8,300 ± 5,500^{a,x}	14 ± 19^{a,b}	12 ± 7.0^{a}	0.17 ± 0.063	4.7 ± 1.2^{x}	5.6 ± 3.7	0.79 ± 0.50^{x}	5.6 ± 1.7^{x}
Upstart	0.067 ± 0.044^{y}	8,700 ± 7,600^{y}	5.1 ± 2.8^{b}	11 ± 8.6^{x}	0.16 ± 0.10	5.1 ± 2.1^{y}	6.0 ± 5.0	0.69 ± 0.64^{y}	6.0 ± 2.8^{y}

	Fe*	Hg	Mn*	Mo*	Ni*	Pb	Sb	Se	Sn
Howick	1,700 ± 800^{x,y}	<0.029	34 ± 7.6^{x,y}	0.89 ± 0.52^{x,y}	3.1 ± 0.88^{x,y}	1.1 ± 0.32^{x,y}	0.23 ± 0.090	0.56 ± 0.24^{x,y}	0.14 ± 0.065^{x,y}
Cleveland	7,800 ± 5,100^{x}	<0.029	650 ± 220^{x}	2.9 ± 1.8^{x}	5.3 ± 2.3^{x}	3.9 ± 1.8^{x}	0.22 ± 0.077	1.2 ± 0.63^{x}	0.37 ± 0.21^{x}
Upstart	6,300 ± 5,200^{y}	<0.029	640 ± 270^{y}	2.2 ± 1.7^{y}	5.6 ± 4.1^{y}	3.4 ± 2.5^{y}	0.23 ± 0.091	1.0 ± 0.73^{y}	0.30 ± 0.25^{y}

	Sr^	Th	Ti	Tl	U^	V	Zn*
Howick	3,200 ± 730^{x,y}	0.62 ± 0.25^{x,y}	81 ± 40^{x,y}	0.052 ± 0.019	3.1 ± 1.0^{x,y}	7.3 ± 4.1^{x,y}	7.2 ± 4.7^{x,y}
Cleveland	320 ± 220^{x}	1.8 ± 1.2^{x}	170 ± 81^{x}	0.049 ± 0.031	0.84 ± 0.28^{x,z}	18 ± 9.4^{x}	22 ± 7.2^{x}
Upstart	280 ± 130^{y}	1.5 ± 1.2^{y}	220 ± 140^{y}	0.063 ± 0.047	1.3 ± 0.68^{y,z}	15 ± 11^{y}	22 ± 13^{y}

Table 1: Differences between mean total extractable metal concentrations in forage (µg/g DW ± std. dev.) between locations in 2014-2016; x, y, z = p < 0.001; a, b = p < 0.05; *essential elements; ^marine metals. Mercury (Hg) was only monitored once, in 2014.

A meta-analysis is recommended to provide more information about exposure context of global seagrass and macroalgae metal concentration data for the purposes of better identifying levels of potential concern that are relevant to the Great Barrier Reef. For example, Govers et al. (2014) undertook a global meta-analysis of leaf metal concentrations in seagrass for polluted and unpolluted sites (as defined in the papers they accessed for their study). They determined significant statistical differences between 'polluted' and 'unpolluted' sites for several metals, and these are reproduced in Table 3. Comparing the data in Table 3 with location mean values obtained in our study identifies exceedances of reported 'unpolluted' mean + s.d. values for Fe at all three locations. High Fe can be indicative of higher exposure to terrigenous sediments, it is possible Govers' data were derived from predominantly carbonate habitats, which naturally have lower Fe.

By contrast, Conti et al. (2015) report 'baseline' concentration ranges for metals from sites that are not necessarily unpolluted but considered unaffected by anthropogenic activity, and often used as reference sites.

	Seagrasses		Red macroalgae^		Forage	
	Brito	Wilkes	Luy	Bonanno	This study	This study >max
Ag	ND	ND	0.52 – **1.63**	ND	0.014 – 0.15	
Al	ND	ND	20 – 151	19.0 – 149	830 – 37,000	HWK CLV UPB
As^	0.2 – 25.0	1.4 – 7.1	0.89 – 9.44	0.97 – 31.0	1.2 – **108**	CLV
Ba	ND	ND	ND	25.4 – **181**	2.2 – 43	
Cd	0.09 – **541**	ND	1.08 – 4.21	0.01 – 16.9	0.039 – 0.47	
Co*	ND	0.91 – **61.6**	1.56 – 4.49	0.03 – 8.52	0.24 – 10	
Cr^	0.07 – **149**	0.05 – 37	0.16 – 0.60	0.17 – 26.0	0.43 – 26	
Cs	ND	ND	ND	ND	0.066 – **3.1**	n.a.
Cu*	1.6 – **56.0**	0.4 – 44	7.5 – 22.9	1.44 – 45.2	1.1 – 16	
Fe*	ND	186 – 2,676.9	41 – 179	36 – 1,610	550 – 24,000	HWK‡ CLV UPB
Mn*	ND	ND	42 – 93	11.8 – 757	20 – 1,700	CLV UPB
Mo*	ND	28.5 – **133**	1.19 – 27.03	0.29 – 2.17	0.21 – 7.5	
Ni*	0.2 – 48.73	1.17 – 5.54	20 – 48	0.33 – **52.6**	1.7 – 23	
Pb	0.15 – **354**	0.1 – 37.5	1.10 – 4.85	0.02 – 352	0.60 – 13	
Sb	ND	ND	0.159 – **0.699**	ND	0.085 – 0.49	
Se	ND	ND	0.18 – 0.31	2.23 – **11.1**	0.14 – 3.1	
Sn	ND	0.01 – **3.7**	0.017 – 0.115	ND	0.016 – 1.6	
Sr^	ND	ND	ND	20.0 – 679	100 – **4,400**	HWK CLV UPB‡
Th	ND	ND	ND	ND	0.20 – **5.7**	n.a.
Ti	ND	ND	ND	ND	18 – **610**	n.a.
Tl	ND	ND	ND	ND	0.013 – **0.27**	n.a.
U^	ND	ND	ND	0.04 – 0.43	0.40 – **5.4**	HWK CLV UPB
V	ND	2.0 – 10.4	1.6 – 22.9	0.14 – 26.0	3.4 – **52**	CLV UPB
Zn*	13.0 – 248	8 – 175	65 – 1,282	0.14 – 248	1.4 – 56	

Table 2: Minimum and maximum metal concentrations (µg/g dry weight) in seagrass leaves and red macroalgae thalli reported in the literature and this study. Locations which returned values above reported global maxima are also indicated (last column). Maximum reported values are bold; ND = no data; ^non-coralline species; *essential elements; ‡ one sample only; n.a. = comparison not applicable due to lack of reported data; Brito = Brito et al. (2016); Wilkes = Wilkes et al. (2017); Luy = Luy et al. (2012); Bonanno = Bonanno & Orlando-Bonaca (2018).

They report concentrations values for five metals in leaves of the seagrass Posidonia oceanica (Table 3). Upper limits are five- to nine-fold lower than Govers et al. (2014) report for Cd and Cr, comparable for Cu and Pb, and 3.5-fold higher for Zn. Part of the discrepancy could be attributed to the choice of statistic (Govers use means, Conti use medians), or any of several spatio-temporal variables known to influence phytobenthic metal uptake. For example, season influences metal uptake because marine phytobenthos can accumulate higher loads of metals during periods of active growth (the growing season), and as a result of seasonal fluctuations in natural and anthropogenic metal availability (Malea et al. 2013). While temporal variability may be more reflective of large-scale processes, spatial variability is likely to reflect local factors (Roca et al. 2017). Intrinsic factors such as biological specificity will also influence relative bioavailability and accumulation rates across and within forage taxa.

Although metal abundance and chemical form may optimise metal availability, the capacity of aquatic macrophytes to differentially integrate environmental metals means that elevated plant metal concentrations do not necessarily indicate pollution. The phytotoxicity of most metals to seagrass and macroalgae is largely unknown, and validated biomonitoring protocols do not yet exist (Bonanno and Orlando-Bonaca 2018). To determine whether forage metal concentrations are spatially or temporally elevated, they must be compared to a known reference level relative to the background geochemical context.

	Govers et al. 2014		Conti et al. 2015		This study (mean)		
	mean ± s.e.	upper limit (mean + s.e.)	median ± m.a.d	upper limit (median + m.a.d)	HWK	CLV	UPB
Cd	1.41 ± 0.18	1.59	0.36 ± 0.29	0.326	0.22	0.17	0.16
Cr	11.44 ± 4.05	15.49	0.40 ± 0.19	0.59	5.4	5.6	6.0
Cu*	9.12 ± 0.99	10.11	7.96 ± 3.16	11.12	2.4	5.6	6.0
Fe*	766 ± 263	1,029	n.a.	n.a.	1,700	7,800	6,300
Hg	0.03 ± 0.01	0.04	n.a.	n.a.	<0.029	<0.029	<0.029
Ni*	7.97 ± 1.79	9.76	n.a.	n.a.	3.1	5.3	5.6
Pb	5.16 ± 0.61	5.77	3.72 ± 2.71	6.43	1.1	3.9	3.4
Zn*	44.64 ± 5.22	49.86	89.58 ± 94.80	184.38	7.2	22	22

Table 3: Seagrass leaf metal concentrations (µg/g dry weight) of unpolluted sites reported in Govers et al. (2014) and baseline ranges reported in Conti et al. (2015), compared to this study; bold values exceed at least one reported upper limit; *essential element; m.a.d. = median absolute deviation.

In coastal bays, seagrass and algae are expected to be higher in terrigenous metals (such as Al, Fe, Mn, Zn etc.) than offshore islands, and offshore islands are expected to be higher in marine metals (e.g., Sr, U) than coastal catchments, purely as a function of the geochemical environment they are living in; coastal seagrasses in mud, offshore seagrasses in carbonate sands. Similarly, regional variations within coastal catchment geochemistry and mineralogy may create corresponding geochemical fingerprints in associated coastal phytobenthos. Consequently, forage metal concentrations in offshore (HWK) samples cannot be directly compared to those of coastal sites. The sedimentary context affects what can be considered 'normal' metal loads in seagrass and macroalgae, and hence existing baseline reference values that are not normalised for the natural geochemical background signatures are not really functioning as reliable references. The development of suitably normalised baseline/reference values for seagrasses and macroalgae is a non-trivial task but is recommended as an avenue of further research.

Sediment

Surficial sediment was analysed for 22 metals and core sediment for 27 metals (Table 4). Summary statistics for core sample metal concentrations are presented in Table A4 and core carbonate and organic carbon values are presented in Table A5. Seven metals in surficial sediment were consistently below analytical limits of detection (Ag, Cd, Hg, Mo, Sb, Th, Tl) and no samples exceeded Australian Sediment Quality Guidelines (Table 4). In the absence of national guideline values metal concentrations ≥2-fold higher than the accepted median natural background (reference) value can be treated as site-specific exceedances of sediment quality guidelines (Simpson et al. 2013). Site-specific guidelines were derived from mean core metal concentrations.

Site-specific guideline values were lower than Australian sediment guidelines for all metals except Pb. The only sample to exceed Australian Sediment Quality Guidelines was UPB-1CO-2. If this core slice is

omitted from the derivation of the site-specific guideline, the new Pb guideline value becomes 18 mg/kg; no surficial samples would exceed this new value but UPB-1CO-1 (22 mg/kg Pb) would exceed the new value. Extremely low LODs for core samples resulted in derivation of site-specific guideline values that were lower than surficial sample LODs for Ag, Mo, Sb, Th, and Ti preventing exceedances from being determined for those metals. These results highlight the difficulty in determining consistent sediment quality criteria for metals in the absence of broadly relevant data.

With the exception of Mn (max 244 mg/kg), surficial sediment metal concentrations are broadly within the ranges reported previously for the GBR by Haynes and Johnson (2000) for As, Cd, Cr, Cu, Fe, Hg, Mn, Ni, Pb, and Zn. Coastal surficial samples exceeded site-specific guideline value for Sr by six-fold and marginal exceedances were observed for As, Cr, and Mn (Table 4).

The purpose of site-specific guidelines is to develop local reference measures to support the detection of change. Given the geochemical differences between reef/offshore and coastal sediments, the site-specific guidelines are not relevant for HWK, and comparisons are not undertaken here; a core sample from a similar sedimentary environment is required to derive site-specific guideline values for offshore areas. Elevated levels of Sr and U are probably indicative of increased carbonate material in surficial relative to core samples. Adjusting sample results to account for the influence of marine carbonate material would provide more accurate estimates of spatio-temporal changes in metal profiles for all metals.

CLV had concentrations at least 30% higher than UPB for Al, As, Co, Cr, Fe, Mn, Se, Sr, U, V, and Zn. HWK had concentrations of Ba, Sr, and U that were in the order of 5, 40, and 10 times higher than coastal sites, respectively. As, Cr, Sr, and U are associated with marine sediments (Doudou et al. 2016, Brady et al. 2015). Although Cd is described as a lithogenic element in Moreton Bay (southeast Queensland; Brady et al. 2015), in Cleveland Bay Cd and Al are poorly correlated, which suggests natural Cd is either more difficult to extract than Al or Cd is not strongly associated with Al mineral phases in this region (Doherty et al. 2000).

Mn and As are commonly linked to marine sediments possibly due to their co-precipitation in marine environments, caused by Mn hydroxides and oxides in clay minerals acting as nucleation sites for As (Brady et al. 2015). Dissolved Co and Cd in sea water can be fixed by phytoplankton and transported to sediment (Gendron et al. 1986, Saito et al. 2017) and Cu can be associated with antifouling activities (Turner 2010). Although Cd is described as a lithogenic element in Moreton Bay (southeast Queensland; Brady et al. 2015), in Cleveland Bay Cd and Al are poorly correlated, which suggests natural Cd is either more difficult to extract than Al or Cd is not strongly associated with Al mineral phases in this region (Doherty et al. 2000). Our results suggest that Fe is the dominant element in UPB and CLV bays, so perhaps Fe will show stronger correlations with trace elements than Al. More samples are required to undertake these investigations.

High Ba can also be a biomarker of terrigenous sediment (Weber et al. 2006). Metals Al, Ti, and Ni are associated with lithogenic inputs and V, Th, Zn, and Co are likely to be adsorbed to clays, especially if Fe is high (Brady et al. 2015). We observe much higher levels of Ba in cores than either CLV or UPB surficial sediment; which further supports the possibility sediment geochemistry is more heavily influenced by marine signatures in surficial samples than in cores. The reason for the relatively high Ba concentration at HWK is less easily explained but possibly is a residual signal of dilute flood plume waters that have reached the reefs. In Cleveland Bay Co, Cu, Ni, Pb, and Zn are associated with clay sediments high in oxides, sulphides, and organic carbon and low in calcium carbonate (Ward and Larcombe 1996). Doherty et al. (2000) report moderate (1.5-fold) enhancement of Cd, Cu, Pb, and Zn in western but not eastern or central Cleveland Bay. The core represents about 2m of sediment deposition over the 105 ± 15 years prior to collection (i.e. ~1907; Lewis et al. 2014). Statistical analysis of the core slices broadly indicates that the oldest layer (UPB-1CO-1; dated 1903-1920) although most similar to UPB-1CO-2 (1960-1968), generally exhibits a different geochemical profile to the younger layers.

		Ag	Al	As*	Be	Cd	Co	Cr	Cs	Cu	Fe	Hg	Mn	Mo	Ni
Surficial Samples	LOD	0.73	9.5	0.024	0.024	0.021	0.017	0.007		0.085	5.5	0.01	2.4	0.76	0.03
CLV		<LOD	1400	3.0	1.2	<LOD	6.4	4.5		1.1	5600	<LOD	620	<LOD	2.0
UPB		<LOD	1100	2.3	1.2	<LOD	4.7	3.0		1.1	3500	<LOD	440	<LOD	1.6
HWK		<LOD	210	1.2	7.1	<LOD	0.072	4.3		0.10	350	<LOD	220	<LOD	0.51
UPB-1CO Core Samples	LOD	0.003	1.252	0.01	0.003	0.001	0.001	0.007	0.001	0.002	0.127		0.01	0.001	0.015
1CO-1		0.056	31000 [xyz]	1.1 [xz]	11 [x]	0.08 [xz]	7.4 [x]	2.8 [xz]	0.033	14	5200 [xz]		190 [x]	0.11 [xz]	5.0 [xz]
1CO-2		0.073	2800 [x]	2.2	22 [xy]	0.29 [xz]	6.0	3.8 [xz]	0.027	22	8700 [xy]		750 [xz]	0.081	5.7
1CO-3		0.0015	450 [a]	0.30 [a]	5.1 [a]	0.01 [b]	1.1 [a]	0.61 [xz]	0.033	0.94	870 [ax]		41 [b]	0.014 [xz]	0.77 [a]
1CO-4		0.0015	720 [z]	0.43 [b]	5.3 [z]	0.01 [xz]	1.8 [a]	1.1 [xz]	0.037	1.2	1300 [xy]		79 [b]	0.021 [xz]	1.1 [a]
Sediment Quality Guideline		1	None	None	None	1.5	None	80	None	65	None	0.15	None	None	21
Site-specific Guideline		0.066	17000	2.0	21	0.19	8.1	4.1	0.066	19	8000	None	530	0.11	6.3

		Pb	Sb	Se	Sn	Sr*	Th	Ti	Tl	U*	V	W	Zn	Zr
Surficial Samples	LOD	0.027	0.11	0.13		14	1		0.0016	0.022	0.012		4.3	
CLV		4.8	<LOD	2.0		190	<LOD		<LOD	0.52	7.4		8.6	
UPB		4.0	<LOD	1.5		120	<LOD		<LOD	0.34	5.5		6.1	
HWK		0.95	<LOD	0.61		6100	<LOD		<LOD	4.8	2.8		<LOD	
UPB-1CO Core Samples	LOD	0.003	0.001	0.024	0.005	0.017	0.002	0.047	0.0003	0.002	0.006	0.007	0.165	0.003
1CO-1		22 [abc]	0.054	3.4 [ax]	0.12 [ab]	9.0	0.60 [ab]	73	0.0084	0.55 [ab]	30 [ax]	0.020	16 [ab]	0.19 [a]
1CO-2		**130** [abc]	0.19	4.2	0.27 [acz]	22	0.53 [cz]	92	0.0063	0.89	38 [bx]	0.085	54 [cz]	0.38
1CO-3		2.1 [bz]	0.068	0.57 [z]	0.019 [bx]	3.3	0.18 [ac]	6.0	0.0031	0.070 [ax]	2.65 [ab]	0.010	2.3 [ac]	0.064 [a]
1CO-4		3.1 [cz]	0.085	0.98 [a]	0.029 [z]	4.7	0.28 [bz]	7.9	0.0036	0.12 [bx]	3.23 [ac]	<LOD	4.1 [bz]	0.080
Sediment Quality Guideline		50	None	None	9	None	None	None	None	None	None	None	200	None
Site-specific Guideline		78	0.14	4.6	0.22	19	0.79	89	0.011	0.81	37	0.060	38	0.36

Table 4: Mean weak-acid extractable metal concentrations (AEM; mg/kg DW) in surficial (n=1) and core (n=2) sediment to two significant figures. Significant differences between core slices are indicated; a,b,c = p<0.05, x,y,z = p< 0.001. Bold values exceed Australian Sediment Quality Guidelines for protection of 99% of aquatic species (Simpson et al. 2013); shaded values exceed site-specific guidelines; slices are numbered from oldest (1) to youngest (4).

Differences between the 1903-1920 layer and the younger layers are most pronounced for Al, Ba, Cr, Fe, and Se (Table 4). When the two youngest layers are compared to the two oldest layers, statistically significant differences ($p<0.05$) are evident for most metals (but not Al, Cd, Mn, Pb, Sb, Sr, W, Zn, Zr), with the strongest differences ($p<0.001$) apparent for Co, Ni, Se, Th, Ti, and V (data not shown).

The older layer has significantly higher concentrations of Ag, As, Ba, Co, Cr, Cu, Fe, Mo, Ni, Se, Sn, Th, Ti, Tl, and V and significantly lower concentrations of Cs. Sediment deposited during the 1960-68 period (the second-oldest slice analysed) was higher in all metals other than Al, Co, Mo, Th, and Tl, which were highest in the oldest sediments analysed (dated ~1903-20); and Cs, which was highest in the most recently deposited slice that we analysed (~1977-84; Table A4). The oldest layers may reflect the effects of contemporary mining activity, ot legacy effects of the mining that was happening in the 1800s. Other sources of variation could include temporal differences in weathering and erosion processes or depositional chemistry e.g, redox conditions.

At first glance, CLV metal concentrations appear consistently equal to, and generally higher than, equivalent data for UPB (Table 4). Not all sediments are created equally but are unique blends of material of lithogenic or biogenic parentage. This geochemical 'pedigree' governs the capacity of sediment particulate material to release or retain trace elements in different environmental settings. The accepted primary markers of sediment 'lithogenicity' are the terrestrially ubiquitous elements Al and Fe and assorted secondary lithogenic characteristics, the detail of which is initially determined by the geochemical fingerprint of the parent rock. Sediments which geochemically deviate from parent rock profiles have either been diluted by material from other (natural or anthropogenic) sources, or have undergone physically, chemically or biologically-induced geochemical change.

The Enrichment Factors in Table 5 normalise sample element concentrations relative to the most abundant element to facilitate comparisons between different sediments by removing some of the noise from the geochemical profile. Since Fe was most abundant in all samples we used Fe as a normalising element, but the Al is also commonly used. The only sample with a dominant Al profile is the oldest core slice, dated ~1903-1920, which has concentrations an order of magnitude higher than the 1960-1968 slice, and two orders of magnitude higher than the two youngest slices (Table A4). The mean core Al data are strongly driven by this sample. The possibility of contamination by a shard of core tubing during sample collection cannot be discounted, however many other metals are also elevated in this slice so we accept the value as real. Consequently, the core mean is dominated by Al whereas the surficial samples are dominated by Fe. Because the choice of reference metal can affect results we have presented results for Al- and Fe-normalised data.

As expected, Al-normalised Enrichment Factors indicate higher levels of enrichment than Fe-normalised data. Both Al and Fe calculations indicate that Ag, Mo, and Sr are highly enriched ($EF > 5$) in surficial relative to core samples, and As is picked up in Al but not Fe-normalised data. Patterns of lesser enrichment vary between Al- and Fe-derived EFs; Al data picks up Co, Cr, Fe, Mn, which are commonly associated with Fe, and Th, Tl, and U.

When Fe-normalised UPB and CLV Enrichment Factors in Table 5 are compared, the pattern of relative enrichment observed from non-normalised data in Table 4 is moderated or reversed; UPB Fe-normalised (or Al-normalised) metal concentrations are consistently equal to or higher than corresponding data for CLV except for Cr, Sr and U which remain higher at CLV. In other words, although CLV has higher concentrations of Al and Fe, when concentration data are considered relative to Al or Fe, UPB is more enriched than CLV for most metals. This is likely due to differences in grain size profiles between CLV and UPB; we would expect UPB to have a higher proportion of the smallest grain sizes (Bainbridge et al. 2017; Delandmeter et al. 2015), which retain higher concentrations of elements, especially transition metals such as Co, Cr, and Mn.

Levels of Mo, Sb, Sr, Th, Tl, and Zn in UPB surficial relative to core sediment map reasonably well to relative abundance patterns observed in HWK surficial sediment (Table 4) and may reflect higher levels of available biogenic/authigenic material in UPB surficial samples relative to cores.

Element	Al normalised			Fe-normalised		
	Core	CLV	UPB	Core	CLV	UPB
Ag	3.76E-05	19	28	8.22E-06	9.0	13
Al	1.0	0.3	0.3	2.2	0.1	0.1
As	1.16E-04	5.2	5.6	2.52E-04	2.4	2.6
Ba	0.0012	0.2	0.3	0.0027	0.1	0.1
Cd	1.11E-05	0.2	0.3	2.40E-05	0.1	0.1
Co	0.00047	2.7	2.9	0.0010	1.3	1.3
Cr	2.34E-04	3.9	3.7	5.08E-04	1.8	1.7
Cu	0.0011	0.2	0.3	0.0024	0.1	0.1
Fe	0.46	2.2	2.2	1.0	1.0	1.0
Mn	0.031	4.0	4.1	0.067	1.9	1.9
Mo	6.41E-05	12	17	1.39E-05	5.5	7.8
Ni	3.61E-04	1.1	1.3	7.84E-04	0.5	0.6
Pb	0.0045	0.2	0.3	0.0098	0.1	0.1
Sb	7.91E-05	1.4	2.0	1.72E-05	0.6	0.9
Se	2.63E-04	1.5	1.6	5.72E-04	0.7	0.8
Sr	0.0011	34	30	0.0024	16	14
Th	4.58E-05	2.3	3.2	9.95E-05	1.1	1.5
Tl	6.18E-07	2.5	3.6	1.34E-06	1.2	1.7
U	4.68E-05	2.3	2.1	1.02E-04	1.0	1.0
V	0.0021	0.7	0.7	0.0046	0.3	0.3
Zn	0.0022	0.8	0.8	0.0048	0.4	0.4
Mean EF		4.6	5.2		2.1	2.4
MPI		25	22		11	10

Table 5: Fe-normalised Enrichment Factor and Metal Pollution Index values for UPB and CLV surficial sediment relative to mean core values normalised to either Al or Fe; EFs > 5 are in bold; EFs between 2 and 5 are in italics; values are mg/kg dry weight.

Given the high values recorded in some of the core layers, it is also possible that historical activities could have contributed elevated metal loads. It is also feasible that locally elevated levels of some metals could a be caused by disturbance, resuspension and subsequent dissociation of metals from previously buried sediment.

Trigger values for the MPI have been structured such that any values above 10 are considered indicative of 'severe pollution' (Brady et al. 2015). Ascribing elevated levels observed in our samples to pollution is premature an ill-advised for several reasons. The MPI is based on a weighted average and its trigger thresholds are lower than other indices, consequently it is among the most conservative methods for assessing sediment quality and tends overall to indicate higher contamination for suites of elements than single elements (Brady et al. 2015). The MPI threshold categories were not necessarily derived to reflect ancient Australian geologies, and it is unclear how this affects results. Also, few previous studies assess or compare both Al- and Fe-derived EFs. Our result demonstrate that the choice of reference metal can have

substantial influence upon which metals are elevated. Finally, our study did not seek to identify sources of pollution, and we have insufficient evidence to clearly identify whether elevated metals are the result of natural processes, in which case they are just elevated, or the result of anthropogenic processes, in which case they may be potential pollutants. Bearing these considerations in mind, the apparently high values derived for the MPI in this study should be considered not as the final assessment of sediment quality, but as an indication that understanding the nature, causes and ecological risks of marine sediment metal chemistry in dynamic coastal environments is complicated and, we believe, worthy of more detailed and rigorous investigation.

Bioaccumulation from sediment

Bioaccumulation of metals from sediment into forage (Table 6) can provide an indication of their long-term cycling, fate, and distribution in turtle habitats. Metals Al, Cd, and Cu bioaccumulated in forage by over an order of magnitude at HWK (Table 6). The highest was Cu, with BAF of 24, which was the highest BAF recorded in this study. Cadmium (Cd) bioaccumulated to the greatest extent at the coastal locations and was slightly higher in CLV. Also, Ba was roughly an order of magnitude higher in forage at coastal sites, UPB slightly lower, and Al, Cu, Mo, and Tl had BAFs between 5 and 10 at both coastal sites.

	Howick Group			Cleveland Bay			Upstart Bay		
	forage	sediment	BAF	forage	sediment	BAF	forage	sediment	BAF
Ag	0.017	0.38	0.046	0.058	0.36	0.16	0.066	0.36	0.18
Al	3,000	209	14	8,400	1,400	5.8	8,800	1,100	8.0
As^	6.0	1.2	5.1	14	3.0	4.6	5.1	2.3	2.2
Ba	7.5	7.1	1.1	12	1.2	10	11	1.2	9.3
Cd	0.20	0.011	19	0.18	0.011	17	0.16	0.011	15
Co*	0.52	0.072	7.2	4.6	6.4	0.73	5.0	4.7	1.1
Cr^	5.4	4.3	1.3	5.7	4.5	1.3	6.0	3.0	2.0
Cu*	2.5	0.10	24	5.5	1.1	5.1	6.0	1.1	5.6
Fe*	1,700	350	4.8	7,700	5,000	1.6	6,300	3,500	1.8
Mn*	35	23	1.5	630	620	1.0	630	440	1.5
Mo*	0.81	0.38	2.1	2.8	0.38	7.2	2.1	0.38	5.6
Ni*	3.0	0.51	5.9	5.4	2.0	2.7	5.5	1.6	3.4
Pb	1.1	0.95	1.2	3.9	4.8	0.81	3.4	4.0	0.88
Sb	0.23	0.055	4.2	0.22	0.055	4.0	0.23	0.055	4.2
Se	0.52	0.61	0.85	1.2	2.0	0.60	1.0	1.5	0.69
Sr^	3,200	6,100	0.53	370	190	2.0	280	120	2.4
Th	0.61	0.52	1.2	1.8	0.52	3.4	1.5	0.52	2.8
Tl	0.052	0.0078	6.7	0.049	0.0078	6.3	0.063	0.0078	8.1
U^	3.2	4.6	0.69	0.86	0.52	1.6	1.3	0.34	3.8
V	7.5	2.6	2.9	18	7.4	2.4	15	5.5	2.8
Zn*	7.2	2.2	3.3	21	8.6	2.5	22	6.1	3.6

Table 6: Metal bioaccumulation factors (BAFs) for forage (mean µg/g DW) relative to sediment (mg/kg DW) at each location between 2014 and 2017. Bioaccumulation factors (BAFs) between 5 and 10 are *bold italics* and BAFs >10 are shaded. Censored data (<LOD) were used at 50% of the LOD, <LOD = all samples were below the limit of detection, *essential metals; ^marine metals.

Thallium (Tl) was measured in a similar range at HWK, and Ni, Co, and As bioaccumulated by 5-10-fold at the offshore but not coastal locations. The high BAFs returned for Cu may be explained by preferential root uptake of metals by aquatic macrophytes which can occur for essential elements over non-essential elements, even at toxic concentrations (Stanley et al. 1974).

Dissolved metal ion concentrations inside the plant are usually higher than substrate metal concentrations (Andresen et al. 2018), and higher BAFs can be expected for substrates where essential metals are relatively deficient compared to those that are abundant. Additionally, considerable interspecific variability exists in essential metal requirements and metal uptake capacity for both seagrass and macroalgae, driven by internal (e.g., genotypic and phenotypic capacity) and external factors (e.g., season, temperature, etc.; Bonanno & Orlando-Bonaca 2018).

Patterns of bioaccumulation in Table 6 are generally similar for both coastal locations, except for U, and perhaps As. BAFs for Cd, V, Zn, Tl, Th, Se, Sb, Cr, are similar across all three locations. Coastal locations have higher BAFs than offshore for marine metals U and Sr, the essential element Mo, and Ba. At UPB the BAF for As was lower than both CLV and HWK. No accumulation was recorded in forage relative to sediment for some non-essential metals; at HWK for marine metals U and Sr, as well as Se and Ag; and at coastal locations for Se, Pb, and Ag. Coastal BAFs for Co indicate that UPB was close to equilibrium (BAF = 1.1) and possibly in surplus relative to forage species' uptake requirements whereas the HWK result indicates that Co may be limiting (BAF = 7.2).

Offshore locations

BAFs are higher than coastal locations where preferential uptake of essential metals is likely e.g., for Fe, Cu, and Co. Patterns of bioaccumulation for Al were similar to those for essential elements, being higher offshore, and Pb and the micronutrient Ni were also slightly higher offshore. Higher levels of accumulation for some non-essential metals from sediments that are relatively deficient in these metals may be explained by the fact that essential metals are actively transported across membranes for uptake, but ions with similar chemical properties, including non-essential metals, often share some of the transport routes (Andresen et al. 2018).

Sea Water

DGTs provide excellent conservative measures of bioavailable (labile) metals (Baeyens et al. 2011). DGTs were deployed in 2014 and all metals except Zn were above LOD at all locations (Table 7). Exceedances of 99th but not 95th percentile trigger values (TVs) for Australian marine water quality (ANZECC and ARMCANZ 2000) were observed for Co in all samples at all locations. The 99th percentile TV for Cu was exceeded in CLV and UPB, and at least one sample in HWK, and at least one sample in UPB exceeded the 95th percentile TV (Table 7). Exceedances of moderate-to-low reliability or 95th percentile TVs were observed for Al at all locations for all samples. Aluminium (Al) was higher in UPB relative to CLV sea water. Cr was elevated in UPB and CLV water relative to HWK. Manganese (Mn) was 7-fold higher in CLV than HWK. Maximum Cd values were approximately one order of magnitude higher at HWK relative to coastal sites (Table 7).

	Location	LOD	n	Mean	Median	Std. Dev.	Min	Max	WQG	
									99%	95%
Al	CLV	2.2	9	6.0	4.7	3.7	2.9	14	0.5*	n.a.
	HWK		7	3.7	3.4	1.5	1.2	5.5		
	UPB		9	29	19	28	7.9	84		
As^ (AsIII)	CLV	3.6	9	1.0 [a]	1.1	0.16	0.78	1.3	2.3*	n.a.
	HWK		7	0.82	0.91	0.19	0.56	1.0		
	UPB		9	0.82 [a]	0.84	0.055	0.75	0.88		
Cd	CLV	0.0050	9	0.0030 [a]	0.0031	0.00046	0.0021	0.0036	0.7‡	5.5‡[c]
	HWK		7	0.013 [a,b]	0.012	0.0074	0.0044	0.023		
	UPB		9	0.0037 [b]	0.0031	0.0012	0.0023	0.00 56		
Co	CLV	0.00050	9	0.045 [a]	0.044	0.0068	0.034	0.065	0.005	1
	HWK		7	0.018 [a,b]	0.020	0.0053	0.010	0.024		
	UPB		9	0.08 [a]	0.066	0.057	0.021	0.17		
Cr^ (CrIII)	CLV	0.025	9	0.19 [a]	0.18	0.05	0.14	0.28	8 (Cr/VI = 0.14)	27 (Cr/VI = 4.4)
	HWK		7	0.030 [a,b]	0.026	0.010	0.016	0.045		
	UPB		9	0.15 [b]	0.15	0.03	0.12	0.21		
Cu #	CLV	0.0080	9	0.16 [a]	0.16	0.018	0.13	0.19	0.1	0.8
	HWK		7	0.087 [a]	0.081	0.039	0.043	0.14		
	UPB		9	0.16	0.10	0.13	0.057	0.46		
Mn	CLV	0.018	9	14 [a,y]	13	3.4	10	19	80*	n.a.
	HWK		7	2.0 [a,x]	2.2	0.74	1.0	2.9		
	UPB		9	5.5 [a,y]	3.8	3.5	2.4	11		
Ni	CLV	0.0040	9	0.15	0.15	0.014	0.13	0.17	7	70[c]
	HWK		7	0.20	0.13	0.10	0.12	0.34		
	UPB		9	0.19	0.15	0.088	0.10	0.32		
Pb	CLV	0.00040	9	0.011 [a]	0.013	0.0043	0.0052	0.018	2.2	4.4
	HWK		7	0.018 [a]	0.018	0.0023	0.014	0.021		
	UPB		9	0.026	0.018	0.027	0.0054	0.080		
V^	CLV	0.029	9	3.7 [a]	3.5	1.5	2.0	6.4	50	100
	HWK		7	7.8 [b]	6.2	3.8	2.7	13		
	UPB		9	2.0 [a,x]	2.0	0.54	1.4	3.0		
Zn #	CLV	0.0030	9	<LOD	<LOD	<LOD	<LOD	<LOD	3.1	6.5[c]
	HWK		7	<LOD	<LOD	<LOD	<LOD	<LOD		
	UPB		9	<LOD	<LOD	<LOD	<LOD	<LOD		

Table 7: Summary statistics for metal concentrations collected by DGT samplers in 2014, by location and rounded to two significant figures. Values in bold exceed the Australian Water Quality Guidelines (WQGs) for protection of 99% or 95% of marine species, or *interim moderate- or low-reliability trigger values (where no 99% trigger values exist; ANZECC and ARMCANZ 2000). All data are µg/L; ‡ recently revised guideline value (NIWA 2017); ^marine metals; superscript letters indicate significant differences between locations where [a], b = p < 0.05 and x, y, z = p < 0.001. [b] chemicals for which possible bioaccumulation and secondary poisoning effects should be considered; [c] figure may not protect key test species from chronic toxicity.

Zn was below LOD at all locations due to a relatively high reading in the field blank, resulting in negative values. This is common for Zn, which is a very common contaminant and difficult to analyse at trace concentrations with any method. The DGT membranes are extremely sensitive and will collect and retain metals from perspiration and exhalation. The risk of sunburn is extremely high during field sampling, and in the first round of deployment, some team members were wearing zinc-based sunscreen. It is possible that Zn from sunscreen was transferred from sampling personnel to the samplers. Sunscreens free from all targeted metals were used for all subsequent sampling rounds, and extra care was taken to keep samples upwind during sampling.

Elevated Al results in UPB were driven by two sub-replicates (i.e. two of the three discs in one of the three DGT trays) which were 5- to 6-fold higher than all other replicates. The third disc on this tray was located within 10cm of the others but did not contain a comparably high concentration of Al. Given the longshore flow of water from UPB to CLV and the lack of any Al signal in CLV, contamination for example if discs touched the aluminium boat so this data point is questionable and cannot be further considered in detail. A polypropylene boat was used for all subsequent sampling and other matrices with much lower detection sensitivity are unlikely to be affected.

The most recent data for metal concentrations in tropical Australian coastal and offshore surface waters were collected almost two decades ago and cover a handful of metals (Munksgaard and Parry 2001, Esslemont 2000; Table 8). Consequently, no comparative data are available for offshore values of Al, Cr, and Mn or V in either coastal or offshore environments. Background marine water metal concentrations derived from samples collected along the NSW coast are available for eight metals (ANZECC and ARMCANZ 2000); these are also listed in Table 8 for comparison. Background data are currently unavailable for Al, Co, Mn, and Fe.

All results are within the upper limit of ANZECC and ARMCANZ (2000) background ranges except Cr, which exceeded the background maxima by less than a factor of two at CLV and UPB (Table 8). Other Cr measures are reported <LOD in a single Australian study but the LOD (0.4 ug/L; Table A6) is an order of magnitude greater than this study (0.03 ug/L), which makes comparisons uninformative. Chromium (Cr) was more than twofold lower than ANZECC and ARMCANZ (2000) background minima at HWK, and As approximated or was slightly lower than the background minima at all sites. Our observation of values below previously recorded background minima is likely to reflect the increasing accuracy of metal analytical techniques rather than depletion.

Marine surface water results are also within the upper limit of reported north Australian concentration ranges for As, Cr, Cu, Mn, and Ni (Table 8). Comparable data for Al, Cr, and Mn are not available for offshore lcations and no tropical Australian data are available for V. UPB was 30% higher than the maximum surface water concentration previously reported for Co, at Gladstone Harbour a busy industrial port. Co values for CLV were third-highest on record, approximately 50% higher most other coastal values. Offshore Co concentrations reported in this study are comparable with data collected in the Gulf of Carpentaria over a decade ago.

Surface water concentrations of Mn were more than twice as high in CLV as reported for Cleveland Bay in the late 1970s. UPB concentrations of Mn were also high. UPB Pb concentrations were higher than recent data for Gladstone Harbour, and threefold higher than older data for the Timor Sea and the Gulf but much lower than older Townsville region data (Table 8). HWK concentrations of Cd and Pb were about 50% higher than literature maxima, reported for the Norman Channel. HWK Ni concentrations were roughly twice as high as reported in the 1980s for nearby Lizard Island but this could be due to improvements in analytical limits of detection. Coastal Cu results are comparable to previous results for the region and markedly lower than reported recently for Gladstone Harbour, Darwin Harbour, and the Gulf (Table 8).This analysis highlights the relative paucity of baseline metal data for tropical north Australian surface waters. Our observation of potential increases in coastal Co concentrations relative to the busy industrial port of Gladstone Harbour is concerning, especially given the lack of suitable background data for reference and comparison. Metals may require routine monitoring to establish reliable estimates which allow trends with potential to harm ecological systems to be confidently detected and promptly addressed.

Location		Region	Date	Season	n	Element (µg/L)									Literature Source
						Al	As	Cd	Co	Cr	Cu	Mn	Ni	Pb	
Background values for Australian marine waters						n.a.	1.0-1.6	0.002-.7	n.a.	0.067-0.1	0.025-0.58	n.a.	0.13-0.3	0.004-0.03	ANZECC & ARMCANZ 2000
Coastal/terrigenous															
	Cleveland Bay	Central GBR	2014	dry	3	6.0	1.0	0.0030	0.045	0.19	0.16	14	0.33	0.011	This study
	Upstart Bay	Central GBR	2014	dry	3	**29**	0.62	0.0037	**0.080**	0.15	0.16	5.3	0.39	0.026	This study
	Gladstone Harbour	Rockhampton	2011	wet	32	14	0.90	0.005	0.064	<0.4	**0.79**	1.8	**0.59**	<0.016	Angel et al. 2012
	Tannum Sands	Rockhampton	2011	wet	2	3.5	1.2	<0.003	0.024	<0.4	0.13	0.075	0.21	<0.016	Angel et al. 2012
	Rodd's Bay	Rockhampton	2011	wet	3	2	**35**	<0.003	0.029	<0.4	0.12	0.68	0.20	**6.3**	Angel et al. 2012
	Darwin Harbour	Timor Sea	1997-00	wet	16		1.2	0.0058	0.025		0.28		0.23	0.0086	Munksgaard & Parry 2001
	Bing Bong Coast	Gulf of Carpentaria	1996-99	dry	29			0.0041	0.027		0.31		0.37	0.0077	Munksgaard & Parry 2001
	Pioneer Bay	Townsville	1993	dry	4			<0.90			0.37			3.9	Esslemont 2000
	Nelly Bay	Townsville	1993	dry	4			<0.90			0.27			0.93	Esslemont 2000
	Cleveland Bay	Townsville	1993	dry	4			<0.90			0.64			1.9	Esslemont 2000
	Orpheus Island	Townsville	1982-83	both	32			<0.01			0.18		0.12	<0.06	Denton & Burdon-Jones 1986
	Cleveland Bay	Townsville	1976-77	wet	9			**0.32**			0.38	**5.7**	0.28	0.72	Burdon-Jones et al. 1982
	Tickleberry Bay	Townsville	1976-77	wet	9			0.22			0.16	3.2	0.23	0.77	Burdon-Jones et al. 1982
Offshore/carbonate															
	Howick Islands	Northern GBR	2014	dry	3	3.7	0.62	0.013	0.018	0.03	0.087	2.0	0.2	**0.018**	This study
	Karumba Offshore	Gulf of Carpentaria	1996-99	both	12		**1.8**	0.0054	0.017		0.20		0.18	0.011	Munksgaard & Parry 2001
	Norman Channel	Gulf of Carpentaria	1996-99	both	17		0.68	**0.0081**	**0.024**		**0.40**		**0.27**	0.012	Munksgaard & Parry 2001
	Various	Torres Strait	1994	wet				0.0018			0.16		0.16		Apte & Day 1998
	Lizard Island	Northern GBR	1982-83	both	32			<0.01			0.13		0.09	<0.06	Denton & Burdon-Jones 1986
	Heron Island	Southern GBR	1982-83	both	32			<0.01			0.14		0.08	<0.06	Denton & Burdon-Jones 1986

Table 8: Mean metal concentrations (ug/L) reported in the literature for north Australian marine coastal and offshore surface water rounded to two significant figures; maxima for each habitat are in fold font; n = number of samples. Refer to original texts for analytical methods and Table A6 for literature LODs.

River Water

Dissolved metal concentrations in Burdekin River water are shown in Table 9. Concentrations of Al, Cu, and Zn at Northcote and Home Hill, Ag at Home Hill and Fe at Northcote exceeded the relevant 95th percentile Australian freshwater guideline values for dissolved metals (ANZECC and ARMCANZ 2000). It is standard practice to use international toxicant guidelines if we don't have one in Australia – we used the Canadian Environmental Quality Guidelines (CCME 1999). Summary statistics can be found in the Appendix at Table A7.

Element	Barratta Creek at Northcote		Burdekin River at Home Hill		Trigger Value[a] PC95 (µg/L)
	µg/L	Dissolved % of total	µg/L	Dissolved % of total	
Ag	0.025	17%	0.053	67%	0.05
Al	165.8	1%	68.9	0.1%	55
As	2.26	82%	1.761	36%	24
B	59	97%	46.6	91%	370
Ba	118.6	78%	53.85	21%	None
Be	0.1	10%	0.13	10%	None
Cd	0.02	20%	0.02	20%	0.2
Co	0.5	12%	0.126	1%	1.4[b]
Cr	0.24	2%	0.335	0.5%	1
Cu	3.3	28%	1.8	6%	1.4
Fe	320.2	2%	131.5	0.2%	300[c]
La	0.758	6%	0.22	1%	None
Li	2.5	18%	2.5	33%	None
Mn	131.4	34%	13.01	2%	1900
Mo	1	90%	0.8	53%	73[c]
Ni	1.8	22%	0.5	1%	11
Pb	0.534	4%	0.153	1%	3.8
Sb	0.156	32%	0.163	12%	None
Se	0.2	20%	0.2	37%	11
Sn	0.6	6%	0.5	33%	None
Ti	18.08	3%	5.377	1%	None
Tl	0.288	6%	0.129	34%	None
U	0.476	49%	0.49	41%	15[c]
V	4.996	16%	6.723	7%	100[d]
Zn	9.06	36%	10.06	4%	8

Table 9: River water 95th percentile dissolved metal concentrations (µg/L) between July 2014 and June 2017 for the Burdekin River at Home Hill (n = 93) and Barratta Creek at Northcote (n = 183). Results were assessed and compared with the Australian and New Zealand freshwater quality guidelines using 95 % level of protection (ANZECC and ARMCANZ 2000). Dissolved as a percentage of total metal concentration is shown for each metal (calculated on the 95th percentile). Bold values indicate exceedance of the trigger value; shaded metals indicate the dissolved portion is ≤ 1% of the total metal load. [a] Trigger values were obtained from Australian and New Zealand Guidelines for Fresh and Marine Water Quality water quality guidelines using 95% level of protection (ANZECC and ARMCANZ 2000). [b] A low reliability Australian guideline was available for cobalt (Co) which is comparable to the Canadian Environmental Quality Guidelines (CCME 1999) Federal Water Quality Guideline of 2.5 µg/L. [c] The current Canadian Environmental Quality Guidelines (CCME 1999) Federal Water Quality Guideline were used in the absence of Australian TV . [c] Marine trigger value from Australian and New Zealand Guidelines for Fresh and Marine Water Quality water quality guidelines using 95% level of protection (ANZECC and ARMCANZ 2000). [*] total metals were only collected from June 2014 to July 2016.

Annual metal loads were calculated to investigate their total metal input to the system for the 14 metals that occur primarily in particulate form (Figure 2). It is important to note that between 2014-2017 the Burdekin River discharge ranged between 5 to 27% of the previous 8 years' (2006 to 2014) annual average discharge (Table 10). Burdekin River flow rates are extremely variable, ranging from near-zero in dry years to almost 40,000 m^3/sec in major floods (Orpin et al. 2004). In sampled years, flow volumes in the Burdekin were small (~900 – 4400 GL) thus measured metal loads (Table 10) may be below average, and below levels observed during the stranding event.

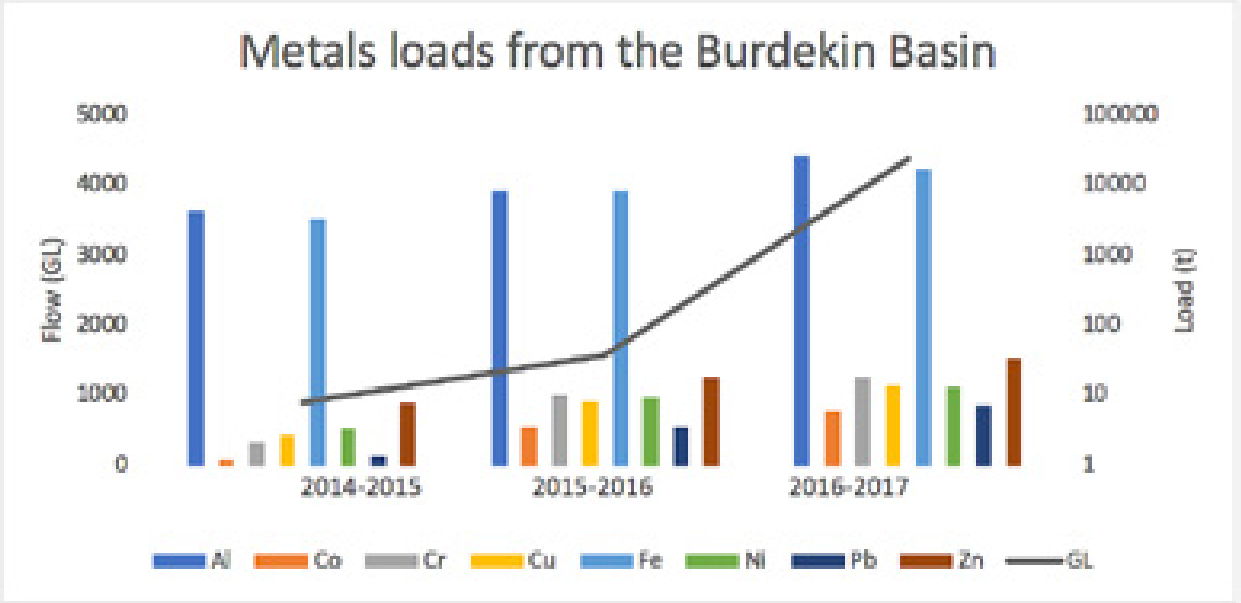

Figure 2: Increased loads of metals observed with increasing flow in the Burdekin River. Note log scale is used for loads.

Water Year	Discharge (GL)	Al	Co	Cr	Cu	Fe	Ni	Pb	Zn
2014-15	892	4,400	1.2	2.1	2.7	3,400	3.3	1.3	7.6
2015-16	1,565	8,400	3.6	9.9	8.6	8,100	9.1	3.7	19
2016-17	4,385	26,000	5.8	18	14	17,000	13	7.1	34

Table 10: Calculated loads in kilograms for the Burdekin River at Home Hill.

Loads of some metals are strongly correlated with flow volume, as is the case for suspended sediment from the Burdekin (Kuhnert et al. 2012). Table 9 shows that a large fraction of most metals are discharged at Home Hill in the particulate form. For 12 metals including Cu and Zn the dissolved fraction comprised no more than 7% of its total metal load at Home Hill. For seven of these (Al, Co, Cr, Fe, Ni, Pb, and Ti); the dissolved fraction comprised no more than 1% of its total load. These metals are highlighted grey in Table 9. Dissolved metal fractions were higher at Northcote, for seven metals including Cr, Pb, and Sn, the dissolved fraction comprised no more than 7% of its total metal load at Northcote and for Al the dissolved fraction contributed no more than 1% of the total load.

Most elements associated with riverine suspended and bedload material are likely to be released in significant quantities upon entering coastal waters, most significantly from suspended particulates (Oelkers et al. 2012). The components of suspended particulates that are most significant for trace element transport are clay minerals; Fe, Al, and Mn (hydr)oxides, and particulate organic matter (Viers et al. 2009; Doherty et al. 2000). The Upper Burdekin and Bowen-Bogie sub-catchments are the dominant source of the <10 µm sediments being delivered to the Great Barrier Reef (delivering 72-85% of total; Furuichi et al. 2016; Bainbridge et al. 2014). Cattle grazing is the dominant land-use in all Burdekin

sub-catchments not just the Upper Burdekin and Bowen-Bogie (Figure A3). Grazing is thus unlikely to be the sole direct cause of the elevated sediment loads in these sub-catchments relative to others in the Burdekin, but differences in the interaction between land-use. Landscape, and lithology will affect sensitivity to erosion at the sub-catchment scale. The Upper Burdekin and Bowen-Bogie systems have steeper gradients and higher rainfalls which increase susceptibility to erosion (Furuichi et al. 2016, Croke et al. 2015) and basaltic (high in Fe and Mg), granitic (high in Si and Al), and sedimentary (high in clay minerals) geologies that drive end-of-river and flood plume fine sediment loads (Figure A4; Bainbridge et al. 2016; Croke et al. 2015). Current data indicate that most (67%) of the Burdekin River suspended sediment load is deposited or retained within Upstart Bay and resuspended but not removed by (sub-cyclonic) wind-driven events, however clay-sized particles can be retained in flood plume waters as organic flocs and transported large distances (Bainbridge et al. 2017; Delandmeter et al. 2015; Bainbridge et al. 2012).

Basaltic landscapes are highly associated with elevated downstream fine sediment loads in the Upper Burdekin, Bowen, and Suttor sub-catchments (Bainbridge et al. 2017). Geochemical tracing data show that the Upper Burdekin has the highest concentrations of Al, As, Co, Cr, Cu, Ni, Pb, Th, and U, (Furuichi et al. 2016; reproduced in Table 11). The Bowen and Bogie sub-catchments have the highest concentrations of Mn, Sr, V, and Zn (Furuichi et al. 2016). Catchments with highest Fe concentrations were Upper Burdekin, Bowen, and Suttor (Table 11). Medan concentrations of Al and Ti from the Burdekin River are more than double those reported for world rivers, and As and Pb are less than half the world rivers values (Table 11). The Upper Burdekin and Lower Burdekin (Bowen-Bogie) sub-catchments deliver the largest volumes of fine sediment, which contain the highest concentrations of many trace elements. Most elements are likely to be released from particulates upon entry to the coast but the quantitative relationship between river-borne metals and observed coastal geochemical profiles in water and sediment remains unclear.

Element	Burdekin catchments							Burdekin mean	World Rivers mean
	Belyando	Suttor	Cape	Upper Burdekin	BFD Overflow	Bowen	Bogie		
Al_2O_3 (%)	20.4	20.3	24.4	24.1	22.1	23	20.1	22.1	8.72
As	7	6	13	19	13	9	4	10	36.3
Ba	471	382	567	601	616	501	511	518	522
Co	15	26	27	33	22	26	25	25	22.5
Cr	74	176	83	119	91	92	72	101	130
Cu	30	42	52	70	48	46	60	50	75.9
Fe_2O_3 (%)	5.3	7.7	7.6	9.9	7.9	9	7.7	7.9	5.81
MnO (%)	0.08	0.12	0.133	0.160	0.240	0.136	0.231	0.160	0.168
Ni	31	69	36	73	55	57	48	53	74.5
Pb	21	18	35	35	29	23	26	27	61.1
Sr	101	123	93	136	138	209	243	149	187
Th	20.8	14.9	21.6	21.7	20.6	12.6	13.4	17.9	12.1
TiO_2 (%)	1.03	1.78	1.16	1.15	1.01	1.09	1.09	1.19	0.44
U	3.77	2.66	4.53	4.54	4.47	2.97	2.69	3.72	3.30
V	112	147	152	173	135	176	148	149	129
Zn	87	118	128	168	130	126	177	133	208

Table 11: Mean metal concentrations in Burdekin catchment suspended sediments <10 µm (Furuichi et al. 2016) and world river suspended sediments (Viers et al. 2009). Major elements are wt% and trace elements are mg/kg. Burdekin sub-catchment maxima are in bold font. Mean Burdekin values that are greater than world river mean values are in italics.

Overview

This study adopts a screening approach to identify a broad range of metals across several environmental matrices. The data presented here for river water, sea water, sediment, and forage provide a comprehensive snapshot of the state of metals at the study sites (Table 12). Environmental metals observed at concentrations above 'background' levels and/or relevant ecological guidelines may be ecologically harmful and require further investigation. Our results show that Al exceeds fresh and marine water quality guidelines at all locations tested (Table 12); Co exceeds marine water quality guidelines at all marine locations tested; and Cu exceeds water quality guidelines in river and coastal surface water; Ag and Zn exceed guidelines for river water. No sample metal concentrations exceeded national sediment quality guidelines, however for 13 of the tested metals no guideline was available. Consequently, site-specific sediment quality guidelines were derived from local core samples, surficial samples exceeded site-specific sediment guidelines for As, Cr, Mn, and Sr at coastal locations (Table 12). Our results suggest that Ag, Al, As, Co, Cu, Cr, Mn, Mo, Sr, and Zn exceed guidelines values and may be present in the Great Barrier Reef at ecologically harmful concentrations.

Comparing our results to other systems also indicated that some metals could be present at potentially concerning levels in the Great Barrier Reef. We observed the highest coastal surface water concentrations of Al, Co, and Cr yet reported in Australian tropical surface waters, and higher seagrass or macroalgae concentrations of Al, As, Cr Fe, Mn, U, and V than reported in four recent literature reviews. Similarly, Furuichi et al. (2016) report comparatively high values of suspended particulate Al, Co, Fe, Th, Ti, and U, which points to the possibility that the Burdekin River is a key source of elevated elements to UPB.

Substrate geochemical profiles change along an inner-to-outer shelf gradient, such that terrigenous lithogenic sediments are gradually diluted by marine biogenic sediments (Doherty et al. 2000). Seagrasses and macroalgae accumulate metals from their surrounding environments and are often used as bioindicators of metal pollution (Bonanno and Orlando-Bonaca 2018). Correspondingly, 'marine metals' are expected to be observed in offshore phytobenthic samples at concentrations higher than reported at coastal locations. Metals that have no biological role at any dose, e.g. Pb, As, Hg, Cd, are sometimes referred to as 'xenobiotic metals', or metals that are foreign to the organism (Solenkova et al. 2014). In principle, there is no reason to expect to see elevated levels of these metals in forage samples, however some non-essential metals can act as replacement essential metals in some plants, especially when essential metals are limiting (Andresen et al. 2018). Processes that create periods of essential metal limitation in coastal areas are too complex to consider here. Forage accumulation patterns may become clearer if examined under the lens of relative essentiality and co-limitation.

Statistically significant elevations in marine metals were observed in HWK forage for Sr and U but not As and Cr. High BAFs were not observed for any marine metals. Elevated levels of Sr and U are likely related to the presence of marine carbonates, for example, foraminifera incorporate both Sr and U into their tests (Russell et al. 1994; Kastner 1999). To better understand metal profiles in marine (as opposed to coastal) environments, it is recommended that future studies use multiple marine sites and develop geochemical profiles which use Ca as a reference element, to reflect the strong biogenic influence on marine geochemistry (McMahon et al. 2013).

We found non-essential metals in forage include Ag, Al, As, Ba, Cs, Pb, Se, Sn, Th, Ti, and V were significantly higher in coastal versus offshore samples. Offshore forage strongly accumulated Al, Cd and Cu, Al and Cu are expected to be limiting in the offshore environment, Cd accumulation requires further interrogation. Coastal forage samples were above literature seagrass or macroalgae baselines for Cr and Fe, but baseline data were unavailable for many metals, and the relative exposure risk for those metals remains unquantified. Coastal forage samples strongly accumulated Cd, and Ba (CLV only), however matching patterns of abundance were not found for associated sediment. We find that Ba and U are higher in UPB sediment and forage than CLV. Barium (Ba) concentrations in riverine and coastal areas are commonly high compared to slope and oceanic waters (McMahon et al. 2013) as it is a common constituent of marine phytoplankton (Collier and Edmonds 1984) although is also associated with coral skeletal aragonite (Kastner 1999).

Comparison type	River Water	Sea Water	Sediment		Forage
			surficial	core	
<LOD	nil	nil	Ag Cd Hg Mo Sb Th Tl (all)	W (1CO-4)	nil
Mean > guideline	Ag, Al, Cu, Zn No guideline: Ba, Sb, Sn, Tl	Al Co* (all) Cu (CLV, UPB)	nil No guideline: Al, Ba, Co, Fe, Mn, Mo, Sb, Se, Sr, Th, Tl, U, V	Pb (1CO-2)	n.a.
Mean > site-specific guideline	n.a.		As, Sr (CLV, UPB) Cr, Mn (CLV)	Ag, Al, As, Ba, Cd, Cu, Fe, Mn, Pb, Sb, Sn, Tl, U, W, Zn, Zr (1CO-2)	n.a.
Dissolved ≤ 1% total (for sediment, AEM ≤1% TRM)	Al, Co, Cr, Fe, Ni, Pb, Tl	n.a.	n.a.	Cs, Zn (all) Th (1CO-2, 1CO-4) Tl (1CO-3, 1CO-4)	n.a.
Spatial comparisons					
CLV > UPB	n.a.	As^, Mn*	Al, As, Co, Cr, Fe, Mn, Se, Sr, U, V, Zn	n.a.	As^
UPB > CLV	n.a.	nil	nil	n.a.	Ba, U^
offshore > coast	n.a.	Cd, V	Ba, Sr, U (both); Cr (UPB)	n.a.	Sr^, U^
coast > offshore	n.a.	Co*, Cr*, Mn* (both) Cu*, Pb (CLV)	All except the above which were >LOD	n.a.	Ag, Al, As^ (CLV only), Ba, Co*, Cs, Cu*, Fe*, Mn*, Mo*, Ni*, Pb, Se, Sn, Th, Tl, V, Zn*
Temporal comparisons				Multiple differences, 1CO-2 highest	
(Fe) Enrichment Factor >5	n.a.	n.a.	Ag, Mo, Sr	n.a.	n.a.
BAF ≥ 10	n.a.	n.a.	n.a.	n.a.	Cd (all) Al, Cu* (HWK) Ba (CLV)
Exceeds national or global values	Al, Co, Fe, Th, Tl, U (external particulate data)	Al, Co (UPB); Cd, Pb (HWK); Cr (CLV, UPB)	Mn	n.a.	Al (all), As^, Fe*, Mn*, U^, V

Table 12: Summary of comparisons between results from this study and relevant reference values; n.a. = not assessed; ^marine metals; *essential elements.

We also find that As is higher in CLV than UPB. Mn and As are commonly linked to marine sediments possibly due to co-precipitation in marine environments (Brady et al. 2015). Differences between the two coastal locations could also be indicative of different local metal inputs. It is important to note that Al is generally considered to be of low toxicity, since it has a strong tendency to form insoluble hydroxo complexes at physiological pH values but can exert toxicity externally if exposure occurs under mildly acidic conditions (Nieboer & Richardson, 1980). Ti is similarly unlikely to be soluble enough to access to reactive sites in living organisms (Nieboer & Richardson, 1980). Tissue residues have been observed about reference intervals for As, Cd, and Co in turtles in UPB and CLV (Villa et al. 2017), suggesting that trophic accumulation may be occurring from forage or sediment to turtles.

Although Co did not exceed freshwater guidelines, ~99% of total Co was present in particulate form (Table 9) and marine water quality guidelines were exceeded in all three locations, with coastal sea

water concentrations were 2.5 – 4-fold higher than offshore locations. Some particulate Co in river water may become dissolved when it enters the marine environment. This could in part explain the sea water exceedances in the absence of marked elevation in sediments relative to background at coastal areas. Alternatively, sediment EFs may not reflect the water quality guideline results if background levels are high – this would indicate that Co is naturally elevated in coastal bays. The enrichment of coastal bay sediments in terrestrially-derived minerals such as Co, Al, Fe, Mn, etc., relative to offshore is natural, the latter receiving negligible fluvial metal input from terrestrial sources and Co etc. not being naturally dominant in carbonate sediment geochemistry. This however does not explain the Co exceedance observed at the offshore location. Co is within reported global Co values for seagrass and macroalgae, however these reports do not discriminate between 'polluted' and 'unpolluted' contexts in their analyses and more intensive investigation into the range of reported values is required to determine an effective upper limit for Co in forage that has received little anthropogenic impact. Cobalt is a seagrass micronutrient, and showed substantial bioaccumulation from sediment at HWK, where it was probably in limiting supply, since forage concentrations in offshore forage were still an order of magnitude lower than coastal forage. Nutrient-like elements such as Ba and Co are expected to fluctuate dynamically in response to biological uptake and release processes. These processes could not be considered in detail here.

The geology of the Burdekin catchment is diverse. Collectively our results indicate the dominance of grain-size dependent Fe signatures across all matrices and, presumptively, Fe-related signatures for transition elements like Co, Mn, and Cr which are commonly associated with Fe in fine particulate material. Previous studies show three sub-catchments of the Burdekin deliver disproportionately high volumes of fine sediment to Upstart Bay. These loads are associated with disproportionately high concentrations of many metals we observed at elevated concentrations across various environmental matrices, including green turtles (Villa et al. 2017). This material will preferentially settle in UPB and will consequently have a greater influence upon metal profiles in UPB than CLV. Due to the complex relationships between the standard normalisation elements Al and Fe in the GBR, future studies are recommended to investigate using Ti as the reference element for normalising lithogenic geochemical profiles (Lam et a. 2015).

The increased erosion in the Burdekin catchment has led to a substantial increase in the discharge of fine sediment from the river over the last 150 years (Lewis et al. 2007; Kuhnert et al. 2012; Kroon et al. 2012). Current best estimates conclude that fine sediment loads have increased, on average, by eight times from about 0.5 million tonnes in 1850 to 4 million tonnes currently (Kroon et al. 2012). Such large increases in fine sediment loads are likely to also result in large increases in the discharge of metals associated with soils in the catchment. Most of the fine sediment discharged from the Burdekin River is deposited in UPB with smaller amounts in Bowling Green Bay and even smaller amounts in CLV (Lewis et al. 2014). Thus, increased fine sediment discharge associated with higher metal concentrations might be expected in UPB. However, this does not show up as higher concentrations of metals in UPB seagrass compared to CLV. The reasons for this are unclear but could be related to marine chemical speciation processes affecting bioavailability or other differences in local metals inputs to each bay.

The lower Burdekin floodplain drains to UPB, it is Australia's largest, most intensively developed agricultural floodplain and is dominated by sugar cane (Davis et al. 2013). Creeks on the southern headlands of Upstart Bay are in the Don River basin but drain into Upstart Bay, but the main northern part of the bay comprises the mouth of the Burdekin River (Brodie et al. 2014). The catchment is dominated by grazing with horticulture, sugarcane, small areas of state forest and timber reserve, and small urban settlements. Upstart Bay may also receive small discharges from Barratta Creek, which along with the Haughton River largely drains irrigated sugarcane into Bowling Green Bay. Intensive use of groundwater for irrigation has exacerbated soil erosion and the expansion of irrigation has resulted in risk of increased groundwater contamination (Baskaran et al., 2001; GBRMPA, 2012).

Potential sources of industry-related pollution within the Don River basin are mainly associated with the township of Bowen (Brodie et al. 2014). Potential metal sources also exist in association with Port of Abbot Point operations including dredge disturbance and spoil dumping and coal dust (e.g., Cd), wastes from recreational marinas and harbours, the sewage outfall, a copper refinery (now closed; potential

legacy metals are As, Bi, Cd, Sb), and a coke works (Brodie et al. 2014). By comparison, potential metals sources to Cleveland Bay include the Ross River and smaller waterways which largely drain urban and light industrial areas, the presence of the commercial Port of Townsville operations, and minor variations in local catchment geology.

Our literature comparisons were preliminary, and a detailed review of global literature databases will provide more insight into whether observed metal concentrations are higher than expected relative to global values for all matrices. Such a review will also serve to clarify the degree to which contextual factors such as substrate geology can be expected to influence metal concentrations in near-pristine environments. These findings should be used to inform future research on metals in the Great Barrier Reef. Further research is warranted to disentangle the biogeochemical processes that enable catchment, river, sediment, and surface water metals to accumulate in forage at levels that may be harmful to higher trophic orders. This research will require close consideration of the role that essential vs. non-essential metals may play in accumulation potentials, as well as analysis of the influence of substrate geochemistry on expected tissue concentrations in forage species.

Our results indicate that metal concentrations in the Great Barrier Reef are variable but often higher than national guidelines, national background values, and/or national or globally comparable ecosystems. There is a real risk that many metals the Great Barrier Reef World Heritage Area are present at ecologically harmful concentrations. Our data are limited for sediment and marine surface waters, and our estimates are unlikely to capture the full range of variability in metal concentrations in these matrices. Further examination of all metals will confirm concentration ranges with more accuracy than was possible in this study.

Conclusions

Our results suggest that Ag, Al, As, Co, Cu, Cr, Mn, Mo, Sr, and Zn may be present in the Great Barrier Reef at elevated concentrations relative to comparable systems. Lithogenic metals were observed at higher concentrations in the coastal locations of UPB and CLV compared to HWK water, sediment and forage. Al, As, Fe, Mn, U, and V were measured above the range of previously reported values in the literature reviews examined, Al and Cu exceeded freshwater guidelines, and Co and Al exceeded marine water guidelines in all locations. Al was elevated in most comparisons which may be indicative of local soils rich in these elements, however this requires further investigation. Metals which exceeded the global forage values (e.g., Al, As, Fe, Mn, U, V) require closer examination within the context of source geochemistry. Terrigenous metals that are high at coastal compared to offshore locations (Al, Fe, Mn, V) may be associated with discharge from the adjacent Burdekin River, in particular sub-catchment with steeper slopes and reactive basalt soils which are more susceptible to erosion. Unusually high concentrations of Co (river and coastal sea water, and coastal forage) could be due to clay and sulphide minerals acting as nucleation sites for the sorption of these metals. Fine sediment discharge from the Burdekin River has increased greatly since the introduction of grazing on the catchment 150 years ago and this may be a key source of metal loads. We recommend a tiered risk assessment focusing on the following metals, at minimum; Al, As, Ba, Cd, Co, Cr, Cu, Fe, Mn, U, V, however a full-scale screening assessment of all relevant matrices is advised. A systematic review and meta-analysis of global seagrass and macroalgae metal baseline values within the context of the pollution status and geochemical environment of each sample would support the risk assessment and provide a consistent and rigorous resource for ongoing assessment of metals to the Great Barrier Reef, and further afield.

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Appendix

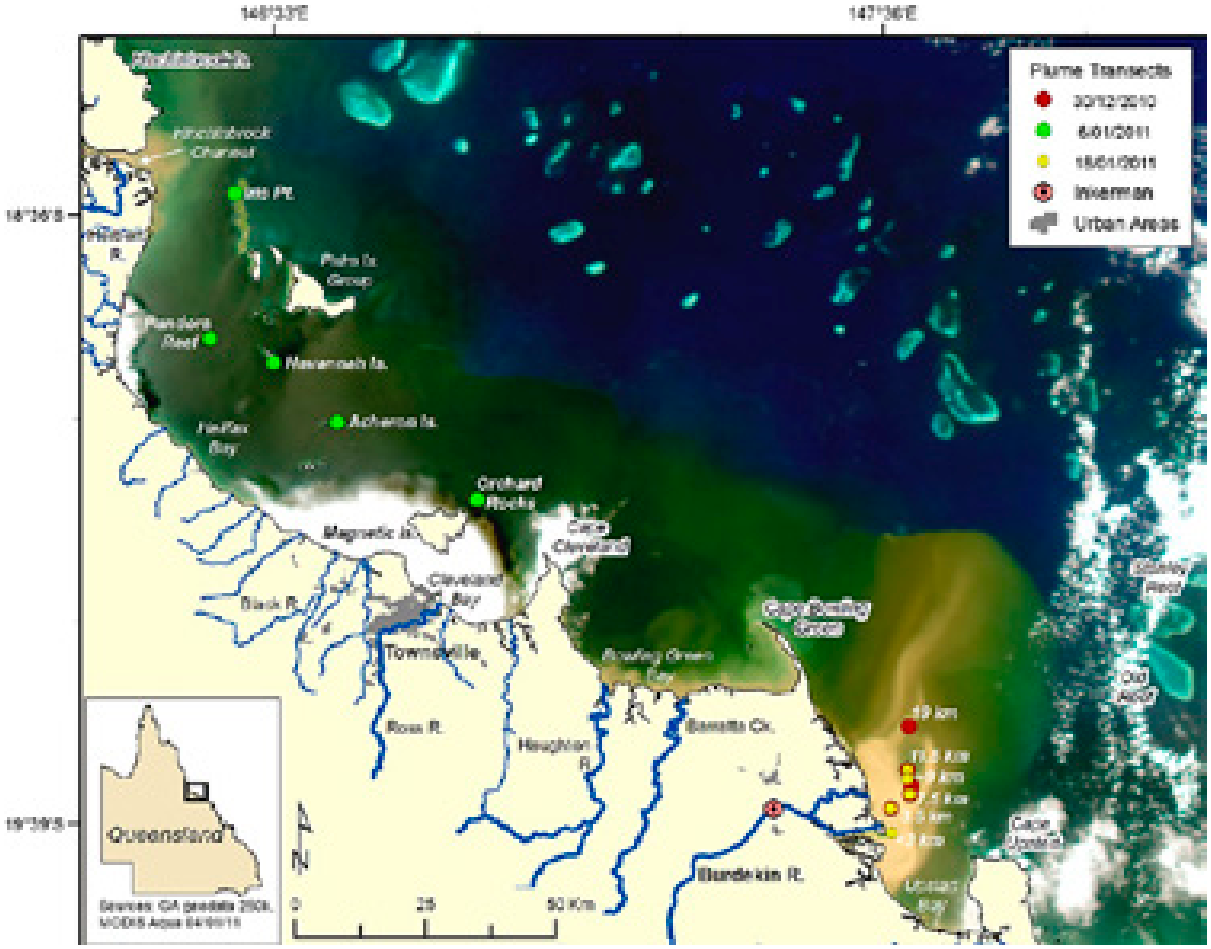


Figure A1: Burdekin River discharge plume in January 2011 showing transport of material into Upstart and Cleveland Bays (Bainbridge et al. 2012).

Inputs	Upstart	Cleveland	Howick
Agricultural – grazing, sugarcane	High river input from ag	Moderate river input	Very low
Urban	Low population	High population & STP discharge	Zero
Port including metal product and ore handling	Very low – no port in bay	High – major port in bay	Zero – No ports nearby
Industry- metal refining	Very low – limited heavy industry in adjacent catchment area	High – 2 mineral refineries and assorted commercial industries	Zero
Oceanic – upwelling/tidal jets*	Very low	Very low	Moderate

Table A1: Likely influences on the three sampling locations due to surrounding geographical features.

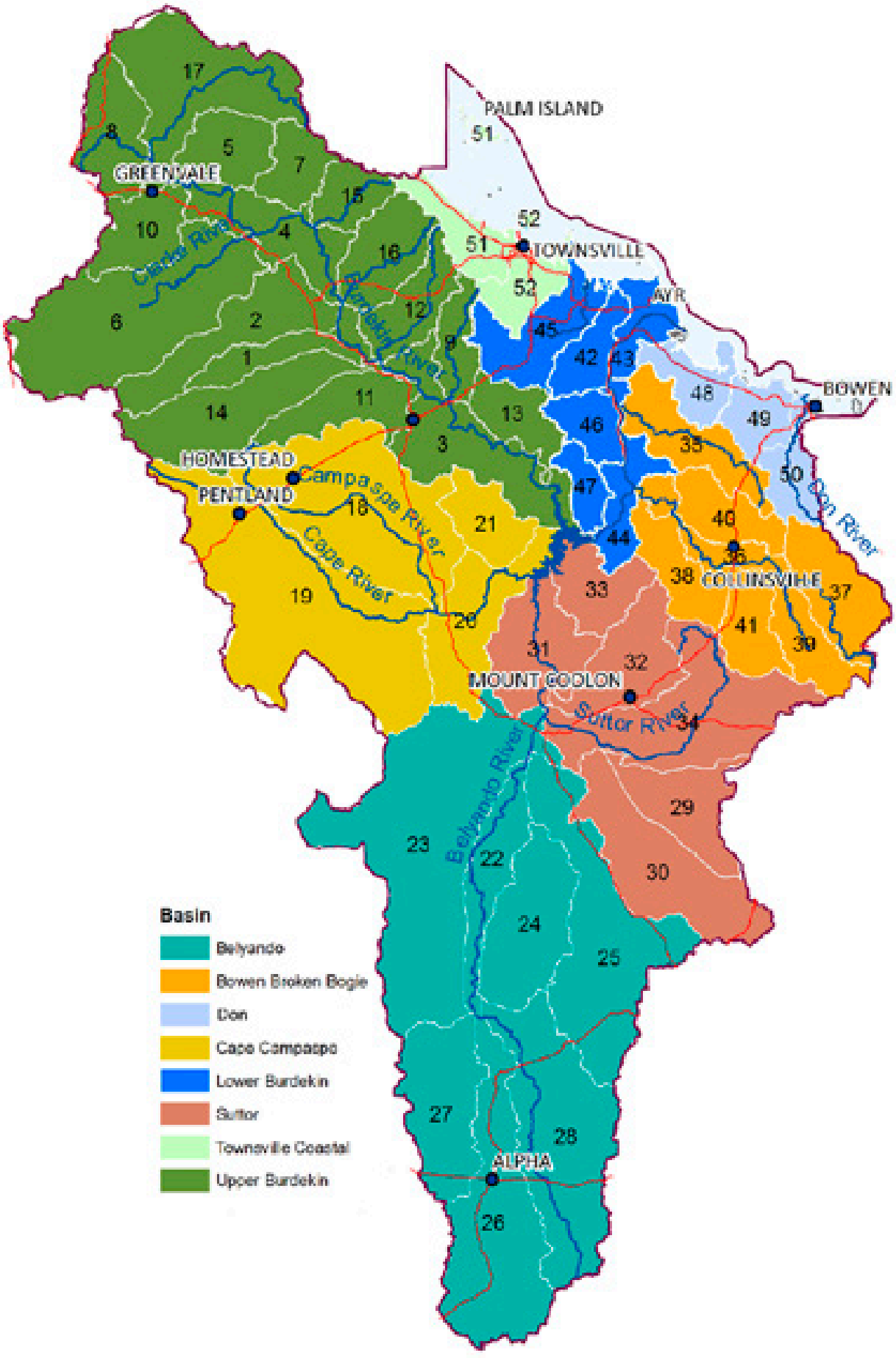


Figure A2: Burdekin catchment and sub-catchments (NQDT 2018).

* Wolanski et al. (1988)

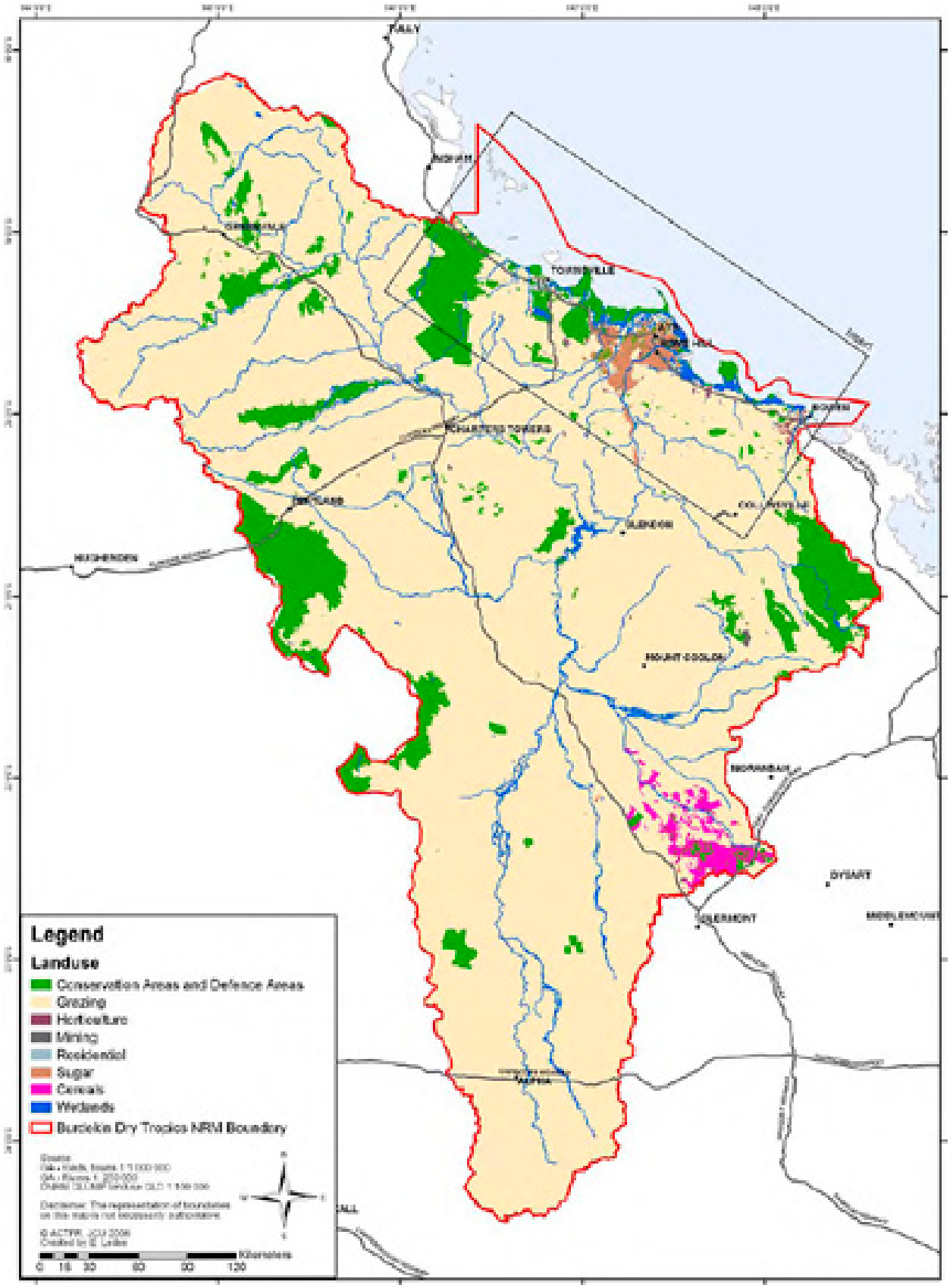


Figure A3: Land use on the Burdekin catchment (Lewis et al. 2006).

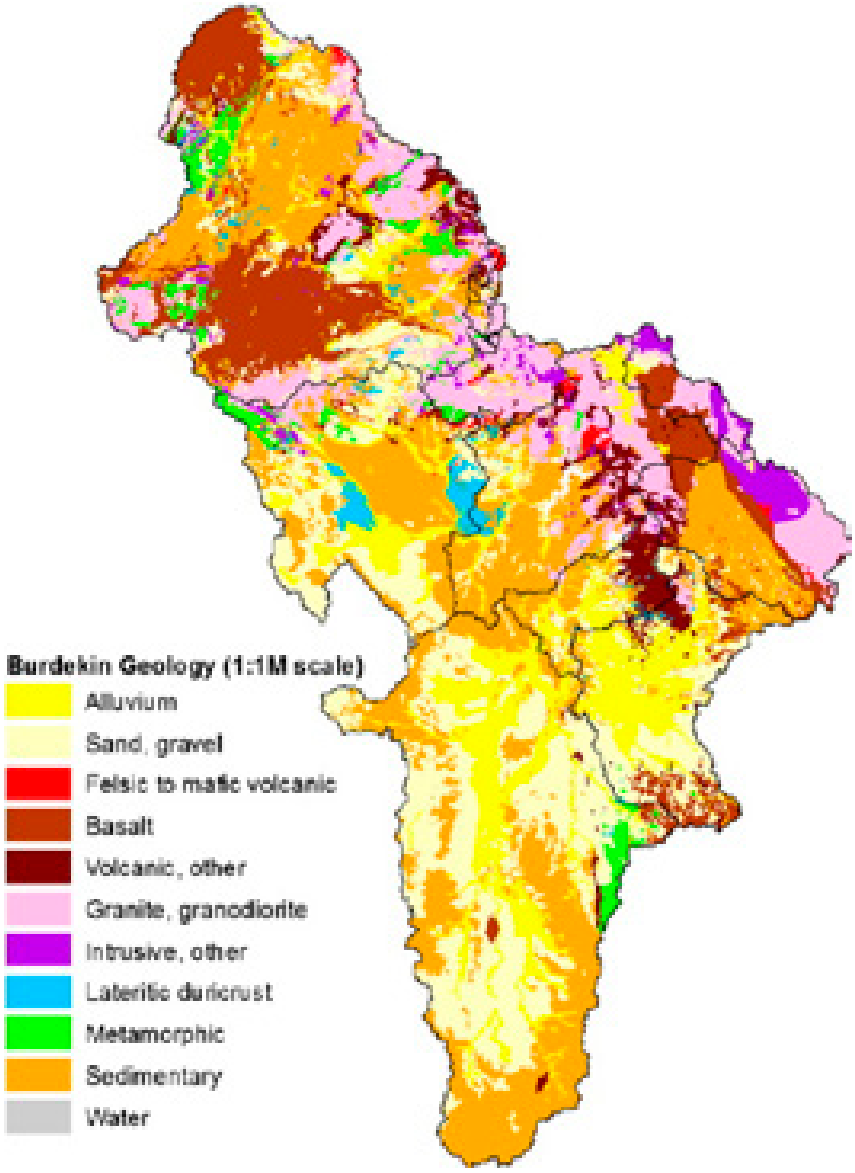


Figure A4: Geology of the Burdekin catchment (Croke et al. 2015).

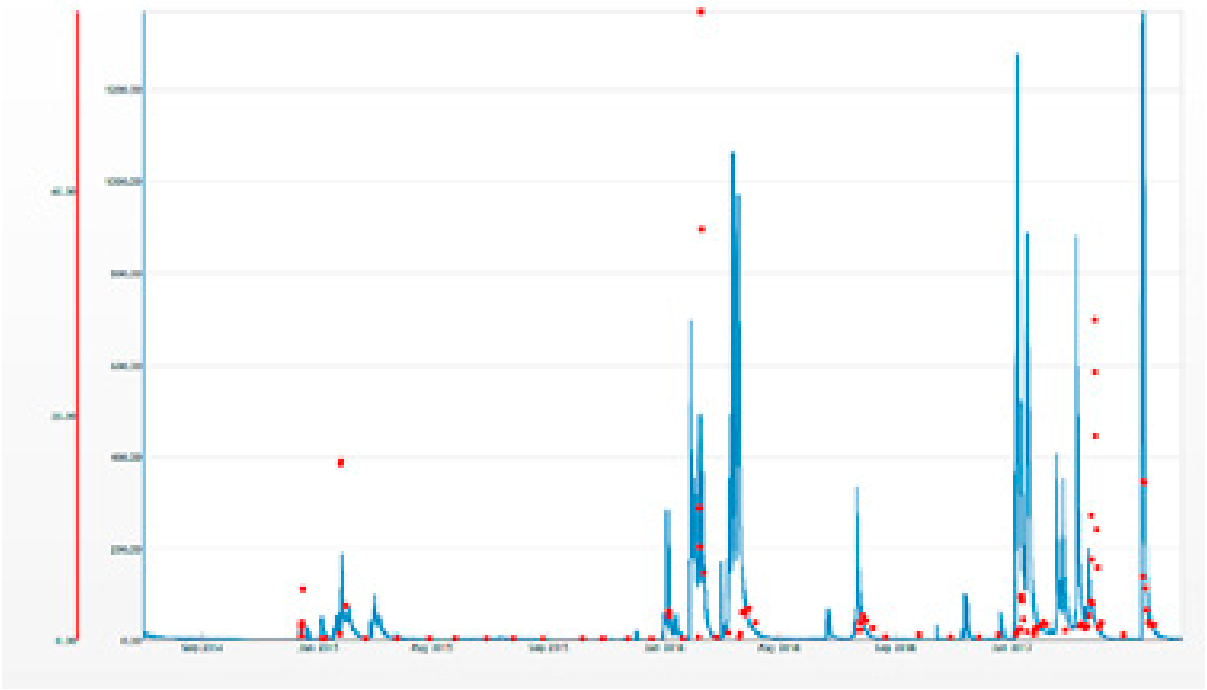


Figure A5: Burdekin River hydrograph and sampling points

	Ag	Al	As	Ba	Cd	Co	Cr	Cs	Cu
Certified value	0.054	1 200	1.1	98	0.17	0.23	1.3	0.14	6.6
Our value 1	0.052	670	1.1	108	0.24	0.18	1.0	0.140	7.0
Our value 2	0.071	690	1.3	115	0.23	0.20	1.2	0.151	7.8
Mean value	0.058	680	1.2	111	0.24	0.19	1.1	0.146	7.4
% recovery	120	59	110	113	140	83	89	104	110
	Fe	Mn	Mo	Ni	Pb	Sb	Se	Sn	Sr
Certified value	460	31	0.20	1.1	9.7	0.2	0.17	3.8	170
Our value 1	490	34	0.24	0.80	10.3	0.256	0.10	4.83	170
Our value 2	550	40	0.23	1.0	11.0	0.253	0.069	5.17	180
Mean value	520	37.2	0.24	0.92	10.7	0.254	0.086	5.00	180
% recovery	110	122	120	84	110	127	51	132	100
	Th	Ti	Tl	U	V	W	Zn	Zr	
Certified value	0.14	38	0.05	0.045	1.2	nd	18	nd	
Our value 1	0.122	16	0.056	0.033	0.97		21		
Our value 2	0.118	16	0.060	0.031	1.0		20		
Mean value	0.120	16	0.058	0.032	1.0		21		
% recovery	86	42	97	72	87	nd	110	nd	

Table A2: Evaluation of analytical accuracy of total extractable metals from forage through certified reference materials (CRM).

NOTE: Where certified values are below the LOD, recovery is not reliable.

	Location	n	Mean	Std. Dev.	Median	Min	Max	90%ile	95%ile
Ag	CLV	57	0.058	0.027	0.052	0.014	0.15	0.092	0.13
	HWK	20	0.017	0.013	0.014	0.0056	0.056	0.035	
	UPB	58	0.066	0.043	0.052	0.014	0.23	0.12	0.20
Al	CLV	58	8,400	5,500	7,600	1,500	37,000	14,000	16,400
	HWK	20	3,000	1,600	2,900	830	7,500	5,000	
	UPB	58	8,600	760	6,500	1,200	37,000	20,000	26,000
As	CLV	58	14	18	8.1	1.2	108	37	55
	HWK	20	6.0	5.8	3.9	1.4	23	19	
	UPB	58	5.1	2.9	4.5	1.3	15	10	11
Ba	CLV	58	12	7.0	11	2.3	43	19	24
	HWK	20	7.6	2.2	7.8	3.5	12	10	
	UPB	58	11	8.7	8.5	2.2	36	28	34
Cd	CLV	58	0.18	0.068	0.18	0.042	0.33	0.26	0.31
	HWK	20	0.20	0.068	0.21	0.080	0.31	0.28	
	UPB	58	0.16	0.10	0.14	0.039	0.47	0.32	0.43
Co	CLV	58	4.6	1.3	4.5	2.6	7.7	6.6	7.2
	HWK	20	0.52	0.21	0.51	0.24	1.1	0.75	
	UPB	58	5.0	2.1	4.7	1.8	10	8.0	9.0
Cr	CLV	58	5.7	3.7	5.2	1.5	26	9.2	12
	HWK	20	5.4	1.9	5.5	2.2	9.5	8.3	
	UPB	58	6.0	5.0	4.6	0.43	26	14	15
Cs	CLV	58	0.78	0.49	0.72	0.13	3.1	1.4	1.6
	HWK	18	0.29	0.15	0.28	0.084	0.69	0.49	
	UPB	57	0.69	0.64	0.50	0.066	2.5	1.7	2.4
Cu	CLV	58	5.5	1.7	5.0	3.7	13	7.7	8.5
	HWK	20	2.5	3.3	1.5	1.1	16	3.7	
	UPB	58	6.0	2.8	5.4	1.5	14	11	12
Fe	CLV	57	7,700	5,000	7,200	900	24,000	16,000	16,000
	HWK	20	1,700	830	1,600	550	4,000	2,700	
	UPB	58	6,300	5,300	4,800	930	24,000	14,000	19,000
Mn	CLV	58	630	230	560	280	1,700	890	1,100
	HWK	20	35	7.7	34	20	51	45	
	UPB	58	630	270	610	230	1,500	980	1,100
Mo	CLV	58	2.8	1.7	2.0	0.84	8.5	5.4	8.1
	HWK	20	0.81	0.44	0.80	0.21	1.9	1.6	
	UPB	58	2.1	1.7	1.5	0.62	7.5	4.4	7.3

Table A3: (Part 1) Summary statistics for metal concentrations in forage samples (2014-2017) by location, rounded to two significant figures. All values are µg/g dry weight.

	Location	n	Mean	Std. Dev.	Median	Min	Max	90%ile	95%ile
Ni	CLV	58	5.4	2.2	4.9	2.3	15	7.9	8.8
	HWK	20	3.0	0.90	3.1	1.7	5.4	4.4	
	UPS	58	5.5	4.1	4.3	2.0	23	12	15
Pb	CLV	58	3.9	1.8	3.7	0.93	11	6.3	7.0
	HWK	20	1.1	0.33	1.1	0.60	1.7	1.7	
	UPS	58	3.4	2.5	2.7	0.73	13	7.5	9.0
Sb	CLV	58	0.22	0.072	0.20	0.11	0.47	0.32	0.32
	HWK	20	0.23	0.088	0.25	0.085	0.32	0.32	
	UPS	58	0.23	0.091	0.22	0.086	0.49	0.35	0.40
Se	CLV	58	1.2	0.64	1.2	0.24	2.7	2.1	2.3
	HWK	20	0.52	0.23	0.50	0.28	0.91	0.87	
	UPS	58	1.0	0.73	0.90	0.14	3.1	2.2	2.8
Sn	CLV	58	0.36	0.21	0.32	0.093	1.6	0.56	0.69
	HWK	20	0.14	0.065	0.13	0.032	0.31	0.22	
	UPS	57	0.30	0.25	0.26	0.016	1.0	0.71	0.90
Sr	CLV	58	370	350	260	150	2,100	660	1,200
	HWK	18	3,200	660	3,200	2200	4,400	4,200	
	UPS	58	280	130	260	100	920	440	470
Th	CLV	57	1.8	1.2	1.7	0.20	5.7	3.3	3.9
	HWK	20	0.61	0.25	0.58	0.20	1.2	0.96	
	UPS	58	1.5	1.2	1.1	0.14	5.0	3.3	4.3
Ti	CLV	58	170	79	160	38	480	270	330
	HWK	18	81	40	92	18	180	139	
	UPS	57	220	140	200	34	610	449	520
Tl	CLV	58	0.049	0.031	0.038	0.013	0.21	0.080	0.11
	HWK	20	0.052	0.019	0.047	0.026	0.10	0.088	
	UPS	58	0.063	0.048	0.038	0.013	0.27	0.14	0.16
U	CLV	57	0.86	0.29	0.74	0.40	1.5	1.3	1.5
	HWK	20	3.2	1.0	2.8	1.9	5.4	5.0	
	UPS	58	1.3	0.69	1.1	0.57	5.0	2.1	2.4
V	CLV	58	18	9.3	17	3.4	49	28	33
	HWK	20	7.5	4.3	5.9	3.9	21	14	
	UPS	58	15	11	12	3.4	52	33	39
Zn	CLV	58	21	7.3	21	8.2	43	29	33
	HWK	20	7.2	4.7	7.5	2.4	24	9.7	
	UPS	58	22	13	19	1.4	56	45	53

Table A3: (Part 2) Summary statistics for metal concentrations in forage samples (2014-2017) by location, rounded to two significant figures. All values are µg/g dry weight.

Element	Slice	N	Mean	Std. Deviation	Std. Error	Minimum	Maximum
Ag	USB-ICO-1	2	0.056	0.012	0.0081	0.048	0.064
	USB-ICO-2	2	0.073	0.023	0.016	0.057	0.089
	USB-ICO-3	2	0.0023	0.00038	0.00027	0.0020	0.0026
	USB-ICO-4	2	0.0017	0.00077	0.00055	0.0011	0.0022
Al	USB-ICO-1	2	30700	260	180	31000	31000
	USB-ICO-2	2	2800	288	200	2900	3000
	USB-ICO-3	2	450	33	23	430	480
	USB-ICO-4	2	720	140	97	620	820
As	USB-ICO-1	2	1.1	0.041	0.029	1.1	1.1
	USB-ICO-2	2	2.2	0.38	0.24	2.0	2.5
	USB-ICO-3	2	0.30	0.0067	0.0047	0.30	0.31
	USB-ICO-4	2	0.43	0.017	0.012	0.42	0.44
Ba	USB-ICO-1	2	11	0.59	0.42	10	11
	USB-ICO-2	2	22	0.13	0.089	22	22
	USB-ICO-3	2	5.1	0.14	0.096	5.0	5.2
	USB-ICO-4	2	5.3	0.082	0.058	5.2	5.4
Cd	USB-ICO-1	2	0.078	0.0058	0.0048	0.073	0.083
	USB-ICO-2	2	0.29	0.0079	0.0056	0.29	0.30
	USB-ICO-3	2	0.0074	0.0023	0.0016	0.0057	0.0090
	USB-ICO-4	2	0.0057	0.0029	0.0020	0.0037	0.0077
Co	USB-ICO-1	2	7.4	0.11	0.074	7.3	7.5
	USB-ICO-2	2	6.0	0.37	0.26	5.7	6.2
	USB-ICO-3	2	1.1	0.039	0.027	1.1	1.1
	USB-ICO-4	2	1.8	0.048	0.034	1.8	1.8
Cr	USB-ICO-1	2	2.6	0.059	0.042	2.5518	2.6
	USB-ICO-2	2	3.8	0.029	0.021	3.7916	3.8
	USB-ICO-3	2	0.61	0.042	0.030	0.5772	0.64
	USB-ICO-4	2	1.1	0.048	0.034	1.0626	1.1
Cs	USB-ICO-1	2	0.033	0.0019	0.0014	0.0312	0.034
	USB-ICO-2	2	0.027	0.0024	0.0017	0.0254	0.029
	USB-ICO-3	2	0.035	0.0030	0.0021	0.0328	0.037
	USB-ICO-4	2	0.037	0.0023	0.0017	0.0355	0.039
Cu	USB-ICO-1	2	14	1.0	0.72	13.4134	15
	USB-ICO-2	2	22	3.6	2.5	18.9688	24
	USB-ICO-3	2	0.94	0.045	0.032	0.9091	0.97
	USB-ICO-4	2	1.2	0.031	0.022	1.1786	1.2
Fe	USB-ICO-1	2	5200	160	110	5038.6736	5300
	USB-ICO-2	2	8700	31	22	8658.3938	8700
	USB-ICO-3	2	870	59	42	830.0950	910
	USB-ICO-4	2	1300	45	32	1229.8226	1300
Mn	USB-ICO-1	2	190	31	22	171.7945	220
	USB-ICO-2	2	760	35	25	728.1397	780
	USB-ICO-3	2	41	8.	5.7	35.3432	47
	USB-ICO-4	2	79	3.8	2.5	76.8272	82
Mo	USB-ICO-1	2	0.11	0.0029	0.0020	0.1044	0.11
	USB-ICO-2	2	0.081	0.024	0.017	0.0643	0.097
	USB-ICO-3	2	0.014	0.00052	0.00037	0.0139	0.015
	USB-ICO-4	2	0.021	0.00043	0.00030	0.0204	0.021

Table A4: (Part 1) Summary statistics for acid-extractable metal (AEM) concentrations in slices from Upstart Bay core UPB-1CO (n = 2), reported to two significant figures; ^marine metals; all values are mg/kg dry weight.

Element	Slice	N	Mean	Std. Deviation	Std. Error	Minimum	Maximum
Ni	USB-ICO-1	2	5.6	0.080	0.056	4.9651	5.1
	USB-ICO-2	2	5.7	0.36	0.26	5.3952	5.9
	USB-ICO-3	2	0.77	0.0035	0.0025	0.7644	0.77
	USB-ICO-4	2	1.1	0.054	0.038	1.0337	1.1
Pb	USB-ICO-1	2	22	0.86	0.61	21.7559	23
	USB-ICO-2	2	130	4.6	3.3	125.6758	130
	USB-ICO-3	2	2.1	0.10	0.073	2.0544	2.2
	USB-ICO-4	2	3.1	0.042	0.029	3.0522	3.1
Sb	USB-ICO-1	2	0.064	0.011	0.0078	0.0564	0.072
	USB-ICO-2	2	0.20	0.050	0.035	0.1596	0.23
	USB-ICO-3	2	0.0068	0.0010	0.00073	0.0061	0.0076
	USB-ICO-4	2	0.0085	0.00074	0.00052	0.0079	0.0090
Se	USB-ICO-1	2	3.4	0.10	0.071	3.3112	3.5
	USB-ICO-2	2	4.2	0.028	0.020	4.1858	4.2
	USB-ICO-3	2	0.57	0.090	0.063	0.5110	0.64
	USB-ICO-4	2	0.96	0.055	0.039	0.9369	1.0
Sn	USB-ICO-1	2	0.12	0.00081	0.00058	0.1156	0.12
	USB-ICO-2	2	0.27	0.032	0.023	0.2507	0.30
	USB-ICO-3	2	0.019	0.00047	0.00034	0.0183	0.019
	USB-ICO-4	2	0.029	0.0022	0.0016	0.0272	0.030
Sr	USB-ICO-1	2	9.0	0.32	0.22	8.7398	9.2
	USB-ICO-2	2	22	0.48	0.34	21.1761	22
	USB-ICO-3	2	3.3	0.66	0.47	2.8283	3.8
	USB-ICO-4	2	4.7	0.012	0.0087	4.6393	4.7
Th	USB-ICO-1	2	0.60	0.016	0.011	0.5850	0.61
	USB-ICO-2	2	0.53	0.12	0.084	0.4448	0.61
	USB-ICO-3	2	0.18	0.014	0.010	0.1733	0.19
	USB-ICO-4	2	0.28	0.0065	0.0046	0.2755	0.29
Ti	USB-ICO-1	2	73	1.16	0.82	71.6820	73
	USB-ICO-2	2	92	4.8	3.4	88.1762	95
	USB-ICO-3	2	6.0	0.36	0.25	5.7311	6.2
	USB-ICO-4	2	7.9	0.45	0.32	7.5962	8.2
Tl	USB-ICO-1	2	0.0084	0.00049	0.00035	0.0081	0.0088
	USB-ICO-2	2	0.0064	0.00096	0.00068	0.0057	0.0070
	USB-ICO-3	2	0.0031	0.00058	0.00048	0.0026	0.0036
	USB-ICO-4	2	0.0036	0.00060	0.00042	0.0031	0.0040
U	USB-ICO-1	2	0.55	0.012	0.0087	0.54	0.56
	USB-ICO-2	2	0.89	0.17	0.12	0.77	1.0
	USB-ICO-3	2	0.070	0.00045	0.00032	0.070	0.071
	USB-ICO-4	2	0.12	0.00050	0.00035	0.12	0.12
V	USB-ICO-1	2	30	0.14	0.099	30	30
	USB-ICO-2	2	38	1.3	0.89	37	39
	USB-ICO-3	2	2.7	0.080	0.056	2.6	2.7
	USB-ICO-4	2	3.2	0.032	0.023	3.2	3.3
W	USB-ICO-1	2	0.020	0.0091	0.0065	0.013	0.026
	USB-ICO-2	2	0.085	0.011	0.0076	0.077	0.093
	USB-ICO-3	2	0.014	0.010	0.0071	0.0071	0.021
	USB-ICO-4	2	0.0051	0.0019	0.0013	0.0038	0.0064

Table A4: (Part 2) Summary statistics for acid-extractable metal (AEM) concentrations in slices from Upstart Bay core UPB-1CO (n = 2), reported to two significant figures; ^marine metals; all values are mg/kg dry weight.

Element	Slice	N	Mean	Std. Deviation	Std. Error	Minimum	Maximum
Zn	USB-ICO-1	2	16	0.36	0.25	16	17
	USB-ICO-2	2	54	2.8	2.0	52	56
	USB-ICO-3	2	2.3	0.037	0.026	2.3	2.3
	USB-ICO-4	2	4.1	0.26	0.18	3.9	4.3
Zr	USB-ICO-1	2	0.19	0.014	0.0097	0.18	0.20
	USB-ICO-2	2	0.38	0.31	0.22	0.16	0.60
	USB-ICO-3	2	0.065	0.012	0.0083	0.056	0.073
	USB-ICO-4	2	0.080	0.00059	0.00042	0.079	0.080

Table A4: (Part 3) Summary statistics for acid-extractable metal (AEM) concentrations in slices from Upstart Bay core UPB-1CO (n = 2), reported to two significant figures; ^marine metals; all values are mg/kg dry weight.

Core	USB-2CO data (Lewis et al. 2014)			USB-1CO data (this study)	
	Sediment type	Est. age (yr)	Est. date	% organic	% $CaCO_3$
USB-1CO-1	silty clay	100 ± 10	1903-1920	10.32	4.23
USB-1CO-2	silty clay	49 ± 4	1960-1968	7.28	4.67
USB-1CO-3	silty clay	42 ± 7	1964-1978	0.81	0.48
USB-1CO-4	sand	30 ± 4	1977-1984	1.05	0.71

Table A5: Mean carbonate and organic carbon characteristics of the USB-1CO core samples (n = 2) and matching stratigraphic regions and depth-age relationships in USB-2CO (Lewis et al. 2014).

Source	Elemental limits of detection (µg/L)									
	Al	As	Cd	Co	Cr	Cu	Mn	Ni	Pb	V
This study	2.2	3.6	0.0050	0.005	0.03	0.008	0.02	0.004	0.004	0.029
Angel et al. 2012	1	0.09	0.003	0.002	0.4	0.02	0.1	0.032	0.016	n.a.
Munksgaard & Parry 2001	n.a.	0.1	0.001	0.001	n.a.	0.005	n.a.	0.004	0.002	n.a.
Eisklement 2000	n.a.	n.a.	n.r.	n.a.	n.a.	n.r.	n.a.	n.a.	n.r.	n.a.
Apte & Day 1998	n.a.	n.a.	0.0008	n.a.	n.a.	0.013	n.a.	0.031	n.a.	n.a.
Denton & Burdon-Jones 1986	n.a.	n.a.	0.01	n.a.	n.a.	n.r.	n.a.	n.r.	0.06	n.a.

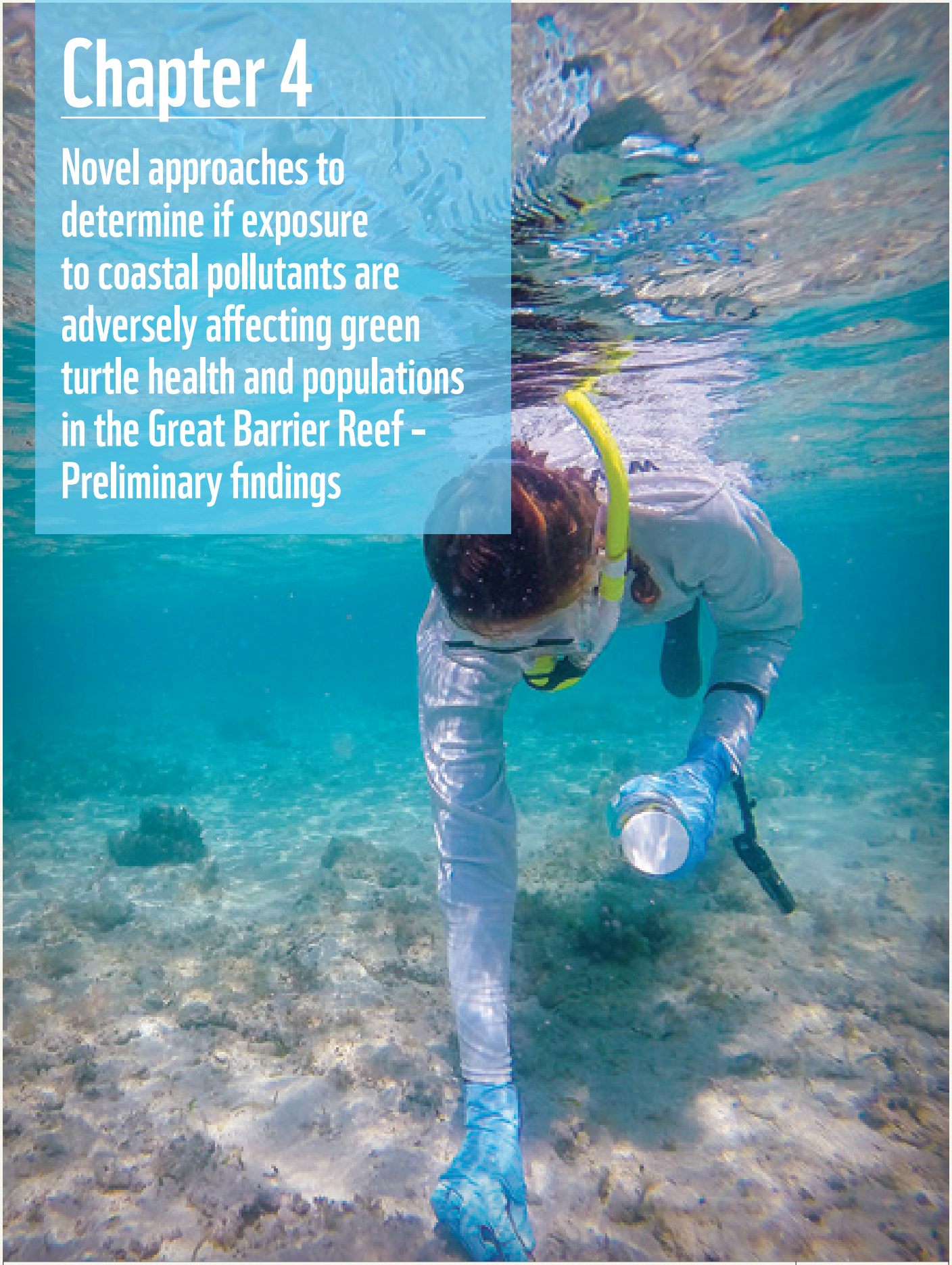
Table A6: Reported marine sea water limits of detection; n.r. = not reported; n.a. = not assessed.

	DISSOLVED METALS								TOTAL METALS							
	Barratta Creek (n=183)				Burdekin River (n=93)				Barratta Creek (n=106)				Burdekin River (n=44)			
	Min	Max	Med	Mean	Min	Max	Med	Mean	Min	Max	Med	Mean	Min	Max	Med	Mean
Ag	0.025	0.11	0.025	0.025	0.03	0.15	0.03	0.03	0.15	0.3	0.15	0.15	0.15	0.3	0.15	0.16
Al	3.0	410	34	56	0.52	130	18	22	310	33000	4700	7000	64	96300	3000	11000
As	0.45	2.9	1.1	1.2	0.51	2.6	1.0	1.07	1	3.3	1.7	1.7	1.2	11	1.6	2.5
B	27	82	43	44	21	56	38	38	30	69	46	47	23	59	40	40
Ba	28	160	68	73	16	130	39	39	58	220	97	110	38	680	68	100
Be	0.10	0.10	0.10	0.10	0.10	0.50	0.10	0.11	0.5	1.4	0.5	0.54	0.5	3.8	0.5	0.72
Cd	0.02	0.06	0.02	0.02	0.02	0.10	0.02	0.02	0.1	0.2	0.1	0.10	0.1	0.24	0.1	0.11
Co	0.08	2.1	0.23	0.29	0.03	0.34	0.03	0.05	0.3	5.6	1.15	1.6	0.15	56	0.7	4.4
Cr	0.05	0.67	0.11	0.12	0.05	0.41	0.10	0.11	0.3	18	2.7	4.3	0.3	135	2.8	12
Cu	0.40	360	2.0	4.8	0.50	2.4	1.10	1.19	0.5	14	4.2	5.2	0.5	99	4	9.8
Fe	12	500	85	120	5.20	180	17	33	360	16600	2900	4500	47	110000	2100	10000
La	0.02	0.96	0.27	0.33	0.02	0.35	0.05	0.07	0.2	16	2.6	3.8	0.1	61	1.3	6.2
Li	2.5	2.5	2.5	2.50	0.50	2.5	2.5	2.5	2.6	14	14	14	2.6	48	14	15
Mn	1.3	2900	22	54	0.20	160	1.7	6.3	40	640	92	120	11	2600	64	240
Mo	0.20	1.5	0.60	0.62	0.20	0.90	0.50	0.52	1	3	1	1.0	1	3	1	1.1
Ni	0.50	2.9	1.1	0.98	0.50	2.4	0.50	0.53	3	11	3	3.7	3	120	3	10
Pb	0.025	1.4	0.12	0.17	0.025	5.5	0.06	0.13	0.15	8.4	1.5	2.3	0.15	45.4	1.2	4.6
Sb	0.05	0.30	0.05	0.06	0.05	0.44	0.05	0.07	0.3	1.1	0.3	0.34	0.3	4.3	0.3	0.58
Se	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	1	2	1	1.0	1	2	1	1.1
Sn	0.05	0.80	0.05	0.13	0.05	0.80	0.05	0.10	0.3	2.5	0.3	0.4	0.3	0.8	0.3	0.33
Ti	0.12	25	6.4	7.4	0.09	6.6	0.71	1.3	4.6	1200	66	170	1.3	1100	76	210
Tl	0.025	1.1	0.025	0.061	0.025	2.0	0.025	0.057	0.15	1	0.15	0.19	0.15	0.7	0.15	0.21
U	0.07	1.2	0.16	0.21	0.04	0.86	0.15	0.21	0.1	1.2	0.4	0.49	0.1	2.4	0.5	0.57
V	1.2	5.5	3.7	3.67	1.6	7.1	3.2	3.6	2.1	36	9.2	12	1.8	240	8	26
Zn	0.50	49	1.8	2.93	0.50	18	1.1	2.13	3	42	11	12	3	220	10	23

Table A7: Summary statistics of total and dissolved metal concentrations (µg/L) in river water collected from Burdekin River at Home Hill and Barratta Creek at Northcote, rounded to two significant figures where possible.

Chapter 4

Novel approaches to determine if exposure to coastal pollutants are adversely affecting green turtle health and populations in the Great Barrier Reef - Preliminary findings



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4. Novel approaches to determine if exposure to coastal pollutants are adversely affecting green turtle health and populations in the Great Barrier Reef – Preliminary findings

C. Gallen[1,*], A. Heffernan[1,2], S. Kaserzon[1], G. Dogruer[1,3], K. Thomas[4], M.J. Gomez-Ramos[1], J.F. Mueller[1]

[1] Queensland Alliance for Environmental Health Sciences, The University of Queensland, 39 Kessels Road, Coopers Plains, Qld 4108;
[*] Corresponding author (C. Gallen): c.bentley@uq.edu.au; Phone +61428532053
[2] The Florey Institute of Neuroscience & Mental Health, Melbourne
[3] Institute for Environmental Research, RWTH Aachen University, Germany
[4] Norwegian Institute for Water Research (NIVA), Oslo

Abstract

Marine organisms are exposed to diverse mixtures of both known and unknown chemicals in their natural environments. In 2012, a mass stranding of green turtles (*Chelonia mydas*) occurred in a coastal bay within the Great Barrier Reef Marine Park. In 2014, a case-control sampling approach was used to investigate the exposure of wild green sea turtles to anthropogenic pollutants at two coastal 'case' sites influenced by primarily urban/ industrial and agricultural activities, respectively; and a remote, offshore 'reference site. Water and sediment samples collected from the three sampling locations showed differences in pollutant profiles that reflected the dominant land uses in the adjacent catchment. Target analysis of a range of pesticides, industrial chemicals, pharmaceuticals and personal care products found the concentrations and frequencies of detections highest in the coastal sampling locations, which are influenced by river and urban discharge. Non-target analysis for 'unknown' chemicals of water and sediment detected few chemicals of interest. However, they showed clear differentiation of the urban/ industrial location from the other two locations, as did non-target analysis of turtle blood also collected from the same locations In-vitro assays of pooled sediment samples from each location showed overall a higher induction in four of the five assays by at least one of the coastal locations compared to the control site. This study was part of a larger multi-disciplinary investigation working to link the external exposure of turtles to a range of turtle health parameters measured during the same sampling events. This study provides evidence that green turtles foraging in coastal areas are exposed to a range of anthropogenic pollutants derived from the adjacent coastal catchment areas and naturally produced pollutants that are yet to be fully characterised. Despite the overall very low concentrations of many of these detected chemicals, the cumulative effects of long-term exposure remain unknown and of increasing concern for this iconic species.

Keywords:

Time-of-flight mass spectrometry; Chemical exposure; Non-target screening; Marine wildlife; Passive sampling; Great Barrier Reef

Introduction

The World Heritage Great Barrier Reef Marine Park covers an area of 344,400 km^2 and spans 2,300 km along the Queensland coast in Eastern Australia. Green turtles (*Chelonia mydas*) are one of its iconic species, and are the most abundant of the six marine turtle species known to reside on the Reef (GBRMPA 2016a). The conservation status of green turtles has been assessed by IUCN (the World Conservation Union), and by the Australian and Queensland governments as either endangered or vulnerable (GBRMPA 2016a). Following a period known as the 'lost years' where juvenile turtles are thought to reside in the open ocean, sub-adult and adult green turtles show strong fidelity and return to shallow inshore foraging areas where they spend approximately two to eight years between breeding cycles, primarily feeding on seagrass (preferentially) or algae (GBRMPA 2016a). Living in these inshore coastal

areas leaves them susceptible to longer-term exposure to land-derived (anthropogenic) contaminants (such as sediments and chemical pollutants) as well as at higher risk of injury through encountering human activities (for e.g. boat strikes, ingestion of litter).

The Great Barrier Reef (GBR) receives run-off from 35 river basins (or catchments) which drain approximately 424,000 km^2 of coastal Queensland. Currently, approximately 80% of the entire Reef catchment area is used for agricultural activities with grazing, forestry and cropping the primary land uses (DSITIA 2012b). Pesticide run-off from diffuse agricultural land uses has been identified as a high risk threat to GBR ecosystems and heritage values (GBRMPA 2014), with the sugar cane industry the major contributor of loads of photosystem II (PSII) inhibiting herbicides into the marine environment (Kroon et al. 2013). PSII herbicides have been recognised as priority pollutants of interest due to their heavy usage within Reef catchments and their demonstrated effects on GBR marine photosynthetic organisms such as microalgae, coral and seagrass (Davis et al. 2013). These and other agricultural chemicals used in adjacent catchments are introduced into the inshore waters of the GBR in river run-off primarily during the wet season, and can reside at elevated concentrations in the marine environment for extended periods of time (Devlin and Schaffelke 2009, Grant et al. 2017). To date, they have been the major focus of monitoring activities on the GBR, although research into other groups of pollutants (such as pharmaceuticals and personal care products (PPCPs)) is starting to emerge (Kroon et al. 2015). Other activities occurring within GBR catchments such as aquaculture, ports, urban and industrial development represent smaller point sources of other land-based chemical pollutants such pharmaceuticals and personal care products (PPCPs) and may also impact on the health and resilience of Reef ecosystems.

Over June and July 2012, approximately 100 mostly adult green turtles were reported washed up dead or dying in Upstart Bay, within the GBR. Several of the stranded turtles that were still alive appeared to be suffering neurological symptoms (head sensitivity, uncontrolled head movements and seizures) despite appearing otherwise outwardly healthy with no evidence of fibropapilloma, injury or starvation. No other fauna suffered obvious illness or death during this period. Again in 2013 and 2014, a small number of green turtles with similar neurological symptoms were found, with the cause of their symptoms still unknown. In 2014, the authors were part of a multi-disciplinary team charged to investigate and identify whether anthropogenic pollutants may be adversely affecting the health of resident green turtles. In parallel, a turtle toxicology and health sampling program was undertaken with the ultimate goal of determining if correlations exist between any identified pollutant exposure and turtle heath baseline parameters. In the event of another mass stranding event of green turtles in Upstart Bay, a rapid environmental sampling response would be mobilised in an effort to identify any potential organic causative neurotoxic agent/s responsible.

The objectives of this environmental sampling campaign were to:

- Broadly screen for organic pollutants present in water and sediment in Upstart Bay.
- Compare the pollutant profiles of water and sediment in Upstart Bay to two reference locations using a ‘case-control’ approach and attempt to identify chemicals unique to the stranding site.
- Investigate whether there are seasonal trends in organic pollutant profiles at each location as a result of the increased river discharge or urban run-off during the wet season.
- Investigate potential trends with other datasets (such as analysis of turtle blood for organic pollutants) collected by collaborators.

Materials and methods

High purity solvents (HPLC grade) and sulfuric acid were purchased from Merck (Darmstadt, Germany). Ultrapure water (Millipore, 0.22 μm filtered, 18.2 $m\Omega\ cm^{-1}$) was used for chemical analysis. Mobile phases were filtered using Millipore 0.45 μm DuraporeTM filter membranes. Laboratory glassware, accelerated solvent extraction (ASE) cells and passive sampler housings were rinsed with appropriate solvents (acetone/ methanol and/ or hexane) prior to use. Styrenedivinylbenzene Reverse Phase Sulfonated

(SDB-RPS) EmporeTM Extraction Disks (EDs) were purchased from Phenomonex. Diffusion limiting polyether sulfone membranes (diameter 47 mm, pore size 0.45 µm) were purchased from Sartorious. Polydimethylsiloxane (PDMS) sheets (thickness 500 µm) was purchased from Purple Pig, Brisbane. Syringe filter units (0.22 µm and 0.45 µm; PTFE and 0.2 µm; RC) were purchased from Phenomenex. Sodium sulphate was purchased from Thermo Fisher Scientific.

Aluminium oxide and silica was purchased from Sigma. Empty 6cc cartridges, Strata-X solid phase extraction (SPE) cartridges (200 mg; 6 mL) and frits were purchased from Phenomenex. Copper powder and florisil were purchased from Sigma Aldrich. Amber glass vials (2 mL) and hydromatrix were purchased from Agilent. Isotope-labelled internal standards were purchased from Wellington Laboratories (Guelph, Ontario, Canada), Novachem (Collingwood, Victoria, Australia) or AccuStandard (New Haven, Connecticut, USA).

Sampling sites

Two sites were selected for comparison to the stranding site. Both sites support foraging areas of green turtles and no known turtle deaths occurred at these two locations over the same period as the Upstart Bay stranding.

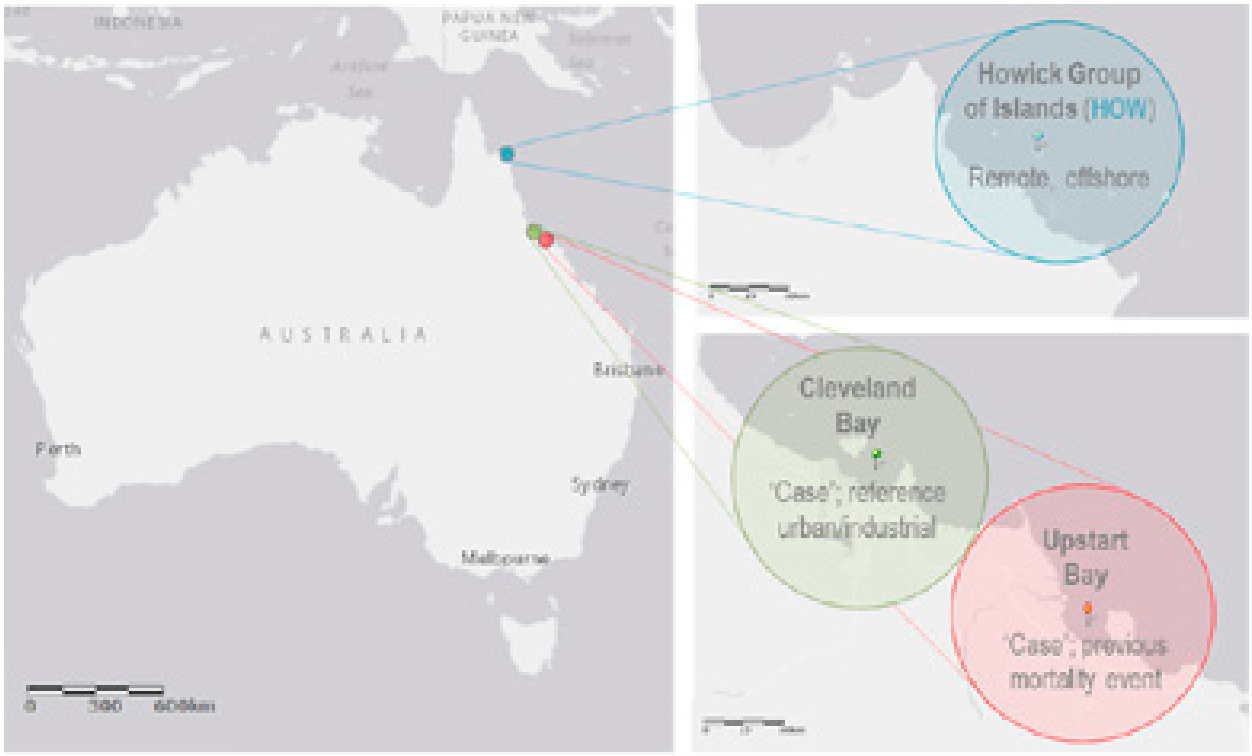


Figure 1: The three sampling locations; the'control' Howicks Group of Islands (top right) and the two coastal 'case' sites Cleveland Bay and Upstart Bay (bottom right).

The Howicks Group of Islands was selected as the pristine control location. This collection of unpopulated islands is located approximately 100 km off-shore and adjacent to the Cape York region, which is the least developed of the entire GBR catchment (DSITI 2015). Its remote location and large distance from land-based contaminant point sources such as river run-off should result in very low background levels of environmental contaminants. The three primary sampling sites at this location were Ingram Island, Coombe Reef and Outer Reef.

Cleveland Bay was selected as a coastal 'case' location and receives urban/ industrial input from the city of Townsville and agricultural input from the vast Burdekin region of the Reef catchment which supports grazing, sugar cane farming and horticulture land uses (Gunn and Manning 2009) (DSITIA 2012a). This bay is geographically similar to Upstart Bay where the mass stranding occurred, being only 100 km north and experiencing similar environmental input in terms of weather events and adjacent land use.

The sampling sites were located approximately 20 km southeast of Townsville city (CB1 – 3), at Bedwell, Cockle Bay and near the waste water treatment plant (WWTP).

The location of the 2012 stranding was Upstart Bay, a north facing bay also located in the Burdekin region (GBRMPA 2013a). Land use is primarily grazing however it supports various aquaculture activities as well as horticulture. The Burdekin River (historically discharging the largest annual volumes of freshwater into Reef inshore areas) (Gallen et al. 2014) discharges into the north of this bay area, with the Burdekin Delta floodplain characterised by large areas of irrigated cropping and grazing (Dight 2009, DSITIA 2012a). The sampling sites visited were UB1 - 3.

Sampling Campaigns

Sampling activities were carried out twice yearly over a three-year period between 2015 – 2017, to capture potential temporal and seasonal differences in organic pollutant profiles. A summary of the samples collected and analysed in the first year of sampling are detailed in the Supporting Information (SI) (Table A1, Table A2 and Table A3). Sampling at these locations was repeated in years two and three and archived at -20°C in the Environmental Specimen Bank located at QAEHS. Extraction and analysis of samples from years two and three was not pursued based on the low concentrations detected during year one and the absence of any further turtle stranding or major rainfall/ weather events occurring.

Passive Samplers

Water quality monitoring was conducted using both grab 'snap-shot' sampling as well as passive sampling techniques, whereby chemicals accumulate into a sorbing material from water via passive diffusion. The passive samplers utilized included:

SDB-RPS Empore™ Disk (ED) polar passive samplers for relatively hydrophilic organic chemicals with relatively low octanol-water partition coefficients (logKOW). These were deployed in both the routine and naked configurations (Stephens et al. 2009). Only the naked EDs (which accumulate chemicals at a faster rate) were extracted and analysed. Routine EDs were archived at -20°C.

Polydimethylsiloxane (PDMS) non-polar passive samplers for organic chemicals that are relatively more hydrophobic (higher log KOW). At least two PDMS strips were deployed in a cage at one site per sampling location. During extraction, two strips were combined per sample to increase the amount of chemicals accumulated and surpass the detection limits of analysis.

Passive flow monitors (PFMs) are deployed with passive samplers to provide an estimate of the site-specific flow conditions. The loss of plaster from the PFM can be used to predict changes in the uptake of chemicals into a sampler dependent on flow and turbulence (O'Brien et al. 2011).

The methods for preparation, transportation and extraction of passive samplers have been extensively reported in numerous publications (Page et al. 2014, GBRMPA 2016b, O'Brien et al. 2016a).

Sampling Methods

Grab water samples (1 L) were collected directly into high density polyethylene bottles. The bottle was attached to a sampling pole and dipped underwater to an approximate depth of 50cm. The bottles were capped, covered in foil to prevent photo-degradation and frozen within 24 h of collection to preserve the integrity of the sample. Field blank samples (MilliQ water) were uncapped for the duration of sampling activities. Sediment samples were collected using gloved hands from the surface at low tide within the turtle foraging areas. Sediment was scooped into acetone-rinsed glass jars (375 mL), which were wrapped in foil to prevent photo-degradation and frozen within 24 h of collection.

Extraction Methods

Grab water samples

500 mL of water from each sampling location was measured and spiked with a mixture of isotope labelled herbicides and personal care products (PPCPs). Field blanks and laboratory blank water samples were also included. Samples were loaded onto Strata-X 200mg 6cc cartridges (Phenomenex) that had been

pre-conditioned with 4 mL methanol and 4 mL of MilliQ water. Cartridges were dried under vacuum for approximately 1 h and eluted with two x 2 mL of methanol. Eluents were reduced in volume under N2 to approximately 1 mL, filtered (0.2 µm; regenerated cellulose) and adjusted to a final volume of 500 µL (20 % methanol; 80 % water) in a 2 mL LC vial. Isotope labelled recovery standards were spiked into each sample prior to analysis, however it must be noted results have not been recovery corrected.

Sediments

Each pooled sample or sub-sample was thawed, thoroughly mixed and then an aliquot transferred into a 50 mL falcon tube. Samples were re-frozen and then freeze-dried to remove all water. 20 g of sediment from each sampling location was weighed into pre-cleaned ASE (ASE, Thermo Scientific 350; Dionex, USA) cells with 5 g of florisil and hydromatrix. Cells were spiked with a surrogate to account for losses during extraction and exhaustively extracted using hexane: dichloromethane (1:1). Extracts were reduced in volume to approximately 3 mL and subjected to further cleanup using 3 % deactivated silica and 6 % deactivated aluminium oxide, eluted with 40 mL of hexane: dichloromethane (1:1). Samples were reduced to a final volume of 200 µL in hexane. Results have not been corrected for recoveries.

Following an initial screen for organic pollutants, a more targeted 'in-cell' clean up approach was trialled, to increase the probability of detection of pollutants by using larger masses of sample, sulfuric acid clean up to remove interference and provide a quantitative analysis. Pooled freeze-dried samples were weighed into pre-cleaned ASE cells. ASE cells were packed with multiple layers of silica, sulfuric acid treated silica, copper powder, and florisil. Samples (50 g) were spiked with a mixture of isotope labelled standards (pesticides, polychlorinated dibenzodioxins/ furans (PCDD/Fs), polychlorinated biphenyls (PCBs) and polybrominated diphenylethers (PBDEs)) and extracted using hexane: dichloromethane (1:1). Extraction was performed using two static cycles, each lasting 5 min at 100 °C and 1500 psi. The flush volume was regulated at 60 % and the purge time was 90 s.

Extraction of sediment for bioassay

Extraction of sediment samples followed a standardised US EPA Method 3545A using accelerated solvent extraction (ASE). Prior to extraction, the ASE was run with diatomaceous earth (DE) (Sigma-Aldrich) to flush any residual contamination. In brief, DE (approx. one third of the cell`s volume) was loaded into stainless-steel extraction cells (33 mL) prepared with a cellulose filter (Dionex™ ASE™). Six g of each sediment sample was then added and topped with DE. Furnaced DE (550 °C for 24 h) served as process control for QA/QC. Extraction was performed during three static cycles, each lasting 5 min at 100 °C and 1500 psi using hexane and acetone as solvents (1:1, v:v, LiChrosolv®). The Flush volume was regulated at 60 % and the purge time was 90 s. The extraction efficiency of this ASE method was tested previously and ranges from 80-120 % for chlorinated pesticides, semi-volatile organics and PCBs (Li et al. 2013). The extracts were concentrated with a rotary evaporator and further blown gently down under a stream of nitrogen to near dryness, then resolubilized in 60 µL DMSO and stored at -20 ˚C until analysis. DMSO extracts were directly used for analysis on the in vitro bioassays with no further clean-up procedures.

Analytical Methods

Target analysis for herbicides and PPCPs in EDs and water samples was done using an AB Sciex QTRAP 6500 mass spectrometer (AB Sciex, Concord, Ontario, Canada) equipped with an electrospray (TurboV) interface coupled to a Shimadzu Nexera HPLC system (Shimadzu Corp., Kyoto, Japan).

Non-target screening for unknown chemicals in water samples was carried out using a Shimadzu Nexera X2 ultrahigh-pressure liquid chromatography (UPLC) system equipped with a binary pump and a reverse-phase Gemini-NX C18 column (3 µm × 2.0 mm × 50 mm, Phenomenex. The UPLC was coupled to a hybrid quadrupole time-of-flight mass spectrometer (QTOF-MS) system, (Triple-TOF 5600, Sciex), with an electrospray (ESI) interface operating in positive and negative ionization mode. Full details regarding the processing of raw data files with PeakView® software (v2.2,Sciex, Melbourne, Aus) have been reported (Heffernan et al. 2017).

Target analysis for PDMS samplers was performed using Thermo Fisher trace GC ultra-TSQ triple quadrupole Quantum XLS system for PCBs, PBDEs and pesticides and a Thermo Scientific DFS High Resolution GC/MS in splitless injection mode for polyaromatic hydrocarbons (PAHs), dioxins and furans. Non-target screens for chlorinated and brominated chemicals were done using a Shimadzu GC-2010 gas chromatography coupled with QP-2010 mass spectrometer operating in NCI-mode and for masses using a single quadrupole mass spectrometer (ISQ LT. Thermo Fisher Scientific) operated in EI mode. For the advanced sediment clean up analysis, a Thermo Scientific DFS High Resolution GC/MS in splitless injection mode was used for the analysis of PBDEs, acid-resistant organochlorine pesticides (OCPs), PCBs, polychlorinated napthalenes (PCNs) and PCDD/Fs.

For the non-target screening of sediment and PDMS samples at the Norwegian Institute of Water Research (NIVA) a Thermo Fisher Scientific Q Exactive GC Orbitrap MS system was used. The separations were carried out on a BD-5 column (30 m 0.25 m 0.25 mm, Agilent). All the injections were performed in splitless mode having an injection volume of 1 µL. Helium was used as the carrier gas. The oven, initially, was kept at 60 °C for 2 min. The oven temperature was ramped to the final temperature of 310 °C with a ramp rate of 5 °C/min. The temperature of injection port was kept at 280 °C during the runs. Both transfer line and the source temperatures were set to 280 °C. After each injection the column was baked out at 310 °C for 30 min in order to avoid any carry over from previous injections. The TOFMS collected two spectra every second between 50 and 600 m/z. The detector exhibited a resolution of ~ 8000 at half width full range (i.e. 50 to 600 m/z). The detector was operated at 2850 V and a filament current of ~ 1 mA. Lists of target analytes are provided in the SI (Table A4, Table A5 and Table A6). For the target analysis, if the amount quantified in the field blank was <5 % of the amount quantified in the sample, no blank correction was made. If the field blank amount was 5 – 20 % of the sample amount, then it was subtracted from the sample prior to calculation of concentration. If the field blank amount was >20 % of the sample amount, then the sample was assumed to be below the limits of quantification (<LOQ). It must be noted that corrections for losses during extraction have not been made.

Results and discussion

A range of chemical pollutants were identified at all three sampling locations using a combination of passive and grab water sampling techniques. Overall, estimated water concentrations were very low, with the exception of various personal care products for which contamination either in the field or in the laboratory cannot be excluded. This apparent contamination occurred despite the diligent use of gloves and the avoidance of personal care products (such as sunscreens) during sampling and extraction activities. As expected the pristine reference site Howicks Islands had relatively few detections, when compared to samples collected at the inshore Cleveland Bay and Upstart Bay locations, which are more directly influenced by terrestrial sources of chemical pollutants. In the first year of sampling, PDMS samplers accumulated very low levels of PAHs across all three sites, and only two pesticides (chlorpyrifos and trans-chlordane) were detected above detection limits. A screen for chlorinated chemicals found unique peaks at Howicks Islands and Cleveland Bay (yet unidentified).

Water (Passive samplers - EDs)

Overall, for both the pre-wet and post-wet sampling periods, the estimated concentrations of target chemicals detected across all three sampling locations were low (i.e. < 3 ng/L). For both sampling campaigns, contamination of laboratory blanks with several PPCPs occurred including DEET, caffeine and triclosan, which are present in many personal care products that may have been in use by sampling personnel. Thus, any results of these detected in deployed samplers have been excluded. Despite this, the profiles of chemicals differed both between sites and between sampling campaigns (Table 1).

For both sampling campaigns, the trend in total number of chemicals detected was Howicks Islands < Upstart Bay < Cleveland Bay. Water concentrations could be estimated for several of the detected chemicals (Table 1) due to the availability of calibration data (i.e. sampling rates). As the samplers were deployed in the naked configuration (i.e. without flow limiting membranes), a correction factor based

on Shaw and Mueller (2009) was applied. For chemicals where a correction factor was not available, the correction factor for atrazine was used.

	Howicks		Upstart Bay		Cleveland Bay	
Sampling Campaign	Pre-Wet 2014	Post-Wet 2015	Pre-Wet 2014	Post-Wet 2015	Pre-Wet 2014	Post-Wet 2015
Est. concencration range (ng/L)						
Ametryn	n.d.	n.d.	n.d.	n.d.	0.07	n.d.
Atrazine	n.d. - 0.10	n.d. - 0.07	0.28 - 0.29	0.19 - 0.23	0.91 - 1.6	0.61 - 0.66
Atrazine desethyl	n.d.	n.d.	n.d.	n.d. - 0.05	0.20 - 0.28	n.d.
Diuron	n.d. - 0.06	n.d. - 0.03	0.29 - 0.30	0.16	0.25 - 0.30	0.2
Hexazinone	n.d.	n.d.	0.11	0.14 - 0.18	0.10 - 0.15	0.14 - 0.15
Metolachlor	n.d.	n.d.	0.05 - 0.06	0.03	0.18 - 0.36	0.15
Simazine	n.d.	n.d. - 0.02	0.06 - 0.08	n.d. - 0.02	n.d. - 0.12	0.04 - 0.10
Tebuthiuron	n.d.	n.d.	n.d.	n.d.	n.d.	n.d. - 0.01
Imadicloprid	n.d.	n.d. - 0.03	n.d.	n.d.	n.d.	n.d. - 0.02
Desisopropyl Atrz.	n.d.	n.d.	n.d.	n.d. - 0.01	n.d.	n.d. - 0.01
24-D	n.d. - 0.03	n.d.	n.d.	n.d.	n.d. - 0.08	n.d.
Chlorpyrifos	n.d.	n.d.	n.d.	n.d.	n.d. - 0.10	n.d.
MCPA	n.d.	n.d.	n.d.	n.d.	0.05	n.d.
Also detected^						
Carbamazepine						x
Citalopram						x
Codeine						x
Desmethyl Citalopram						x
Ioprocmide						x
Methomyl				x		
Paracetamol	x					
Tramadol						x
Venlafaxine						x
DCPMU						x
Ametryn hydroxy				x		x
Triclopyr		x				
Triclosan						x
Hydrochlorthiazide						x

Table 1: Estimated water concentrations (ng/L) of selected chemicals (top) and positive detections of other chemicals (bottom) detected using EDs

^ Calibration data not available for these chemicals. Water concentrations not calculated.

Catchment-specific profiles of organic contaminants were detected. As expected at the Howicks locations, very few chemicals were detected in both sampling periods and estimated concentrations were very low (<0.1 ng/L). The PSII herbicides atrazine and diuron (both used extensively in GBR catchments and frequently detected in existing an marine monitoring program (Grant et al. 2017)) were common to both sampling periods. Only five herbicides were detected in total over both sampling campaigns at this location.

The coastal site Cleveland Bay had the greatest number of chemicals (20 herbicides and PPCPs) detected in total over both sampling periods. This included ten chemicals (carbamazepine, citalopram, codeine, desmethyl citalopram, ioprimide, tebuthiuron, tramadol, venlafaxine, DCPMU and hydrochlorothiazide) that were not been detected at either Upstart Bay or the Howicks Islands (Figure A1). On average, concentrations of the herbicides atrazine and metolachlor in Cleveland Bay exceeded Upstart Bay by approximately three and five times respectively, a trend observed over both sampling campaigns. Diuron and hexazinone were present in similar concentrations in both Upstart Bay and Cleveland Bay, and were all < 1 ng/L (Table 1).

The land area that drains to Cleveland Bay supports approximately 20% of the GBR population in the city of Townsville (Gunn and Manning 2009). Land use activities in close proximity to Cleveland Bay include urban/residential land use, grazing, nature conservation, other minimal use activities (e.g. golf courses), and small areas of manufacturing, industry and waste treatment. Storm water, urban run-off, and

effluent discharged from industry and wastewater treatment plants are all potential sources of chemical compounds identified in water and sediment samples collected at this location.

A total of nine herbicides were detected in EDs over both sampling campaigns at Upstart Bay. Despite being located almost 10 km apart, the chemical profile of samplers deployed at both the UB 1 and UB 2 sites remained very similar. Atrazine, diuron and hexazinone were the most abundant herbicides detected (maximum estimated concentrations of 0.29, 0.30 and 0.18 ng/L respectively). These three herbicides are consistently the most frequently and abundantly detected herbicides during the wet season of the GBR Marine Monitoring Program, which is conducted also using passive sampling techniques (Gallen et al. 2014). In grab samples, two atrazine breakdown products (desethyl atrazine and desisopropyl atrazine), ametryn-hydroxy and simazine were detected at very low concentrations at UB 2 only (< 0.05 ng/L) in the post-wet season sampling (Table 1; Figure A1). The carbamate insecticide methomyl was detected only at Upstart Bay and not any of the other locations ((Table 1; Figure A1).

Intensive sugar cane cultivation occurs in the Burdekin Delta which drains into the north of Upstart Bay. The Don River Basin is located adjacent to the southern part of Upstart Bay and supports nature conservation land uses, grazing as well as small areas of irrigated cropping and aquaculture (GBRMPA 2013a). In contrast to Cleveland Bay, only a small urban population is situated in proximity to the Upstart Bay sampling activities. The profiles of pollutants detected in water and passive samplers are largely dominated by agricultural uses.

Non-target analysis of EDs from both sampling campaigns was also undertaken however the high level of background interference (i.e. any material sorbed to and extracted from the EDs in parallel with the desired organic pollutants) meant that little data could be generated. This method of analysis may not be appropriate for these types of samples.

Water (Passive samplers – PDMS)

The PDMS sampler deployed at each of the sampling locations underwent both targeted analysis (for known chemicals) and a non-targeted screen in both EI and NCI mode (for unknown chemicals based on mass range and the presence of bromine/ chlorine atoms). Target analysis included a range of polychlorinated biphenyls, pesticides and polycyclic aromatic hydrocarbons (SI, Figure A5).

In the pre-wet season 2014 sampling, four PAHs were detected at levels just surpassing the LOD all three of the sampling locations. Re-analysis of these samples on a high-resolution GC-MS/MS further confirmed the presence of the insecticide chlorpyrifos in Upstart Bay, and low concentrations of trans-chlordane at both Upstart Bay and Cleveland Bay. Chlorpyrifos has been previously detected in PDMS samplers deployed as part of the MMP (Gallen et al. 2013, Gallen et al. 2014). Despite widespread detection across the Wet Tropics, Burdekin and Mack Whitsunday regions, estimated water concentrations are typically very low (<1 ng/L). Trans-chlordane is an organochlorine pesticide (OCP), banned for use since the 1980s. As OCPs are relatively resistant to degradation processes, and thus long-lived in the environment, their sporadic detections at very low concentrations may occur as erosion of farming soil occurs over time (Cavanagh et al. 1999).

The reanalysis found five PAHs were also identified (above blank concentrations) at Cleveland Bay, and four PAHs identified at Upstart Bay. The use of the HR-GC-MS/MS can eliminate some of the interferences that may occur during standard low-resolution analysis.

In the post-wet 2015 season, perylene (at Cleveland Bay) was the only PAH detected above blank levels.

The non-target screen for PDMS samplers was conducted over a large m/z range of 50 – 600, and yielded no specific peaks of interest. Comparison of NCI screening for chlorinated chemicals in PDMS samples deployed over the two sampling campaigns found unique chlorinated peaks at the Howicks Islands and a separate unique peak at Cleveland Bay (Figure A2). (No comparison was able to be made with Upstart Bay). These chlorinated peaks remain unidentified.

PDMS extracts from both sampling campaigns were also sent for non-target GC/MS analysis to potentially identify unknown chemicals (Section 3).

Water (Grab samples)

Similarly to the passive samplers for both sampling campaigns, a total of eleven chemicals (mostly PPCPs including caffeine, DEET, paracetamol, salicyclic acid and ibuprofen) were detected in blank samples and were disregarded from further consideration. Trace analysis of chemicals is inherently effected by even very low levels of blank contamination, that may be associated with laboratory equipment, the working environment or the technician, even despite considerable efforts to minimise it (Capdeville and Budzinski 2011). Following blank subtraction, ten chemicals were detected in the pre-wet sampling campaign (eight herbicides, and two PPCP) and 16 chemicals in total in the post-wet sampling campaign (13 herbicides, and three PPCPs). Again, the sites located in the Howicks had the least number of chemical detections for both sampling campaigns (3 and 6 respectively), followed by Upstart Bay (5 and 11 respectively), and Cleveland Bay had (7 and 8 respectively) (Figure 2).

Seven of twelve grab water samples (post-wet season) from Howicks Island had at least one chemical detected. Four herbicides were detected at the Howicks Islands, ranging from 0.14 ng/L (propoxur) to 0.65 ng/L (ametryn). The articificial sweetner acesulufame was detected at the highest concentration (1.4 ng/L) in a single sample from Howicks Island.

Anthropogenic chemicals were detected in all grab water samples (pre- and post-wet) collected at Upstart Bay. Atrazine was the most frequently detected chemical in samples (0.30 – 0.46 ng/L; 100 % of samples), followed by diuron (0.26 – 0.68 ng/L; 83 % of samples). Chlorpyrifos, citalopram, prometryn and ametryn, were detected only at site UB 2. Bromacil and DCMPU were detected only at site UB 3. No concentrations detected exceeded 1 ng/L.

Chemicals were detected in all water samples collected at Cleveland Bay (pre- and post-wet). Similarly to Upstart Bay, atrazine was the most frequently detected (0.41 – 0.55 ng/L; 100 % of samples), followed by desethyl atrazine and ametryn hydroxy (also known as atrazine hydroxy) which were both found in 67 % of samples. Metolachlor was detected in two samples (maximum concentration 0.25 ng/L), and not detected at either Howicks Islands or Upstart Bay.

Recognising that diffuse pesticide run-off was a significant contributor to the declining quality of water entering the Reef, environmental monitoring and research activities on the GBR have focused primarily on only a small number of heavily used agricultural anthropogenic pollutants with a large focus on five priority PSII herbicides (ametryn, atrazine, diuron, hexazinone and tebuthiuron) (GBRMPA 2013c) (Turner et al. 2012, Turner et al. 2013, Wallace et al. 2014, DEHP 2015, Garzon-Garcia et al. 2015, Wallace et al. 2015). Recently as part of best farming practices, the use of alternative (non-priority PSII herbicides including 2,4-D, acifluorfen, imazapic, imazethapyr, isoxaflutole, metribuzin, trifloxysulfuron-Na, metolachlor, trifluralin, pendimethalin) has been encouraged (Smith et al. 2015). However, these too have been exported to marine environments and found in detectable levels in passive samplers (Garzon-Garcia et al. 2015), with 2,4-D detected at all locations in the current study (maximum of 0.19 ng/L at Upstart Bay).

The types and concentrations of herbicides detected in water and passive samplers are within the range also been observed in other marine monitoring activities (Gallen et al. 2014) which assess concentrations and trends of herbicides and pesticides only. Passive samplers are routinely deployed monthly (wet season) or bimonthly (dry season) at up to twelve inshore marine locations as part of this Marine Monitoring Program (MMP; under Reef Plan).

Two of these routine passive sampler locations (Cape Cleveland and Magnetic Island) are close to those in the current project. Similar to this study, atrazine (together with diuron) dominate the herbicide profiles at many of the MMP sites, including Cape Cleveland and Magnetic Island. Overall, lower levels are observed during the dry season (May – October), and comparatively higher levels observed in the wet season (November – April). Concentrations observed in this current study were lower than those observed during the same periods for the MMP, however it must be noted that samplers are configured differently (with respect to the use of flow limiting membranes and deployment times) and are in locations that may be less directly influenced by river flow.

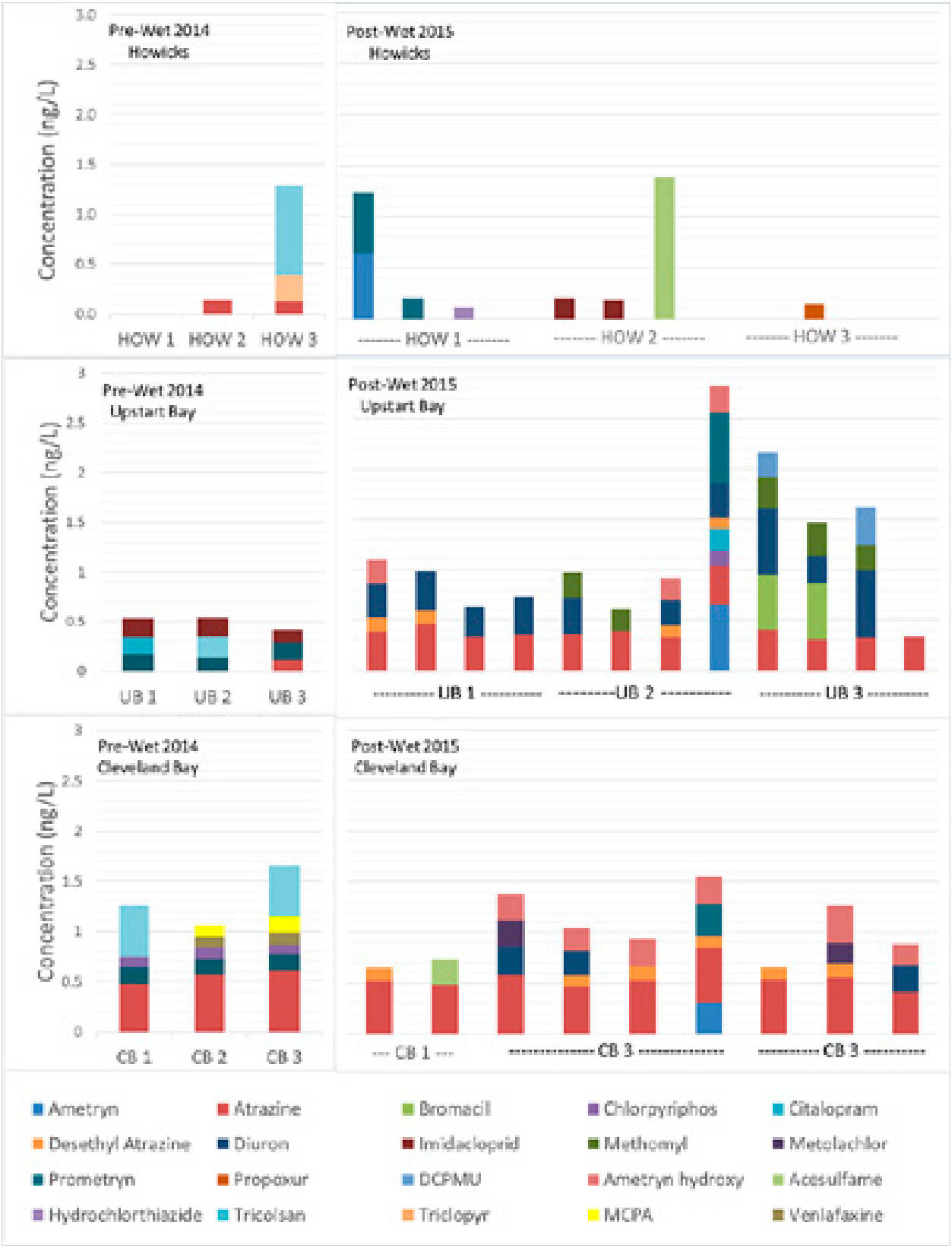


Figure 2: Estimated water concentrations (ng/L) of chemicals in extracted water samples during the pre-wet 2014 season (left section) and post-wet season 2015 (right section). Upstart Bay (top), Cleveland Bay (middle) and Howicks group of Islands (bottom)

The observed low concentrations in this study are also consistent with the trend of low rainfall and river discharge from adjacent catchments. All of the herbicides observed in passive samplers in this study have been previously detected and reported by the MMP. There are no routine monitoring programs that

monitor water concentrations of PPCPs or PAHs in the GBR, and thus this dataset reports some of the first marine detections of such chemicals.

Non-target analysis: Correlating data from blood and water samples

Water (grab samples), and ED passive samplers (post-wet season, 2015) underwent non-target analysis, including: (1) screening against commercial libraries for known compounds; and (2) non-target structural characterisation for unknown compounds. The library screening results were compared to non-target analysis of whole turtle blood collected at the same three sampling locations, also as part of this study (Heffernan et al. 2017). A comparison for the three sample types are presented in Figure 2.

Screening against commercial compound library

Screening of blood samples against commercial libraries generated few positive matches, dominated by endogenous biological compounds (Heffernan et al. 2017). Of these, 20:4 long chain fatty acid, and the DNA adduct n-ethylguanine were also detected in passive samplers from all three locations; the fatty acid was also detected in grab samples from all three locations. Few PPCPs were identified in blood samples: allopurinol and milrinone in Cleveland Bay, and azaelic acid in both Cleveland Bay and Upstart Bay but these compounds were not detected in the corresponding water samples.

Pesticides (herbicides and insecticides) were identified in water samples from Cleveland Bay (atrazine and TEPP), and Upstart Bay (cyromazine), but not from Howicks. This correlates with identification of an insecticide metabolite (ethiofencarb sulfone) in blood samples from turtles in Cleveland Bay (Heffernan et al. 2017). It is important to note that this is not a metabolite of the insecticide measured in water samples, but it does provide moderate evidence that insecticides are used in the adjacent land in Cleveland Bay, and these compounds are being metabolised by local turtle population.

Additionally, five pharmaceuticals used to treat human health conditions were identified in blood and water samples from Cleveland Bay, but no one pharmaceutical was detected in both matrices. This is likely due to chemical properties that see individual compounds compartmentalise differently into water, sediment and serum. Additionally, we would expect many compounds to be metabolised and excreted by turtles, so detecting these possibly short-lived metabolites is challenging. This may also correlate with the lipid peroxidation products, neuroinflammatory markers and oxidative stress previously identified in Cleveland and Upstart Bay, which showed moderate-to-strong correlations with clinical measures of inflammation and liver dysfunction. Note that all identifications should be regarded as tentative until mass, retention time and fragmentation pattern can confirmed with an analytical standard.

Compound		Howicks			Cleveland			Upstart		
		Blood		Grab H2O	Blood		Grab H2O	Blood		Grab H2O
Endogenous compounds										
2-Octenoyl-carnitine	Lipid; fatty ester	-	✓	-	-	-	-	-	-	-
Adenine	Nucleotide precursor	-	-	-	✓	-	-	✓	-	-
Adenosine	Nucleotide precursor	-	-	-	✓	-	-	✓	-	-
Dopamine	Neurotransmitter	-	-	-	✓	-	-	-	-	-
Fatty acid (C20:4)	Long chain fatty acid; phospholipid	-	✓	✓	✓	✓	✓	✓	✓	✓
Inosine	Nucleoside	-	-	-	-	-	-	-	-	✓
N-ethylguanine	DNA adduct	-	✓	-	✓	✓	-	✓	✓	-
Nicotinamide	Vitamin B3 derivative	-	✓	✓	-	✓	-	✓	✓	-
Pharmaceuticals & personal care products										
Allopurinol	Pharmaceutical; used in humans to treat excess uric acid e.g. gout	-	-	-	✓	-	-	-	-	-
para-aminomethyl-benzoic acid	Antifibrinolytic agent used to control bleeding	-	-	-	-	✓	-	-	-	-

Compound		Howicks			Cleveland			Upstart		
		Blood		Grab H2O	Blood		Grab H2O	Blood		Grab H2O
Azelaic acid	Industrial: lubricants, plasticizers. PPCPs: e.g. acne cream	-	-	-	✓	-	-	✓	-	-
Cyclopentamine	Over-the-counter vasoconstrictor, largely discontinued in Australia	-	-	-	-	-	-	-	-	✓
DEET	Common ingredient in insect repellent	-	✓	✓	-	✓	✓	-	✓	✓
Milrinone	Vasodilator, used to treat heart failure	-	-	-	✓	-	-	-	-	-
salicylic acid	Primary metabolite of aspirin, and ingredient in topical acne products	-	-	✓	-	-	✓	-	-	-
Viloxazine	Pharmaceutical; selective norepinephrine reuptake inhibitor used as an antidepressant	-	-	-	-	✓	-	-	-	-
Pesticides and industrial products										
8-hydroxy quinolone	Diverse applications e.g. chelating agent, antiseptic and pesticide	-	-	-	-	-	-	✓	-	-
Atrazine	Triazine herbicide, used in sugarcane crops and turf	-	-	-	-	✓	-	-	-	-
Cyromazine	Insecticide and acaricide	-	-	-	-	-	-	-	-	✓
Tetraethyl pyrophosphate (TEPP)	Organophosphate compound, used as an insecticide	-	-	-	-	-	✓	-	-	-
Other										
Acesulfame	Artificial sweetener	-	-	-	-	-	-	-	✓	-
Hordenine	Naturally-occurring plant product	-	-	-	-	-	-	-	✓	-

Table 2: Summary of compounds tentatively identified by library screening in Cleveland and Upstart Bay. Library match was based on accurate mass, isotopic distribution and fragmentation profile); or compound identified manually as a structural isomer of a library compound.

Principal component analysis and unknown analysis

Principal component analysis (PCA) of turtle blood showed thrent individuals from the same site clustered together in the two-dimensional plot (Figure 3A) demonstrating that between-individual variation in chemical profile was smaller than between-site differences. This clustering was different depending of mass features identified in positive or negative ionisation mode, and differed between blood and water samples, as expected. This distinction was particularly evident for Cleveland Bay, where samples were grouped together and clearly distinct from samples originating from Howicks and Upstart Bay (Figure 3A, C). This grouping allowed us to analyse the results as a function of site-specific xenobiotic contaminant profile, and not inter-individual variability.

In blood samples, the main class of chemicals responsible for differences between Upstart Bay and Cleveland Bay were lipids, specifically long chain fatty acids (Figure 3A); and lipid metabolites, and are likely derived from dietary sources. The group of chemicals responsible for differences between Cleveland Bay and Howicks (Figure 3B) were industrial chemicals, including sulfonic acids, which have broad applications as detergents and surfactants, dyes and acid catalysts for industrial processes (Heffernan et al. 2017) Formulas were generated for an additional 37 compounds (23 in Upstart Bay and 14 in Cleveland Bay), including one polybrominated compound, but no structure or compound name could be identified. It's possible that this brominated compound is natural in origin, with location-specific brominated signatures also detected in sediment samples (see Section 3)

Similarly, grab water samples from the 2015 post-wet season from Cleveland Bay were clearly differentiated from the other two sites. Unknown identification of mass spectral features is ongoing, and the causative agent(s) are unknown at this time.

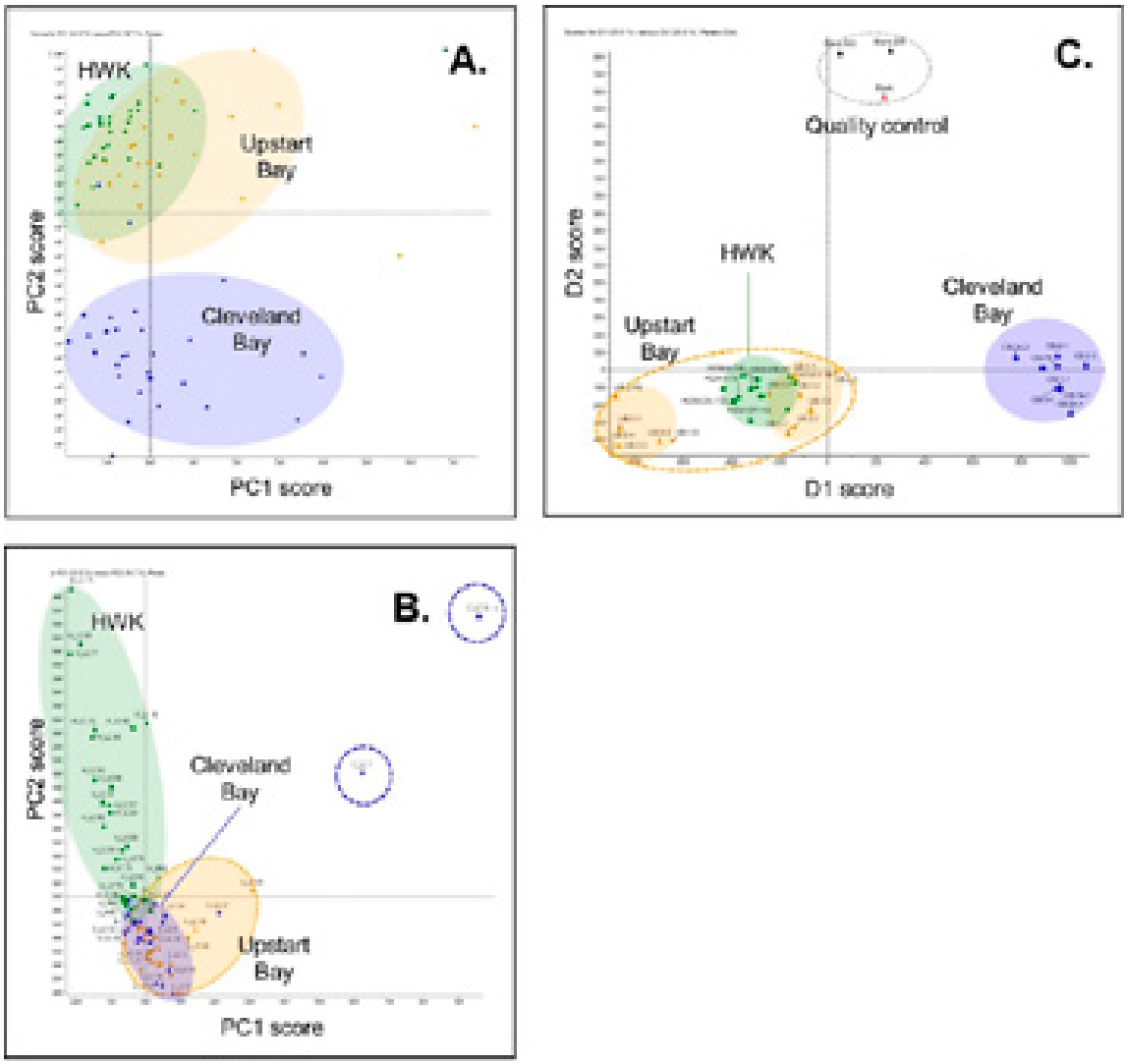


Figure 3: Multivariate analysis of data acquired through non-targeted mass spectrometry; Cleveland Bay (blue), Upstart Bay (orange) and Howicks Group of Islands (green). Pareto-scaled score plot from principle component analysis (PCA) of whole turtle blood in positive ionisation mode (A. PC1 (24.8%) vs. PC2 (19.1%)) and negative ionisation (B. PC1 (27.6%) vs. PC2 (19.7)); and PCA discriminate analysis of grab water samples in positive acquisition mode (C), D1 (25.0%) vs. D2 (25.0%).

Sediment

Pooled sediment samples from each investigation site from both sampling campaigns were analysed for the same suite of target analytes as the PDMS samplers (Table A5). Analysis yielded few results above detection limits although relatively higher amounts of the PAH perylene was observed in Cleveland Bay compared to other sampling locations. In the Pre Wet 2014 samples, nine PAHs were detected, with an increasing trend in the number and concentration of detections from the Howicks < Upstart Bay < Cleveland Bay. Re-analysis of the same samples using high-resolution GC-MS/MS also confirmed the presence of chlorpyrifos (an organophosphate pesticide used by the sugarcane industry) in both the Upstart Bay and Cleveland Bay samples, in addition to low levels of alpha endosulfan (an organochlorine pesticide) at Cleveland Bay.

In the Post Wet 2015 samples, eight PAHs were detected. Two PAHs (fluoranthrene and pyrene) were detected in all three samples but did not sufficiently exceed blank concentrations and were disregarded. No PAHs were detected above blank concentrations at Howicks Islands. Six PAHs were detected at very

low concentrations at Upstart Bay, and two PAHs were detected in Cleveland Bay. A distinctive spike in the concentration of perylene at Cleveland Bay was similar to that observed in the previous sampling campaign. These exceedingly low/ non-detectable concentrations are consistent with previous studies undertaken on coastal GBR sediments adjacent to the Burdekin region (Cavanagh et al. 1999, Müller et al. 1999, Davis et al. 2012)

A more comprehensive clean-up of a single sediment sample (Cleveland Bay from pre-wet season 2014) was trialled, and analysis undertaken for a larger suite of chemicals (PBDEs, PCNs, selected OCPs and PCBs) using HR/GCMS-MS. Very low concentrations (<100 pg/g) of PCB-118,-153, -138 and-180 were the only chemicals detected. PCBs have historical uses as coolants and lubricants in electrical equipment (such as transformers and capacitors), hydraulic fluids, additives in paint, carbonless copy paper, plasticisers and dye carriers (NPI 2014). This group of chemicals was banned by the Stockholm Convention on Persistent Organic Pollutants in 2001.

Distinct differences in the profiles of brominated chemicals in sediments were observed between locations (Table A5). Overlaying chromatograms from the two sampling campaigns at each location found good reproducibility at Howicks Islands and Cleveland Bay, however samples collected from Upstart Bay were the least reproducible (possibly due to differences in sampling protocol between periods). Further interrogation of the Upstart Bay sample using full scan in NCI mode identified one peak as a dibromodiphenol, most likely 2,4-DBP, which can naturally occur in molluscs and crustaceans (PubChem) (PubChem) or as breakdown product of the UV irradiation of once widely used flame retardants (Bendig and Vetter 2013).

Natural halogenated chemicals have been previously identified in passive samplers deployed on the GBR including at Magnetic Island located within Cleveland Bay (Vetter et al. 2009). To date, several thousand natural halogenated products have been identified from algae, marine sponges and other primarily marine organisms with many exhibiting interesting biological activity (antibacterial, antifungal, anti-parasitic, antiviral, antitumor, anti-inflammatory, antioxidant, and enzymatic) (Vetter et al. 2009, Gribble 2015). They share similar chemical structures, physicochemical properties and environmental fates as those of persistent organic pollutants, however their level of toxicity is relatively unknown. Of concern, they have been identified in water, sediments and marine wildlife worldwide in similar concentrations to anthropogenic organohalogen compounds, showing evidence of bioaccumulation in higher marine organisms such as dolphins and dugongs (Vetter et al. 2009, Gribble 2015). It is possible that the unidentified chlorinated and brominated chemicals detected in both sediment and PDMS samplers are from natural producers located in the bays and reefs within the sampling locations.

Non-target screening of sediment and PDMS samples

Passive sampler extracts (PDMS) and sediment extracts from the Pre-wet 2014 and Post-Wet 2015 sampling periods were transferred to Norwegian Institute for Water Research (NIVA) for non-target analysis of GC amenable chemicals. Using MassLynx software, the chromatograms were first deconvoluted using the retention time of the extracted ion chromatograms. This resulted in a long list of components detected within each chromatogram, including blank samples. These components were then filtered out by applying the minimum threshold for the ion count (100), removing NIST match factors that were deemed less reliable. A NIST library search of all the remaining components was then undertaken followed by a final Match filter, which was set to minimum value of 750. This last filter reduced the number of components from approximately 1000 to <100 per sample. The final compounds identified have a confidence level of 3, i.e. are only considered 'tentative candidates' as evidence exists for possible structure(s), but insufficient information for one exact structure only (e.g., positional isomers) (Schymanski et al. 2014). These identifications are regarded as tentative until confirmed with a high purity analytical standard.

A number of unidentified spectral features in the blank samples confounded analysis; additional enrichment and purification of sample extracts is warranted to aid future analysis. Any compounds that were detected in the blanks were disregarded from further consideration in the samples. After blank

subtraction a range of chemicals including pesticides, herbicides, human and veterinary pharmaceuticals, fragrances, flavours, industrial hydrocarbons were tentatively identified in all locations. There is considerable uncertainty associated with these tentative identifications. Complete identification can only be assured by comparison of samples with a high purity analytical standard. A selection of the identified compounds (based on a match factor > 750 and a known use) are provided in Table 3 below. For all samples, a much larger range of organic compounds (including long chain aliphatic and aromatic hydrocarbons, alcohols and ketones) were identified, however, it was not feasible to individually investigate their sources/uses. Whilst spills, leaks and discharge from vessels, ports, agriculture and urban sources are considered the main sources of hydrocarbon exposure on the Reef, it cannot be excluded that some of the hydrocarbons identified are of natural origin, synthesised by algae and phytoplankton.

		Sediment		Approximate Number Identified	PDMS		Approximate Number Identified
	Location	Name of selected chemicals	Use		Name of selected chemicals	Use	
Pre-wet 2014	Howicks Group of Islands	Acetophenone Ethyl 2-hexanoate	Precursor to resins and fragrances, flavouring agent Fragrance and flavouring agent	6	Ribavirin	Anti-viral medication	6
	Upstart Bay			1	Imipramine Tyramine	Antidepressant Fat Burning agent, Veterinary drug	7
	Cleveland			20			9
Post-wet 2015	Howicks Group of Islands	Benzophenone Disperse Orange 3 Lamotrigine Methaqualone Metolachlor	UV stabiliser in personal products Azo dye for polyester and acetate fibres anticonvulsant Sedative Herbicide	46	Acetophenone Arginine Cinobufotal Flurbiprofen Naproxen Perphenazine Pyrantel embonate Simazine	Precursor to resins and fragrances, flavouring agent Amino acid beta-adrenergic agonist nonsteroidal anti-inflammatory drug nonsteroidal anti-inflammatory drug antipsychotic medication Tuberculosis medication Selective herbicide	46
	Cleveland	Arginine Aspergin Benzothiazolemethyl Disperse Orange 3 Enalapril Fenfluramine Imipramine Ramelteon Terbinafine	Amino acid Antibiotic Selective herbicide Azo dye for polyester and acetate fibres Hypertension medication Appetite suppressant Antidepressant antipsychotic agent Antidepressant	58	Clozapine Diuron Diethyl Phthalate Fulvene	antipsychotic medication insecticide plastics manufacture Flavouring agent and perfumery	62
	Upstart	Ajmaline Amidosulfuron Carbendazim Chlortoluron Isoproturon Mefenamic Acid Phenytoin Physostigmine Risperidone	antiarrhythmic agent Broad spectrum herbicide Broad spectrum fungicide Urea herbicide systemic herbicide nonsteroidal anti-inflammatory drug anticonvulsant cholinesterase inhibitor antipsychotic medication	69	Sample lost		

Table 3: Selected compounds tentatively identified in sediment and PDMS samples using non-target GC-MS/MS

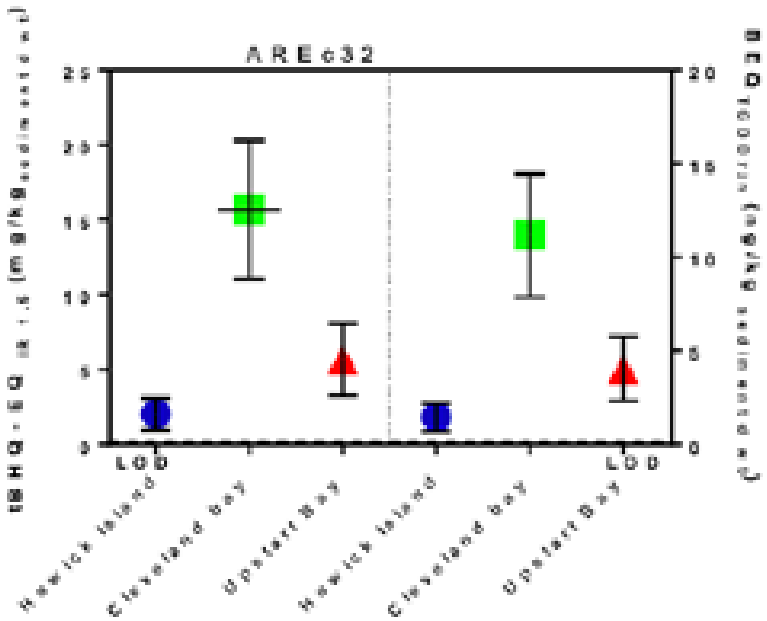
Overall the (approximate) number of compounds identified in the post wet 2015 samples were greater than the pre wet 2014 samples (for both sediment and PDMS). A similar increase in the number of compounds detected between the two sampling campaigns was also observed in the ED passive samplers and extracted water samples (Figure 3 and Table A3). Upstart Bay showed markers of both agricultural and urban input into the marine environment, and Cleveland Bay showed primarily urban markers – consistent with adjacent land uses. Despite its relatively remote location, numerous pharmaceuticals were also identified in the Howicks location. Both hydrocarbons and PPCPs have been named as emerging chemicals of concern in the GBR (Kroon et al. 2015). These results join the growing body of evidence demonstrating their presence in GBR waters.

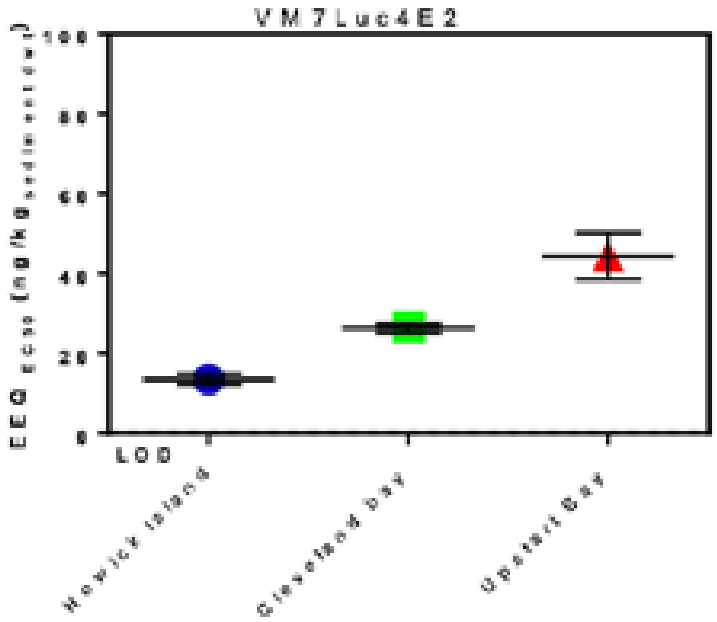
Correlating data from sediment chemical analysis and bioassay results

Green turtles have a unique and complex life history with several developmental stages including an open water stage as hatchlings and juveniles, and a coastal stage as subadults and adults (Limpus and Chaloupka 1997). This ontogenic shift is not only associated with major habitat changes, it implements also dietary shifts with an omnivorous pelagic stage and an herbivorous coastal stage (Arthur et al. 2008). The herbivorous coastal stage brings them in close contact with sediments (Hermanussen 2009); as turtles forage for seagrass they may ingest sediment incidentally, thus accumulting a diverse mixture of known and unknown chemicals over a prolonged time (decades) (Gaus et al. 2001, 2004, Hermanussen et al. 2004, 2006, Gaus et al.). Sediments can act as a sink for many chemicals in the aquatic environment, thereby being a major chemical source for aquatic wildlife. Resuspension of sediment-bound chemicals is known to be particularly evident after dredging activities or flood events (Hollert et al. 2000, Hollert

et al. 2002, Brinkmann et al. 2010, 2010). To characterise these diverse mixtures using traditional target analysis is difficult, time consuming and costly. To overcome these limitations, an effect-based approach uses a battery of in vitro bioassays (based on the activation of cellular pathways at the early stage of toxicity), to provide information on the potential internal chemical exposure of turtles at each sampling location as a result of sediment ingestion. Previous studies have shown that in vitro bioassays are a valuable tool to assess sediments toxicity and understand the adverse impact of these complex chemical mixtures on biological systems (Hollert et al. 2000, 2002, Legler et al. 2002, Brack 2003, Brinkmann et al. 2010, Li et al. 2013, Smital et al. 2013).

Pooled sediment samples from each sampling location from the pre-wet 2014 sampling campaign were subjected to a battery of in vitro bioassays (AhR-CAFLUX, AREc32, NFκB-bla, VM7Luc4E2, p53- bla), each with a different mode of action (MOA) (e.g., Ah receptor mediated xenobiotics, Nrf2-mediated oxidative stress, NFκBmediated response to inflammation, estrogen activity and DNA damage, respectively) in order to cover the potential biological effects from a wide spectrum of chemicals (Dogruer et al. 2018). Generally, Ah receptor mediated dioxin-like response and estrogen activity are two typically tested endpoints in sediments due to the fact that biological effects of sediment contaminants are mostly driven by sediment-bound persistent organic pollutions (POPs) (Eichbaum et al. 2014).





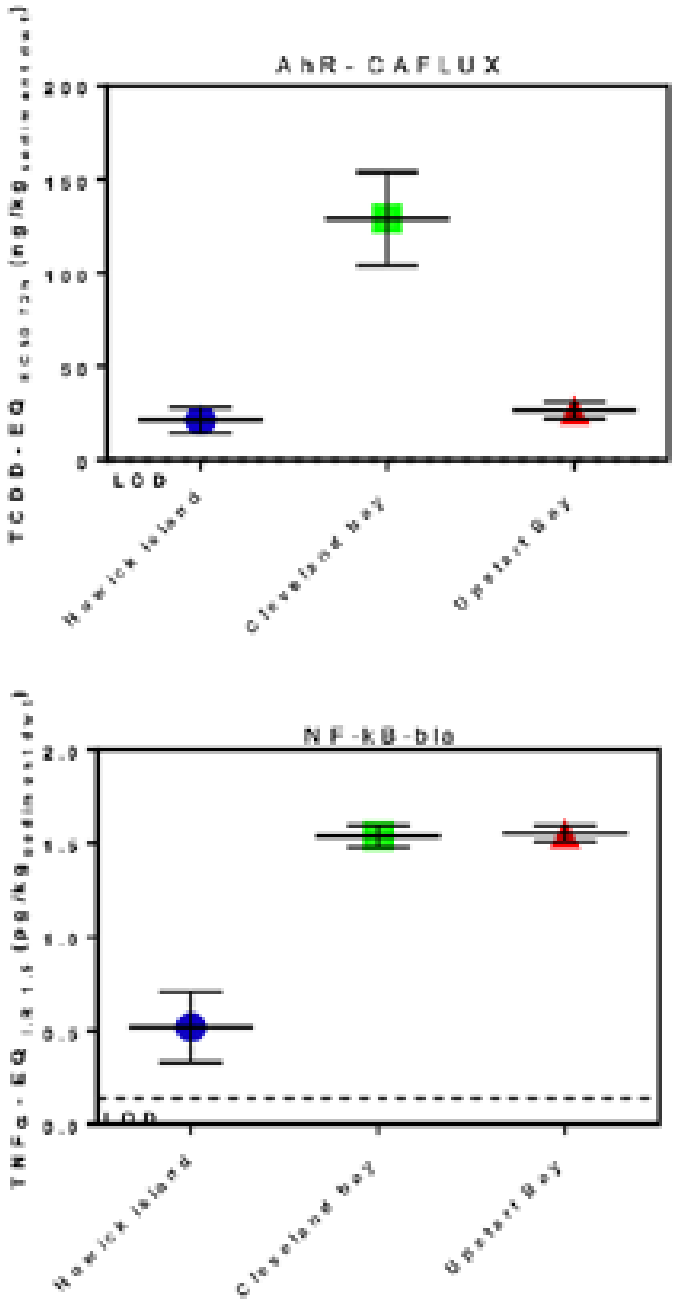


Figure 4: BEQ (TCDD (2,3,7,8- Tetrachloro-dibenzo-diozin), tBHQ (t-Butylhydroquone), TNF-alpha (Tumor necrosis factor- alpha), and E (17-beta estradiol) equivalent (EQ)) values of sediments (dry weight; dwt) from each site for a battery of bioassays tested in this study. For assays with specific (VM7Luc4E2, AhR-CAFLUX,) MOA, 50% effect concentration of the maximum effect (EC_{50}) were calculated, and for assays with reactive (AREc32, NFκB-bla, , p53- bla) MOA induction ratios 1.5 (IR 1.5) were determined. The results showed that the internal exposure of green turtles through (hypothetical) sediment ingestion differed between sampling locations for several of the bioassays. In the p53-bla assay none of the sites showed any significant effect induction, and no cytotoxicity was observed across the tested concentrations range.

Many AhR-ligands are also hormonally active agents, endocrine disrupting chemicals (EDCs), such as PCBs, PAHs, dioxins as well as many organochlorine pesticides (e.g. p,p'-DDT). These have been banned for decades, but due to their high persistence, are still present in the environment and continue to be detected on the GBR (Cavanagh et al. 1999). The results of the bioassays are presented in Figure 4 given in relation to the nominal (spiked) concentrations of the potent reference compounds of each assays (bioanalytical equivalent concentrations (BEQs) values) (Escher et al. 2013, Dogruer et al. 2018).

For the AhR–CAFLUX assay we evaluated the data after an exposure time of 24 h as well as after 72 h to reduce the contribution of potentially labile AhR-active compounds present in the samples (e.g. PAHs).

The impact of such labile compounds can be assessed by comparing the effect concentrations for both exposure times. After 24 h, the urban/industrial site Cleveland Bay showed the highest induction with 559 ng TCDD-EQ/kg sediment dry weight (dwt) followed by the control site Howick Island (BEQTCDD = 283 ng/kg dwt) and the agricultural site Upstart Bay BEQ (BEQTCDD= 202 ng/kg dwt).

Recent studies analysed ASE extracted sediments from Queensland in a similar battery of bioassays including the AhR-CAFLUX assay (with an exposure time of 24 h) (Li et al. 2013). The authors observed the highest induction in the Brisbane River with a BEQTCCD concentration of 927 ng/kg dwt, followed by Oxley Creek with 360 ng/kg dwt and Port of Brisbane with 352 ng/kg dwt. The BEQTCDD in our urban/industrial site (Cleveland Bay) was higher than the reported BEQTCDD values from Oxley Creek and the Port of Brisbane, but lower than the results from Brisbane River. Sediments from Upstart Bay and Howick Island showed lower BEQTCDD concentrations compared to the Brisbane River, Oxley Creek, and Port of Brisbane. Surprisingly, the differences between the relatively pristine sediments from Howick Island and Upstart Bay area, which is surrounded by agricultural industry and located close to a major river embayment, were not significant despite previous studies reporting high concentrations of dioxin-like chemicals in soil and sediment in coastal areas of Queensland as a result of the extensive use of pesticides in agriculture (Holt et al. 2008).

A longer exposure time of 72 h isolates the effects triggered by the more persistent compounds in the AhR-CAFLUX assay. The BEQ concentrations after 72 h were in all samples lower compared to the 24 h values ranging from 21 ng TCDD-EQ/kg dwt (Howick Island), 26 ng/kg dwt (Upstart Bay) to 128 ng/kg dwt (Cleveland Bay). These results suggest that, overall, labile substances such as PAHs have a considerable impact on the cell responses in the bioassays and that highly persistent POPs such as organochlorine pesticides (OCPs) play a smaller role.

These findings correspond with the results from chemical analysis conducted in sediment samples. Whilst few chemicals were detected through the target chemical analysis of sediment, the rigorous clean-up and quantitative analysis of a single Cleveland Bay sample detected low levels of PCBs, including a dioxin-like congener PCB-118. Further, a semi-quantitative analysis using high resolution GC-MS/MS detected ten PAHs above blank concentrations at Cleveland Bay, in comparison to only six at comparatively low concentrations at Upstart Bay and a single detection at Howicks. These polycyclic and halogenated aromatic hydrocarbons (potentially together with other unknown chemicals sharing the same mode of action) are known high-affinity ligands of the Ah receptor and are likely contributing to the response seen in the AhR-CAFLUX assay. Whilst there are only limited studies examining the turtle-specific effects of dioxin-like chemicals, immune suppression, reproductive and developmental effects and oxidative stress have been observed (Bishop et al. 1998, de Solla et al. 1998, Keller and McClellan-Green 2004, Keller et al. 2006, Tremblay et al. 2016). Bioassay results of turtle whole blood from the same locations did not reflect the same trend, with the three locations exhibiting similar biological responses with the exception of six turtles from Upstart Bay (Dogruer et al. 2018).

As it is known that many AhR ligands can also act as EDCs, the same trends were expected in the estrogen-response indicative VM7Luc4E2 assay. Surprisingly, the results did not show the same pattern. Here, Upstart bay showed the highest induction with 44 ng EEQ/kg dwt followed by Cleveland bay with 26 ng/kg dwt and the lowest induction was observed in Howick Island sediments with 13 ng/kg dwt. Xenoestrogen mixtures can be released into the aquatic environment via industrial effluent as well as from agricultural runoff released and can potentially accumulate in the sediments (Legler et al. 2002). Elevated levels of EDCs are well known to have the potential of causing adverse effects on biological systems (Leusch et al. 2010, 2014), including marine wildlife populations (Colborn et al. 1993, Ankley et al. 1998, Simon et al. 2011, 2013). For green turtles as coastal benthic organisms, estrogenic compounds can be a concerning chemical group.

For the AREc32 assay, we determined a BEQtBHQ concentration for Howick Island of 2.6 mg/ kg dwt, for Upstart Bay 5.6 mg/ kg dwt and for Cleveland Bay, 15.7 mg/ kg dwt. Although the AREc32 assay has become recently more popular and is commonly applied for risk assessment of water and wastewater, there is to our knowledge no literature data for sediments apart from the study from Li et al. (2013). In

comparison to the Li et al. (2013) study, all sites of our study had relatively low BEQtBHQ values, in particular also for sediment from the Howick Islands which had even lower inductions in comparison to the control site Wivenhoe Dam in Li et al. (2013) (BEQtBHQ =3.1 mg/kg dwt). The broad conclusion from our study is that the sediments from Howick Islands, Cleveland Bay and Upstart Bay contain low levels of chemicals that trigger the oxidative stress response in the AREc32 assay.

To our knowledge, this study is the first to apply the NF-κB-bla assay to sediment extracts. The endpoint of this reactive MOA provides information about the key transcriptional driver of immune and inflammatory response. Interestingly, Howick Island samples showed the lowest induction with TNF-alpha equivalent concentration of 0.68 pg/kg dwt in comparison to both Cleveland Bay 2.13 pg/kg dwt and Upstart Bay with 2.19 pg/kg dwt. This bioassay is, however, indicative for a wide array of chemicals. As current methods in chemical analysis are not suitable yet to fully characterize chemical mixtures, it is impossible to assign the responses of the NF-κB-bla assay to specific chemicals or chemical groups.

Overall, the results showed differences in the chemical mixture among the three turtle habitats. Howicks Island sediments had low BEQ results in all bioassays in comparison with the two other study sites (Cleveland Bay and Upstart Bay). Considering the feeding behaviour of marine sea turtles and the previously documented correlation of contaminant levels in sea turtles with sediments of their foraging ground, sediments represent an important contaminant uptake route (Hermanussen et al. 2004, 2006). Based on the bioanalytical screening results, Howick Island appears to be a suitable control site for investigating internal exposure of green turtles. However, different extraction methods were used for sediment and blood samples (Dogruer et al. 2018), thereby making it hard to directly extrapolate results from the sediments to the blood samples. Sediment samples served in this study as a guide to gain insight into the external exposure of benthic marine wildlife.

Summary of factors that may influence chemical pollutant concentrations in the marine environment

Anthropogenic pollutants derived from either diffuse agricultural or other point sources have been identified as a high risk threat to the maintenance of GBR ecosystem habitats, the biodiversity they support and the Reef's heritage values (GBRMPA 2014). Key factors that may influence the ability to detect pollutants in the marine environment may include (but are not limited to): pesticide usage, land use area, rainfall, timing and method of pesticide application, pesticide run-off behaviour, pesticide persistence, rates of pesticide loss from paddocks, volume of water discharged from rivers, end of catchment pesticide loads, proximity of sampling activities to rivers and frequency of flood plume impacts at the sampling site, the timing of sampling and adoption rates of best management practices for land management (including chemical use, surface and irrigation water management). Quite often, the necessary data needed to interpret the presence/ absence or trends in chemical pollutant detections (particularly pesticide usage in GBR catchments and their application rates) are either not available or only updated periodically.

A wide range of land uses occur in the GBR catchments, with great diversity between regions (DSITIA 2012b). Certain regions and/or smaller coastal catchments supporting intensive activities (for example sugar cane farming in the Mackay Whitsunday region) or concentrated urban populations can represent areas of higher localised risk of land-based run-off or discharge, in part due to the higher average rainfall occurring in coastal areas (GBRMPA 2013b). Many herbicides are readily water soluble, permitting their transport long distances from their original area of application through groundwater, creeks and rivers, into the marine environment, particularly following large rainfall events (Devlin et al. 2015). The profiles of herbicides and pesticides detected at the end-of-catchment river systems or in near shore marine areas as part of other monitoring programs show chemical signatures associated with the land uses of the adjacent catchment areas (Figure A4) (Gallen et al. 2014, O'Brien et al. 2016b, Wallace et al. 2016). The total loads of herbicides and pesticides exported into the marine environment are highly variable and reflect variations in annual climatic conditions, catchment rainfall and river discharge volumes as well as land use (Figure A4).

The Burdekin River flows into the northern reaches of Upstart Bay and represents one of the major sources of freshwater discharge into the GBR lagoon. It has contributed to some of the greatest historical peak discharge volumes and associated loads of sediments, nutrients and pesticides exported to the GBR marine environment (Devlin et al. 2012). The mass stranding event in June 2012 followed a peak in daily discharge in the Burdekin River in March and sustained elevated flow from mid-January to end of April (SI Table A5 and Table A6). Rainfall in the lower Burdekin and Upstart Bay catchments exceeded long term means by approximately three fold (BOM 2016). For five out of the six years preceding the mass stranding event, the Burdekin River had discharge volumes of between 1.8 to 5 times the long term median (SI, Table 7). The cumulative impacts of pesticide, freshwater, nutrient, sediment, and reduced light conditions are not well understood but all of these factors have likely contributed to the decline in seagrass and coral metrics observed in the Burdekin region (Thompson et al. 2014, McKenzie et al. 2015), which in turn created pressure on the resident green turtle population. A simultaneous pulse of a catchment-derived contaminant/s (such as metals or organics) on top of these stressors could have been the tipping point for the stranding event.

Emerging anthropogenic pollutants of concern in the GBR

To date, the majority of research and monitoring for chemical pollutants on the GBR has focused on the five priority PSII herbicides. But more recently, research into other chemical pollutant groups such as PAHs, PPCP and alternative herbicides is beginning to emerge, and the risk of exposure in marine environments assessed (O'Brien et al. 2014, Garzon-Garcia et al. 2015, Kroon et al. 2015, Smith et al. 2015).

The known presence of pharmaceuticals in the GBR is limited to data from the effluent of two WWTPs and one river, located primarily in the Wet Tropics region (i.e. north of both Upstart Bay and Cleveland Bay) (O'Brien et al. 2014, Scott et al. 2014). A total of 26 pharmaceuticals were detected in treated effluent (maximum concentrations of low ug/L) and included venlafaxine, hydrochlorathiazide and citalopram, all of which were detected in the current study. Our observed concentrations of pharmaceuticals in the marine environment were typically < 1ng/L demonstrating substantial dilution occurs following effluent release into rivers and the marine environment.

Similarly, little research has been conducted on the GBR for the presence of personal care products (i.e. chemicals used in products such as cosmetics, shampoo, body wash such as musks, anti-microbials, plasticisers and UV filters). A single study of waste water effluent in the Wet Tropics found acesulfame (an artificial sweetener) and triclosan (anti-microbial) at maximum concentrations of 4.4 ug/L and 30 ng/L respectively (O'Brien et al. 2014). More broadly, in 19 rivers across Queensland that were analysed for 42 herbicides, industrial chemicals and PPCPs; caffeine, paracetamol and salicylic acid were most frequently detected (in up to 60% of samples) (Scott et al. 2014). PPCPs including caffeine, paracetamol, triclosan, carbamazepine and salicylic acid appear widely spread across Australian waterways, being the most frequently detected chemicals across 73 river sites, located in areas of differing land uses (Scott et al. 2014). In the current study, the PPCPs caffeine, DEET and triclosan were detected in several field blank and unknown samples, suggesting that contamination from human contact occurred during sample collection and an estimate of water concentration could not be reasonably established.

Other chemicals associated with urban run off, household use or industrial activities are also in use in GBR catchments yet marine monitoring data is scarce. Petroleum hydrocarbons which include the PAHs have been assessed as an emerging contaminant in the GBR with chronic input from terrestrial activities such as ports the most likely source. The limited sediment monitoring data available for PAHs indicates that the trend in contamination was highest in busy ports > urban-influenced rivers > island locations visited by small boats > pristine offshore coral reefs (Kroon et al. 2015). The sediment and PDMS samplers from this study also reflected this trend.

Through normal daily use or slow release from the products themselves, other chemicals associated with household products (such as flame retardants) may also enter wastewater but, unlike pharmaceuticals which are relatively water soluble, they may also accumulate into the biosolids (the solid waste product

from WWTPs) (Ying and Kookana 2007, Langdon et al. 2011, Gallen et al. 2016). The re-use of biosolids as a soil improver in agriculture that may contain these chemicals creates an additional pathway for re-entry into the environment. The extent of biosolids use in GBR catchments is unknown. .

As wastewater is considered one of the most significant sources of pharmaceuticals, personal care products, and other chemicals associated with household consumer products, the risk is greatest around urban centres and areas of human in-water activities (Wet Tropics to the Burnett-Mary region) (Kroon et al. 2015). At least 50 WWTPs are operational in the GBR catchments that discharge effluent into the environment. As the population of Townsville and other GBR towns increase over the coming decades, chemicals associated with urban and industrial uses may become of greater concern to coastal marine wildlife. Land management practices in the Reef catchments also continue to change in response to Reef Plan initiatives (ABS, 2014; DSITIA, 2012a), and thus the impacts of these activities on water quality in the near shore marine environment are also changing. For example, the increasing use of alternative herbicides can increase pesticide loads exported by up to 21 %. Little ecotoxicology data of these complex mixtures of herbicides exists (Smith et al. 2015).

There are no recent and reliable figures available for the current local-scale usage of agricultural chemicals in the Reef catchments. End-of-catchment pesticide loads monitoring demonstrates that usage is dynamic and can fluctuate yearly based on specific pest pressures, climatic conditions, regulatory action (such as the APVMA restriction on diuron use in 2012), use of resistant crop varieties or the development of herbicide resistance in weeds (Devlin et al. 2015). Because there is a lack of knowledge surrounding exactly what types of pesticides and herbicides are in use, environmental monitoring using non-target high resolution mass spectrometry and effects-directed analysis is the most informative approach of determining the exposure of marine wildlife to largely unknown anthropogenic contaminant mixtures.

Conclusions

This study presents an innovative approach to monitoring the exposure and health impacts of organic environmental pollutants on marine wildlife. A combination of effect-based and non-targeted chemical analysis screening tools, allowed detection of a large number of chemicals that are not targeted by the existing marine monitoring program. The ‘case-control’ approach to sampling and comparative analysis confirmed spatial differences in the external exposures of resident coastal versus offshore green turtles. As no other turtle stranding events occurred for the duration of the project, no outstanding causative agent that may have been responsible for the 2012 mass stranding could be identified. Sampling activities closely following similar environmental conditions that preceded the mass stranding (above average catchment rainfall and river discharge) may identify possible candidates in future.

Overall low concentrations of pesticides, pharmaceuticals and personal care products associated with known uses in the adjacent catchments were detected in water, passive samplers and sediment. Despite these low concentrations, the cumulative effects of exposure on such long-lived species such as the green turtle are unknown. Long-term exposure to organic pollutants may be a tipping point for turtle health, reducing their resilience to the multiple local, regional and global stressors they are already faced with (such as feminisation induced by climate change, increasing frequency and severity of cyclones, increasing coastal development). Future monitoring programs assessing ecosystem health would benefit from adopting this method of screening by:

1. Using a battery of effect-based bioassays of blood to identify markers of pollutant exposure or effect
2. Cost effective sampling of environmental matrices focussed in appropriate locations, and
3. Non-target high resolution mass spectrometry chemical analysis to identify unknown chemicals of biological concern.

Despite the sampling activities occurring during a period where there were no major disturbances to turtle foraging areas such as high catchment rainfall, cyclones or high volumes of riverine discharge, hundreds of chemicals were detected in this study. Arguably this represents the ‘best case scenario’ for

these populations of turtles. However, despite appearing outwardly healthy there is evidence to suggest that catchment-specific pollution is having an effect on resident coastal turtle populations.

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Appendix

Sampling Location	Site Description	Sampler types	Date Deployed	Date Retrieved	Latitude	Longitude
Pre wet season 2014						
CB 1	Site near creek	rED, rED, PFM, PDMS	13/10/2014	17/10/2014	19° 15.732;	146° 58.862
CB 3	Site near beach	rED, rED, PFM	13/10/2014	17/10/2014	19° 14.853;	146° 58.615
UB 1	Knobbys	rED, rED, PFM, PDMS	21/10/2014	27/10/2014	19° 47.448;	147° 45.585
UB 2	Rocky Ponds	rED, rED, PFM	21/10/2014	27/10/2014	19° 49.345;	147° 41.013
HOW 1	Coombe Reef	rED, rED, PFM, PDMS	18/8/2014 (cage) 21/8/2014 (EDs)	31/08/2014	14° 23.967°S;	144° 55.700°E
HOW 2	Outer Reef	rED, rED, PFM	21/08/2014	31/08/2014	14° 22.197°S;	144° 57.981°E
HOW 3	Ingram Island	rED, rED, PFM	21/08/2014	31/08/2014	14° 25.241°S;	144° 52.460°E
Post wet season 2015						
CBWT	offshore of the WWTP	rED, PFM	29/05/2015	5/06/2015	19° 16.778	147° 52.186
CB 2	Middle	rED, PFM, PDMS	29/05/2015	5/06/2015	19° 14.500	147° 57.674
UB 1	Knobbys	rED, PFM, PDMS	14/06/2015	21/06/2015 (EDs) 14/7/15 (Cage)	19° 47.344	147° 45.404
UB 2	Rocky Ponds	rED, PFM	14/06/2015	21/06/2015	19° 49.267	147° 40.887
HOW 1	Coombe Reef	rED, PFM, PDMS	25/7/2014 (cage) 9/8/2014 (EDs)	16/08/2016	14° 23.955°S;	144° 55.702°E
HOW 2	Outer Reef	rED, PFM	9/08/2015	16/08/2016	14° 22.197°S;	144° 57.981°E
HOW 3	Ingram Island	rED, PFM	9/08/2015	16/08/2016	14° 25.241°S;	144° 52.460°E

Table A1: Details of passive sampler deployments

Sampling Location	Sample composition	Date	Latitude	Longitude
Pre wet season 2014				
Cleveland Bay (Pooled)	CB1	17/10/2014	19° 15.838	146° 58.965
	CB2	17/10/2014	19° 15.553	146° 58.345
	CB3	17/10/2014	19° 14.987	146° 58.765
Upstart Bay (pooled)	UB1	23/10/2014	19° 47.679	147° 45.848
	UB2	23/10/2014	19° 49.585	147° 40.921
	UB3	23/10/2014	19° 45.001	147° 36.109
Howicks Gp (HOW1)	Coombe Reef	21/08/2014	14° 23.967	144° 55.700
Howicks Gp (HOW2)	Outer Reef	21/08/2014	14° 22.197	144° 57.581
Howicks Gp (HOW3)	Ingram Island	21/08/2014	14° 25.241	144° 52.499
Post wet season 2015				
Cleveland Bay (Pooled)	CB2	29/05/2015	19° 14.500	146° 57.674
	Cockle Bay	1/06/2015	19° 14.863	146° 59.615
	Offshore of the WWTP	29/05/2015	19° 16.889	146° 52.075
	Offshore of the WWTP	29/05/2015	19° 16.878	146° 51.966
	Offshore of the WWTP	29/05/2015	19° 16.898	146° 51.971
	Offshore of the WWTP	29/05/2015	19° 16.892	146° 52.085
Upstart Bay (UB1)	Knobbys	14/06/2015	19° 47.679	147° 45.848
Upstart Bay (UB2)	Rocky Ponds	15/06/2015	19° 49.585	147° 40.921
Upstart Bay (UB3)	Wunjunga	14/06/2015	19° 45.001	147° 36.109
Howicks Gp (HOW1)	Coombe Reef	13/08/2015	14° 24.279	144° 55.628
			14° 24.461	144° 55.972
Howicks Gp (HOW2)	Outer Reef	12/08/2015	14° 21.905	144° 57.511
			14° 22.300	144° 57.875
Howicks Gp (HOW3)	Ingram Island	14/08/2015	14° 25.242	144° 52.693
			14° 25.417	144° 53.083

Table A2: Details of sediment sample collection

Sampling Location	Site Description	Date	Latitude	Longitude
Pre wet season 2014				
Cleveland Bay (CB1)	Site near creek	17/10/2014	19° 15.888	146° 58.965
Cleveland Bay (CB2)	Middle	17/10/2014	19° 15.553	146° 59.345
Cleveland Bay (CB3)	Site near beach	17/10/2014	19° 14.987	146° 59.765
Upstart Bay (UB1)	Knobbys	23/10/2014	19° 47.679	147° 45.848
Upstart Bay (UB2)	Rocky Ponds	23/10/2014	19° 49.585	147° 40.921
Upstart Bay (UB3)	Wunjunga	23/10/2014	19° 45.001	147° 36.109
Howicks Gp (HOW1)	Coombe Reef	21/10/2014	14° 23.967	144° 55.700
Howicks Gp (HOW2)	Outer Reef	21/10/2014	14° 22.197	144° 57.581
Howicks Gp (HOW3)	Ingram Island	21/10/2014	14° 25.241	144° 52.469
Post wet season 2015				
Cleveland Bay (CB1)	Site near creek	5/06/2015	19° 15.969	146° 55.595
Cleveland Bay (CB1)	Site near creek	5/06/2015	19° 15.002	146° 59.610
Cleveland Bay (CB2)	Middle	5/06/2015	19° 17.024	146° 57.767
Cleveland Bay (CB2)	Middle	1/06/2015	19° 14.533	146° 57.674
Cleveland Bay (CB3)	Site near beach	5/06/2015	19° 16.084	146° 58.848
Cleveland Bay (CB3)	Site near beach	5/06/2015	19° 13.964	146° 58.634
Upstart Bay (UB1)	Knobbys	21/06/2015	19° 46.478	147° 44.810
Upstart Bay (UB1)	Knobbys	21/06/2015	19° 47.319	147° 45.298
Upstart Bay (UB1)	Knobbys	19/06/2015	19° 47.286	147° 45.396
Upstart Bay (UB1)	Knobbys	19/06/2015	19° 46.729	147° 45.031
Upstart Bay (UB2)	Rocky Ponds	14/06/2015	19° 49.267	147° 40.887
Upstart Bay (UB2)	Rocky Ponds	14/06/2015	19° 49.257	147° 40.887
Upstart Bay (UB2)	Rocky Ponds	21/06/2015	19° 49.284	147° 40.767
Upstart Bay (UB2)	Rocky Ponds	21/06/2015	19° 49.125	147° 40.337
Upstart Bay (UB3)	Wunjunga	14/06/2015	19° 45.336	147° 36.491
Upstart Bay (UB3)	Wunjunga	14/06/2015	19° 45.167	147° 36.320
Upstart Bay (UB3)	Wunjunga	19/06/2015	19° 45.343	147° 36.678
Upstart Bay (UB3)	Wunjunga	19/06/2015	19° 44.962	147° 36.598
Howicks Gp (HOW1)	Coombe Reef	9/08/2016	14° 20.950	144° 55.691
Howicks Gp (HOW1)	Coombe Reef	9/08/2016	14° 23.896	144° 55.672
Howicks Gp (HOW1)	Coombe Reef	16/08/2016	14° 23.961	144° 55.687
Howicks Gp (HOW1)	Coombe Reef	16/08/2016	14° 23.960	144° 55.684
Howicks Gp (HOW2)	Outer Reef	9/08/2016	14° 22.165	144° 57.576
Howicks Gp (HOW2)	Outer Reef	9/08/2016	14° 22.152	144° 57.555
Howicks Gp (HOW2)	Outer Reef	16/08/2016	14° 22.192	144° 57.572
Howicks Gp (HOW2)	Outer Reef	16/08/2016	14° 22.173	144° 57.588
Howicks Gp (HOW3)	Ingram Island	9/08/2016	14° 25.244	144° 52.442
Howicks Gp (HOW3)	Ingram Island	9/08/2016	14° 25.207	144° 52.409
Howicks Gp (HOW3)	Ingram Island	16/08/2016	14° 25.253	144° 52.469
Howicks Gp (HOW3)	Ingram Island	16/08/2016	14° 25.240	144° 52.454

Table A3: Details of water sample collection

Analytes	
2,4 DB	Hexazinone
24 D	Hydrochlorthiazide
245T	Ibuprofen
3,4 DiCl Aniline	Imazapic
Acesulfame	Imazethapyr
Ametryn	Imidadopri d
Ametryn hydroxy	Iopromide
Asulam	Malathion
Atenolol	MCPA
Atorvastatin	Mecoprop
Atrazine	Methiocarb
Bromadi l	Methomyl
Bromoxynil	Metolachlor
Caffeine	Metribuzin
Carbamazepine	Metsulfuron-Methyl
Carbofuran	Naproxen
Chlorpyriphos	Paracetamol
Citalopram	Paraxanthine
Clopyralid	Pendimethalin
Codeine	Pi doram
DCPMU	Prometryn
DCPU	Propazine
DEET	Propiconazole
Desethyl Atrazine	Propoxur
Desisopropyl Atrazine	Salicylic acid
Desmethyl Citalopram	Sildenafil
DesmethylDiazepam	Simazine
Diazinon	Simazine hydroxy
Dicamba	Tadalafil
Dichlorvos	Tebuconazole
Diuron	Tebuthiuron
Fenamiphos	Temazepam
Fluazifop	Terbuthylazine
Flumeturon	Terbuthylazine desethyl
Fluoxetine	Terbutryn
Fluroxypyr	Tramadol
Furosemide	Tri dopyr
Gabapentin	Tri closan
Haloxyfop	Venlafaxine

Table A4: Target analytes using LC/MS analysis for water and ED passive samplers

Highlighted analytes have been added for analysis for post wet season 2015 samples.

Furans	PAHs	Pestidides	PCBs
2,3,7,8-Tetrachlorodibenzofuran	acenaphthene	Pentachlorobenzene	PCB-52
1,2,3,7,8_Pentachlorodibenzofuran	acenaphthylene	a-HCH	PCB-81
2,3,4,7,8-Pentachlorodibenzofuran	fluorene	b-HCH	PCB-77
1,2,3,4,7,8-Hexachlorodibenzofuran	phenanthrene	r-HCH (lindane)	PCB-101
1,2,3,7,8,9-Hexachlorodibenzofuran	anthracene	d-HCH	PCB-123 + 118
2,3,4,6,7,8-Hexachlorodibenzofuran	fluoranthene	HCB	PCB-114
1,2,3,4,6,7,8-Heptachlorodibenzofuran	pyrene	Heptachlor	PCB-105
1,2,3,4,7,8,9-Heptachlorodibenzofuran	benzo[a]anthracene	Chlordane	PCB-126
1,2,3,4,6,7,8,9-Octachlorodibenzofuran	chrysene	DDE (o,p + p,p)	PCB-153
Dioxins	benzo[b + k]fluoranthene	DDT (o,p + p,p)	PCB-138
2,3,7,8-Tetrachlorodibenzo-p-dioxin	benzo (e) pyrene	DDE (o,p + p,p)	PCB-167
1,2,3,7,8-Pentachlorodibenzo-p-dioxin	benzo (a) pyrene	Mirex	PCB-156
1,2,3,4,7,8-Hexachlorodibenzo-p-dioxin	perylene	Permethrin	PCB-157
1,2,3,6,7,8-Hexachlorodibenzo-p-dioxin	benzo[ghi]perylene		PCB-169
1,2,3,7,8,9-Hexachlorodibenzo-p-dioxin	indeno[1,2,3-cd]pyrene		PCB-180
1,2,3,4,6,7,8-Heptachlorodibenzo-p-dioxin	dibenzo[a,h]anthracene		PCB-189
1,2,3,4,6,7,8,9-Octachlorodibenzo-p-dioxin			

Table A5: Target chemicals analysed in PDMS and an initial screen of sediments using GC-MS/MS
Note that Post wet season 2015 samples were not analysed for furans and dioxins.

Polybrominated diphenyl ethers (PBDEs)	Organochlorine pestidides (OCPs) (add resistant)	Polychlorinated naphthalenes (PCNs)	Polychlorinated biphenyls (PCBs)
BDE-28	PeCB	PCN-11	PCB-13
BDE-47	a-HCH	PCN-28	PCB-36&28
BDE-100	HCB	PCN-52	PCB-27
BDE-99	b-HCH	PCN-101	PCB-48
BDE-154	r-HCH	PCN-81	PCB-46
BDE-153	d-HCH	PCN-77	PCB-52
	t-chlordane	PCN-123	PCB-50
	op-DDE	PCN-118	PCB-53
	c-chlordane	PCN-114	PCB-66
	pp-DDE	PCN-153	PCB-69
	op-DDD	PCN-105	PCB-72
	pp-DDD	PCN-138	PCB-73
	op-DDT	PCN-126	PCB-75
	pp-DDT	PCN-167	
	mirex	PCN-156	
		PCN-157	
		PCN-180	
		PCN-169	
		PCN-189	

Table A6: Target chemicals analysed in method development of single sediment sample using high resolution GC-MS/MS

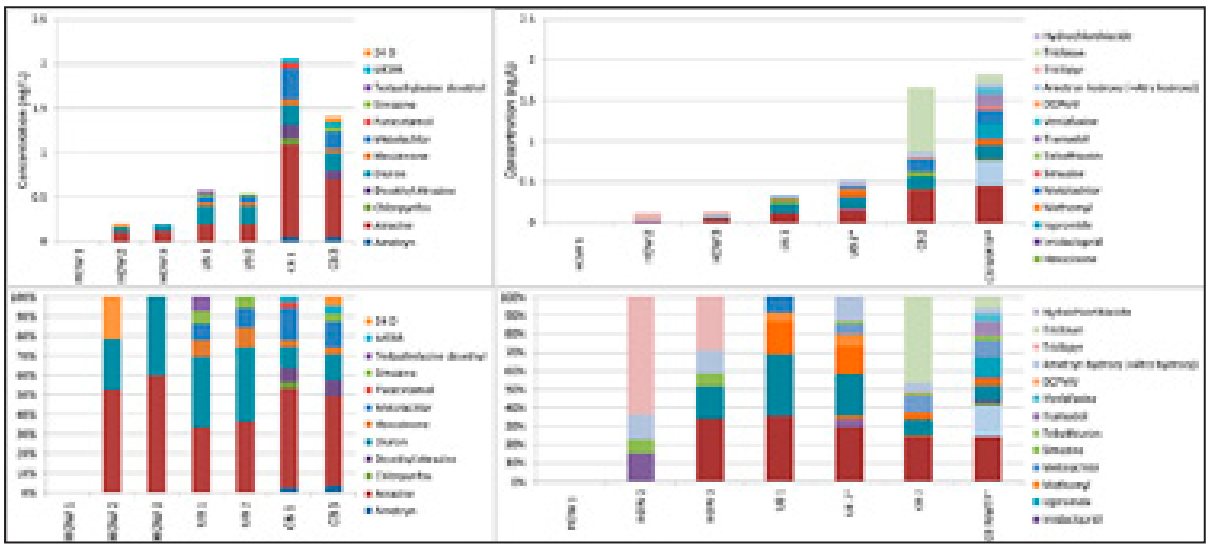

Figure A1: Rates (ng/sampler/day) (top) and profiles (bottom) of all chemicals detected in water using EDs. Pre-wet season 2014 sampling (left); Post-wet 2015 sampling (right); *Indicates mean of replicate samplers.

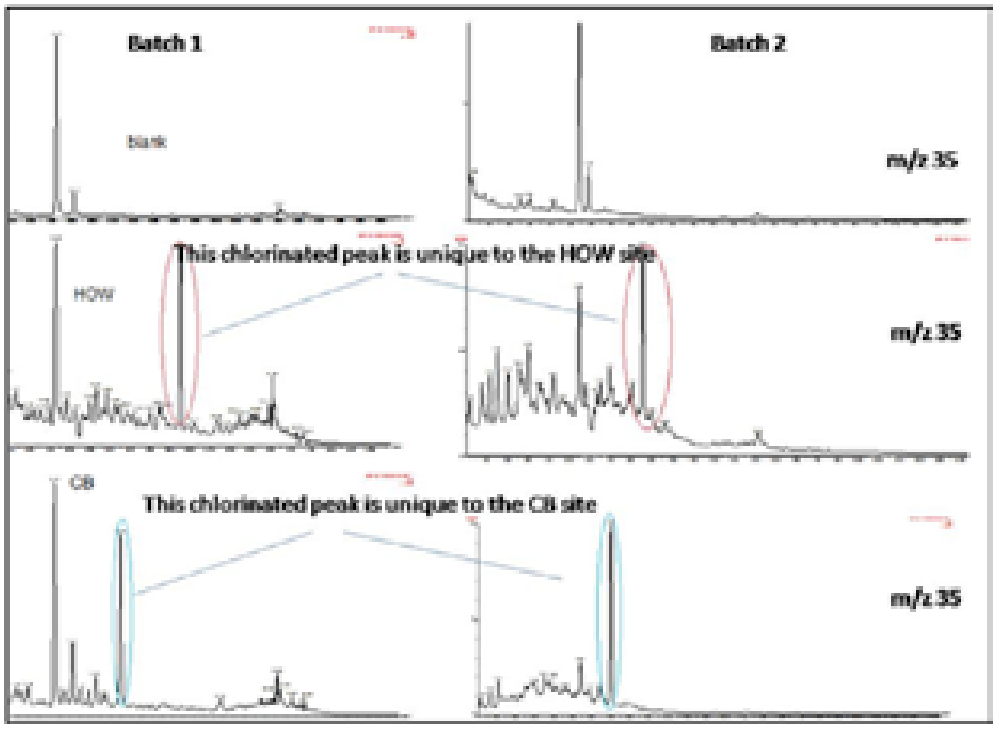

Figure A2: Comparison of chromatograms screening for chlorinated chemicals between the pre-wet season 2014 and post-wet season 2015 sampling periods at the Howicks group of Islands (middle) and Cleveland Bay (bottom)

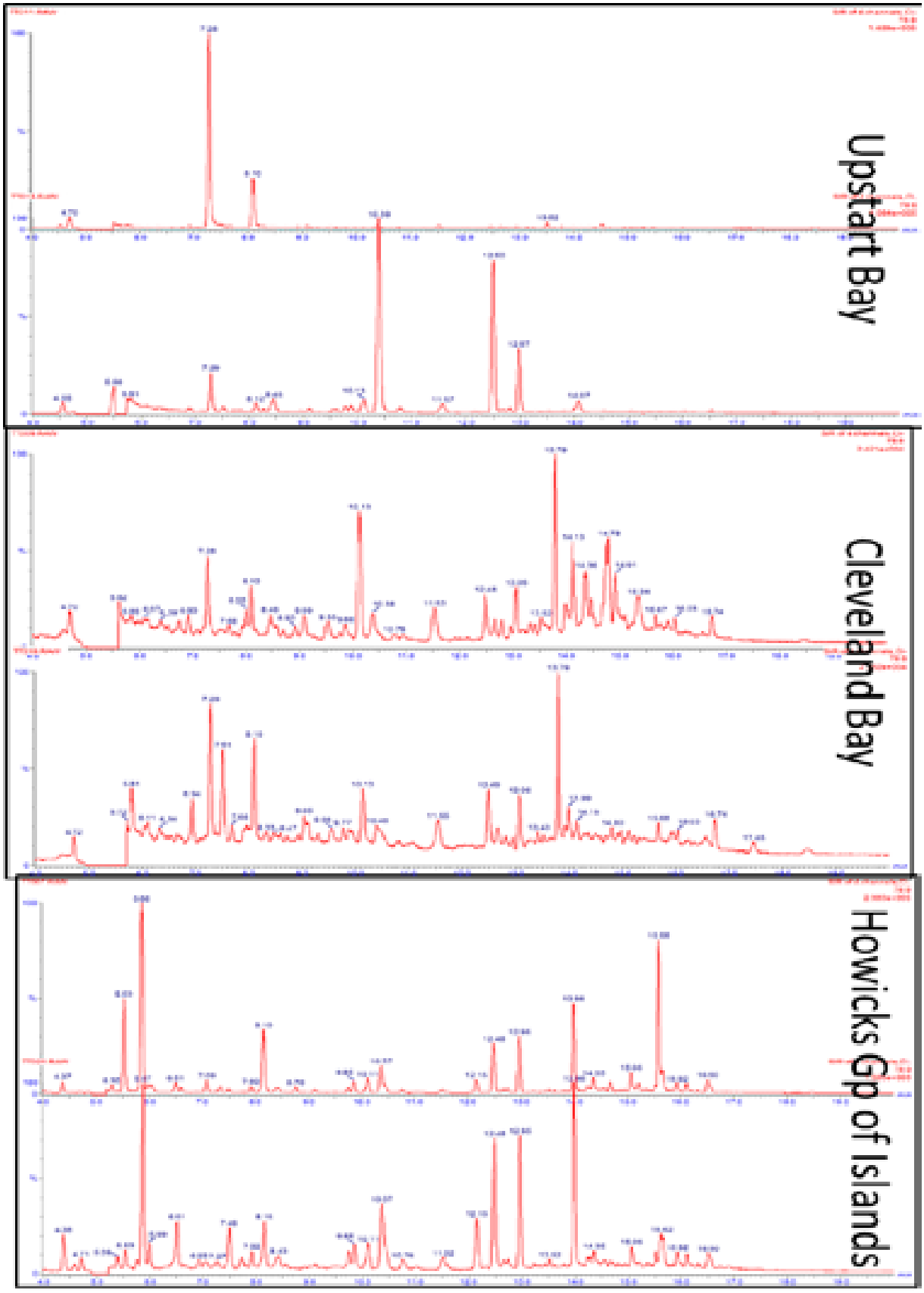

Figure A3: Comparison of chromatograms screening for brominated chemicals in sediments collected in the pre-wet and post-wet sampling periods (top and bottom of each section respectively)

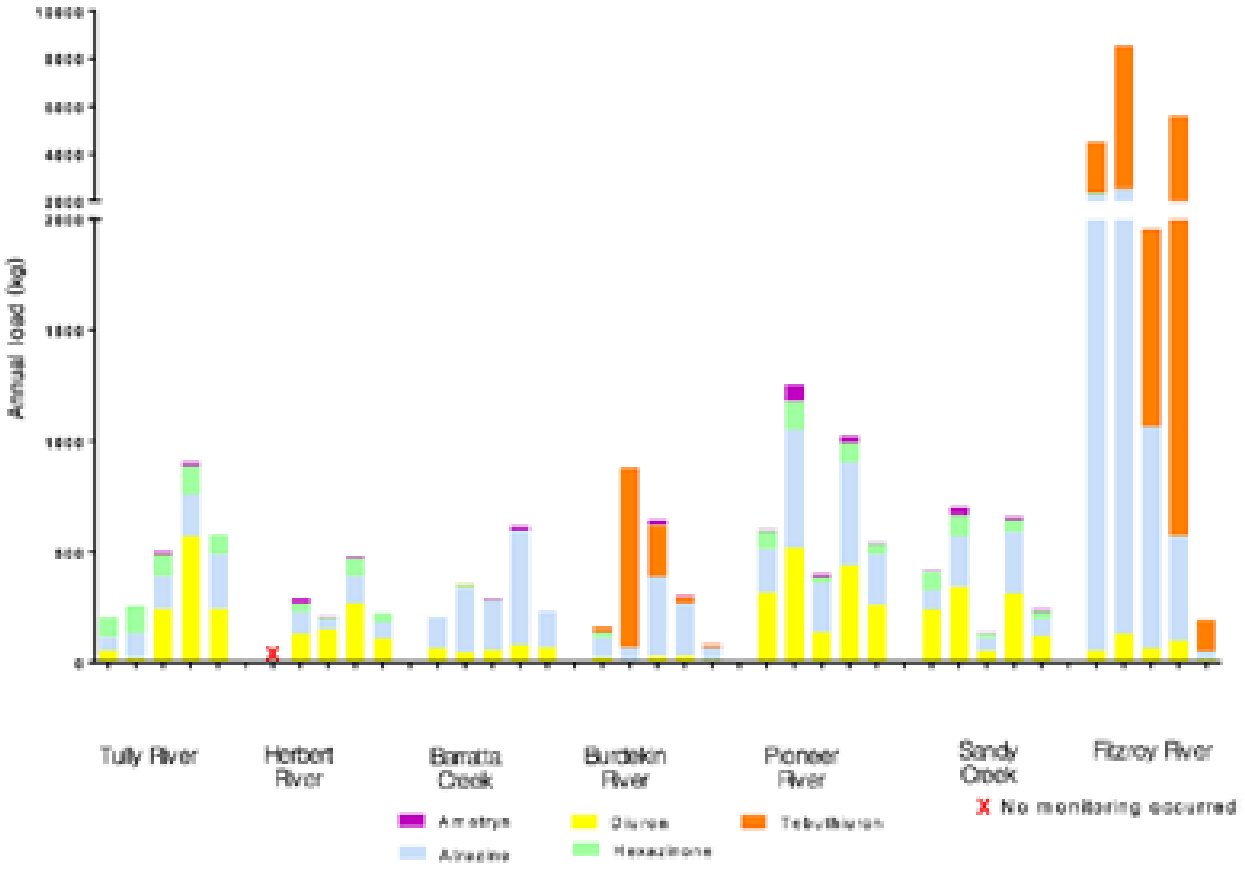

Figure A4: Annual loads of PSII herbicides from selected GBR rivers between 2009 and 2014

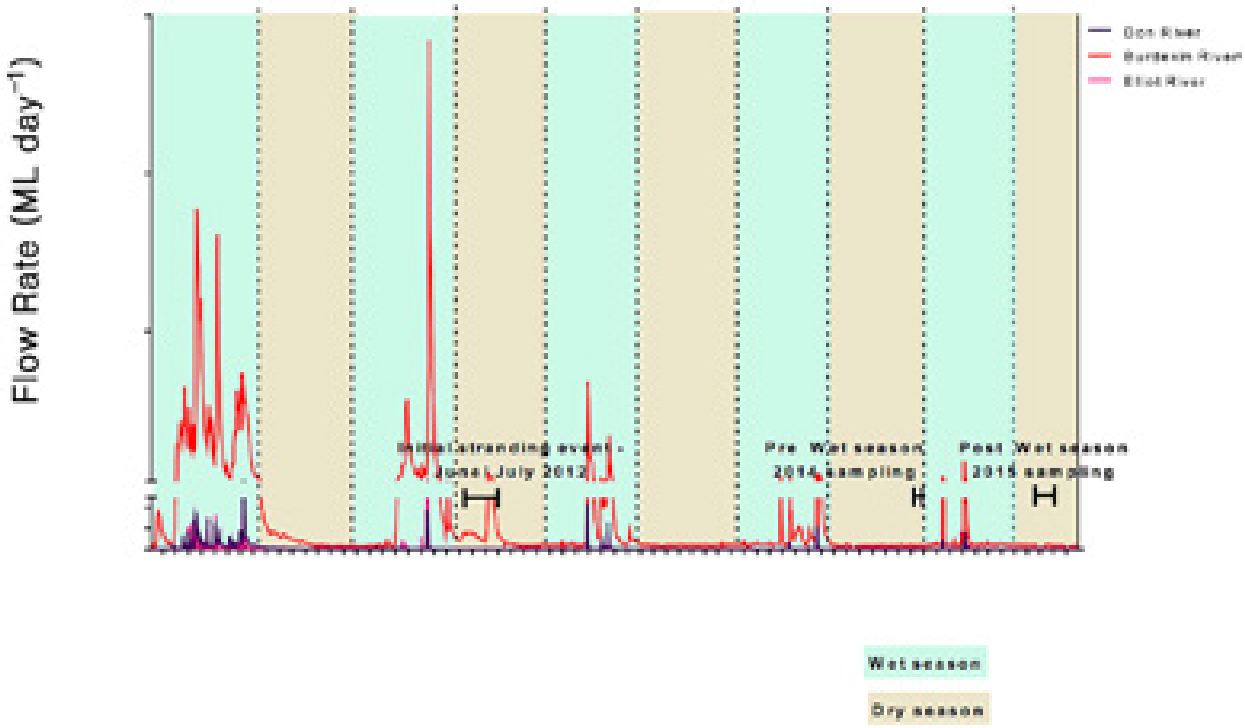

Figure A5: Volume (ML) of freshwater discharge from rivers in proximity to Upstart Bay between 2010 and 2015

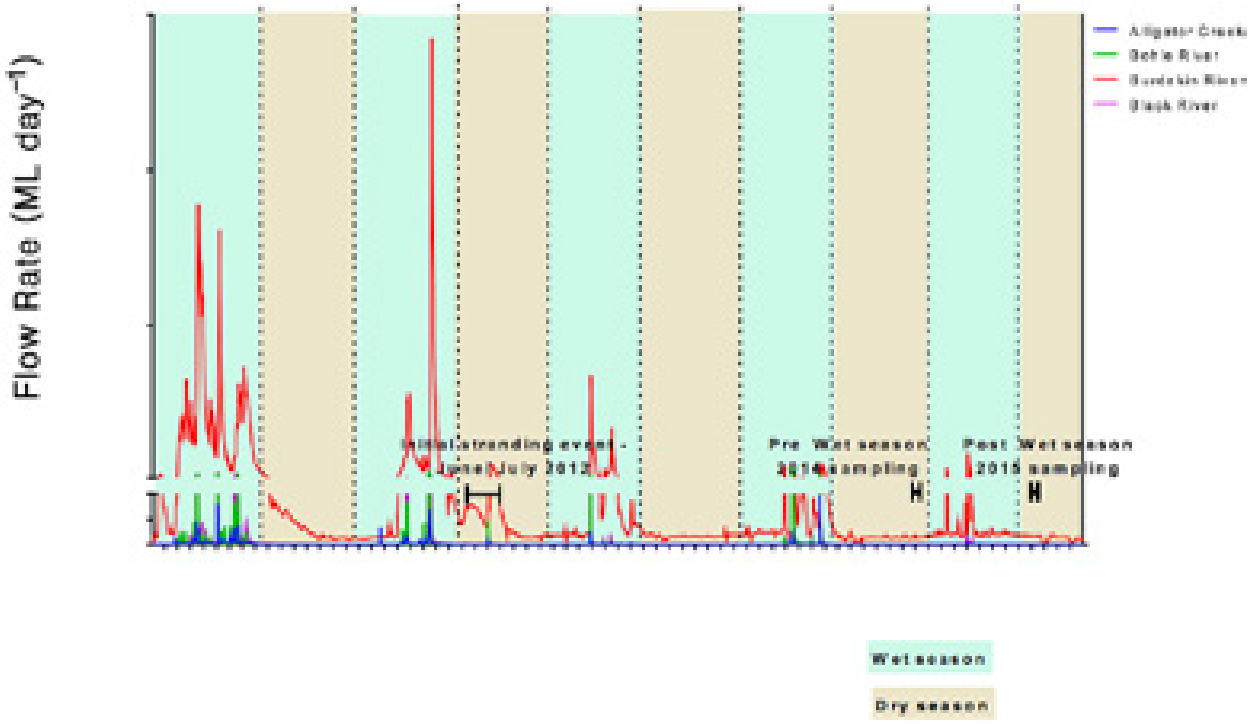

Figure A6: Volume (ML) of freshwater discharge from rivers in proximity to Cleveland Bay between 2010 and 2015

River	LT median	2001 - 2002	2002 - 2003	2003 - 2004	2004 - 2005	2005 - 2006	2006 - 2007	2007 - 2008
Black	4.58E+04	4.04E+04	1.04E+04	4.54E+04	2.77E+04	5.36E+04	1.39E+05	1.81E+05
Ross/Bohle	2.12E+04	4.75E+04	7.85E+03	5.60E+04	2.17E+04	4.28E+04	1.35E+05	1.61E+05
Burdekin	5.31E+06	4.49E+06	2.05E+06	1.52E+06	4.33E+06	2.20E+06	9.77E+06	2.75E+07
Don	5.12E+04	3.86E+04	4.37E+04	5.46E+04	9.74E+04	4.12E+04	1.65E+05	4.62E+05

River	LT median	2008 - 2009	2009 - 2010	2010 - 2011	2011 - 2012	2012 - 2013	2013 - 2014	2014 - 2015
Black	4.56E+04	2.99E+05	1.49E+05	3.47E+05	1.82E+05	4.60E+04	1.02E+05	4.31E+03
Ross/Bohle	2.12E+04	2.31E+05	1.48E+05	2.43E+05	1.54E+05	3.22E+04	1.37E+05	
Burdekin	5.31E+06	2.94E+07	7.95E+06	3.48E+07	1.56E+07	3.42E+06	1.46E+06	8.81E+05
Don	5.12E+04	2.45E+05	1.44E+05	8.48E+05	2.17E+05	1.58E+05	8.76E+04	4.63E+04

Figure A7: Annual freshwater discharge volumes (ML) from GBR rivers nearby to Cleveland Bay and Upstart Bay

Yellow indicates 1.5 – 2 times the long term median, orange indicates 2 to 3 times the long term median, red indicates >3 times the long term median; water years are from 1 Oct to 30 Sept. Table provided by Eduardo da Silva (James Cook University).

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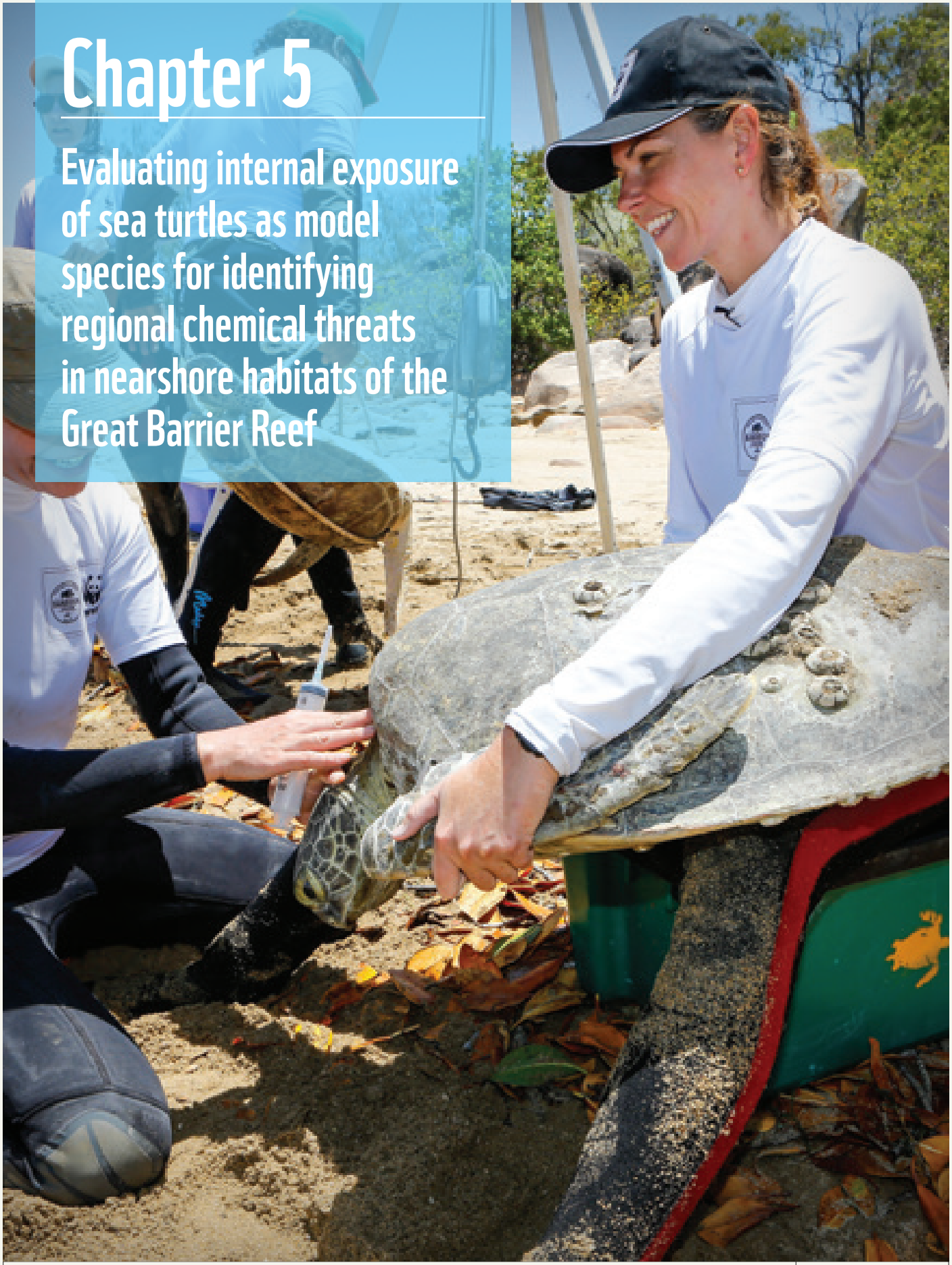
Chapter 5

Evaluating internal exposure of sea turtles as model species for identifying regional chemical threats in nearshore habitats of the Great Barrier Reef



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5. Evaluating internal exposure of sea turtles as model species for identifying regional chemical threats in nearshore habitats of the Great Barrier Reef

Caroline Gaus[1,*], Alexander Cesar Villa[1], Gulsah Dogruer[1], Amy Heffernan[1], Soumini Vijayasarathy[1], Chun-Yin Lin[1], Mark Flint[2], Christine Hof[3], Ian Bell[4]

[1] Queensland Alliance for Environmental Health Sciences (QAEHS), The University of Queensland, 39 Kessels Road, Coopers Plains, Queensland 4108, Australia
[*] Corresponding author: c.gaus@uq.edu.au
[2] School of Forest Resources and Conservation, University of Florida, The Florida Aquarium's Center for Conservation, Apollo Beach, FL 33572, USA
[3] Species Conservation, World Wildlife Fund for Nature, Australia
[4] Department of Environment and Heritage Protection, Threatened Species Unit, Townsville, Australia

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contaminants, bioanalytical screening, non-target screening, multi-element screening, blood

Abstract

Marine megafauna that forage in proximity to land can be exposed to chemical mixtures made up of numerous organic and inorganic contaminants that - individually or combined - have the potential to affect their health. Characterizing such exposure and examining associations with health, however, still poses considerable challenges associated with sampling, analysis and interpretation. The present study summarizes the development and application of novel approaches and methods to identifying chemical hazards and their potential impacts on the health of coastal wildlife, using green sea turtles as model species. We used and epidemiological study approach to collect blood and keratinized scute samples from free ranging turtles foraging in coastal areas and an offshore control site, in combination with non-targeted, effect-based and multi-chemical analytical screening approaches to assess internal exposure to a wide range of chemicals. The screening phase identified a suite of trace elements as priority for further investigation. Many of these elements are not commonly analyzed in marine wildlife, illustrating that comprehensive screening is important where exposure is unknown or uncertain. In particular, cobalt was present at highly elevated concentrations, in the order of those known to elicit acute effects across other vertebrate species. Several trace elements, including cobalt, were significantly correlated with clinical indicators of impaired turtle health. In addition, biomarkers of oxidative stress identified in the blood of turtles showed significant correlations with clinical health, as well as with cobalt. Due to the lack of sufficient information on turtle exposure and reptile toxicity of such elements we established exposure reference intervals using a healthy control population. In addition, exposure history was investigated by establishing temporal exposure indices for prioritized trace elements using steady-state blood and scute relationships. Overall, the data provide a strong argument for the notion that trace element exposure is having an impact on the health of coastal sea turtle populations.

Introduction

Nearshore marine environments are highly diverse systems and support some of the World's most unique habitats [1]. They are increasingly subject to human impacts associated with urban, agricultural, industrial, or port developments [2]. Many megafauna that rely on such habitats, such as dugongs, dolphins and sea turtles, are today classified as threatened, endangered or vulnerable to extinction.

Increasing trends in mass mortalities and incidences of disease, as well as the occurrence of new diseases, are observed in marine megafauna around the world [6, 7]. These are most evident along heavily polluted coastal areas and adjacent to urban, agricultural or industrial lands, particularly in semi-enclosed waters as opposed to offshore habitats [6-9]. Unusually high numbers of morbidity and mortality have also been

reported in populations associated with mining and dredging impacted areas [8, 10, 11]. Species that depend on such environments as forging grounds can be exposed to high loads of contaminants composed of complex mixtures of mostly uncharacterized organic and inorganic compounds, some of which may be present at harmful levels, or act together to elicit combined effects [54]. There is strong laboratory and field-based evidence that exposure to a wide range of contaminants can cause adverse effects at environmentally relevant levels [13-17]. These range from subtle biochemical changes that can compound to result in reduced population resilience or permanent physiological damage (e.g., neurotoxicity, cancer, behavioral changes and impairment of the endocrine, developmental, reproductive and immune systems) [17, 18].

Despite mounting evidence that chemical pollution is an issue of concern for nearshore wildlife, research on threatened species faces numerous constraints related to ethical, logistic, time and cost considerations [18]. These are associated with i) difficulties obtaining suitable samples, ii) challenges associated with assessing exposure to the wide range of chemical mixtures present, and iii) interpretation of exposure in the absence of comprehensive toxicological data. These constraints are further detailed below, and for most marine megafauna, they give rise to a stark lack of even the most fundamental understanding regarding exposure and its toxicological relevance [19].

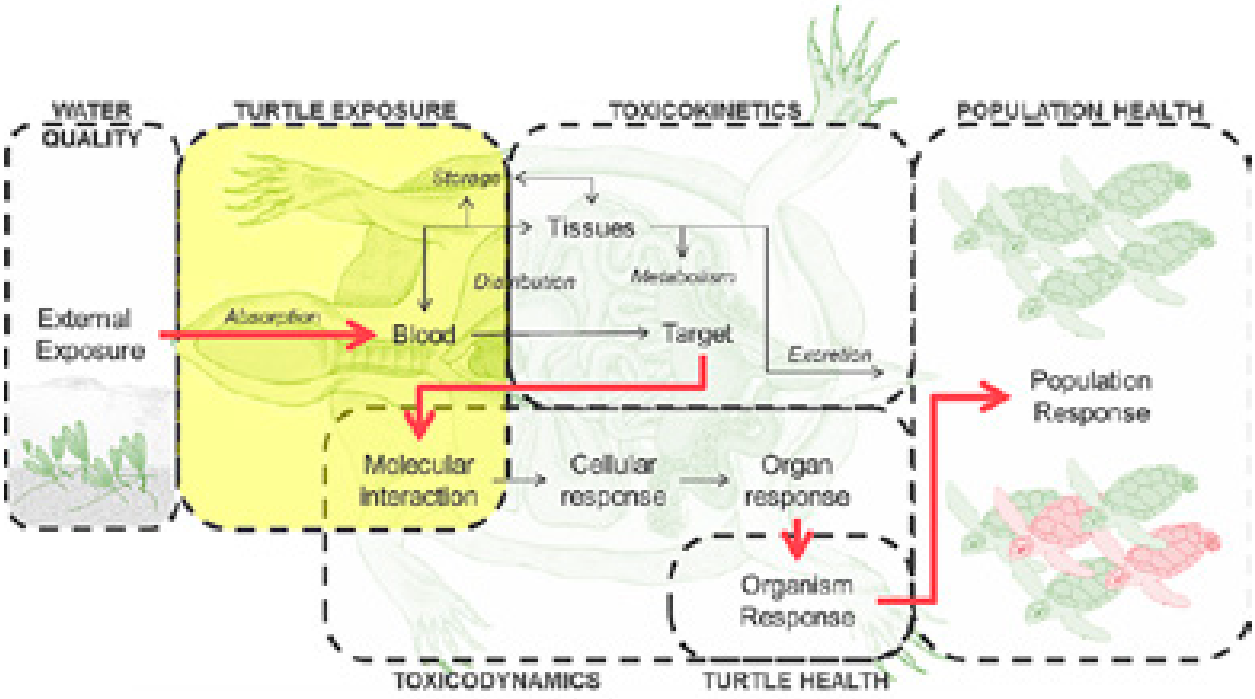


Figure 1: Conceptualized framework of the RRT program *turtle exposure* (yellow highlight) and its links (red arrows) with other RRT program components. The program components *toxicokinetics* and *toxicodynamics* are part of a future Phase II of the RRT.

Here we provide an overview on all studies carried out within one (*turtle exposure*) of four disciplines (*turtle exposure, turtle health, population dynamics, and water quality*) under the umbrella of the Rivers to Reef to Turtle Program (RRT), which was initiated by WWF Australia in response to increased turtle strandings along the Queensland coastline. This *turtle exposure* component of the RRT sought to characterize the internal exposure of green turtle to contaminants that may affect the health of exposed individuals and their respective subpopulations. It employed novel blood and tissue based biomonitoring methods to measure the internal concentrations of contaminants, their biomarkers and mixtures. The links of this component within the RRT program are conceptualized in Figure 1. In brief, contaminants present in blood or tissues are the result of external contamination (*water quality*) and reflect all exposure routes (e.g. water, sediment, food) and take into account chemical intake and absorption. The internal contaminant concentrations are governed by both the physico-chemical properties of the

compounds and the physiology of the species (*toxicokinetics*; future RRT Phase II) which determines the concentrations at the target site of action. Upon reaching the target site, chemicals can initiate a toxicity pathway – a sequence of causally linked events that regulate normal biology, and when sufficiently perturbed, lead to an adverse outcome at the cellular, and possibly the physiological level (*toxicodynamics*; future RRT Phase II). This sequence of events can affect the health of the organism (*turtle health*) and its population (*population dynamics*). Activation of a toxicity pathway upon exposure to physical or chemical insult is controlled by regulatory proteins (transcription factors), which bind to a specific DNA sequence (regulatory elements), thus initiating the transcription of RNA. This sequence of events and their dose-dependent nature can be assessed using *in-vitro* cell based bioassays to signal the toxic potential of chemicals in a mixture (here included within *turtle exposure*).

In its initial phase (2014-2017), the RRT focused on developing suitable methodologies, tools and approaches, and on addressing major data gaps for evaluating links between water quality, green turtle exposure and health. The ultimate goal of the RRT – which extends into a future phase II – was to integrate the outcomes across all disciplines to test the hypothesis (A) that *land-based contaminants adversely impacts the health of resident green turtle populations*. A second hypothesis (B) that *neurotoxicity associated morbidity and mortality in Upstart Bay turtles was due to acute exposure to contaminants*, was incorporated in response to an unusual mass stranding event at a coastal foraging ground in North Queensland (Upstart Bay) in 2012, during which turtles presented with signs of neurotoxicity. Since suitable samples from the event were not available and it is unlikely that links between exposure and the mass stranding could be ascertained retrospectively, hypothesis (B) was included in case additional events of similar nature would present during the study duration.

Materials and methods

Study design

An epidemiological study design was adopted whereby populations were selected to address the two working hypothesis detailed above:

- *Link between exposure and adverse health*. Populations were selected according to a cohort-type study approach (Figure 2) whereby subjects were identified by their foraging grounds near or distant to land-based pollutant sources, their (non-target and target) chemical exposure was characterized, and differences in their health status was evaluated.

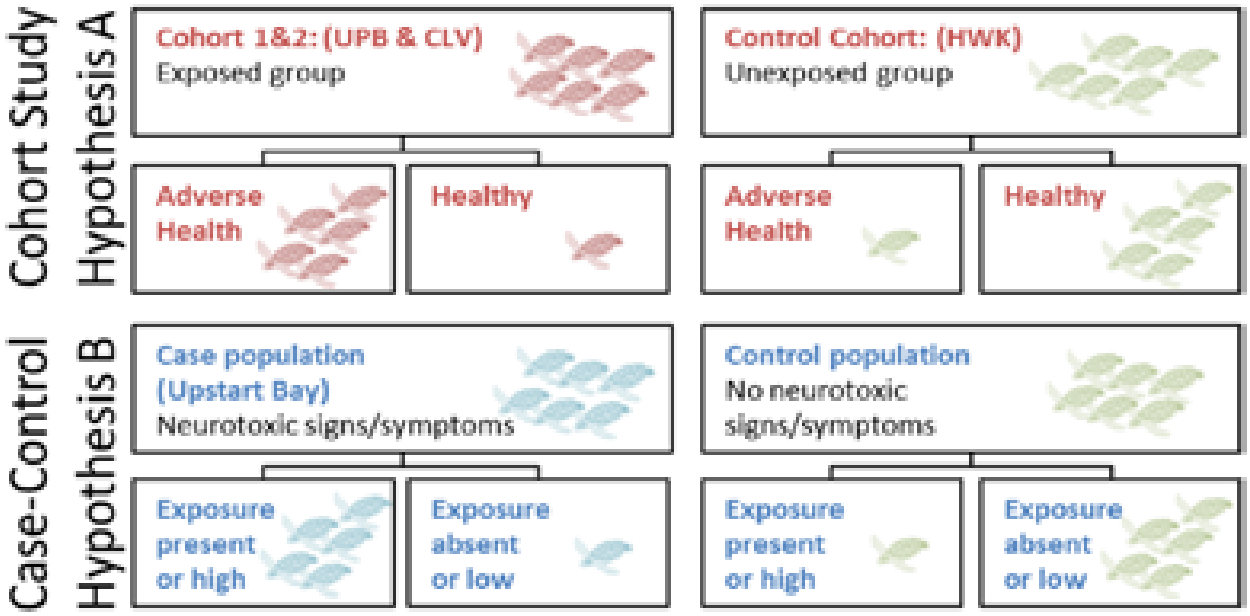


Figure 2: Case-control-type and cohort-type study designs to address the two working hypotheses A (exposure-health) and B (neurotoxicity – exposure).

- *Link between local mass stranding and contaminant exposure*. Populations were selected according to a case-control-type study approach (Figure 2). Providing the event recurred during the study duration, subjects would be identified by the presence or absence of neurotoxic signs and symptoms and differences in their contaminant exposure would be evaluated.

To minimize variability due to age, gender and breeding migrations, animals were selected based on curved carapace length (CCL) to target older (subadult) individuals that had foraged for 10-20 years at the foraging site, but were still sexually immature (≥65 cm to ≤90 cm CCL [20]).

Site selection

Due to expected high spatial variability of contaminants in nearshore environments, the study was conducted at a regional scale incorporating distinct turtle foraging grounds at two adjacent coastal sites with different anthropogenic activities, and one remote and offshore reference site in the northern regions of the Great Barrier Reef (Figure 3).

Resident turtles from the Upstart Bay (UPB; -19.767554 °S, 147.702955 °E) were selected as coastal cohorts for Hypothesis A and served as the primary target for Hypothesis B due to the previous mass stranding event in 2012. UPB is a rural coastal area, approximately 100 km south of the urban/industrial center of Townsville, and 50 km north of an international coal terminal. It receives discharges from the Burdekin River (Australia's largest river by peak discharge volume and catchment area) and a system of mostly ephemeral creeks dominated by intensive agriculture, grazing and mostly legacy mining [21].

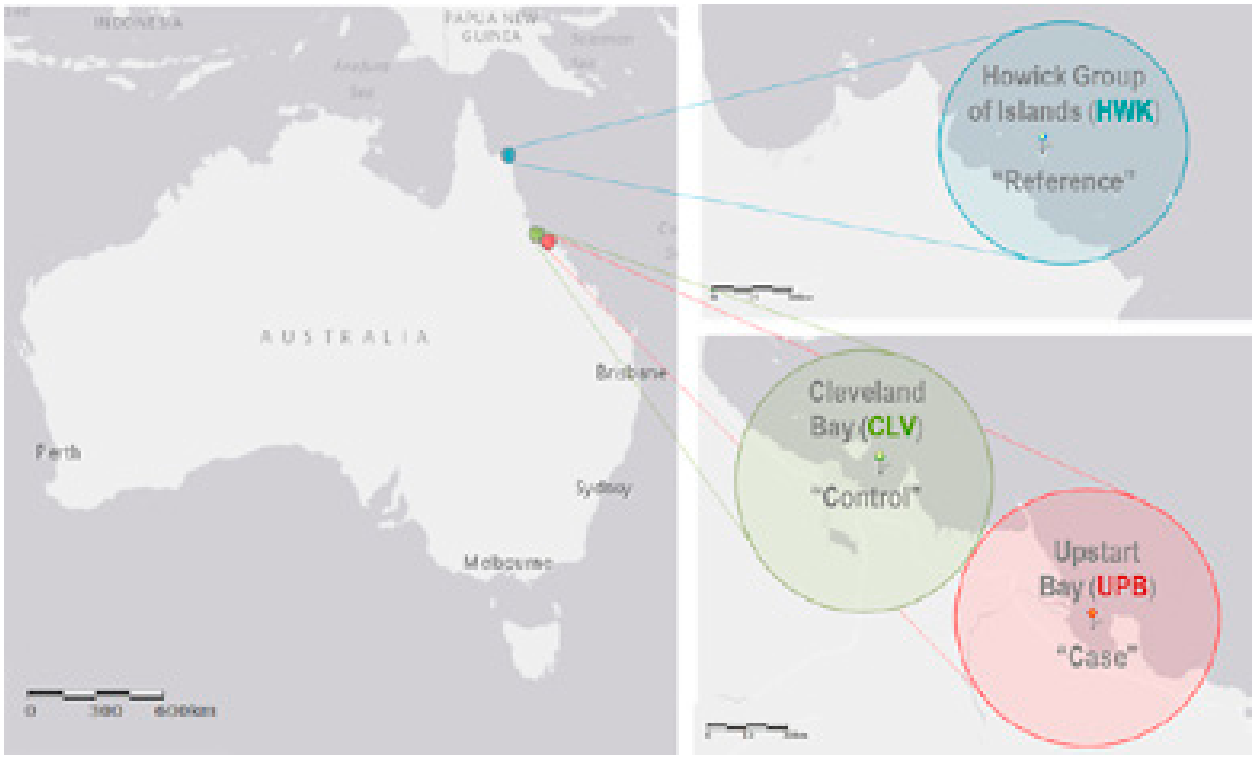


Figure 3: Map of turtle foraging areas included in this study with Upstart Bay (UPB) as the target ("Case"), Cleveland Bay (CLV) as control, and the Howick Group of Islands (HWK) as reference site.

Turtles foraging in Cleveland Bay (CLV; -19.235428 °S, 146.938284 °E) were selected as a second coastal site (Hypothesis A), and served as control for UPB (Hypothesis B). CLV is adjacent to the city of Townsville which supports a population of >175,000 as well as metal processing (Zn, Cu, Ni, and Co) and other heavy industries, and is home to an international port [22].

The Howick Group of Islands (HWK; (-14.416695 °S, 144.880484 °E) was selected as reference site (Hypothesis A). HWK is located approximately 30 km offshore the Cape York region catchment. The latter is a remote coastal wetland with low pressures from nutrient, sediment, and pesticide loads, or water

regime changes and habitat alterations [23]. As a collection of sheltered, uninhabited reefs within the remote northern Great Barrier Reef Marine Park, this offshore sea turtle foraging ground was expected to be free from anthropogenic contaminant point sources.

Sampling design and sample collection

The sampling program was designed to facilitate multiple investigations: screening of internal exposure to a diverse range of organic chemicals and trace elements, subsequent target analyses of prioritized analytes, blood biochemistry and hematology based health assessments, as well as future toxicokinetic/ toxicodynamic studies anticipated in phase II of the RRT. Samples were divided into aliquots for immediate analyses and for archiving at -20 °C for future research.

Between 2014-2017, samples for the *turtle exposure* component of the RRT were collected during bi-annual (wet and dry season) field campaigns at both coastal sites (UPB and CLV) and annual field work at HWK. Over the four year sample period, blood and scute were sampled from a total of 507 green turtles: n=182 in UPB (n=73, 64, 43, 2 per year, respectively), n=125 in CLV (71, 39, 15, 0 per year, respectively), and n=200 in HWK (96, 60, 35, 9 per year, respectively). Sampling in the first year focused on obtaining sufficient sample numbers to account for intra-individual variability of various metals and organic contaminants (estimated minimum of n=30 for most analytes using power analysis of literature data). Subsequent years focused predominantly on sampling of recaptures from previous years. A total of 36 animals were recaptured and sampled in 2015-2017 (UPB: n=8, CLV: n=4 and HWK: n=24).

Whole blood, plasma and scute samples were collected from each of the turtles sampled (Figure 4). A subset of these animals (n=202) also underwent lavage sampling to obtain crop (esophagus) contents; these samples were archived for Phase II of the RRT to quantify external exposure (dose). Similarly, organ, blood and scutes were collected from stranded and necropsied animals (n=5) and archived to facilitate future investigations of contaminant tissue distributions and physiologically based toxicokinetic modelling of priority contaminants.

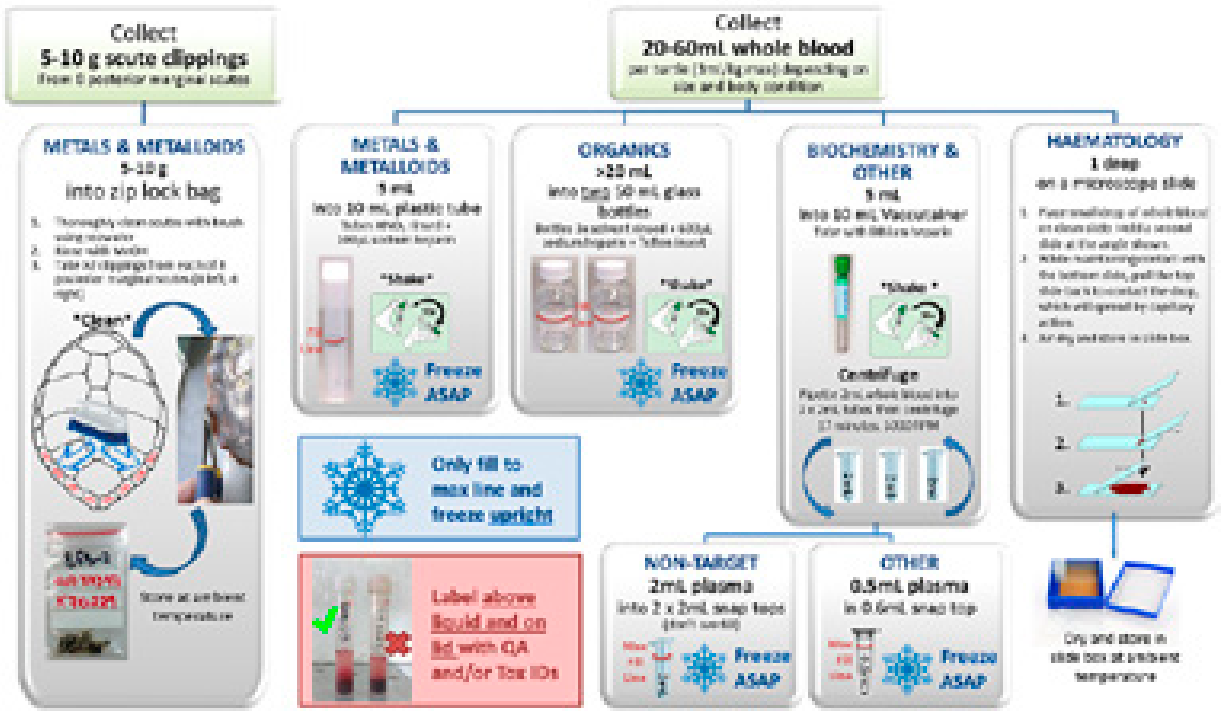


Figure 4: Sampling strategy designed to maximize the analytical scope for both non-target screening and subsequent targeted analyses with limited blood and scute volume available, as well as hematology and blood biochemistry.

Blood was collected from the dorso-cervical sinus using a disposable syringe and 18-21 gauge needle according to methods described in [24]. A total maximum volume of 50-60 mL was collected (ethics

approval NRCET/147/14/APA/WWF), taking care not to exceed the recommended limit of 3 mL/kg [25] and immediately distributed according to analytical requirements. For non-target screening, bioanalytical screening, multi-residue screening and potential follow-up target analyses of a range of organic contaminants, 20-40 mL of whole blood was transferred to solvent washed glassware containing sodium heparin (1,000 IU/ml whole blood). For multi-element screening and potential follow-up target analyses of trace elements, 5 mL of whole blood was transferred into acid-rinsed, sodium heparin dosed polyethylene tubes (1,000 IU/ml whole blood). For clinical blood biochemistry, 5 ml of whole blood was transferred into lithium heparinized tubes (BD Vaccutainer®, Becton, Dickinson and Co., NJ, USA) and centrifuged (2.5 x 106 g·s) to generate plasma as soon as practical in the field (typically within 2-6 hours). For haematology, whole blood smears were air-dried at the time of sampling [26]. All blood samples (except blood smears) were kept on ice during field work and frozen immediately at the end of each day.

Scutes were sampled using techniques to minimize the typically high inter- and intra-scute variability of metal concentrations, as described in detail in [27]. Briefly, scutes were scrubbed thoroughly with seawater to remove extraneous material and rinsed with methanol. Two to four clippings from each of the eight most posterior marginal scutes were then collected and combined into a polyethylene zip log bag to form one composite sample (Figure 4). This sampling strategy considerably reduces uncertainties when predicting mean concentrations across the carapace [27]. Samples were stored at room temperature.

Upon receipt at the laboratory, each sample was cross checked to assure accuracy, logged into a database, and re-captured animals identified. For analysis, samples were selected randomly from each site (within the CCL range specified above) and defrosted.

Analytical framework

The analytical framework is illustrated in Figure 5. It encompassed an initial phase during which methods were developed, tested, optimized and then applied to obtain non-selective exposure information.

In summary, a total of 90 blood (and for trace elements also matched scute samples) from subadult green turtles foraging at each of the three study site (i.e. n=30 per site) underwent analysis using four different analytical screening techniques covering polar and nonpolar organic chemicals, and trace elements. Screening data was interrogated using statistical methods (see below) as well as literature data, and tested for correlations with health indicators generated within the RRT. The outcomes were used to identify priority contaminants for further evaluation using target analysis. Data from target analyses were then interpreted using toxicological information (where available for reptiles, or other marine organisms and vertebrates in general) as well as reference intervals and exposure indices (see below).

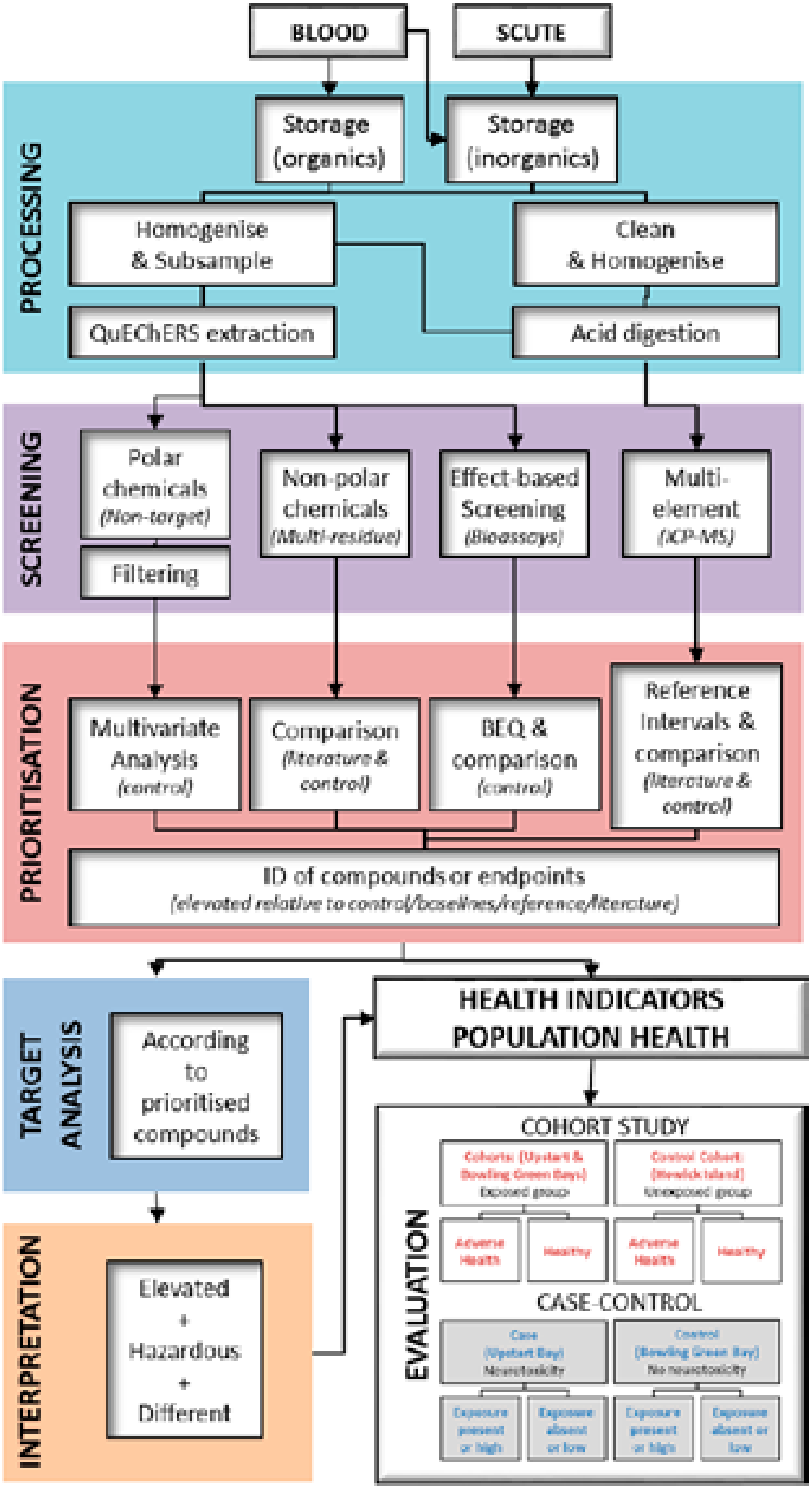


Figure 5: Analytical framework developed for blood and scute sample processing, screening, prioritization and target analysis of contaminants, and illustrating integration with other study components to evaluate links between exposure and health in turtle populations.

Sample extraction

Initially, sample processing and extraction procedures were tested and modified to achieve minimal chemical selectivity, and optimized to meet analytical requirements (focused on the detection of elevated levels). Due to frequent clotting, all blood samples were homogenized prior to subsampling using a TEMA vibrating disc mill after addition of solvent-cleaned polytetrafluorethylene (PTFE) boiling chips. For screening analyses of organic chemicals [28] and effect-based measures [29], 1-4 mL of blood was extracted using a QuEChERS method. Validation demonstrated acceptable recoveries (56-119%) for most model chemicals across a wide spectrum of physico-chemical properties [28]. For multi-element screening, 0.5-1 mL of blood was acid digested according to procedures validated for turtle blood [30], and scutes were homogenized and sonicated in acetone to remove extraneous contamination followed by acid digestion of a 250 mg subsample according to procedures designed to minimize variability [27].

Effect-based screening

Cell based in-vitro bioanalytical methods for screening of the combined effects of unknown and known chemicals were based on approaches described in [31, 32]. These methods were modified and validated using 4 mL of turtle blood and for compatibility with the QuEChERS extraction method. The procedures are described in detail in [29].

In brief, extracts were concentrated to dryness and reconstituted in media for dosing to a battery of cell based bioassays: a) the CAFLUX assay for xenobiotic metabolism indicative of aryl hydrocarbon receptor (AhR)-mediated activity [33, 34], b) the AREc32 assay for Nrf2-mediated oxidative stress response [35, 36], c) the Microtox assay for non-specific modes indicative of baseline toxicity [37, 38], d) the NF-κb-bla assay for modes of action indicative of inflammation [32, 39], d) the p53 assay for DNA damage [32], and e) the VM7Luc4E2 assay for receptor activation in hormone mediated modes of action indicative of estrogenicity [40]. Together, these modes of action cover a comprehensive range of endogenous and exogenous chemical mixtures.

Bioanalytical equivalent concentration (BEQ) was used to quantify the biologically active chemical burden present in blood (BEQbio). BEQbio is the concentration of chemicals in blood causing the same effect as the equivalent concentration of an appropriate reference compound [41].

Non-target polar chemical screening

Whole blood extracts were screened using non-target approaches described in detail in [28], using a UHPLC coupled to a hybrid quadrupole time-of-flight mass spectrometer (TOF-MS) with electrospray ionization in positive and negative ionization modes (TripleTOF 5600; SCIEX). The MS was operated in Information Dependent Acquisition mode, combining simultaneous TOF-MS survey (m/z 100–950) and dependent MS/MS scans (m/z 30–950).

The analytical workflow involved (1) screening against commercial libraries for known compounds (containing >3000 common pesticides, pharmaceuticals, personal care products and forensic compounds); and (2) tentative identification of selected ‘unknowns’ chemicals by generating a molecular formula and structure where possible, which was confirmed by matching fragmentation pattern against theoretical profiles or online databases when available.

A series of filtering strategies were used to isolate MS spectral features of interest and for data reduction, using HWK as a control site to identify endogenous or exogenous chemicals that differed in turtles from the two coastal sites. These criteria included considerations of p values (<0.005) and effect sizes (log fold-changes >0.5) and retention times, and focused on monoisotopic masses (ignoring isotopes, adducts and ion products generated during the ionization processes) using volcano plots. Subsequently, manual visual inspection of the profile plot of each mass was used to compare the relative intensity of a given mass at each of the three sites, taking into consideration procedural and field blanks. Masses were then ranked according to their intensity (i.e. elevation) and abundance in turtles from UPB and CLV compared to HWK and procedural blanks.

Non-polar chemical screening

Blood extracts were analyzed using gas-chromatography tandem mass spectrometry (GC-MS/MS; Thermo Scientific™ TSQ 8000 triple quadrupole) according to recently developed multi-class/multi-residue methods [42].

For this screening phase, we focused on the most common persistent bioaccumulative and toxic chemicals. A total of 56 analytes were analyzed, comprising 15 organochlorine pesticides (dieldrin, HCB, endosulfan, endosulfan sulfate, cis-chlordane, trans-chlordane, o,p-DDD, p,p-DDD, o,p-DDE, p,p-DDE, p,p-DDT, o,p-DDT, aldrin, heptachlor, lindane), 4 non-ortho PCBs (PCB 77, 81, 126, 169), 8 mono-ortho PCBs (PCB 105, 114, 118, 123, 156, 157, 167, 189), 6 marker PCBs (PCB 28, 52, 101, 138, 153, 180 (+118)), 7 polybrominated diphenylethers (BDE 100, 154, 183, 153, 28, 47, 99), and 16 EPA-PAHs (acenaphthene, acenaphthylene, anthracene, benzo[a]anthracene, benzo[a]pyrene, benzo[b/j]fluoranthene, benzo[ghi] perylene, benzo[k]fluoranthene, chrysene, dibenz[a,h]anthracene, fluoranthene, fluorine, indeno[1,2,3-cd]pyrene, naphthalene, phenanthrene, pyrene).

Metal screening

A multi-element method was developed and validated for semi-quantitative screening across 70 elements [30]. Quantification was carried out using inductively coupled plasma mass spectrometry (ICP-MS; Agilent Technologies 7700) and utilizing relative response factors, which achieves accuracy of >67% relative to fully quantitative analysis for many elements [30].

Targeted trace element analysis

On the basis of the screening results, a suit of 26 trace elements were fully quantified as detailed in [43]. Samples were analyzed using an Agilent Technologies 7500cs ICP-MS system equipped with an Octopole Reaction System. The essential and non-essential elements included into the analytical methods were sodium (Na), magnesium (Mg), aluminum (Al), potassium (K), calcium (Ca), titanium (Ti), vanadium (V), chromium (Cr), manganese (Mn), iron (Fe), cobalt (Co), nickel (Ni), copper (Cu), zinc (Zn), arsenic (As), selenium (Se), molybdenum (Mo), silver (Ag), cadmium (Cd), tin (Sn), antimony (Sb), barium (Ba), thallium (Tl), lead (Pb), thorium (Th) and uranium (U).

Green turtles analyzed included the 30 animals previously used for screening plus additional animals and recaptures (total n= 44 from UPB, n= 28 from CLV and n=71 from HWK, including n=16 recaptures from UPB and 11 recaptures from HWK). PCA was undertaken to identify trace elements that are governing differences in exposure for each of the nearshore populations relative to the HWK cohort.

Trace element reference intervals

Metal concentrations quantified in blood were used to establish baseline exposure information for green turtles as detailed in [43]. Upper and lower baseline limits (Reference Intervals) and their 90% confidence intervals were estimated for 20 elements (12 essential, 8 non-essential; Na, Mg, K, Ca, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Mo, Cd, Sb, Ba, and Pb). Blood collected from 49 healthy sub-adult green turtles from HWK defined the baseline cohort.

Trace element temporal exposure indices

Trace element concentrations in blood and scutes were further used to develop Temporal Exposure Indices (TEIs) for Co, As, Mo, Sb, and Cd [27]. This concept capitalizes on the different toxicokinetics in blood and scute and the respective short-term and long-term exposure information that these two matrices can provide.

Steady-state blood:scute relationships under relatively constant exposure regimes were tested using Model II regression models for the HWK cohort (n=71). In order to obtain meaningful data across a range of concentrations, it was also necessary to incorporate turtles from an additional site not targeted by the RRT (Shoalwater Bay = SHL; n=29; -22.370076°S, 150.515198°E). SHL is located within a 4,545 km² Australian Defense Force Training Area which has been both restricted and remote from any human settlement since 1965; its estuarine area is devoid of major riverine inputs, mostly composed of shallow

open water supporting seagrass meadows and rocky reefs, and is subject to higher rates of tidal flushing (>7m tidal range during spring tides) in comparison to any of the other study sites [44].

A two compartment kinetic model was developed to approximate the temporal concentrations of a hypothetical trace-element in blood and scute after an elevated exposure period relative to their steady state ratio in a constant exposure environment. Model parameters are provided in [27].

Results and discussion

Study design and sampling

Selection of green turtles as target species overcame many of the constraints typical for assessing exposure of threatened marine species. Obtaining particular samples (e.g. particular life stages, genders, and healthy specimens) is rarely feasible for most species, particularly in turbid and polluted nearshore environments. Instead, most studies have to rely on stranded animals and are often undermined by the opportunistic sampling basis, lack of associated biometric or habitat information, unknown foraging grounds and other exposure data, poor (decomposed) sample quality, and/or the likely bias towards sick animals [45]. Although green turtles are a rarely considered for examining relationships between chemical exposure and adverse health, or to identify regional chemical threats, these cohorts can serve as model species. When integrated with existing research in wildlife conservation, green turtles offer the ability for systematic studies due their distinct and quantifiable exposure regimes, the access to sufficient and suitable samples, control of confounding factors, information on health and biometric data, and the availability of fresh carcasses:

Distinct and quantifiable exposure regimes

Green sea turtle subpopulations occur in both nearshore and offshore foraging habitats, to which they display exceptionally high fidelity in their subadult and adult life stages [46]. Due to their herbivorous diet (mainly seagrass) and narrow (<10 km^2) foraging home ranges [46], uncertainties and variability encountered in more complex and dispersed food webs are considerably reduced. This offers possibilities to quantify external exposure, or to select sites/populations with particular exposure regimes (e.g. agricultural, urban/industrial in this study) and reference sites (e.g. remote in this study). Note that their low trophic status does not necessarily equate to low exposure as incidental sediment ingestion can contribute substantially to their contaminant intake [47].

Suitable sample volume and type

Despite significant declines over the past decades, abundance of green sea turtles is relatively high in Australia (e.g. estimated 70,000 [48, 49]) and animals are routinely monitored and tagged at many foraging sites [11, 50, 51]. Different matrices can be collected from both healthy and unhealthy specimens (mostly blood and scutes with minimally invasive methods, but if necessary also lavages, faeces, biopsies, egg yolk, eggs). High catch rates allow the ability to obtain samples from approximately 50-100 animals per week and site, and routine field work offers the unique opportunity for repeated collection of samples from the same animals (recaptures) over time for toxicokinetic evaluations.

Control of confounding factors

Size/age relationships are well established for green turtles from various regions [8], and laparoscopy examination can be used to confirm sex and maturity, or to inform on the parity of individuals sampled. Of particular advantage is the long maturation phase of subadult green turtles, which can be selected using size to exclude variability due to breeding and migrations, and to maximize the time of past exposure at their local foraging grounds (>10-20 years).

Information on health and biometric data

Detailed health assessments are routinely carried out by veterinarians during conservation field work. These data are further enriched by established hematological/blood chemistry and other health reference indices [11, 50, 52] to evaluate the health status of individuals and populations. Vast other biometric (e.g.

weight, gender) and physiological (e.g. food intake rates, metabolism) data are additionally available for such species [11, 50, 51].

Availability of fresh carcasses

In addition to free-ranging live specimens, relatively frequent stranding can offer the unique opportunity to access samples from stranded animals of high quality that meet all criteria a) to d) above. These include fresh carcasses of healthy and tagged specimens (i.e. known foraging grounds and life history) which are accidentally killed by e.g. boats or in nets, and undergo full necropsy by qualified veterinarians [53]. Such specimens provide invaluable access to uncompromised tissues, organs and biofluids to determine chemical distributions in target and non-target tissues.

Analytical framework

Traditional methods for assessing internal exposure rely on target analysis of relatively few prioritized chemicals. Such approaches are ill-suited when the chemical composition is complex, variable and largely unknown. Routinely analyzed target compounds represent only a small fraction of the myriad of contaminants that organisms are exposed to and that can elicit effects [55]. Particularly species that forage in coastal environments can be exposed to high loads of chemicals composed of complex mixtures of mostly uncharacterized and variable organic and inorganic compounds, some of which may be present at harmful levels, or act together to elicit combined effects [54]. Such situations call for comprehensive screening that allows analyses without *a priori* chemical selection.

Although still far from routine, instrument based non-target, multi-residue and multi-element analyses, as well as *in vitro* biological test systems are well matched to the task of screening internal exposure comprised of complex chemical mixtures. Collectively, these screening analyses can cover a comprehensive range of (known and unknown) chemicals and their mixtures expected to be present in blood. However, to date such analytical approaches have rarely been applied to biological matrices. A major task of the present study was thus the need to test and validate each analytical screening approach for application to blood.

In the first instance, a blood extraction procedure was required that ensured minimal chemical loss or bias while allowing separation of matrix components (e.g. lipids, proteins) which interfere with chemical detection and quantification. Modification of a recently developed QuEChERS method fulfilled these criteria (covering chemicals across log K_{OW} -0.3 to 10 and a broad activity spectrum) [42]. This extraction method was optimized for UHPLC-ToF-MS non-target screening of polar chemicals [28], GC-QqQ-MS multi-residue screening of non-polar chemicals [42], as well as *in vitro* bioanalytical screening of complex chemical mixtures [29]. These studies provide proof-of-concept that these tools can be adapted to comprehensive screening of body fluids and tissues. A major advantage of the QuEChERS extraction was the ability to use the same extracts for different analytical approaches. The relatively small volumes of blood (1 mL) used for instrument-based analyses so far limits its application to screening for elevated levels. The method could, however, be successfully upscaled (to 4 mL) for bioanalytical testing [29].

Among the widest chemical screening approach applied in this study was the *in vitro* cell-based bioanalytical analysis. Such biological test systems are capable of responding to the totality of chemical mixtures without knowledge of their identity, and many tests are well established for hazard assessment [56]. High sensitivity and high throughput capabilities of *in-vitro* cell-based bioassays in particular facilitate screening of mixture effects using a wide variety of endpoints, ranging from specific receptor mediated (e.g. aryl hydrocarbon receptor (AhR) activity) to non-specific (i.e. baseline) toxicity. The applicability of these tools for mixture toxicity screening has been extensively demonstrated for various environmental matrices [56], and more recently some studies have shown the suitability of selected assays for body fluids and lipid-rich tissues [41, 57]. For the present study, a wide battery of assays was initially selected to cover non-specific, reactive, hormone mediated, and specific modes of action as well as adaptive stress response pathways. Full validation and optimization of the entire battery for screening of all samples, however, proved impractical considering the timeframe, budget and required sample volume. Hence, a pre-screening was employed to identify assays with suitable concentration-response windows

(i.e. respond to the mixtures in the dosed blood before induction of cytotoxicity) for a more limited and manageable ensemble of toxicity pathways: the Microtox assay (which responds to baseline toxicity elicited by a myriad of chemicals), AREc32 (involved in the highly responsive cellular stress pathway as sentinel of chemical insults) and CAFLUX (responding to a specific set of chemicals activating the Aryl Hydrocarbon Receptor). These modes of action can be triggered by a wide range of chemical mixtures, including endogenous and exogenous compounds present in whole blood.

A key disadvantage of bioanalytical tools is their inability to identify the chemicals causing the observed response. Rapidly advancing multiplexed and high resolution technology (e.g. quadrupole time-of-flight), and associated bioinformatic software offer the ability to profile hundreds to thousands of chemicals in a single injection, without a priori knowledge of their identity, and with high sensitivity (ppt-ppb range) [58, 59]. For the present study, non-target analysis employed liquid chromatography (LC)-based methods, which are increasingly applied to non-target approaches [60]. Generally most amenable for polar, thermolabile and higher molecular mass chemicals, these analyses have been successfully used to detect and identify a wide range of biologically active compounds, including toxicants, drugs, metabolites, pesticides, estrogenic compounds, and even highly nonpolar and persistent chemicals such as polychlorinated paraffins [61]. Application of such non-specific screening tools, however, brings its own particular challenges due to the complexity of data generated and the limited availability of databases that can be used to identify environmental contaminants. A variety of tools have been promoted to deal with data rich analytical techniques, and are commonly based on analytical and statistical filtering principles that result in progressive reduction of complexity [59]. In addition, the sampling design of the present study allowed sample stratification via a control group against which the chemical profile could be compared and filtered. This considerably reduced the size of the search space from an initial ~860,000 to a list of <60 spectra of interest for manual interrogation [28].

Although similar non-target analytical tools exist for semi-polar and non-polar chemicals, they could not be tested and validated for the present study due to cost and time limitations. Instead, a multi-residue screening approach was selected using GC-MS/MS, which allowed simultaneous identification and quantification of multiple chemical groups in a sample. While such approaches do require *a priori* selection of chemicals, they can target multiple persistent, bioaccumulative and toxic chemical groups comprising some of the most hazardous chemicals that are prone to accumulate in environmental sinks such as nearshore marine systems. This was considered appropriate for the initial screening purpose of the present study, particularly considering the inclusion of the effect-based screening which also covers semi-polar and non-polar chemical groups, and could be used to inform on the need to integrate non-targeted instrument-based methods.

Unlike the intensive efforts to develop and apply non-targeted methods for characterizing organic chemicals, trace element analysis is still focused on relatively few, mostly non-essential elements with a priority for those presenting highest risks to human health (e.g. Hg, Pb, Cd). Such elements have also been implicated in a number of adverse chronic and acute health effects for marine turtles, including immune suppression [62], lower reproductive success [63], oxidative stress [64], and various other metabolic functions [65]. However, numerous other elements can elicit adverse effects when their intake rates exceed that of excretion and/or detoxification. Coastal development, industrialization, and urban discharges contribute a diverse mixture of trace elements to which resident green turtles are exposed. The sources, composition, bioavailability and toxicity of trace elements for marine megafauna in general are largely unknown and influenced by numerous biotic and abiotic factors. For these reasons, the present study validated and applied a multi-element screening method, covering as many as 70 elements with an accuracy of >67% compared to fully quantitative target analysis [30]. In combination with a suitable reference and multivariate statistical analyses, this approach allows characterization of temporal or spatial differences and unbiased prioritization of trace elements for further investigations [30].

Summary of screening outcomes and prioritization

Results from effect-based pre-screening are provided in detail in [29]. Overall, no responses were observed in assays indicative of endocrine (estrogen) disruption (BG1), DNA damage (p53) or

inflammation (NF-κb-bla), suggesting the presence of relatively low concentrations of relevant chemicals. In contrast, assays indicative of the presence of baseline toxicants (Microtox), compounds acting over the oxidative stress pathway (AREc32) and Aryl hydrocarbon Receptor activity (CAFLUX) responded in a pre-screening of a subset of animals (n=5 per site), and subsequent analysis of all 90 blood samples showed significantly higher induction in turtle blood from UPB ($p<0.05$) compared to CLV (Figure 6); none of the assays differed significantly between CLV and HWK [29]. Approx. 25% of UPB turtles showed moderately elevated TCDD-EQ (range: 50-130 pg/g lw; Figure 6). Considering the outcomes of the non-polar chemical screening (see below), dioxins are likely to contribute the majority to this TCDD-EQ. Similar TEQ (based on target analysis of dioxins) have been observed in some other green turtle populations foraging near urban and agricultural areas of Queensland, and have been implicated in risks for chronic biochemical and immunological effects in these animals [66]. In contrast to the CAFLUX assay, both the Microtox and AREc32 assays respond to a myriad of environmental chemicals and their transformation products with different structures and physico-chemical properties. Considering that the physiology and diet of the three turtle populations are similar, the significantly higher responses in UPB turtles was considered to reflect an overall difference in exposure to more, or more potent biologically active exogenous compounds [29]. The AREc32 results in particular indicated that UPB turtles have a higher need for antioxidant defense. This is consistent with the screening results for polar organics as well as for trace elements (although the recovery of trace elements by acetonitrile was not tested here), which show elevated levels of compounds known to cause oxidative stress, and the presence of biomarkers of oxidative stress in UPB turtles. In addition, these results corroborated with the health [67] and water quality [68, 69] investigations performed as part of the RRT program.

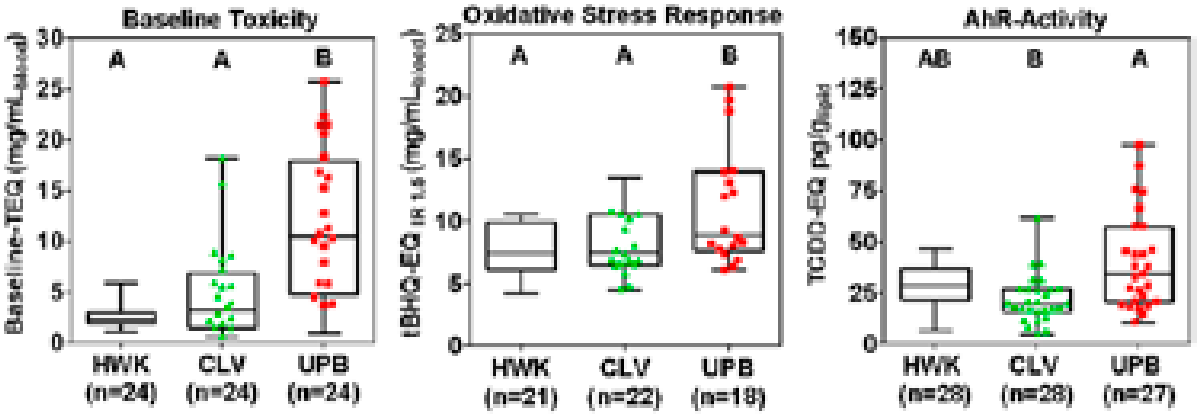


Figure 6: Comparison of the bioanalytical equivalent concentrations (BEQs) in blood of turtles from Howick Island (blue), Cleveland Bay (green) and Upstart Bay (red) for the Microtox (n=72 in duplicate), AREc32 (n=64 in triplicate) and AhR-CAFLUX (n=84 in triplicate) assays. Whiskers = range, boxes = 1st and 3rd quartiles, line = median; significant differences are indicated as letters (Microtox: $p < 0.0001$; AREc32: $p= 0.0038$; AhR-CAFLUX: $p= 0.0007$; Kruskal-Wallis test); Figure modified from [29].

Non-target screening of polar chemicals is detailed in [28]. In summary, PCA of these data showed clear differences in blood of turtles from the three sites (Figure 7), and particularly separated HWK from the two coastal sites. Suspect screening against spectral libraries generated few positive matches with similar relative intensities across CLV and UPB. In contrast, prioritized spectra for manual interrogation showed significant differences in UPB turtles compared to the HWK control for various compounds (reviewed in [28]); particularly for biomarkers of effect (neuroinflammation and oxidative stress): i) benzenetriol-sulfate, a metabolic product of benzene epoxidation by CYP2E1 in the liver followed by hydroxylation of the resulting phenol, which is thought to be mediated by hydroxyl-free radicals; ii) lipid peroxidation products of C18:4, C18:3 and C18:2 long chain fatty acids, which may be the result of oxidative stress following exposure to chemical oxidants or reactive oxygen species; iii) 3-indolepropionic acid, a deamination product of tryptophan with neuroprotective properties against lipid peroxidation and oxidative stress ; as well as iv) vanillylmandelic acid (VMA), a catecholamine metabolite of stress hormones epinephrine and norepinephrine. VMA is also produced by catecholamine-secreting neuroendocrine tumors in humans, such as neuroblastoma. Turtles from the urban/industrial site CLV showed significant differences for a

range of biomarkers of exposure, primarily industrial chemicals and pesticides and their intermediates or metabolites (e.g. sulfonic acids, isoquinoline, guaiacolsulfonate, ethiofencarb sulfone). Overall, these results corroborate with findings from other screening approaches and suggest a prevalence of oxidative stress inducing and industrial chemicals at UPB and CLV, respectively.

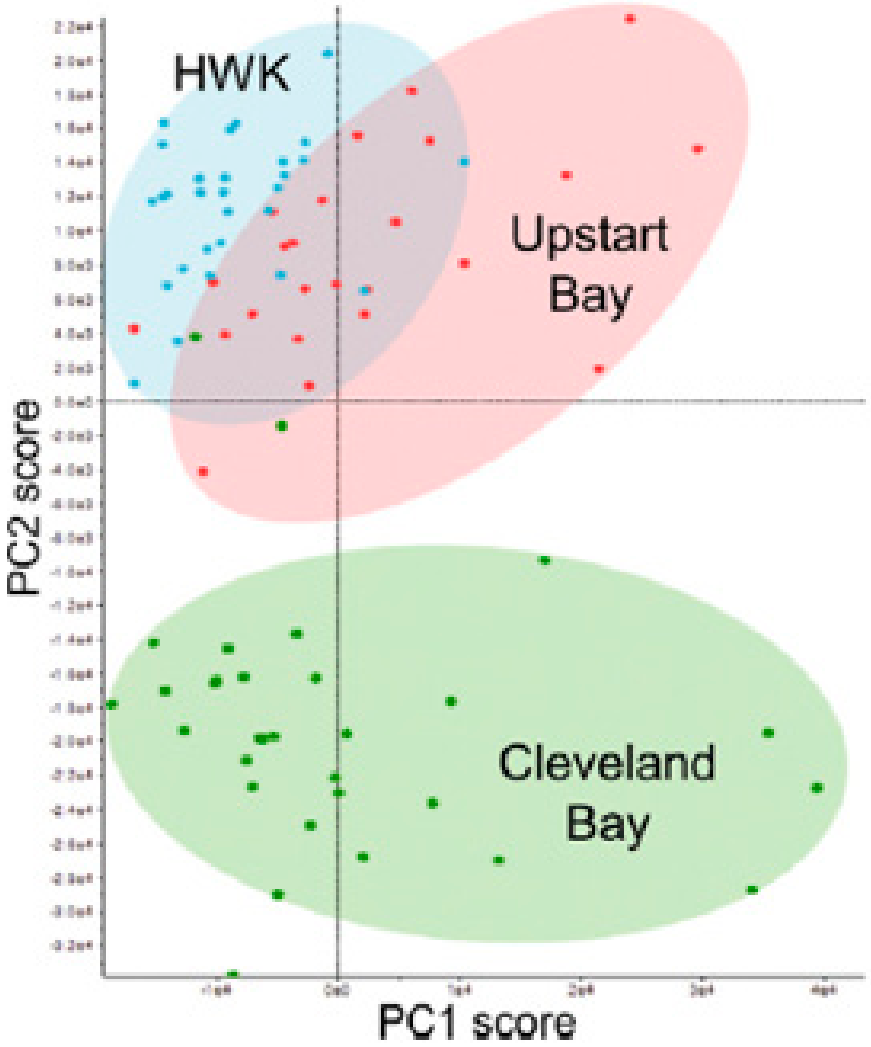


Figure 7: Score plot from principle component analysis of polar organic chemical non-target analysis of turtle blood from CLV (green), UPB (red) and HWK (blue); Figure from [28].

Outcomes of non-polar chemical screening are presented in [70]. Of the 56 chemicals analyzed in whole blood, the majority were present at relatively low concentrations below the MDL [70]. Only some EPA-PAHs were present above the limit of detection, which showed statistically significant ($p<0.05$) differences between sites, with green turtles from HWK generally containing the lowest concentrations (Figure 8). However, compared to literature data from other green turtles, the concentrations detected were overall relatively low or similar to those reported in turtle blood from non-impacted areas (reviewed in [70]), and thus considered unlikely to pose significant risks. On the basis of these screening data, the cost and time involved for undertaking fully quantitative analysis of these hazardous chemicals was unjustified. The decision to exclude further investigation of non-polar chemicals was subsequently made on the basis of the outcomes from all screening approaches, including corroborating data from effect-based screening (see below).

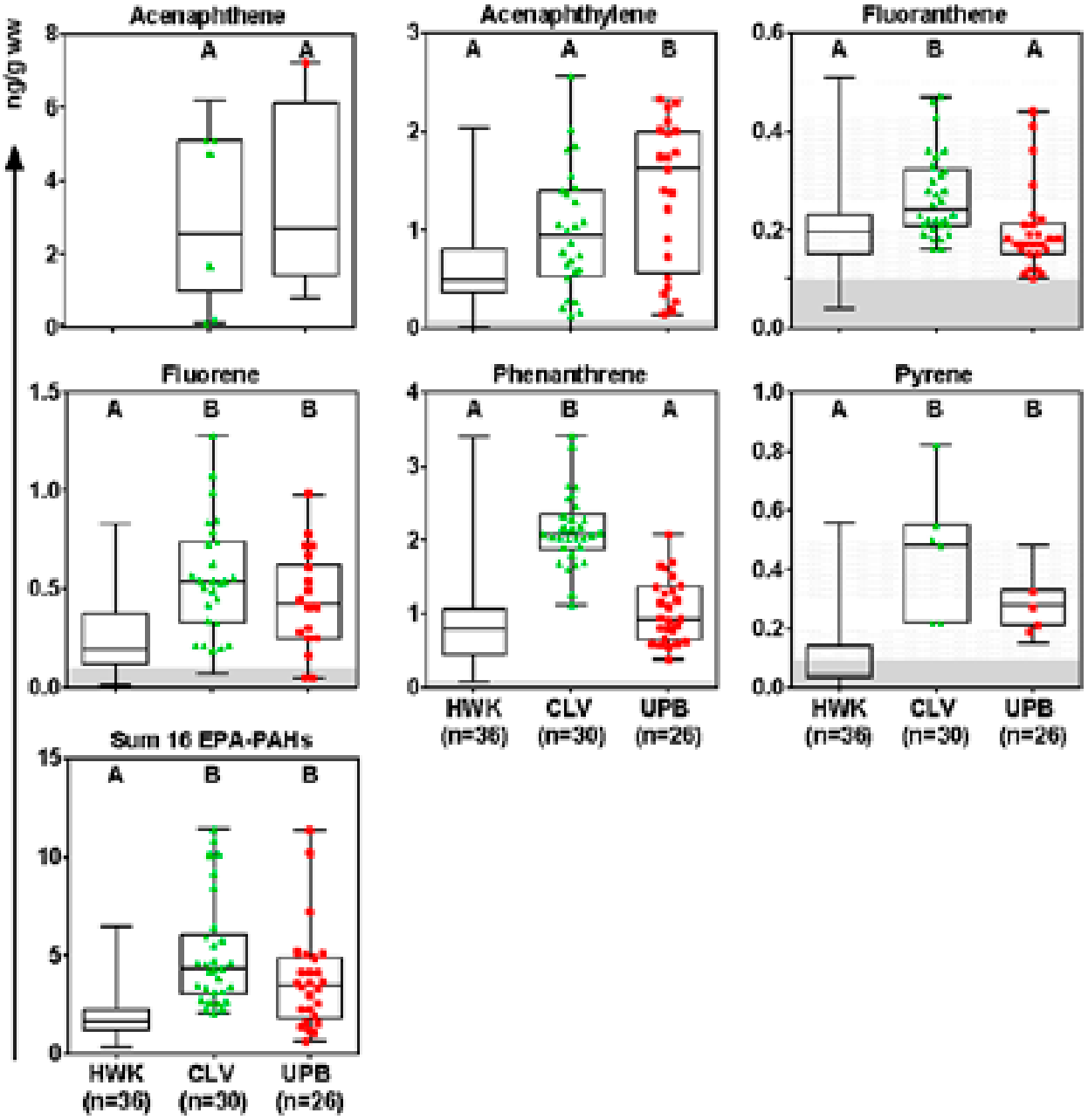
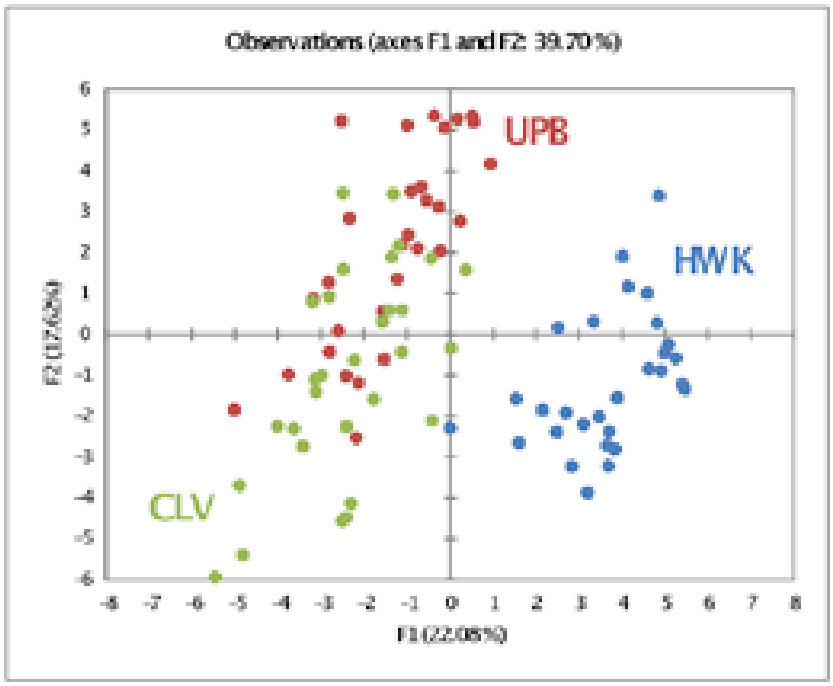


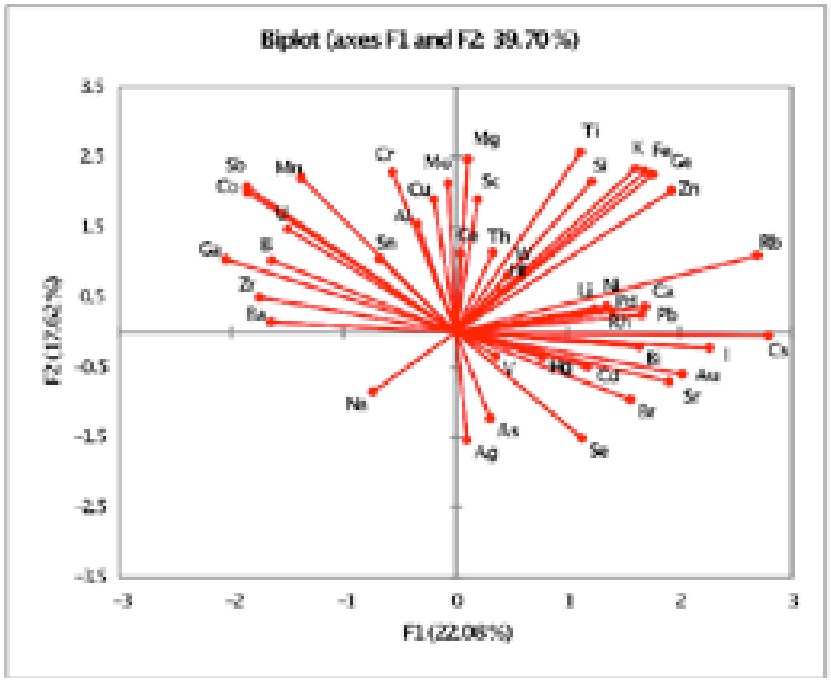
Figure 8: Concentrations (ng/g ww) of EPA-PAHs in blood of green turtles from HWK, CLV and UPB. Grey shaded area = average limit of detection (LOD) for a given analyte; only showing analytes >LOD; any data <LOD was excluded.

Multi-element screening of blood (Figure 9) and scute (Figure 10) showed clear differences between turtles foraging at the different study sites. In blood (i.e. mostly short-term exposure signatures), particularly Ga, Co, Sb, Zr, Ba, B separated HWK from the two coastal sites (PC^1; 22%), while Ti, Mg, K, Fe, Cr, Ge, Mn, Si, Mo, Co, Zn, Sb, Sc, Cu (PC^2; 18%), and Ni, Na, Se, As (PC^3; 9%) governed the differences between UPB and CLV. In scute, where trace elements can accumulate over several years, differences among the three sites were strongly influenced by numerous rare earth elements (Eu > Sm > Pr > Nd > Tb > Y > Ho > La > Tm > Er > Ce > Gd > Yb > Dy > Sc), which were present only at relatively low concentrations in blood. Apart from these, numerous other elements, including Th, Al, Mn, V, Ti, Zr, Nb, Fe, Rb, Li, Ta, Si, Hf, Ga, Ge, Co, Mg, Ba, Cs, Mo, U, Cr, Zn, K, Br, Cu (PC^1; 51%) separated mainly HWK from the two coastal sites, while Sb (PC^3) mainly separated UPB from CLV.

Most of these elements have never been investigated in these areas, nor are they commonly included in megafauna and turtle exposure studies from elsewhere. Albeit semi-quantitative only, the screening results indicated particularly high levels of Co, Mo, Mn, Mg, and Sb concentrations compared to literature data (sea turtles and including where necessary vertebrate species in general), which was subsequently confirmed via target analysis [43]. Some of these elements are known potent inducers of oxidative stress in organisms, and can elicit neurotoxicity as well as a range of other adverse effect at high exposure levels.

Overall, the screening phase strongly suggested the prevalence of chemicals known for their potential to induce oxidative stress at UPB, and industrial chemicals at CLV.





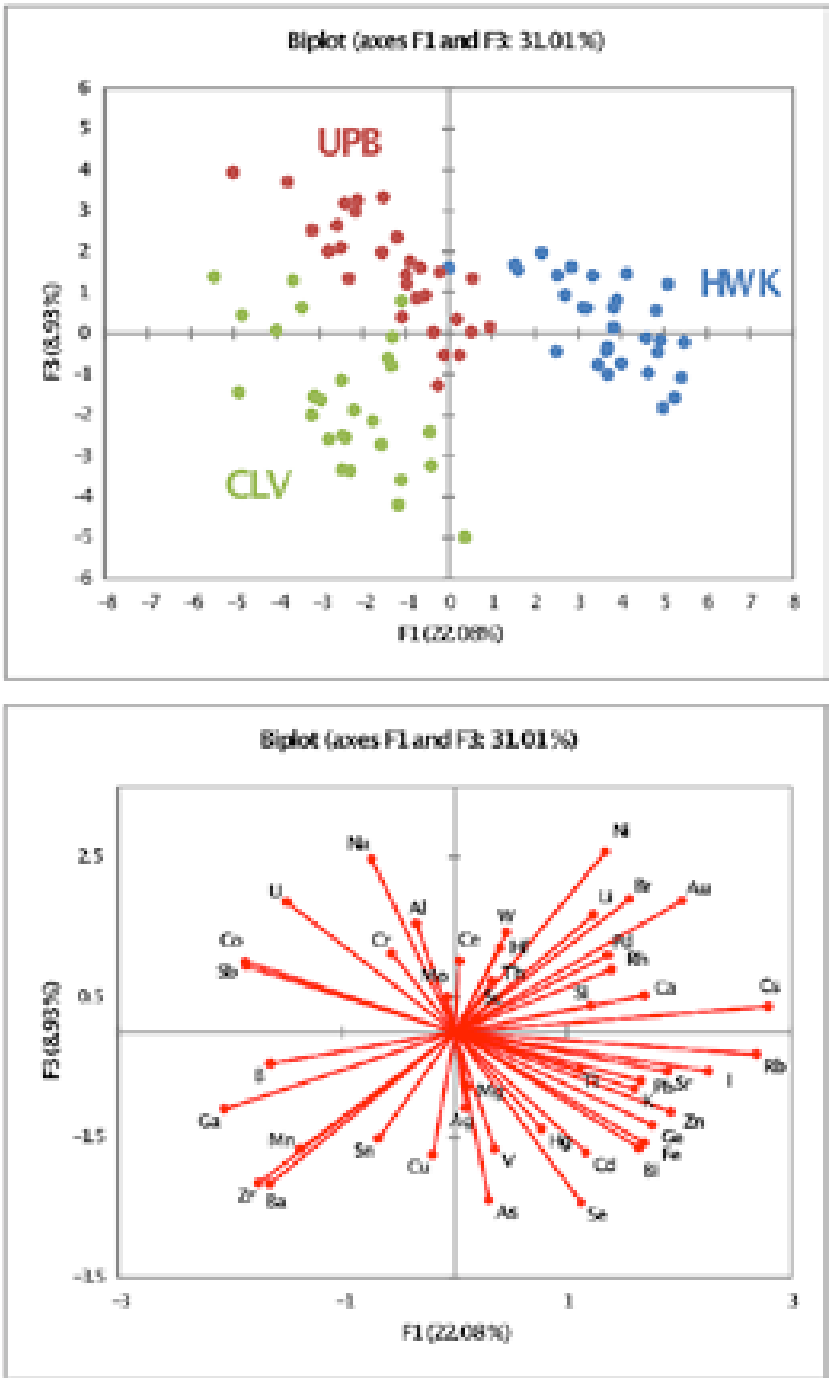
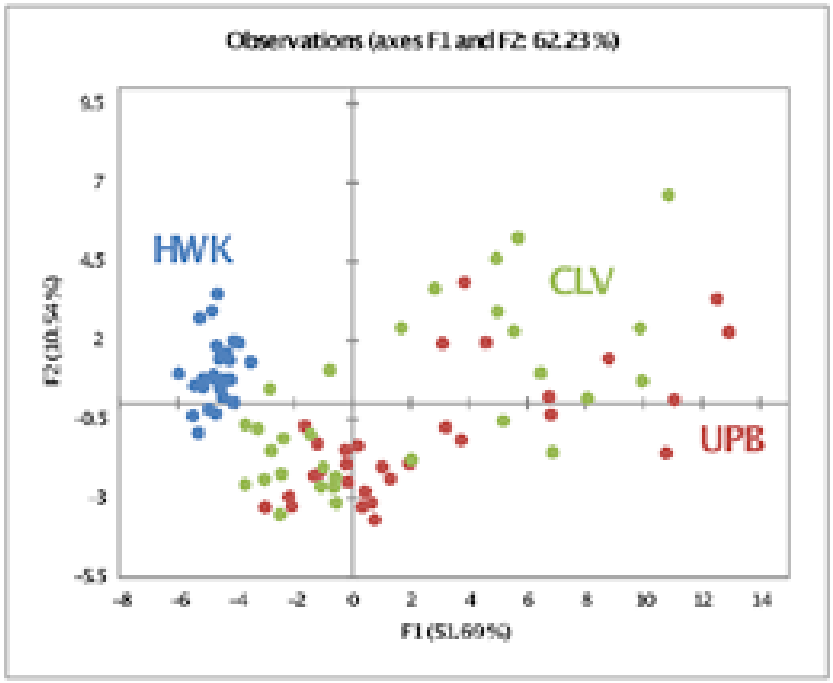
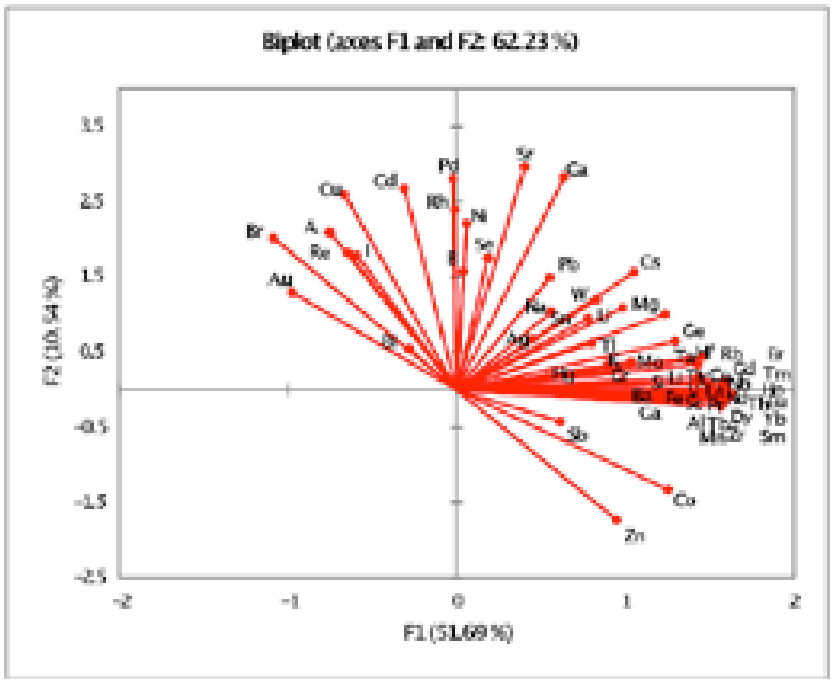


Figure 9: PCA (spearman) bi-plots for trace elements in blood of green turtles from HWK, CLV and UPB (top), and the corresponding loading scores (bottom). Note: outliers (ROUT Q = 0.1%) were removed.

Subsequent multivariate statistical analyses revealed significant correlations between hematological and blood biochemical health indicators and biomarkers of oxidative stress, as well as trace elements (see below). Based on these outcomes and considering the previous mass stranding event at UPB with suspected neurotoxicity, a suite of trace elements were prioritized for further investigation using target analysis.


Observations (axes F1 and F2: 62.23 %)
HWK
CLV
UPB
F2 (10.54 %)
F1 (51.69 %)


Biplot (axes F1 and F2: 62.23 %)
F2 (10.54 %)
F1 (51.69 %)

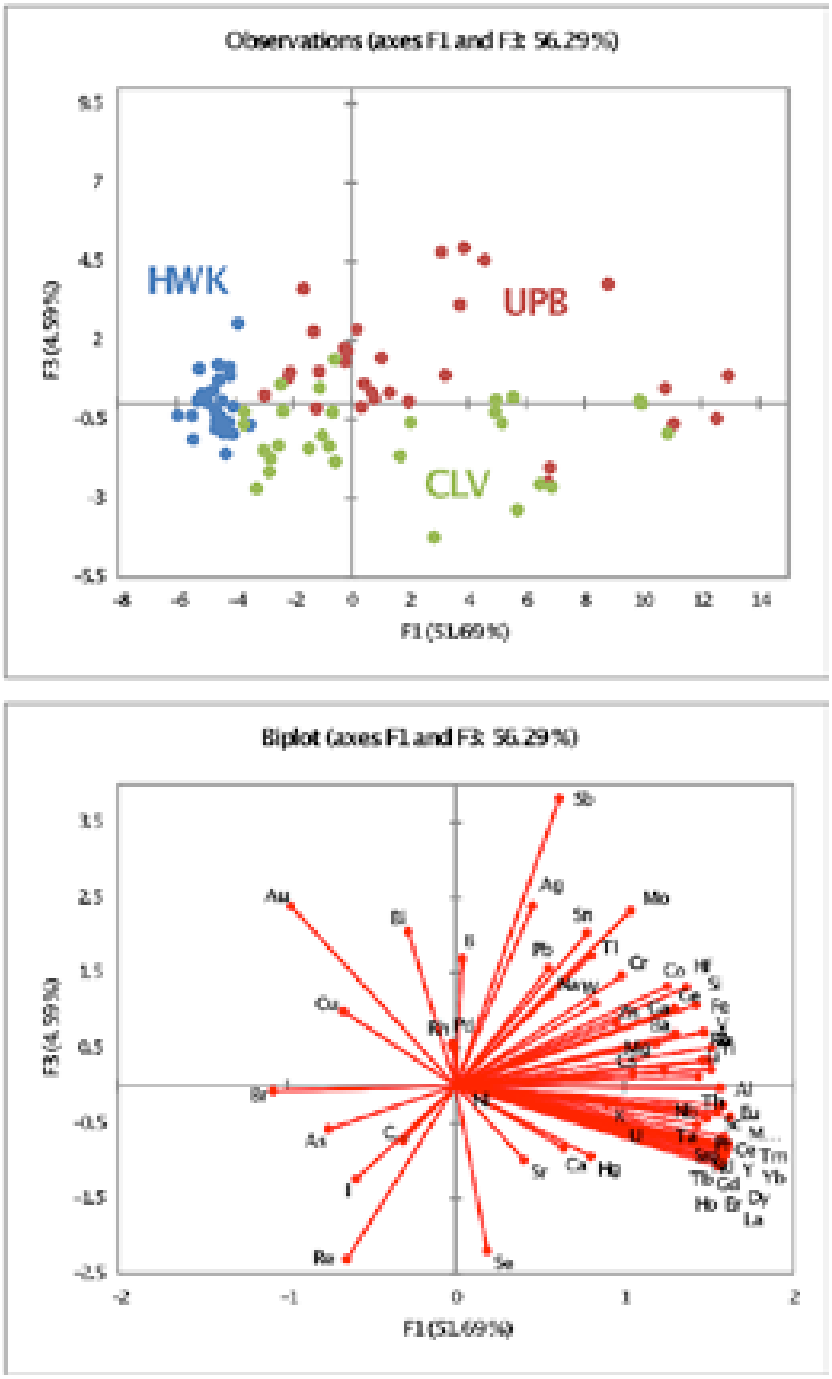


Figure 10: PCA (spearman) bi-plots for trace elements in scute of green turtles from HWK, CLV and UPB (top), and the corresponding loading scores (bottom). Note: outliers (ROUT Q = 0.1%) were removed.

Target analyses and interpretation

While multi-element screening was valuable to identify several trace-elements that should be included into further investigations, many of the elements selected had never been analyzed in sea turtles or similar biota, and both exposure as well as toxicological information is missing for reptiles in general. This makes it challenging to interpret the monitoring data beyond a simple evaluation of univariate differences among sites. To address this, we used clinical reference interval (RI) methods to generate baseline limits for both essential and non-essential elements using blood from healthy subadult turtles foraging at the remote and offshore site HWK [43]. RIs were derived for 20 trace elements (Na, Mg, K, Ca, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Mo, Cd, Sb, Ba, and Pb (Figure 11). Inter-individual variation was low for both non-essential and essential elements, and concentrations of non-essential elements were among the lowest reported in the literature. Hence, the RIs were considered to reflect the optimum ranges of

essential elements for healthy green turtles, and a natural baseline range for non-essential elements [43]. This was further corroborated by the similarity of essential element concentrations reported from other offshore green turtle populations (see detailed review in [43]). Furthermore, trace element concentrations in HWK turtles did not differ significantly between years and fell within the 90% prediction intervals for recaptures.

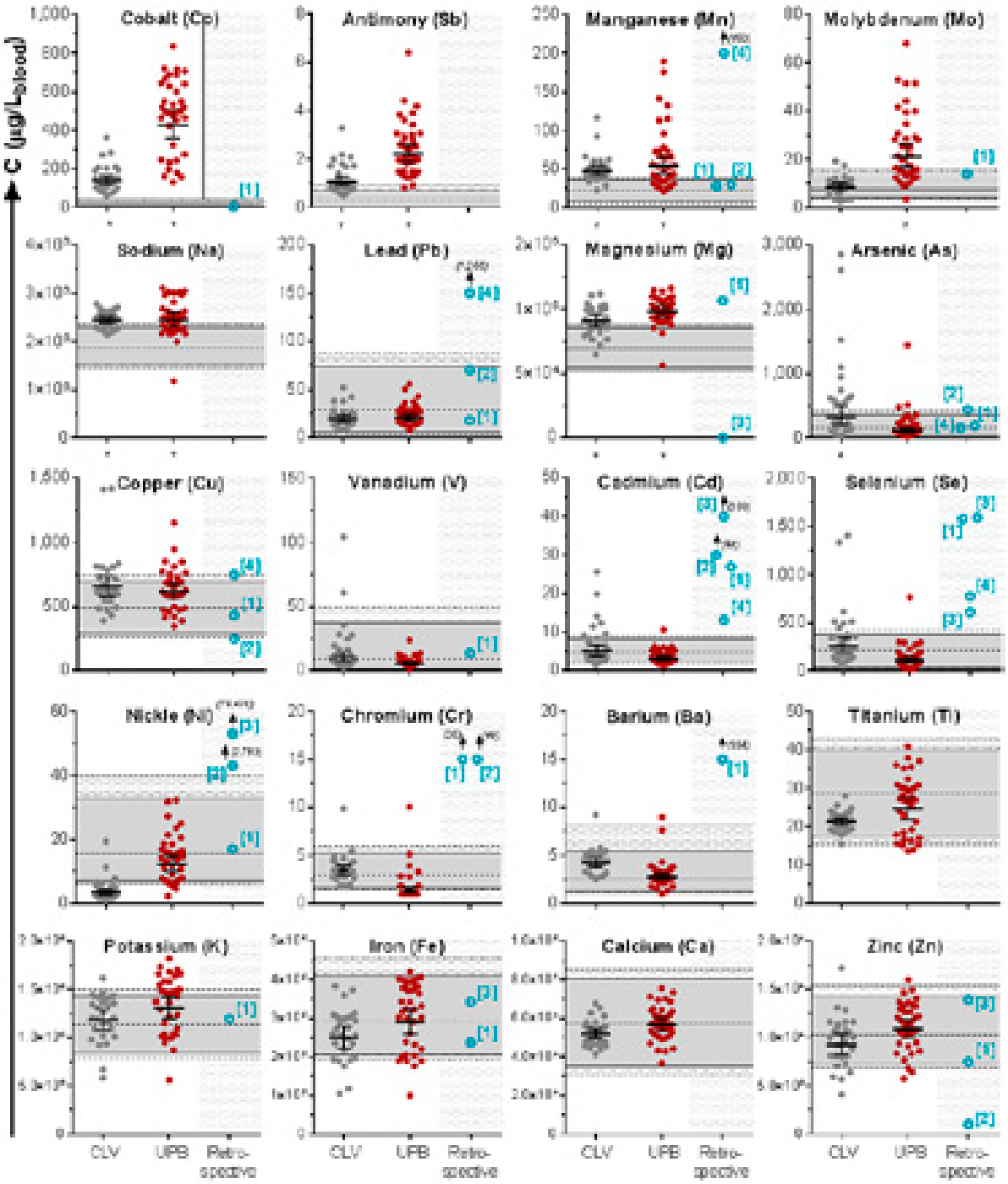


Figure 11: Reference Intervals and 90% CI (horizontal grey shaded area and dotted lines) compared to a) trace element concentrations (µg/L ww) in turtles from SHL, CLV and UPB coastal foraging grounds (left panel; lines = geometric mean and 95%CI), and b) mean levels previously reported from Palmyra Atoll by [1] McFadden et al. (2014); Cape Verede by [2] Camacho et al. (2014a); Punta Abreojos by [3] Labrada-Martagon et al. (2011); San Diego Bay by [4] Komoroske et al. (2011) (right panel). *Significantly different to RI and ≥ 60% of turtles exceed RIs. Figure modified from [43].

Subsequent blood biomonitoring of the two coastal populations and PCA analysis showed clear habitat-specific differences relative to HWK [43] (Figure 12). Some elements clearly exceeded the upper 90% confidence interval of the RI for a large proportion (>60%) of UPB and/or CLV coastal populations

(Figure 11). These were the essential elements Co, Mo, Mn, Mg and Na, and the non-essential elements As and Sb. Among these, Sb, Mo, Mn and Na showed the highest concentrations in UPB turtles, while CLV turtles contained the highest levels of As. Several other trace elements were above RIs, albeit in only a few individuals (e.g. Cu, V, Cd and Se particularly at CLV; Figure 11). Nevertheless, it is noteworthy that Cu exceeded the recently proposed threshold for increased risk of fibropapillomatosis (approx. 1,300 µg/L blood; [71]) in two of the CLV turtles. Overall, these data indicated that the majority of turtles at the nearshore foraging grounds experience elevated exposure to particular elements specific to their habitat, governed mainly by sources of Co, Mo, Mn, Mg, Na, As, Sb, and Pb. Of particular interest were the highly elevated Co in turtles from UPB (ranging from 160 to 840 mg/L; up to 25 times above RI). Although effect thresholds for Co toxicity in green turtles or reptiles in general is lacking, these levels are well within the order expected to elicit acute effects across many other vertebrate species (~300 mg/L blood), and among the highest observed in sea turtles or any vertebrate species globally (see detailed review in [43]). The exact mechanism of action of Co toxicity in vertebrates is not fully understood but is thought to involve induction of a transcription regulatory factor for hypoxic-like responses (HIF-1a), and oxidative stress through the generation of reactive oxygen species [72, 73]. Overall, our results appear to support the notion that the elevated trace element exposure of turtles foraging in coastal areas may pose risks to the health of these resident populations.

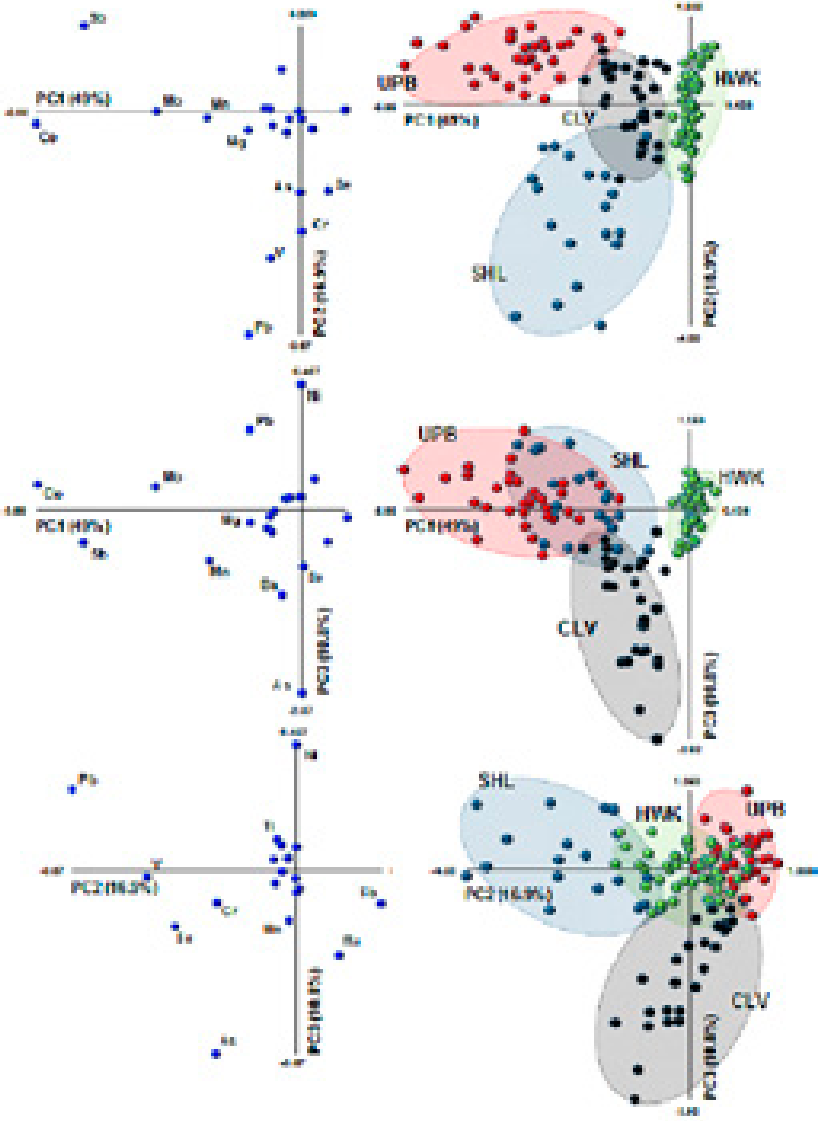


Figure 12: Loading scores (left) and bi-plots (right) for PCAs 1 (49%), 2 (17%) and 3 (11%), centered on the offshore baseline cohort (HWK; green), for populations foraging in coastal areas dominated by agricultural (UPB; red), urban (CLV; grey) and military (SHL; blue) activities. Note: SHL (Shoalwater Bay) turtles were not part of the

WWF study, but were included as a coastal population foraging in a relatively well flushed environments near military activities. Figure modified from [43].

The observed habitat-specific differences in trace element exposure of coastal turtle populations suggests localized influences. Historical reports, past sediment analyses and Australian Government indicate similar natural abundance of trace elements at UPB and CLV, which are located <100 km apart but are influenced by distinct anthropogenic influences (see detailed review in [43]). In contrast, significantly higher Co ($p < 0.0001$), Sb ($p < 0.001$), and Mo concentrations ($p < 0.0001$) were present in turtle blood from UPB compared to CLV. This suggests that the elevated exposure to Co, Sb, and Mo at UPB is a result of the area's anthropogenic activities, warranting further investigations to elucidate the source(s).

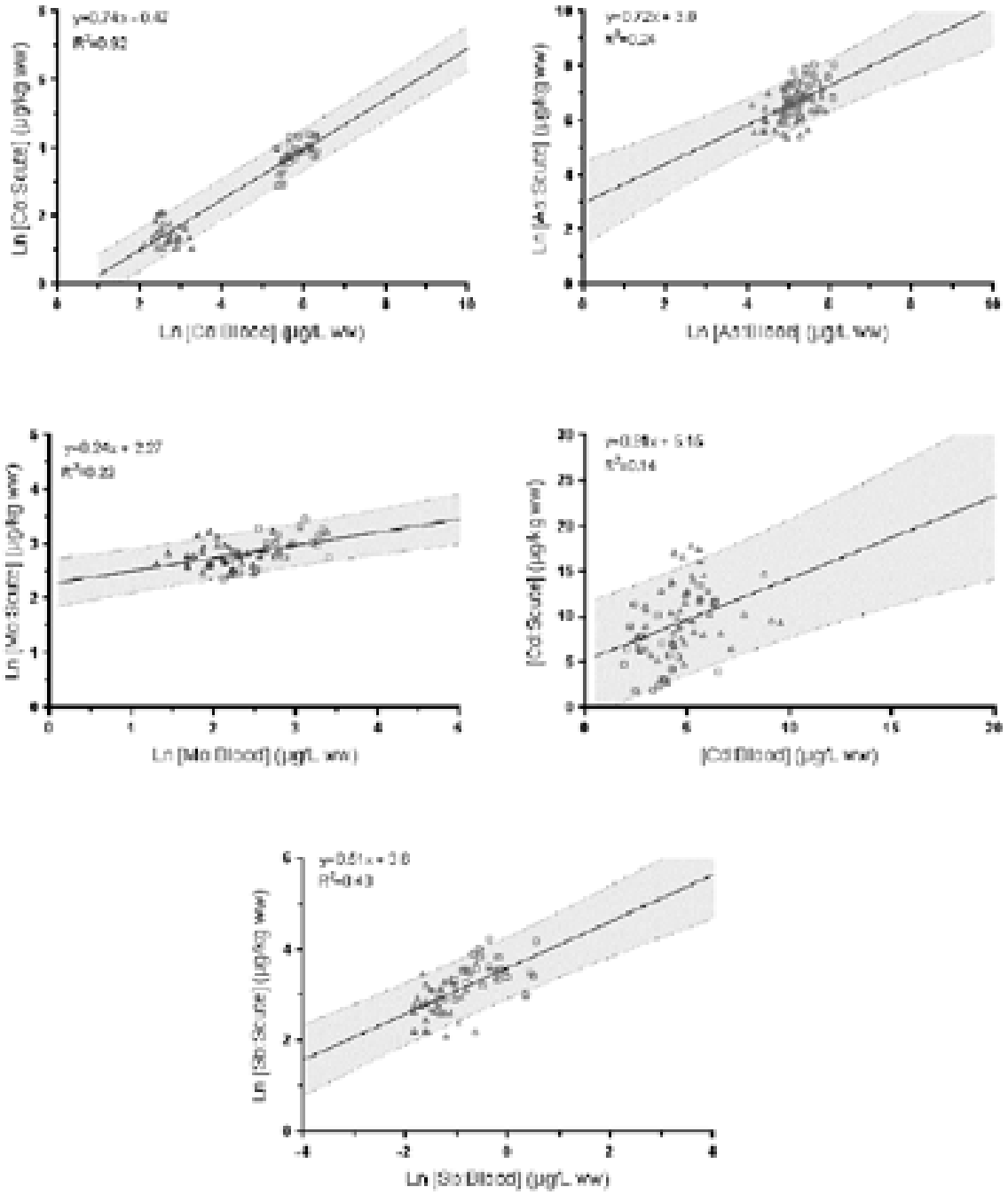


Figure 13: Regression between blood and scute (2014 HWK open triangles and SHL open squares) showing 90% prediction interval (grey area; RPI_{90}) for natural log-transformed Co, As, Mo and Sb concentrations. Concentrations and regression parameter estimates for Cd are for non-transformed data. Figure from [27].

The relatively high concentrations of Co in turtles from UPB and their potential to elicit neurotoxicity raised the question regarding its possible role in the 2012 mass stranding event. Lack of suitable historical samples from 2012, and the absence of a recurring event over the RRT duration to date, however, considerably limited opportunities to address Hypothesis B, i.e. ascertain possible links between trace element exposure and neurotoxic symptoms in this green turtle population. Nevertheless, the different toxicokinetics in blood and scute of green turtles may offer valuable insight into the historical exposure of UPB green turtles [27]. Due to relatively fast blood elimination rates of many trace elements, blood typically reflects the most recent exposure history (generally in the order of days to months; depending on the element and its speciation) [74]. In contrast, accumulation of trace elements in keratinized scute tissue, which is shed over a period of 1.4-6.5 years, can provide time-integrated exposure information for this period [75]. The ratio between blood and scute trace element concentrations can thus help to identify the history of exposure, providing i) the typically high uncertainties for estimating average scute concentrations across the entire carapace can be minimized and quantified, and b) the relationship between blood and scute trace element concentrations at constant exposure regimes (i.e. steady state) can be established.

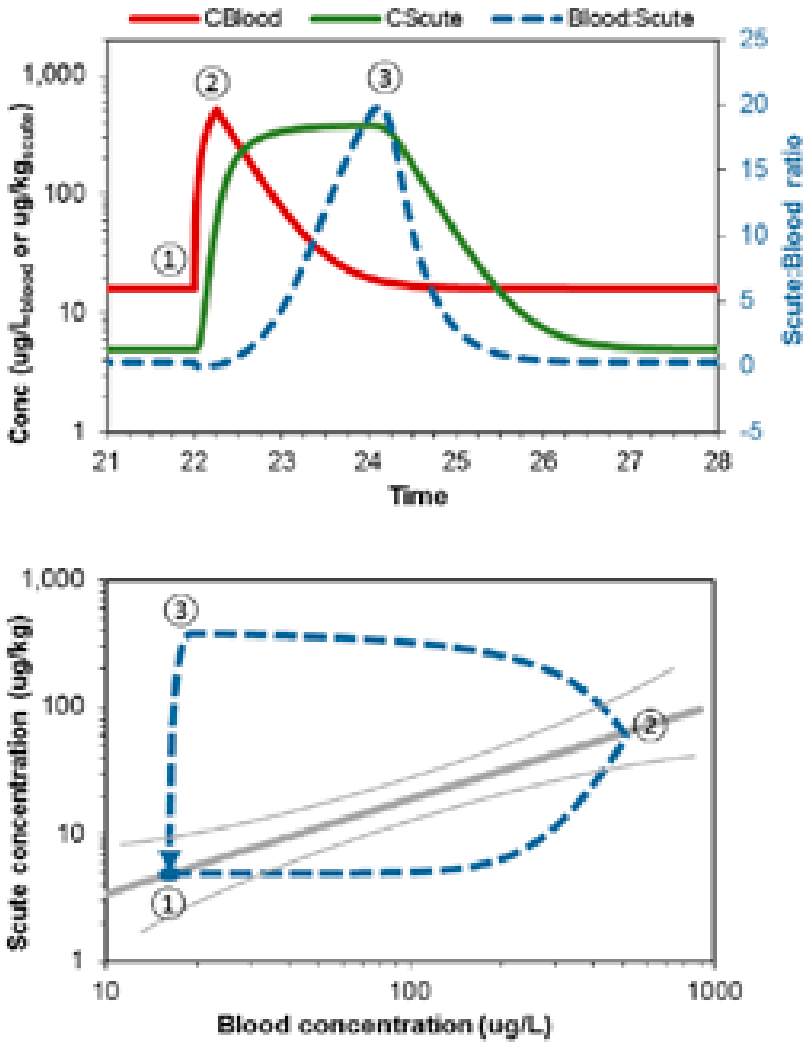


Figure 14: Semi-quantitative physiologically based toxicokinetic model of the temporal changes in cobalt blood and scute concentrations after exposure above baseline levels. Top: Concentrations in blood and scute over time; bottom: temporal changes of blood:scute ratio relative to the steady state relationship.
Baseline and onset of exposure; Peak blood concentration; Peak scute concentration.

To address these prerequisites, scute sampling, sample pooling and sample processing strategies were developed to allow estimation of the mean scute concentration across the carapace with 90-95%

confidence across relevant trace elements [27]. Steady state relationships were then tested using the HWK cohort (no significant differences in blood or scute trace element levels between years), and an additional cohort of healthy subadult green turtles from SHL (see methods). Significant positive correlations and normally distributed standardized regression residuals were obtained for Co, As, Mo, Cd, and Sb in blood and scute, and their linear regressions (within 90% prediction limits) are expected to approximate the steady state relationship between these two matrices [27] Figure 13).

A semi-quantitative kinetic model was developed to understand and approximate temporal changes in blood:scute trace element concentrations relative to their steady state relationship [27] (Figure 14). The model confirmed that the response in the two matrices to changes in exposure may be tracked against their steady-state relationship: elevated blood concentrations (relative to steady state) and corresponding baseline scute concentrations are indicative of recent exposure, and vice versa, baseline blood levels with elevated scute concentrations are indicative of past exposure.

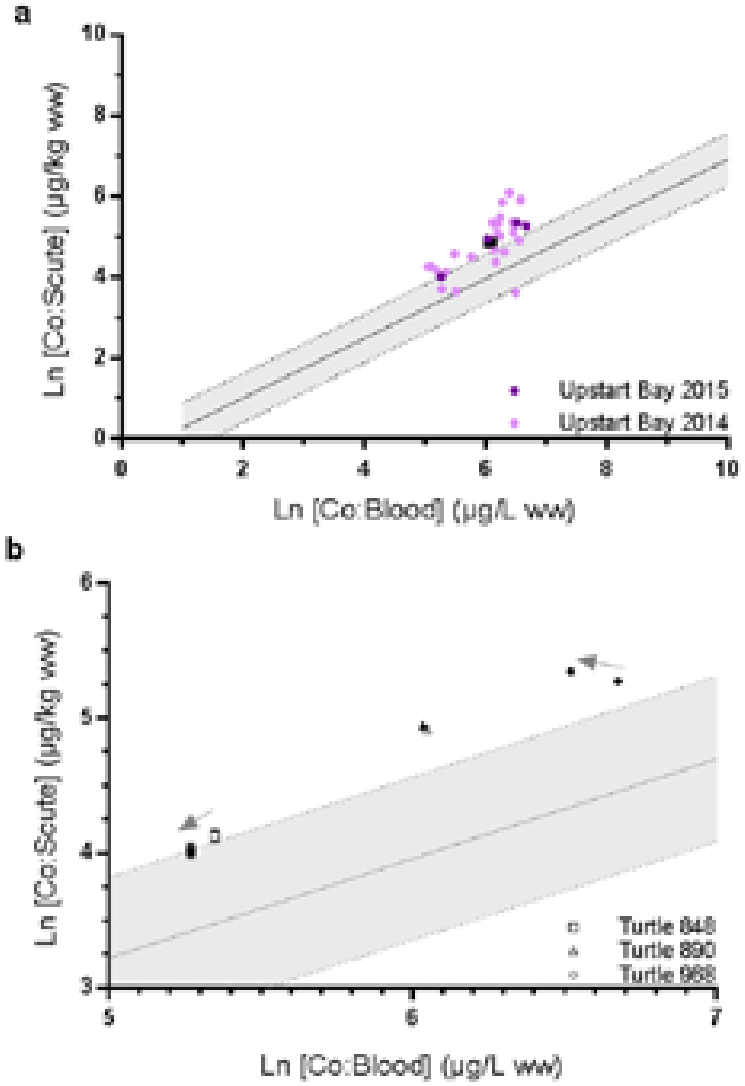


Figure 15: (a) Paired blood and scute cobalt concentrations (individuals = circle; mean = square) for Upstart Bay turtles in 2014 (open) and 2015 (closed) with b) same plot showing only recaptured turtles. Cobalt regression model (HWK and SHL) shown with 90%prediction intervals (grey area). Figure from from [27].

With these tools established, historical exposure of UPB turtles may be evaluated. To date, only a subset of UPB turtle blood and matched scutes have been analyzed (n=28 from 2014 and n=4 recaptures in 2015). These data highlight that Co blood:scute ratios of a large proportion of the UPB cohort (70%) are considerably lower than their predicted steady-state and 90% prediction interval (Figure 15), indicating high Co exposure prior to the first sampling event in 2014 [27]. This is consistent with the hypothesis

B of this study, and suggests that acute cobalt exposure may have played a role in the neurotoxicity and mass stranding event in 2012. However, the samples analyzed to date showed only minor decreases in Co blood concentration over the 1 year period. Co blood elimination rates are not available for reptiles, but are in the order of months in other vertebrate species due to its strong association with erythrocytes [76-78]. Reptiles in general have relatively long erythrocyte lifespans (600-800 days [79]) suggesting slower elimination of Co in reptilian species. Thus, we anticipate further, slow decreasing blood Co concentrations in animals sampled in 2016-2017 and beyond to support these conclusions.

Conclusions

Clinical blood biochemistry and hematology data from the RRT component turtle health were used to test correlations between the health indicators and exposure data from both the screening phase and subsequent target analysis of the present study, providing an additional aide to prioritizing analyses and supporting interpretation of contaminant data.

For turtles from the HWK reference site, clinical indicators (biochemistry and hematology) fell within or only slightly outside previously established clinical biochemical and hematological reference intervals [26], supporting the analytical evidence that this population served as a suitable, clinically healthy reference cohort [43]. In contrast to HWK, clinical data for the two coastal sites suggested the presence of an insipid systemic stressor active at a population level [43]. Turtles at both coastal sites show signs of systemic disease or generalized processes associated with liver dysfunction (UPB) and muscle inflammation (CLV). In addition, the prevalence of an active inflammatory response was evident in a relatively large proportion (44%) of UPB turtles.

We observed associations between blood concentrations of trace elements and several clinical measures (Table 1; [43]). Of particular interest is Co, which showed significant and moderately strong correlations with clinical markers of inflammation (TWCC; p<0.0001). Clinical markers of liver dysfunction were also significantly and moderately correlated with Co, Sb, and Mn (ALP (Co, Sb and Mn) and Tbil (Co and Mn); p<0.0001).

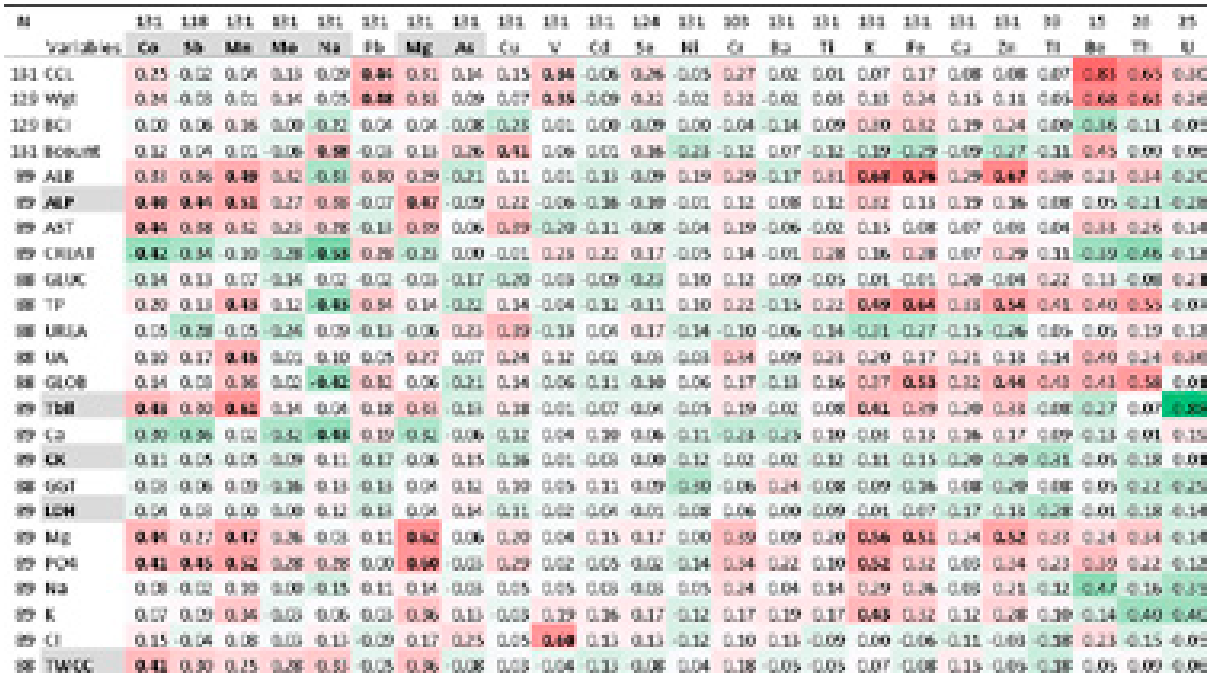


Table 1: Spearman correlation coefficient (rs) between visual and clinical health markers, biometrics and trace element concentration in blood of HWK, CLV and UPB turtle populations. Significant (p≤0.0001) correlations are in bold (red=positive, green=negative), and the color gradient indicates the strength of the correlation (stronger=darker to weaker=lighter colored). Trace elements >RIs for >60% of the populations, and clinical markers which were moderately elevated compared to BHRIs are highlighted in grey. Note: no clinical data were available for SHL. Table modified from [27].

Additionally, 3-Indolepropionic acid was strongly correlated with alkaline phosphatase ($p<0.0001$) and total bilirubin ($p<0.0001$), clinical markers which may be associated with liver dysfunction in reptiles [28]; and had moderate-to-strong correlation with fatty acid and fatty acid oxidation products ($p< 0.004$). Similarly, most lipid peroxidation products were strongly correlated with alkaline phosphatase ($p<0.0004$), and each other. VMA showed a moderate correlation with markers of inflammation (TWCC, $p=0.004$), but was not associated with any fatty acid or lipid peroxidation products.

Furthermore, both 3-indolepropionic acid ($p<0.0001$) and lipid peroxidation products ($p<0.0002$) were strongly correlated with cobalt concentrations (Figure 16). Cobalt toxicity is not well understood, but has been shown to induce an inflammatory response via increased chemokine secretion, and increased oxidative stress in vitro; and has been implicated in oxidative stress and inflammation associated with ischemic brain injury in vivo. There is evidence that cobalt promotes the formation of free radicals, possibly via cobalt mediated Fenton-like chemistry (reviewed in [28], which may explain the presence of lipid peroxidation products in animals with high cobalt blood concentrations. Further analysis e.g. by grouping animals by cobalt concentration, would be warranted to confirm these findings.

These relationships between elevated Co, Sb, Mn exposure as well as biomarkers of oxidative stress with clinical and hematological markers of systemic stressors and acute inflammatory responses provide a strong argument for the notion that trace element exposure is having an impact on the health of these coastal sea turtle populations. The exact effects and their extent, however, require closer examination using targeted diagnostics [43].

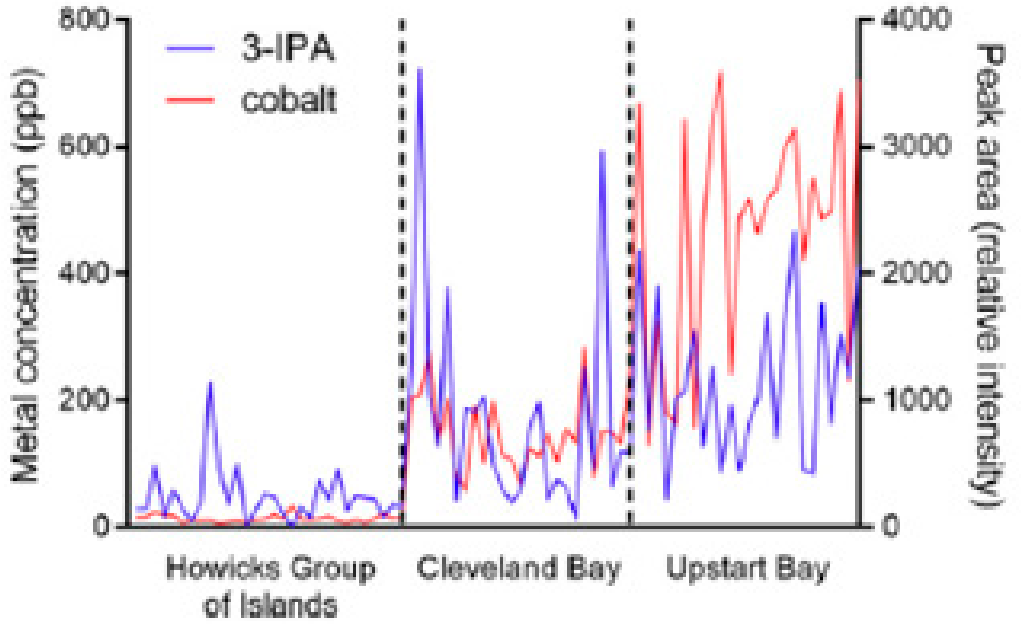


Figure 16: Correlation of tentatively identified 3-indolepropionic acid (blue) and cobalt (red) in turtles from the three study sites ($p<0.0001$). Figure from [28] with cobalt data from [43]

Acknowledgments

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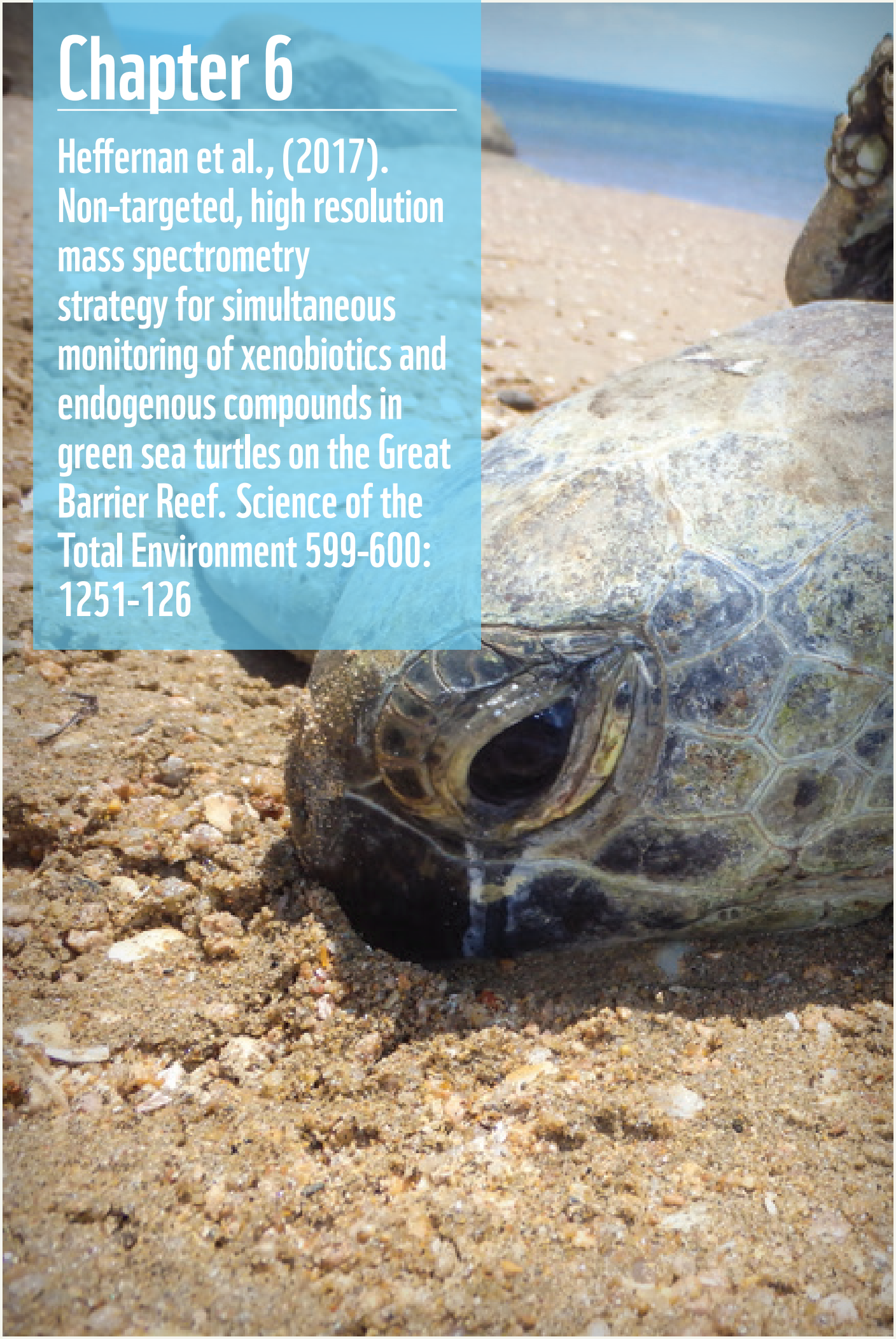
Chapter 6

Heffernan et al., (2017). Non-targeted, high resolution mass spectrometry strategy for simultaneous monitoring of xenobiotics and endogenous compounds in green sea turtles on the Great Barrier Reef. Science of the Total Environment 599-600: 1251-126



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Non-targeted, high resolution mass spectrometry strategy for simultaneous monitoring of xenobiotics and endogenous compounds in green sea turtles on the Great Barrier Reef

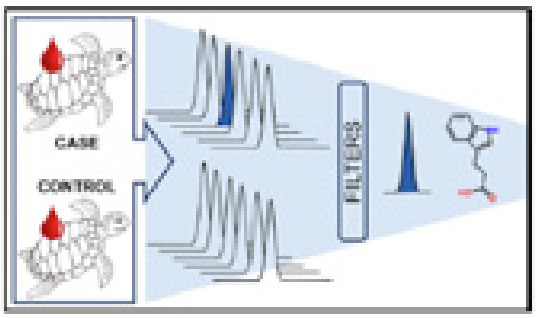
Amy L. Heffernan [a,b,e,1], María M. Gómez-Ramos [c,1], Caroline Gaus [a], Soumini Vijayasarathy [a], Ian Bell [d], Christine Hof [a,f], Jochen F. Mueller [a], María J. Gómez-Ramos [a,c]

[a] Queensland Alliance for Environmental Health Sciences, Faculty of Health and Behavioural Sciences, The University of Queensland, Brisbane, Australia
[b] The Florey Institute of Neuroscience and Mental Health, The University of Melbourne, Melbourne, Australia
[c] Agrifood Campus of International Excellence (ceiA3), Department of Chemistry and Physics, University of Almería, European Union Reference Laboratory for Pesticide Residues in Fruit and Vegetables, Almería, Spain
[d] Aquatic Species Program, Department of Environment and Heritage Protection, Townsville, Australia
[e] Species Conservation and Indigenous Partnerships Unit, World Wildlife Fund for Nature-Australia, Brisbane, Australia

HIGHLIGHTS

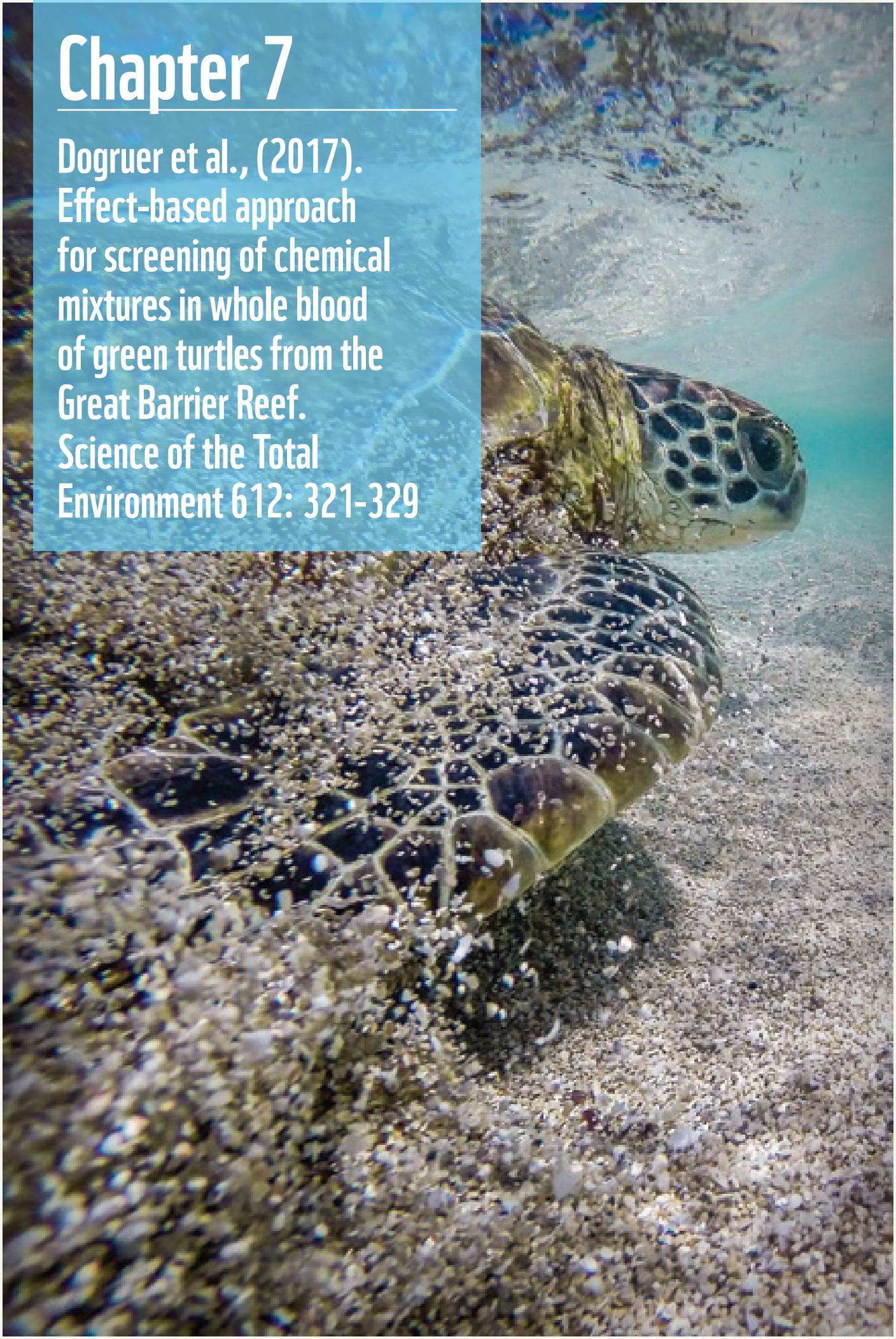
- "Case–control" sampling of green sea turtles in three sites on Great Barrier Reef
- Simultaneous detection of xenobiotics and biomarkers of effect
- Anthropogenic influence, neuroinflammation and oxidative stress in 'case' animals
- Demonstrated utility of green sea turtles as biomonitoring tool

GRAPHICAL ABSTRACT



Chapter 7

Dogruer et al., (2017). Effect-based approach for screening of chemical mixtures in whole blood of green turtles from the Great Barrier Reef. Science of the Total Environment 612: 321-329



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Effect-based approach for screening of chemical mixtures in whole blood of green turtles from the Great Barrier Reef

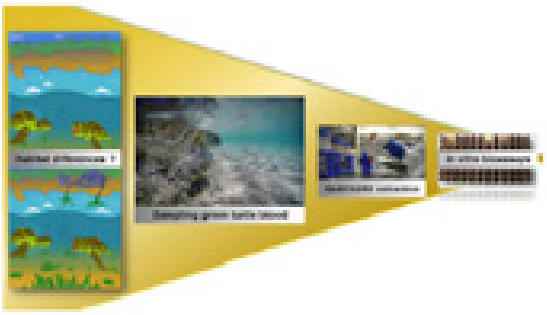
Gülsah Dogruer [a,b,*], Liesbeth Weijs [a], Janet Yat-Man Tang [a], Henner Hollert [b], Marjolijn Kock [a], Ian Bell [c], Christine A. Madden Hof [d], Caroline Gaus [a]

[a] Queensland Alliance for Environmental Health Sciences (QAEHS), The University of Queensland, 20 Kraeb Road, Coopers Plains, Queensland 4108, Australia
[b] Institute for Environmental Research, RWTH Aachen University, Germany
[c] Department of Environment and Heritage Protection, Threatened Species Unit, Townsville, Australia
[d] Species Conservation, World Wildlife Fund for Nature, Australia

HIGHLIGHTS

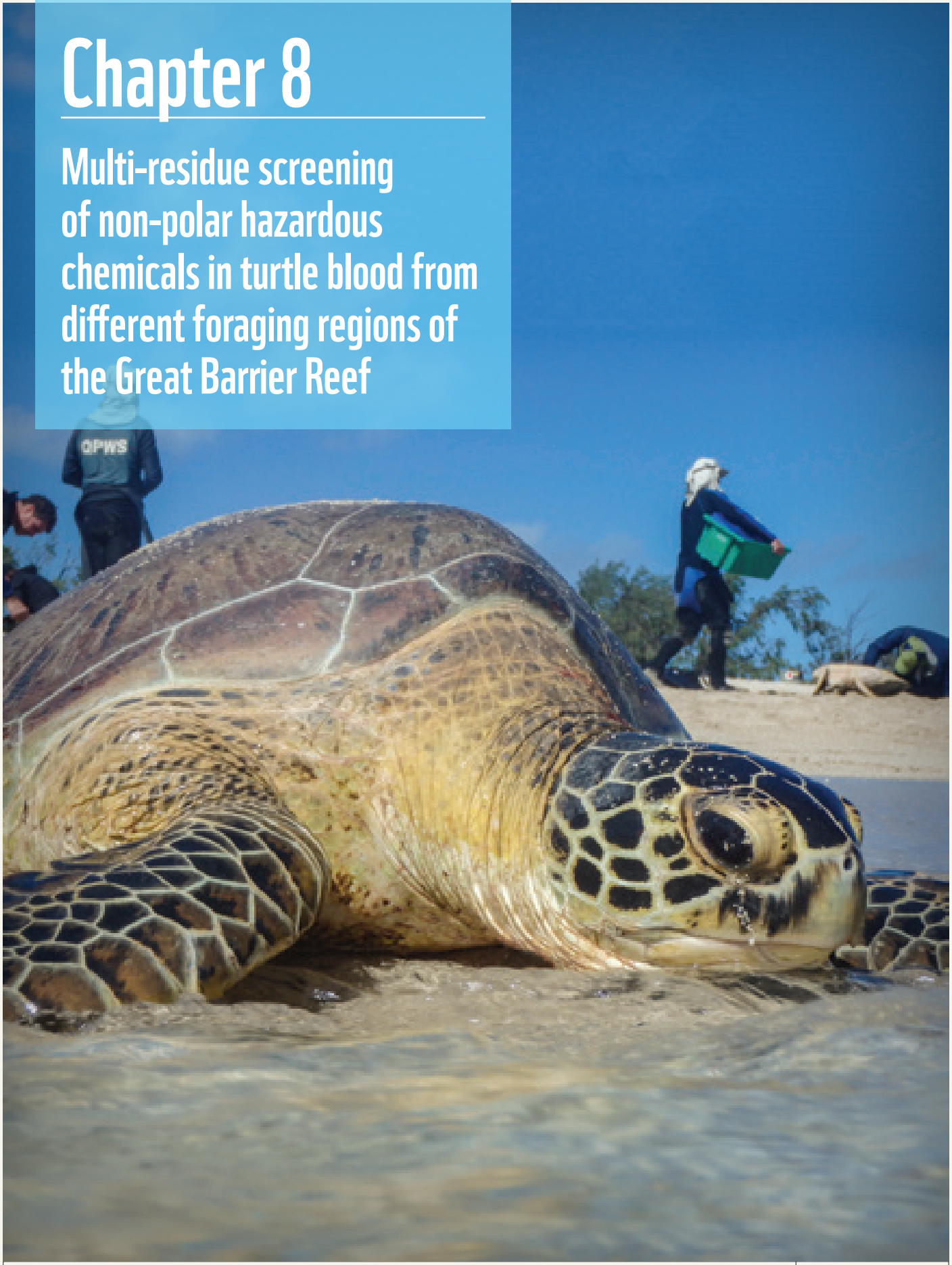
- QuEChERS-extraction of blood is suitable for effect-based chemical mixture screening.
- This approach was applied to sea turtles from agricultural, urban and remote areas.
- Chemical mixture exposure differed significantly in turtles from different areas.
- Turtles from the agricultural area showed higher induction in three in vitro assays.
- Effect-based in vitro screening can help elucidate internal mixture exposure.

GRAPHICAL ABSTRACT



Chapter 8

Multi-residue screening of non-polar hazardous chemicals in turtle blood from different foraging regions of the Great Barrier Reef



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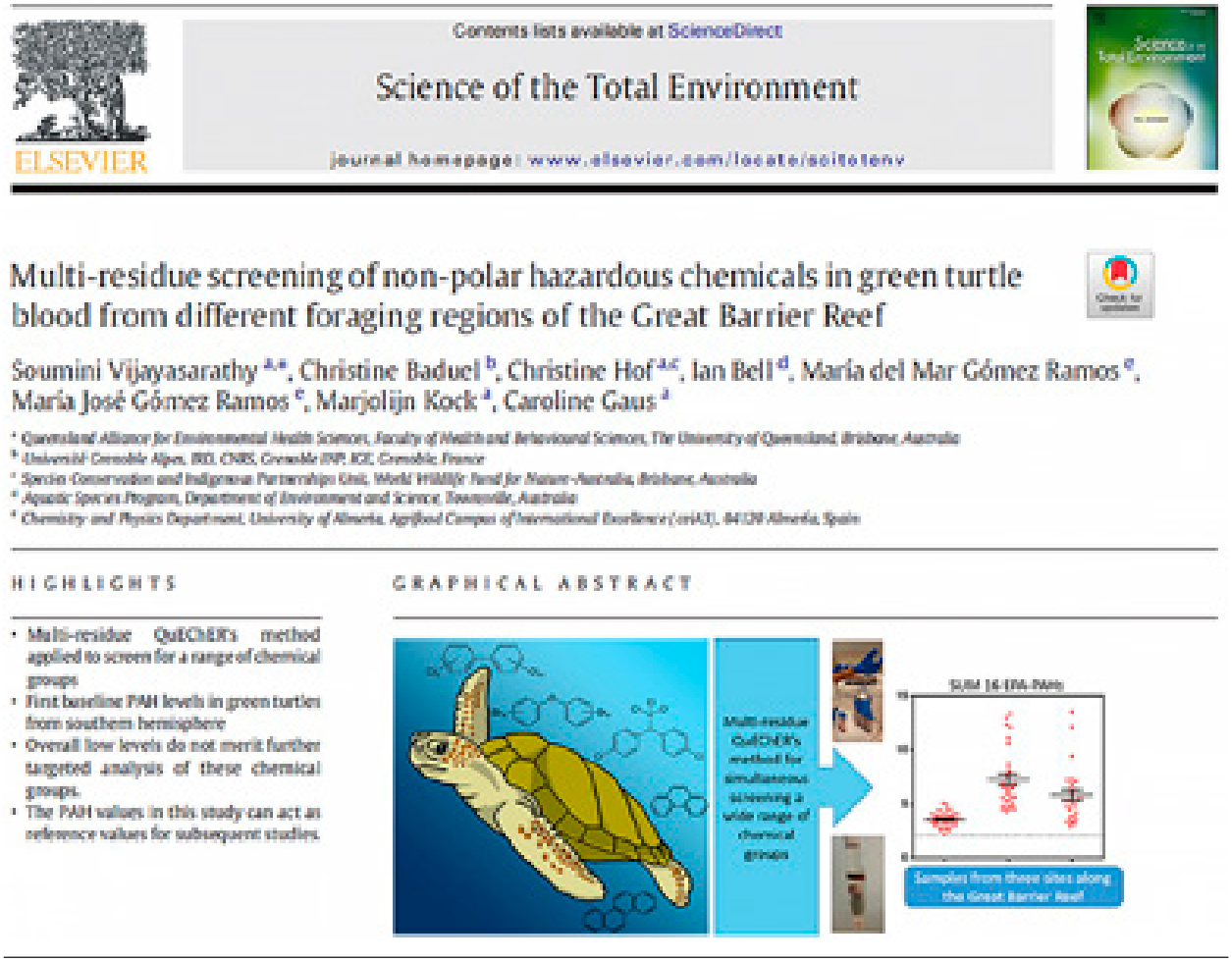
8. Multi-residue screening of non-polar hazardous chemicals in turtle blood from different foraging regions of the Great Barrier Reef



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Multi-residue screening of non-polar hazardous chemicals in green turtle blood from different foraging regions of the Great Barrier Reef

Soumini Vijayasarathy [a,*], Christine Baduel [b], Christine Hof [a,c], Ian Bell [d], María del Mar Gómez Ramos [e], María José Gómez Ramos [e], Marjolijn Kock [d], Caroline Gaus [a]

[a] Queensland Alliance for Environmental Health Sciences, Faculty of Health and Behavioural Sciences, The University of Queensland, Brisbane, Australia
[b] Université Grenoble Alpes, IRD, CNRS, Grenoble INP, IGE, Grenoble, France
[c] Species Conservation and Indigenous Partnerships Unit, World Wildlife Fund for Nature-Australia, Brisbane, Australia
[d] Aquatic Species Program, Department of Environment and Science, Townsville, Australia
[e] Chemistry and Physics Department, University of Almeria, Agrifood Campus of International Excellence (ceiA3), 04120 Almería, Spain

HIGHLIGHTS

- Multi-residue QuEChERS method applied to screen for a range of chemical groups
- First baseline PAH levels in green turtles from southern hemisphere
- Overall low levels do not merit further targeted analysis of these chemical groups.
- The PAH values in this study can act as reference values for subsequent studies.

GRAPHICAL ABSTRACT

Soumini Vijayasarathy[1], Christine Baduel[2], Christine Hof[1,3], Ian Bell[4], María del Mar Gómez Ramos[5], María José Gómez Ramose[3], Marjolijn Kock[*], Caroline Gaus[1]

[1] Queensland Alliance for Environmental Health Sciences, Faculty of Health and Behavioural Sciences, The University of Queensland, Brisbane, Australia
[2] Université Grenoble Alpes, IRD, CNRS, Grenoble INP, IGE, Grenoble, France
[3] Species Conservation and Indigenous Partnerships Unit, World Wildlife Fund for Nature-Australia, Brisbane
[4] Aquatic Species Program, Department of Environment and Heritage Protection, Townsville, Australia
[*] marjolijn.kock@gmail.com
[5] Chemistry and Physics Department, University of Almeria, Agrifood Campus of International Excellence (ceiA3), 04120 Almería, Spain

Keywords:

Green turtle, Great Barrier Reef, PAHs, QuEChERS method, multi-residue method

Abstract

Green turtles spend a large part of their lifecycle foraging in nearshore seagrass habitats, which are often in close proximity to sources of anthropogenic contaminants. As most biomonitoring studies focus on a limited number of targeted chemical groups, this study was designed to screen for a wider range of hazardous chemicals that may not have been considered in prior studies. Whole blood of sub-adult green turtles (*Chelonia mydas*) were sampled from three different locations, a remote, offshore 'control' site; and two coastal 'case' sites influenced by urban and agricultural activities on the Great Barrier Reef in North Queensland, Australia. In order to screen blood samples for chemicals across a wide range of KOW's, a modified QuEChER's extraction method was used. The samples were analysed using a multi-residue GC-MS/MS method that allowed simultaneous quantification of polychlorinated biphenyls (PCBs), polychlorinated diphenyl ethers (PBDES), organochlorine pesticides (OCPs) and polycyclic aromatic hydrocarbons (PAHs). While PBDEs, PCBs and OCPS were below the limits of detection, PAHs were detected in all turtle blood samples. However, PAH levels were relatively low (maximum ΣPAH = 13 ng/mL ww) and comparable to or less than those reported from other green turtles globally. The present study provides the first baseline PAH levels in blood samples from green turtles from nearshore and offshore locations in the Southern Hemisphere.

Introduction

Globally distributed in tropical, subtropical and temperate waters all seven species of marine turtles are currently included in the IUCN Red list under "endangered, critically endangered, vulnerable" or as "data deficient" [1]. Australia is home to six of these seven marine turtle species, including the green sea turtle (*Chelonia mydas*). Despite management and turtle conservation efforts, green turtles continue to be listed as vulnerable in Queensland (Nature Conservation Act 1992) [2], and are ranked as a critical priority under the Department of Environment and Heritage Protection (EHP). Green turtles are subjected to a range of anthropogenic threats including exposure to and accumulation of chemicals. Currently monitoring of key nesting and foraging areas for populations studies are an integral part of the recovery plan for marine turtles in Australia[3], this approach could also be used for biomonitoring purposes. The utilisation of such biomonitoring programs to their full potential may aid in planning and modifying long-term conservation strategies that may help recover turtle populations.

Although many biomonitoring studies analyse only tissues of deceased or stranded marine turtles due to ethical issues, a number of minimally invasive methods for obtaining samples such as blood and scutes are also utilised. Latter methods allow for recapture of turtles availing the opportunity to study temporal trends. While collecting samples from live turtles are extremely useful, the effort involved is expensive and arduous, and such samples are thus relatively difficult to access. Selecting target analytes for biomonitoring in such valuable samples is challenging, particularly as relatively small sample volumes limit the number of chemical analyses. Most monitoring studies in marine turtles to date have focussed on one or two nonpolar bioaccumulative chemical groups using separate sample preparation and analytical techniques. A large number of these studies are on loggerhead turtles [4-22], other studies include hawksbill [18, 23, 24], leatherback [21, 22, 25-27], kemp's ridley [18, 27-29] and olive ridley [19, 20] turtles. Although biomonitoring in green turtles has had some attention globally [17-24, 29-36], the number of studies from Australia are still quite limited [37-44]. Among these studies only two studies employed non-target screening methods, one study employed a multi-residue extraction method (QuEChER's), yet only analysed for one chemical group (OCPs) [35]. The other study employed a broader screen for nonpolar organics (PCBs, OCPs and PBDEs) using ASE and analysis on GC-MS/MS [45]. While targeted analysis is essential to biomonitoring studies, non-target methods that screen for a wider chemical range enable prioritisation of subsequent targeted analysis of valuable samples. Additionally, there is no published data on non-polar contaminants in green turtles from North Queensland. This area supports some of the highest density green turtle foraging populations in the western Pacific Ocean.

The present study was initiated as part of a multi-disciplinary study within the Rivers to Reef to Turtle (RRT) programme headed by WWF in response to increased turtle strandings along the Queensland coastline. Green turtles and their habitats were assessed on their health status, population dynamics, water quality, contaminant sources and internal contaminant exposure [39, 40]. Samples were taken from three different locations in the GBR: i) the Howick Group of Islands (HWK), a remote and pristine location; b) Cleveland bay (CLV), an urban/industrial area, and c) Upstart (UPB) Bay, a rural agricultural area, which was also the site of a mass stranding event in 2012 [46].

The objective of this study was to analyse for non-polar chemicals with a wide range of KOW's as a means of evaluating multiple non-polar chemical groups that may be of concern to green turtles. This would then enable prioritisation of subsequent target analysis. A modified QuEChER's method, was employed as the extraction method to allow screening of multiple chemical groups in the same sample. Extracted samples were analysed for PCBs, OCPs, PBDEs and PAHs on a multi-residue GC MS/MS method. To our knowledge, this is the first study to analyse whole blood for several chemical groups including PAHs not just in green turtles but also in other marine turtles from Australia and the Southern Hemisphere. We also provide the first baseline PAH levels in green turtles for three different areas along the GBR. This study is also the first to use the QuEChER's method to quantify multiple non-polar chemical groups in marine wildlife.

Methods and materials

Sample collection

Whole blood from 86 apparently healthy sub-adults were selected for this study (Howick group of islands, n = 30; Cleveland Bay, n = 30; Upstart Bay, n = 26). Further details on site selection, location, selection of sub-adults, sample collection, archiving of samples and health status determination have been published elsewhere [39, 40, 47]. Briefly, three sites were selected for study, the Howick group of islands (HWK), a remote, offshore site within the Great Barrier Reef Marine park, was selected as a control site. Cleveland Bay (CLV), an urban/industrial site adjacent to the city of Townsville and Upstart Bay (UPB) a rural coastal region dominated by intensive agriculture and legacy mining, and in part, receiving water from the Burdekin river, one of the largest polluted rivers in Queensland.

Method validation

Validation results, determined in whole blood extract, are presented in Table A1. Some compounds in the table were not included in this study. Precision in the chromatographic response for the developed method was determined as repeatability or intra-day (n=5) and reproducibility or inter-day (over 5 days). The relative standard deviations ranged from 1% to 15% and from 3% to 19% for intra- and inter-day studies respectively, except for heavier PBDE congeners (BDE-153, 154 and 183). The recoveries were very good for most of the compounds, they ranged from 60 to 107%, except for benzo(a)pyrene (40%), hexachlorobenzene (54%), naphthalene (33%) PCB-180 (48%), BDE-153 (55%), BDE-154 (39%) and BDE-183 (56%). The recovery values of the 5 replicates presented a relative standard deviation less than 20% in most of the cases, indicating the method's acceptable reproducibility. Linearity was evaluated both in solvent and matrix, by assessing the signal response of the target analytes from matrix-matched calibration solutions prepared by spiking matrix blank extracts at seven concentration levels. Very low difference was observed between matrix matched and solvent calibration standards. The calibration curves were linear over the entire range studied with correlation coefficients higher than 0.991 for most of the compounds. Limit of quantification (LOQ) was determined as the analyte concentration that produced a peak signal ten times the background noise from the chromatograms. LOQ ranged from 0.1 to 2 ng/mL, except for permethrin (5 ng/mL), BDE-153 and 154 (5 ng/mL) and BDE-183 (4 ng/mL).

Sample preparation

All blood samples were subjected to a modified multi-residue QuEChERS method developed and validated for 1 mL of blood. Briefly, frozen whole blood samples were thawed, homogenized and 1 g (± 0.1g) was aliquoted into 15 mL polypropylene centrifuge tubes. The samples were fortified with the mixture of internal standards at 10 ng/mL. Non-fortified samples (blanks) were also prepared to generate blank matrices for matrix-matched calibration standards. For a reagent blank sample, MilliQ water was used in the place of biological sample. The samples were vortexed and allowed to settle for 15 min. To this 3 mL of acetonitrile and 1mL of water was added and the samples were vigorously shaken by hand. The extraction was pursuing by pouring a salt mixture of 1 g $MgSO_4$ (anh) and 0.2 g NaCl into each polypropylene centrifuge tube. A ceramic homogeniser was then added to the tubes and the samples were again shaken vigorously for 1 min. Samples were centrifuged at 3700 rpm for 10 min at 4°C and 5 mL of the supernatant was transferred to a 15 mL centrifuge tube. A freezing out step (low temperature fat precipitation) was then performed by storing the samples below 20°C for at least 4 hours. 1 mL of the supernatant was cleaned up using dispersive solid phase extraction. The supernatant was added to a 2 mL polypropylene centrifuge tube containing 150 mg of magnesium sulfate (anh), 50 mg of primary secondary amine (PSA) and 50 mg of C18 sorbent. The tubes were vortexed for 1 min and centrifuged (9,000 rpm, 8 min). 0.3 mL of supernatant was then transferred into a vial, evaporated to near dryness under a gentle stream of nitrogen, and reconstituted to 100 µL with ethyl acetate (EtOAc). Prior GC-MS/MS analysis, phenanthrene-d10 was added at 5 µg/L, as an injection standard. Validation parameters of the method are provided in Supporting Information.

Chemical analysis

Blood samples were analysed for 16 polyaromatic hydrocarbons (PAHs), 12 dioxin like polychlorinated biphenyls (PCBs), 6 marker PCBs, 17 organochlorine pesticides (OCPs) and 7 polybrominated diphenyl ether (PBDEs) congeners. Samples were analysed using gas chromatography with tandem mass spectrometry system (GC-MS/MS) following a multi-residue method developed by Baduel et al [48]. Analysis was performed using a TSQ Quantum GC (ThermoFischer Scientific) system coupled with triple quadrupole mass spectrometer (QqQ) Quantum and a TRACE GC Ultra equipped with a TriPlus autosampler. Further information on the analytical method have been previously described and validated [48]. Previously unpublished validation details on dioxin like PCBs are given in Table A1.

Calf serum was used a procedural blank in each batch. A matrix-matched calibration was prepared from non-fortified calf serum however, calibration in ethyl acetate was used to quantify as no difference was observed between matrix matched and solvent calibration standards. Calibration curve ranged from 0.1 to 50 ppb for most chemicals. Limit of quantification (LOQ) ranged between 0.1-1.0 ng/mL for OCPs, 0.1-0.5 ng/mL for DL PCBs, marker PCBs, PBDES and 0.1-1.0 ng/mL for PAHs. Recoveries of DDT-d8 ranged between 71-149%. All concentrations are reported in wet weight (ww). All non-detects were replaced with half LOQ values.

Results and discussion

Levels

PBDEs, PCBs and OCPS were below the LOQ in all of the samples in this study. Although the LOQ values obtained with the present multi-residue method are higher than typical for target analyses, the levels of PCBs[41], OCPs[41] [33]and PBDEs [33, 37, 41] reported from green turtle blood globally (including Australia) are above the LOQ values. However, the QuEChERS method employed in this study uses a low sample volume and no concentration step was added, which may have resulted in the <LOQ values noted in this study. This did not impact on the objective of this study to employ an efficient, wide screening method that can facilitate prioritisation of further evaluations.

In contrast to the other chemical groups analysed, all 86 whole blood samples contained at least five or more PAHs (Table 1: Summary statistics for PAHs from all three sites (mean, median, range and standard deviation in ng/mL, ww) (Table 1). Samples were analysed for fifteen PAHs, of which eight were detected in samples from CLV and UPB and five in HWKs (Table 1). Absence of acenapthene at HWK may be due to loss of volatile compounds such as acenapthene via the evaporation step during the sample preparation.

Number of rings	PAHs	CLEVELAND						UPSTART					HOWICKS				
		LOQ	MEAN	MEDIAN	SD	RANGE	DF	MEAN	MEDIAN	SD	RANGE	DF	MEAN	MEDIAN	SD	RANGE	DF
2	Naphthalene		NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
3	Acenaphthene	0.1	1.1	0.05	1.9	0.05-6.2	37	0.7	0.1	1.7	0.05-7.2	19	NA	NA	NA	NA	N/A
	Acenaphthylene	0.1	0.91	0.82	0.43	0.05-2.6	90	1.5	1.5	0.79	0.05-2.3	92	0.42	0.4	0.32	0.013-1.1	77
	Anthracene	0.1	0.3	0.3	0.0	0.25	ND	0.25	0.25	0.00	0.25	0	0.25	0.25	0.00	0.25	ND
	Fluorene	0.1	0.52	0.54	0.31	0.05-1.3	93	0.58	0.63	0.27	0.05-0.98	82	0.23	0.21	0.101	0.01-0.5	80
	Phenanthrene	0.1	2.1	2.07	0.56	1.1-3.4	100	3.0	0.92	0.43	0.38-2.07	100	0.69	0.77	0.36	0.076-0.98	100
4	Benzo[a]anthracene	0.5	0.25	0.25	0.00	0.25	ND	0.220	0.220	0.0768	0.0069-0.25	12	0.25	0.25	0.00	0.25	ND
	Chrysene	1	0.5	0.5	0.0	0.5	ND	0.5	0.5	0.0	0.5	ND	0.5	0.5	0.0	0.5	ND
	Fluoranthene	0.5	0.27	0.24	0.086	0.16-0.47	100	0.20	0.18	0.087	0.102-0.44	100	0.23	0.21	0.10	0.15-0.51	100
	Pyrene	0.5	0.30	0.25	0.078	0.22-0.82	23	0.27	0.25	0.070	0.15-0.68	50	0.11	0.05	0.12	0.0050-0.38	83
5	Benzo[a]pyrene	0.1	0.05	0.05	0.00	0.05	ND	0.05	0.05	0.00	0.05	ND	0.05	0.05	0.000	0.05	ND
	Benzo[b,j]fluoranthene	0.1	0.05	0.05	0.02	0.05-0.13	3	0.05	0.05	0.00	0.05	ND	0.05	0.05	0.000	0.05	ND
	Benzo[k]fluoranthene	0.1	0.05	0.05	0.00	0.05	ND	0.05	0.05	0.00	0.05	ND	0.05	0.05	0.000	0.05	ND
	Dibenz[a,h]anthracene	1	0.5	0.5	0.5	0.5	ND	0.5	0.5	0.00	0.5	ND	0.5	0.5	0.000	0.5	ND
6	Benzo[ghi]perylene	0.1	0.10	0.05	0.12	0.05-0.41	13	0.08	0.05	0.089	0.05-0.28	8	0.05	0.05	0.000	0.05	ND
	Indeno[1,2,3-cd]pyrene	0.5	0.25	0.25	0.00	0.25	ND	0.25	0.25	0.00	0.25	ND	0.25	0.25	0.00	0.25	ND
	Sum 16 EPA PAH	NA	6.7	6.1	2.8	4.4-13		5.8	5.0	2.6	2.9-13		3.6	3.3	0.70	2.8-5.1	

Table 1: Summary statistics for PAHs from all three sites (mean, median, range and standard deviation in ng/mL, ww); CLV – Cleveland Bay, UPB – Upstart Bay, HWK – Howick Islands, DF – detection frequency, ND – not detected, NA – not analysed

Overall, PAH levels were relatively low with maximum ΣPAH concentrations of 13 ng/mL weight wet (ww) (Table 1) from both the urban (CLV) and agricultural sites (UPB). A large percentage of PAHs detected at all three sites were three ringed PAHS (50-60%) followed by four ringed PAHs (25-40%). Though five and six ringed PAHs were detected in UPB and CLV they were absent in samples from HWKs. Besides having similar detection frequency of PAHs in CLV and UPB, samples also had similar PAH levels dominated by three ringed PAHs with acenapthene levels highest at both sites (mean (range); CLV: 1.1 (0.05-6.2) ng/mL ww; UPB: 0.7 (0.05-7.2) ng/mL ww) (Table 1). Most HWK PAH levels were lower or at similar levels compared to UPB and CLV.

Phenanthrene and fluoranthene were detected in all samples, with highest detection frequency of fluorene (>80%) followed by acenapthylene (>77%). Pyrene was the only analyte detected at 2-4 times higher frequency in HWKs (83%) compared to CLV (23%) and UPB (50%). Acenaphthene was present at both CLV and UPB and may have been present in HWKs; however, this could not be confirmed as explained earlier. Three other PAHs were only detected at one or two sites; benzo [a] anthracene in UPB (12%), benzo [b/j] fluoranthene in CLV (3%) and benzo [ghi] perylene in both CLV (13%) and UPB (8%). While fluoranthene levels were in the same range at all three sites, pyrene mean levels in HWK samples (0.11 ng/mL ww) were 2.5 times higher than in UPB (0.27 ng/mL ww) and CLV (0.30 ng/mL ww). This difference in fluoranthene and pyrene levels is discussed further in the profiles section below.

Overall, higher detection frequency (DF) and concentrations of PAHs in samples from CLV and UPB Bays is not surprising as both sites are exposed to higher anthropogenic activity. Results from sediment and water samples from all three sites undertaken at the same time as the turtle blood sampling showed similar results, with <LOQ values for most PAHs and higher detection frequency and concentration of PAHs in CLV and UPB compared to HWKs (Gallen. C in press [49]). As for the presence of low levels of PAHs in turtle blood from the remote HWK islands, long distance airborne transportation of contaminated particles is a well-documented phenomenon for the PAH compound group and the levels observed thus likely represent natural offshore baseline levels [50, 51].

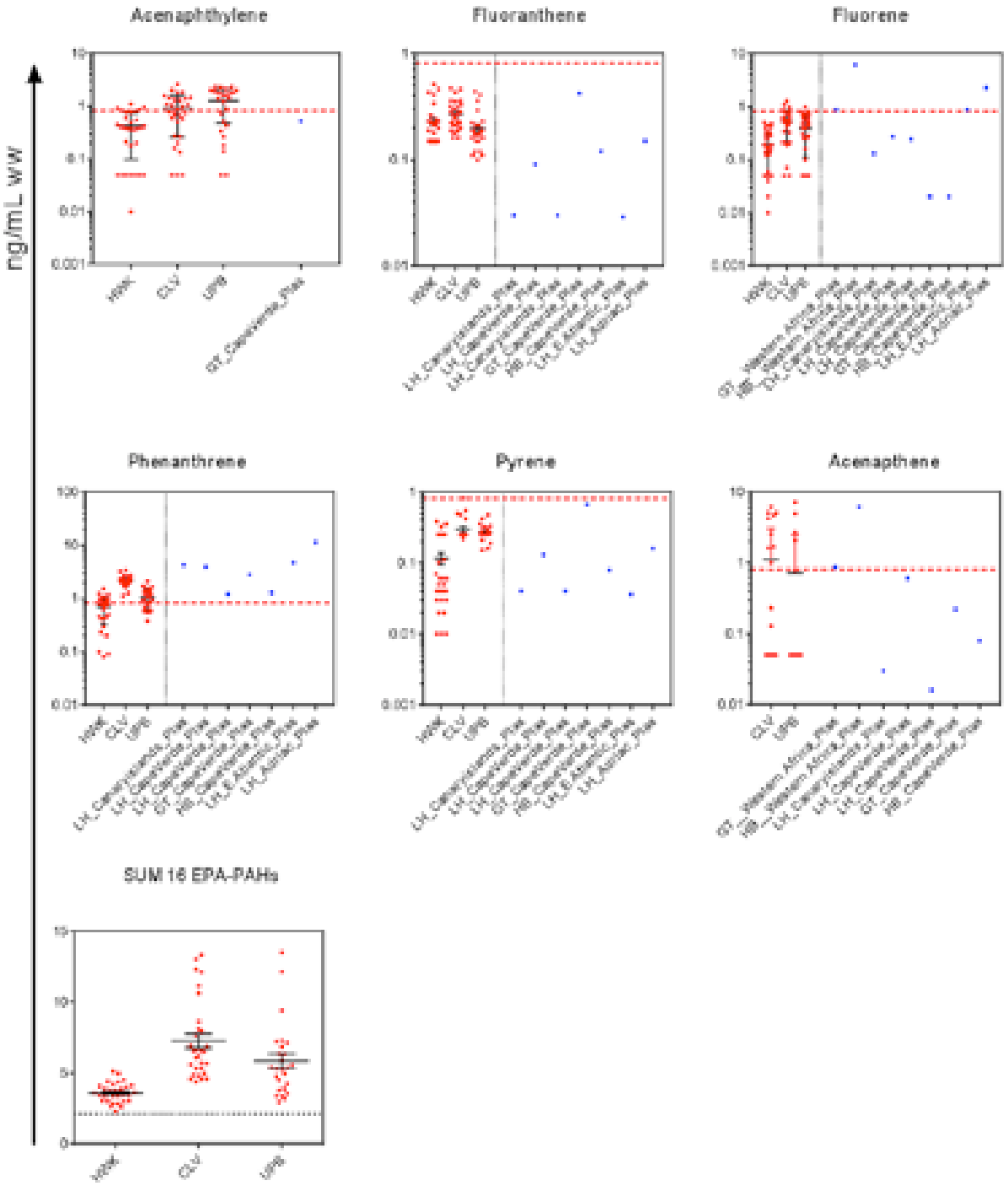

Figure 1: Comparison of PAH levels in blood of green turtles from HWK, CLV and UPB and prior studies [23],[11, 12, 15], Dotted line = average procedural blank, GT-Green turtle, LH-Loggerhead turtle, HB-Hawk's bill turtle

To our knowledge, this is the first study to report whole blood PAH levels not just in green turtles but also in other marine turtles from Australia and the Southern Hemisphere. Even on a global scale, biomonitoring studies on marine turtles are limited with very few studies analysing for PAHs. A mere four studies have previously reported PAH levels in marine turtle blood plasma and were undertaken in marine turtles from the Adriatic sea and Atlantic ocean (Figure 1). Camacho et al 2012, first reported PAH levels in Loggerhead turtles from the archipelagos of Canary Islands and Cape Verde. Phenanthrene was detected at the highest frequency and had the highest concentration from both study sites (median: 5.5 and 4.6 ng/mL in plasma). An ensuing study of PAH levels in green turtles from the same area, revealed that anthracene and phenanthrene were detected in highest concentrations and frequency. In comparison, the PAHs detected

at the highest frequency in this study were fluoranthene and phenanthrene, with acenaphthene showing highest concentrations from two sites. Occasionally, higher PAH levels were noticed in this study compared to previous studies (Figure 1) in Loggerhead, Hawksbill and Green turtles. However, these differences are not significant given that the overall concentrations reported in the case study sites (CLV and UPB) were low, and analogous or even lower than levels reported from relatively un-impacted sites (from Atlantic ocean) and more impacted site (Adriatic sea). The current study thus acts as the first report of baseline PAH levels in green turtle from three sites (inshore and offshore) along the GBR.

PAH Profiles

Figure 2 displays two typical profiles common to all three sites. Three ring PAHs dominated the Σ_{15}PAH concentration profiles, accounting for >55% in CLV and UPB sites and > 40% in HWK. The decrease in three ring PAH percentage contribution at HWK can be explained by the lack of acenapthene data which accounted over for >10% of total Σ_{15}PAH contribution in CLV and UPB. Phenanthrene and acenaphthylene were also major contributors followed by anthracene and fluorene to a lesser extent at all three sites.

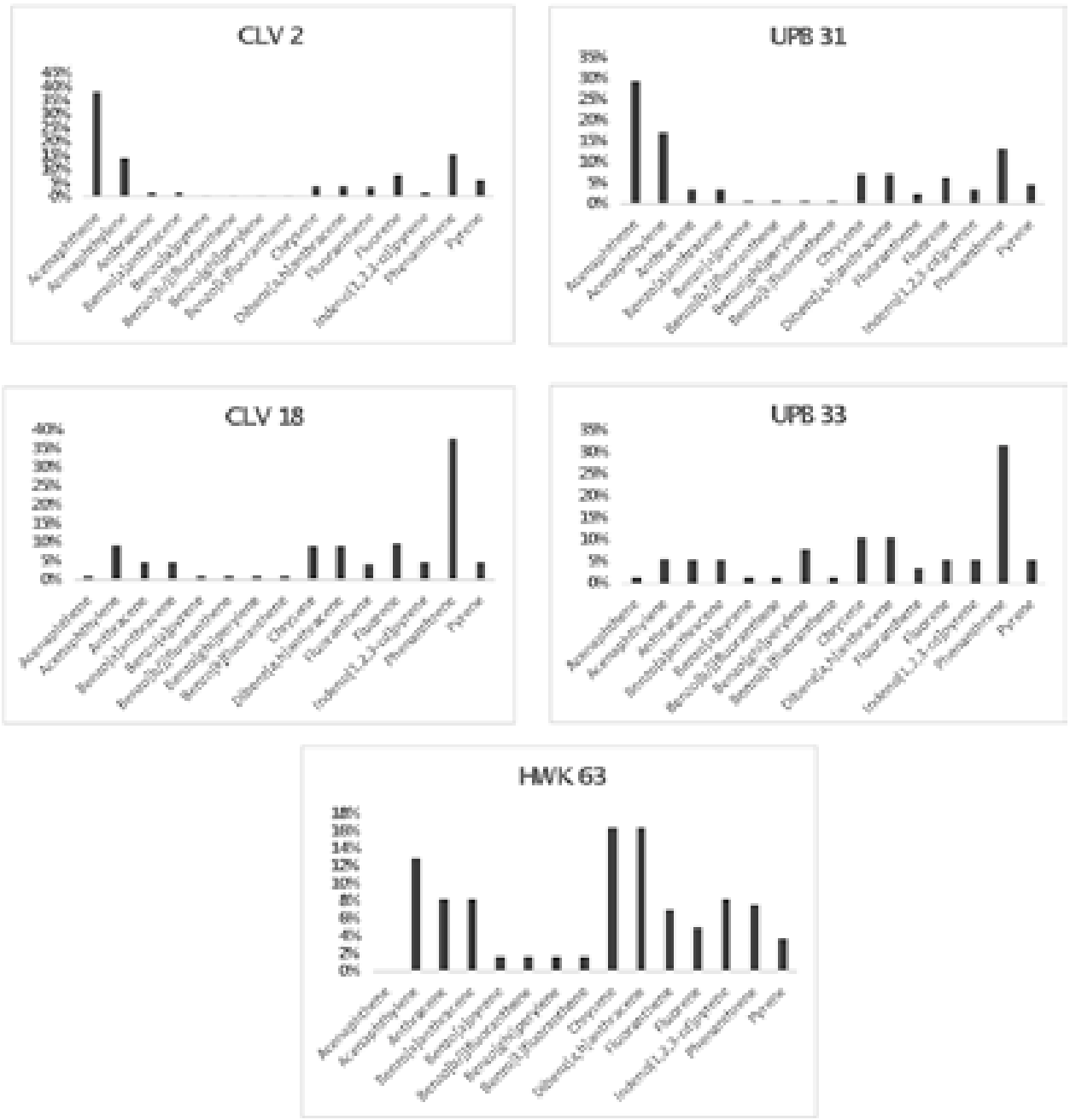

Figure 2: Percent contribution of individual PAH concentrations towards Σ15PAH with examples from all three sites; CLV 2, CLV 18 = samples from CLV, UPB 31, UPB 33 = samples from UPB and HWK 63 – sample from HWKs

While three ringed PAHs had the highest contribution, four ringed PAHs (>20-30%) such as pyrene and fluoranthene closely followed for all three sites. Five and six ring PAHs contributed to less than 5-15% in the three sites.

Previous studies demonstrated that the presence of two, three and four ring PAHs is an indication of a petrogenic source [52]. While this is the general trend seen in all three sites, the ratio of particular PAHs can also indicate the PAH source, such as for example phenanthrene to anthracene ratio (Phe/Ant) of >15 indicates petrogenic sources, whereas a ratio <10 indicates a pyrolytic source [53]. However, all the samples in this study had a very low concentration of anthracene. An alternate diagnostic ratio of fluoranthene/fluoranthene+pyrene, has also been used to determine the source of the PAH contamination in a recent air study [54]. The study showed that fluoranthene was more enriched than pyrene in bushfire samples, in contrast, higher pyrene was measured in areas of high vehicle traffic indicating higher petrogenic source. In this study fluoranthene/fluoranthene+pyrene had mean ratios <0.5 in samples from CLV (0.48) and UPB (0.41) indicating sources were more likely petrogenic, while mean ratio from samples from HWK (0.73) were slightly higher and indicating a pyrogenic source such as wood combustion. Though the similarity between the two studies is interesting, direct comparison of source dependent ratio in air to ratio in turtle blood is not possible as PAHs ratios may change internally in the turtle due to differences in absorption and metabolism.

Conclusion

Obtaining samples from wildlife is an extremely difficult process and once acquired the decision on which chemical group they should be tested for is challenging. Application of a rapid multi-residue QuEChER's method to turtle blood has allowed screening for a large number of chemicals with a wide range of KOW's. Given the countless number of chemical groups that turtles may be exposed to, this study has shown that although PAHs were detected in GBR green turtles, the low levels do not warrant undertaking targeted analysis of this group of chemicals. However, the study does provide first baseline PAH levels in green turtles for three different areas along the GBR. This data can be valuable as a reference for turtles suspected to be exposed to high levels of PAHs, and for subsequent studies that may involve recaptured turtles from the three sites. Having long-term biomonitoring data may give a better understanding of what the turtles are exposed to, and also allow for retrospective analysis in case of future events

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Appendix

Compound	RT (min)	Intra. (%)	Inter. (%)	Recov. (RSD) N=5 (%)	Linearity	Range (ng/mL)	LOQ (ng/mL)
Acenaphthene	9.4	6	6	60 (9)	0.9983	0.1-100	0.2
Acenaphtylene	9.24	4	10	62 (12)	0.997	0.5-100	1
Aldrin	16.45	4	8	72 (8)	0.9914	0.2-250	0.5
Anthracene	13.81	9	12	99 (8)	0.9999	0.2-100	0.5
Benzo(a)anthracene	22.89	3	6	82 (13)	0.9961	0.5-100	1
Benzo(a)pyrene	25.77	10	12	40 (25)	0.9946	0.2-100	0.5
Benzo(b)fluoranthene	25.17	3	3	77 (4)	0.9941	0.1-100	0.2
Benzo(g,h,i)perylene	28.43	2	4	60 (14)	0.9943	0.1-100	0.2
Benzo(k)fluoranthene	25.18	4	3	78 (5)	0.9871	0.1-100	0.2
Bifenthrin*	22.56	4	5	93 (7)	0.9931	0.2-250	0.5
Cadusafos*	12.78	3	9	94 (13)	0.9986	0.5-250	0.8
Chlordane	19	3	8	99 (8)	0.9966	0.5-250	1
Chlorpyrifos-ethyl*	16.5	6	9	103 (10)	0.9991	0.5-250	1
Chrysene	23.12	7	10	100 (13)	0.9915	0.5-100	1
DDD p,p	20.53	4	5	103 (15)	0.996	0.2-250	0.5
DDD, o, p	19.7	4	4	96 (10)	0.9984	0.1-250	0.2
DDE o,p	18.35	3	4	93 (8)	0.9938	0.1-250	0.1
DDE p, p	19.1	4	5	82 (4)	0.9974	0.1-250	0.1
DDT o,p	20.63	4	12	81 (14)	0.9988	0.2-250	0.5
DDT p,p	21.41	2	19	80 (13)	0.9986	0.2-250	0.4
Dibenzo(a,h)anthracene	27.6	2	8	65 (6)	0.9973	0.2-100	0.5
Dieldrin	19.81	6	11	91 (16)	0.998	0.5-250	1
Fluoranthene	18.32	5	6	92 (5)	0.9906	0.2-100	0.4
Fluorene	10.74	4	8	85 (9)	0.9948	0.5-100	1
Galaxolide*	14.43	8	9	86 (13)	0.9904	0.2-100	0.4
Heptachlor	15.4	8	11	77 (13)	0.9981	0.1-250	0.1
Hexachlorobenzene	11.82	7	9	54 (7)	0.9972	0.1-100	0.1
Ideno(1.2.3-cd) pyrene	27.83	2	8	59 (3)	0.9938	0.2-100	0.4
Lindane	13.12	6	8	95 (10)	0.9957	0.2-250	0.4
Naphthalene	6.81	7	11	33 (31)	0.9993	0.2-100	0.4
PCB 101	18.15	2	5	70 (11)	0.995	0.1-100	0.1
PCB 118	20	3	11	61 (7)	0.994	0.1-100	0.2
PCB 153	20.53	11	10	61 (14)	0.997	0.2-100	0.4
PCB 138	21.27	6	9	74 (14)	0.9956	0.1-100	0.2
PCB 180	22.46	6	7	48 (11)	0.998	0.1-100	0.2
PCB 28	14.49	5	8	83 (10)	0.9999	0.1-100	0.2
PCB 52	15.55	1	7	92 (8)	0.9936	0.1-100	0.2
Permethrin peak *	23.83 23.95	11 5	12 11	92 (11) 103 (13)	0.9699 0.9929	3-250 3-250	5 5
Phenanthrene	13.5	9	10	107 (6)	0.9292	0.2-100	0.4
Pyrene	18.8	4	7	90 (1)	0.9999	1-100	2
Terbufos*	13.69	7	13	94 (16)	0.9938	0.2-250	0.5
Tonalide *	14.77	7	11	102 (11)	0.9961	0.2-100	0.5
BDE-28	20.16	15	14	79 (22)	0.9985	0.1-100	0.2
BDE-47	22.65	3	7	83 (14)	0.9981	0.2-100	0.5
BDE-99	23.91	7	13	68 (10)	0.9819	0.5-100	1
BDE-100	24.3	4	7	59 (22)	0.9996	0.2-100	0.5
BDE-153	25.71	11	17	55 (19)	0.9755	3-100	5
BDE-154	25.2	11	4	39 (51)	0.9946	3-100	5
BDE-183	27.28	13	28	56 (20)	0.9956	3-100	4

Table A1: Summary of method performance results

LOQ- Limit of Quantification; Intra.- Intra-day precision, RSD; Inter.- Inter-day precision, RSD; RT- Retention Time;
* - Analysis not included in this study.

Analyte	Quantifier (m/z)	CE (V)	Qualifier 2 (m/z)	CE (V)
dioxin like-PCBs				
PCB 81 (3,4,4',5-Tetrachlorobiphenyl)	290 → 220	26	255 → 220	14
PCB 77 (3,3',4,4'-Tetrachlorobiphenyl)	290 → 220	26	255 → 220	14
PCB 123 (2,3',4,4',5'-Pentachlorobiphenyl)	324 → 254	22	326 → 256	22
PCB 118 (2,3',4,4',5-Pentachlorobiphenyl)	324 → 254	22	326 → 256	22
PCB 114 (2,3,4,4',5-Pentachlorobiphenyl)	324 → 254	22	326 → 256	22
PCB 105 (2,3,3',4,4',-Pentachlorobiphenyl)	324 → 254	22	326 → 256	22
PCB 126 (3,3',4,4',5-Pentachlorobiphenyl)	324 → 254	22	326 → 256	22
PCB 167 (2,3',4,4',5,5'-Hexachlorobiphenyl)	358 → 288	24	360 → 290	24
PCB 156 (2,3,3',4,4',5-Hexachlorobiphenyl)	358 → 288	24	360 → 290	24
PCB 157 (2,3,3',4,4',5'-Hexachlorobiphenyl)	358 → 288	24	360 → 290	24
PCB 169 (3,3',4,4',5,5'-Hexachlorobiphenyl)	358 → 288	24	360 → 290	24
PCB 189 (2,3,3',4,4',5,5'-Heptachlorobiphenyl)	392 → 322	26	394 → 324	22

Table A2: Collision energies (CE), quantifier and qualifier ions of the target compounds analysed using GC-QqQ-MS/MS

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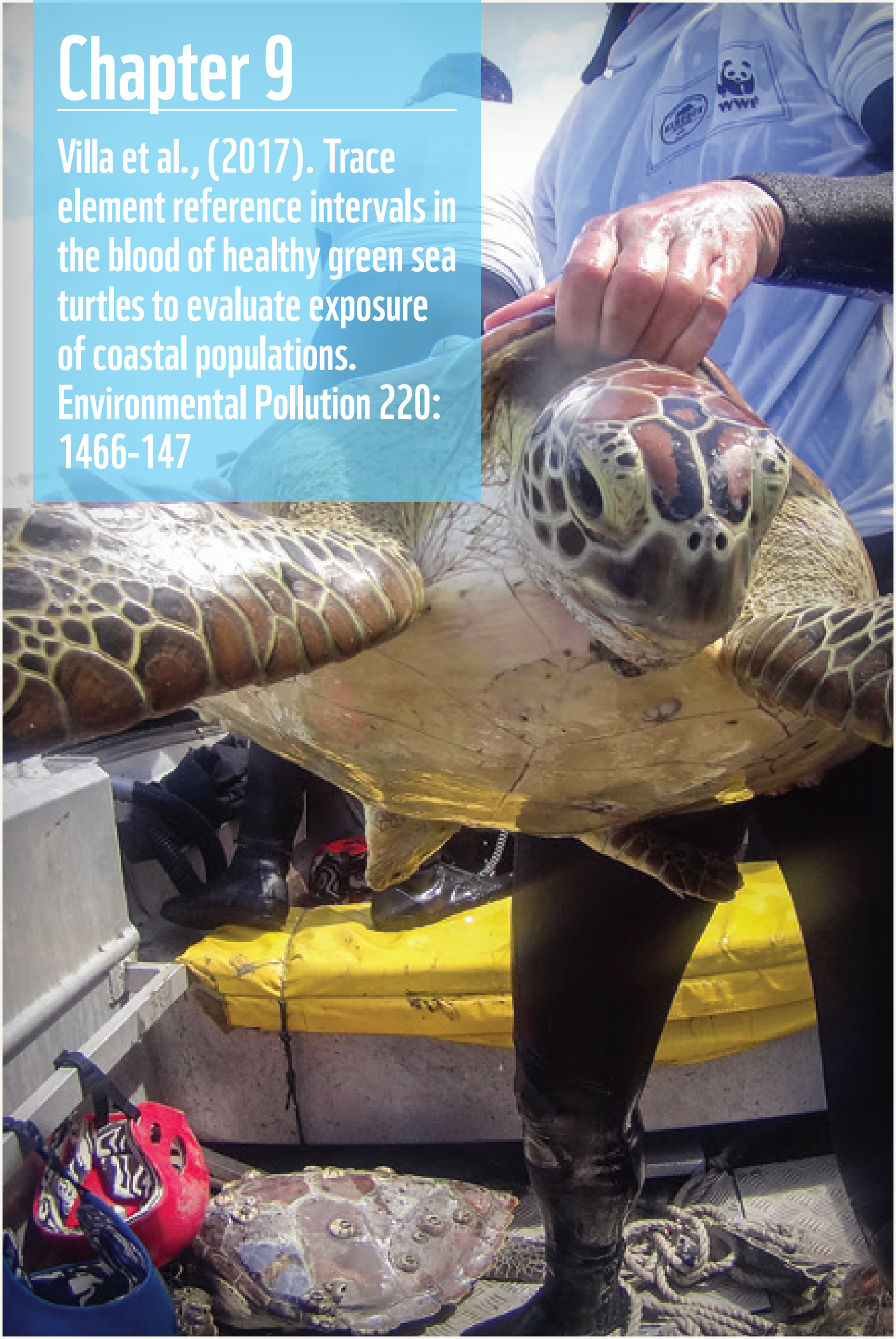
Chapter 9

Villa et al., (2017). Trace element reference intervals in the blood of healthy green sea turtles to evaluate exposure of coastal populations. Environmental Pollution 220: 1466-147



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Trace element reference intervals in the blood of healthy green sea turtles to evaluate exposure of coastal populations*

C.A. Villa [a, *], M. Flint [b, c], I. Bell [d], C. Hof [e], C.J. Limpus [f], C. Gaus [a, **]

[a] The University of Queensland, National Research Centre for Environmental Toxicology (Entox), 39 Kessels Road, Coopers Plains, QLD 4108, Australia
[b] School of Forest Resources and Conservation, University of Florida, The Florida Aquarium's Center for Conservation, Apollo Beach, FL 33572, USA
[c] Vet-MARTI, School of Veterinary Science, The University of Queensland, Gatton Campus, QLD 4343, Australia
[d] Queensland Department of Environment and Heritage Protection, Townsville, QLD 4810, Australia
[e] WWF-Australia, Level 1, 17 Burnett Lane, Brisbane, QLD 4000, Australia
[f] Queensland Department of Environment and Heritage Protection, Brisbane, QLD 4102, Australia

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ABSTRACT

Exposure to essential and non-essential elements may be elevated for green sea turtles (*Chelonia mydas*) that forage close to shore. Biomonitoring of trace elements in turtle blood can identify temporal trends over repeated sampling events, but any interpretation of potential health risks due to an elevated exposure first requires a comparison against a baseline. This study aims to use clinical reference interval (RI) methods to produce exposure baseline limits for essential and non-essential elements (Na, Mg, K, Ca, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Mo, Cd, Sb, Ba, and Pb) using blood from healthy subadult turtles foraging in a remote and offshore part of the Great Barrier Reef. Subsequent blood biomonitoring of three additional coastal populations, which forage in areas dominated by agricultural, urban and military activities, showed clear habitat-specific differences in blood metal profiles relative to the those observed in the offshore population. Coastal turtles were most often found to have elevated concentrations of Co, Mo, Mn, Mg, Na, As, Sb, and Pb relative to the corresponding RIs. In particular, blood from turtles from the agricultural site had Co concentrations ranging from 160 to 840 μg/L (4–25 times above RI), which are within the order expected to elicit acute effects in many vertebrates. Additional clinical blood biochemistry and haematology results indicate signs of a systemic disease and the prevalence of an active inflammatory response in a high proportion (44%) of turtles from the agricultural site. Elevated Co, Sb, and Mn in the blood of these turtles significantly correlated with elevated markers of acute inflammation (total white cell counts) and liver dysfunction (alkaline phosphatase and total bilirubin). The results of this study support the notion that elevated trace element exposures may be adversely affecting the health of nearshore green sea turtles.

Chapter 10

Elucidating temporal trends in trace element exposure of green turtles (*Chelonia mydas*) using the toxicokinetic differences of blood and scute samples

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10. Elucidating temporal trends in trace element exposure of green turtles (*Chelonia mydas*) using the toxicokinetic differences of blood and scute samples

C.A.Villa[1*], I. Bell[1], C. A. Madden Hof[1], C.J. Limpus[2], C. Gaus[1]

[1] Queensland Alliance for Environmental Health Sciences (QAEHS), The University of Queensland, 39 Kessels Road, Coopers Plains, Queensland 4108, Australia
[*] Species Conservation, World Wildlife Fund for Nature, Australia
[2] Department of Environment and Heritage Protection, Threatened Species Unit, Townsville, Australia

Abstract

Blood is considered a suitable biomonitoring matrix for evaluating relatively recent contaminant exposures (including trace elements) since abrupt changes in exposure are rapidly reflected in blood first and can be observed depending on the time of sampling relative to the time of exposure. Green turtles also have external keratinized scutes covering their carapace which has been proposed as an ideal complementary monitoring matrix since it integrates their localised exposure over time. Significant correlations between blood and scute concentrations were found for Co, As, Mo, Sb, and Cd were plotted using simple linear regression for two green turtle populations presumed to experience stable exposure conditions. These regressions were taken to represent the steady-state relationship in trace elements between blood and scute. Altered exposure profiles (recent exposures or past exposures) were evident in paired blood and scute concentrations obtained from turtles from two coastal sites subject to differing anthropogenic stressors. In addition, we propose a conceptual model to predict how an elevated exposure would affect blood and scute concentrations relative to the steady-state regression plot. Individual turtles that had been recaptured and sampled as part of ongoing exposure investigations provided an opportunity to validate the conceptual model predictions. However, the ability to visualise altered exposure appeared to be strongly influenced by blood elimination rates. Although the overall investigation period is still too short and the number of recaptured turtles too low to validate the conceptual model, the recapture data nevertheless allowed us to visualise changes that were generally in-line with the model predictions.

Introduction

Reports of marine turtle strandings in Queensland, Australia have been steadily rising since 1996 with a surge in green turtle strandings from 2009 through 2011 (Meager and Limpus, 2012). Far from being a regional issue, similar findings have been reported from the Hawaiian archipelago where green turtles represented 97% of marine turtle strandings from 1983-2003 (Chaloupka et al., 2008b). In both cases, investigators indicated that about half of these reports had no discernable stranding cause with disease as the second most common cause (Chaloupka et al., 2008b; Flint et al., 2017). Diseases such as fibropapillomatosis and clinical markers of poor health in green turtles have often been reported to correlate with poor water quality (Adnyana et al., 1997; Ariel et al., 2017; dos Santos et al., 2010; Foley et al., 2005; Herbst, 1994; Van Houtan et al., 2010; Villa et al., 2017). For green turtles that forage in shallow coastal embayments adjacent to urban and industrial activity, exposure to pollutants, including trace elements, metalloids and their compounds, can occur as a result of both natural and anthropogenic impacts (e.g. floods, agricultural and industrial runoff, urbanisation, coastal dredging). Understanding of the health and prevalence of diseases in marine turtle populations provides a critical link between ecosystem health and turtle health (Jones et al., 2016). A review of global research priorities for the management and conservation of sea turtles has highlighted key gaps in our ability to evaluate the effects of pollutants on marine turtle development, survivorship, health, reproduction, and critical habitat condition/recovery (Hamann et al., 2010). The first step to help understand the link between exposure to

trace elements and declines in green turtle health is to establish baseline data and develop reliable means to monitor their residues within free-ranging individuals.

Trace element reference intervals for green turtle blood have only recently been developed to help identify elevated concentrations of elements in blood, but depending on their blood elimination rates, trace element biomonitoring using blood is sensitive to the time at which samples are collected (Villa et al., 2017). However, marine turtle scutes, the outermost keratinized layers of the carapace, have been shown to provide long-term exposure information since each layer contains a snapshot of the blood concentrations from when it formed. There are currently no reported methods on ageing individual scute layers in green turtles which would be required to identify an estimated time of exposure. Nonetheless, exposure information can be obtained from the entire scute depth providing a time-integrated trace element accumulation profile for a period spanning 1.4 – 2.8 years (Vander Zanden et al., 2013). Unlike blood, there are no reference intervals to identify elevated trace element concentrations in scute, limiting what can be interpreted from their analysis. One novel approach is to use the exposure information from both blood (short-term) and scute (long-term) to glean some information on the temporal characteristics of trace element exposure.

The distribution of trace elements from blood to all other tissues is governed by toxicokinetic parameters such that under relatively constant exposure conditions turtle blood and scute concentrations should reach a steady-state (Grillitsch and Schiesari, 2010). This steady-state relationship can be modelled using paired blood and scute from green turtle populations that forage in areas distant from point sources and are relatively remote to anthropogenic disturbances or from nearshore protected areas that receive little riverine input and where the catchment is free from most anthropogenic activity. The following conceptual model details how the steady-state relationship can be used to inform on the temporal characteristics of trace element exposure in other populations.

Conceptual model

The following conceptual model illustrates how the different concentrations in blood and scute (driven by toxicokinetics) could be used to identify a recent exposure in other turtle populations. For this conceptual model, we assume a relatively constant exposure (background) to a hypothetical element with a relatively short blood elimination half-life (e.g. a few days). At the onset of increased exposure, blood concentrations rise relatively rapidly (Figure 1), and perfuse the newest forming scute layer. A corresponding scute accumulation occurs, but only within newest layers.

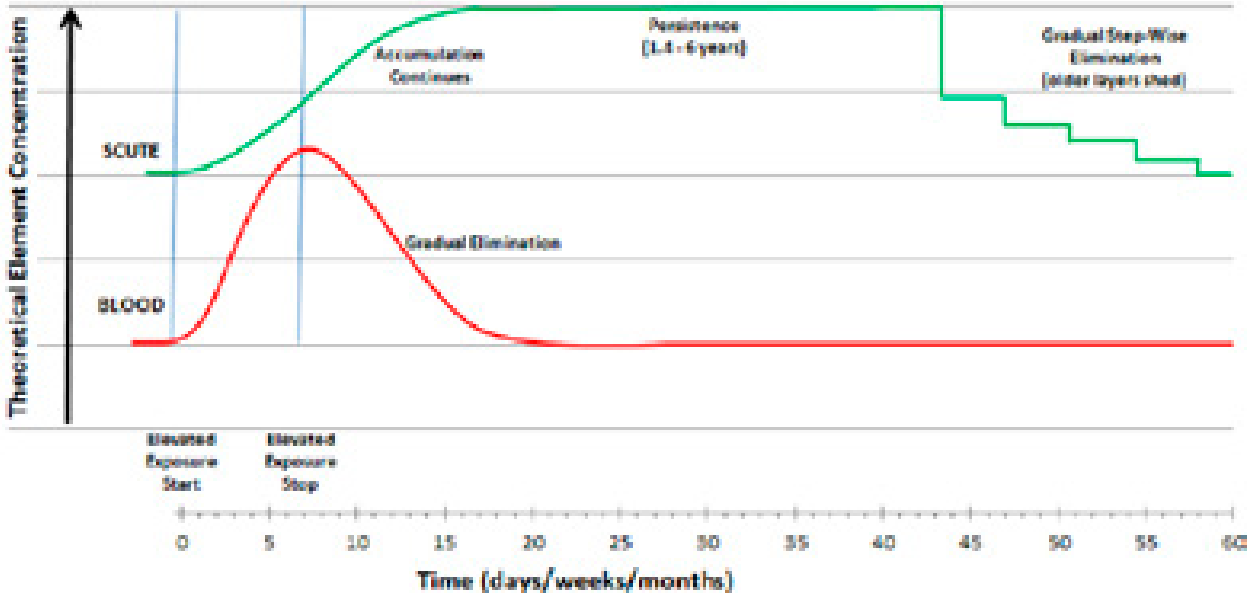


Figure 1: Concentration profiles for blood and scute accumulation of a hypothetical element following a briefly elevated exposure

As a result, the element concentration across the entire scute thickness increases slowly with each scute layer formed during elevated exposure. When exposure ceases, blood concentrations return to near background levels relatively fast, and new scute layers reflect the lower exposure levels. In contrast, the scute layers formed during elevated exposure maintain the accumulated element concentration of that time. As a result, the scute over its entire thickness remains elevated. As old scute layers shed, the layers with high trace element concentrations are transported closer to the surface until they too are ultimately shed. Long after blood has returned to baseline levels, the high element concentration locked within the scute persist until it is gradually eliminated in a step-wise decrease a layer at a time.

Accordingly, the steady-state concentration ratio can be expressed as a linear relationship; the blood-scute ratio (constant slope) remains the same over any exposure magnitude as long as the exposure is constant. This, of course, holds true only when the magnitude of exposure does not fall above or below a concentration that disrupts the distribution kinetics of the element. When an elevated exposure is encountered, the response in each matrix can be tracked against the steady-state relationship (Figure 1). At any given time, an elevated blood concentration with a corresponding baseline scute concentration, relative to the steady-state plot (e.g. points below the regression line) is indicative of a recent exposure (Figure 1 first panel). Conversely, a baseline blood concentration (relative to steady state) with elevated scute concentrations would be indicative of a historically elevated exposure (Figure 1 second and third panels).

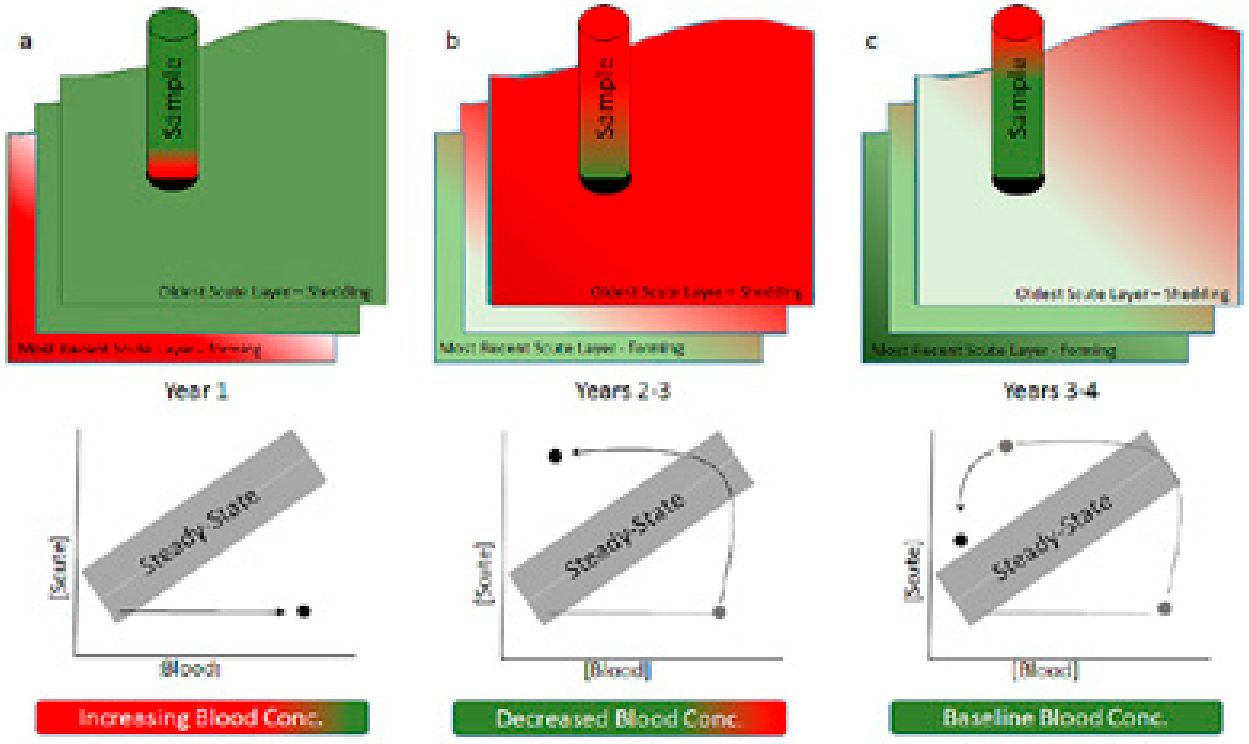


Figure 1: Conceptual model showing the distribution of a hypothetical element between blood and scute layers after a single high exposure scenario. The line and shaded area in the lower graphs represent the blood scute ratio for a baseline scenario. The dots (lower figures) and scute illustrations (upper row) follow an increased exposure scenario in panel a Year 1 back to near background in panel c Years 3-4.

Several studies have shown positive linear correlations between trace elements (Zn, Mn, Hg, in scutes and internal tissues of green turtles (Faust et al., 2014; Komoroske et al., 2011; Sakai et al., 2000; van de Merwe, 2009), loggerheads (Day et al., 2005), Kemps Ridley's (Innis et al., 2008), and other estuarine and freshwater turtles (Dyc et al., 2016; Schneider et al., 2015; Smith et al., 2016). Of these, the use of paired blood and scute to elucidate temporal exposure in marine turtles has only been investigated by Day et al. (2005). The authors used blood and scute to demonstrate that recent accumulations of Hg in loggerheads correlated with the proximity of the capture site to a highly industrialised waterway. Elucidating temporal exposure using blood and scute has not been described for any other species or

element presumably due to the need to first describe the steady-state relationship between these matrices for each element. Such a relationship may be difficult, if not impossible, to extrapolate from the literature since the majority of those reports come from moribund or stranded turtles, from populations close to point sources, and composed of mixed age classes.

Therefore, the aims of this investigation were first to elucidate the relationship between blood and scute for multiple elements using populations of turtles that are expected to experience close to constant exposure conditions, thus approximating a steady state between blood and scute. The resulting steady-state relationships were then used to explore the temporal trace element exposures for turtles from two case study sites, which included individual turtles repeatedly sampled across different years (recaptures).

Materials and methods

Sampling Campaigns and Capture Details

Paired blood and scute tissues samples were collected from subadult green turtles (female CCL ≥60 cm to ≤100 cm; males ≥63 cm to ≤95 cm; unknown sex ≥65 cm to ≤90 cm) captured as part of large collaborative investigations on marine turtle ecology and health at different foraging grounds in Queensland, Australia from 2011 – 2017: Howick Island group (HWK), Shoalwater Bay (SHL), Cleveland Bay (CLV), and Upstart Bay (UPB). With the exception of SHL, each of the foraging grounds was visited multiple times resulting in recaptured individuals both within and across sampling years (Figure 4).

Site	Year	Recaptured
HWK	2014	
HWK	2015	11
SHL	2014	--
CLV	2014	--
CLV	2015	
CLV	2016	4
CLV	2017	
UPB	2013	--
UPB	2014	--
UPB	2015	3
UPB	2016	3
UPB	2017	2

Table 1: Number of recaptured turtles analyzed at each site.

Green turtle foraging grounds from HWK, SHL, CLV, and UPB are described previously in Villa, 2017.

All turtles were captured using rodeo techniques (Limpus and Reed, 1985) and returned to shore for processing (tagged, measured, weighed, visually assessed for health status (Thomson et al., 2009)), in accordance with our ethics approval (AEC Approval Number: NRCET/147/14/APA/WWF). Blood samples were processed according to methods described in Villa et al 2017. Briefly, whole blood was collected from the dorsal cervical sinus using disposable syringes fitted with 21-18 gauge needles. For trace element analyses, a total of 5 mL of whole blood was transferred into acid-rinsed, sodium heparin dosed, polyethene tubes (1,000 IU/ml whole blood; DBL™ Hospira, Australia). Scute samples were collected and treated following a validated method currently being developed into separate manuscript . Briefly, bridge scutes were scrubbed with seawater and briefly rinsed with methanol before collecting approximately 1 gram (total scute fragments) from the posterior three marginal bridging scutes of each side of the carapace-plastron interface, and the supracaudal scutes. Further treatment of scute fragments was performed in the laboratory to reduce residual exogenous contamination; 5ml of acetone was added to

the samples which were then submerged in an ice-water bath and sonicated for 15 min, then sonicated for 15min with Milli-Q water, three times. The process was repeated with one acetone cycle followed by three Milli-Q water cycles. Concerns over damage to the underlying epidermal layers were resolved through anecdotal observation of recaptured turtles (1-year and 2-year post-sampling) where no permanent damage was observed, and new scute material had grown over the sampling areas.

Figure 2: Map of sampling locations for the turtle populations sampled offshore (HWK) and at three coastal sites (CLV, UPB, SHL, and GLD) along the Great Barrier Reef, Queensland, Australia. Inserts: sample numbers (N) for trace element analysis with recaptures in brackets divided by year (see Figure 4).

[Figure will be updated to exclude GLD and revise totals for each WWF location. From CG report: over the four year sample period, a total of 507 green turtles were sampled: n=182 in UPB (n=73, 64, 43, 2 per year, respectively), n=125 in CLV (71, 39, 15, 0 per year, respectively), and n=200 in HWK (96, 60, 35, 9 per year, respectively)]

Trace Element Analysis

Trace element analysis of blood followed methods previously described in Villa, 2015. In general, scute samples were digested with high-purity nitric acid and peroxide solutions and heated (<90°C) on a heating block. Digests were diluted and amended with multi-element internal standards before injection into an ICP-MS. Quality assurance and quality control samples, including blanks, CRMs, and replicates, as well as the calculation of CRM recoveries and MDLs are described in Villa, 2017.

Statistical Analysis

Trace element data distributions were initially assessed for normality using histograms and boxplots and evaluated statistically using the Anderson-Darling test. Outliers were identified visually and through ROUT (False Discovery Rate=1%) and Spearman Rank Correlations performed between blood and scute trace element concentrations. Between year comparisons of blood or scute samples were performed using the Wilcoxon matched-pairs signed rank test (α=0.05). Data visualisation, outlier detection, normality tests, paired comparisons and correlations were performed using GraphPad Prism, version 7.00 for Windows (GraphPad Software, San Diego California, USA). One-way analysis of variance comparing group means and subsequent posthoc tests were performed in using IBM SPSS Statistics for Windows, Version 22.0 (Armonk, NY).

For those elements with significant positive correlations Model II regression (ordinary-least-squares [OLS] and Major Axis [MA]) models were used to evaluate the relationship between paired blood and scute trace elements. Model II regression methods offer the advantage of incorporating the analytical measurement error in both independent and dependent variables for line fitting. Since analytical measurement error can greatly affect regression variable estimations and are rarely estimated directly from the data, we utilised repeated measurements of blood CRM (n=6 replicate of Seronorm 2) and duplicate measures of two randomly selected scute sample (n=4 replicates) to independently estimate the combined analytical and sampling measurement error of each matrix. Per the regression selection criteria outlined by Legendre and Legendre (2012), the OLS method was chosen when the error in the independent variable (scute) is >3x that of the dependent variable (blood). The analytical measurement error values were also used to produce graphical error bars in the log-log regression plots between blood and scute.

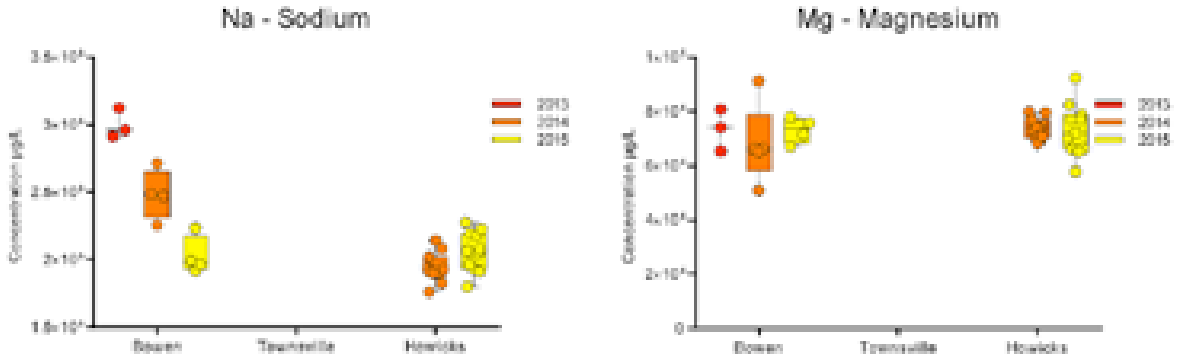
The regression variable estimates were evaluated using D'Agostino & Pearson omnibus K2 test for normality of residuals and a test for homoscedasticity. Regression estimates, evaluations and 90% prediction intervals (PI_{90}) were determined using GraphPad Prism. Significance was defined as $p \leq 0.05$ for all of the above tests.

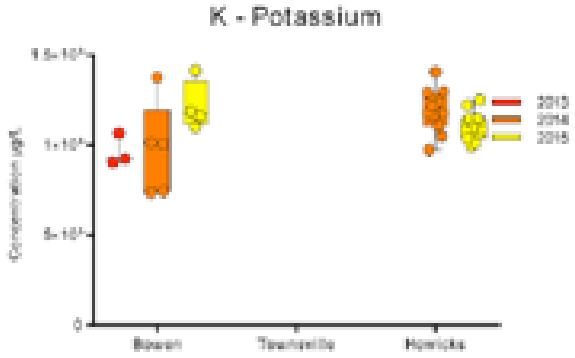
Results and discussion

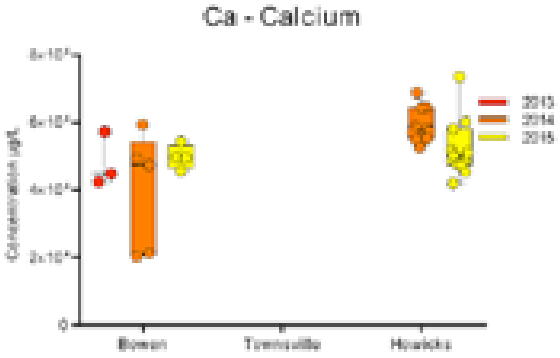
Blood and scute concentration in all turtles were determined for 24 elements (Na, Mg, K, Ca, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Mo, Cd, Sb, Ba, and Pb). Concentrations of the elements Be, Al, Sn, Hg, Tl, Th, and U were mostly <MDL and were excluded from further analysis. Coastal sites adjacent to greater levels of anthropogenic activities had elevated trace elements in blood relative to baselines as discussed in detail for samples obtained in 2014 for HWK, SHL, CLV, and UPB in Villa, 2017. Briefly, trace elements in blood from CLV turtles sampled in 2014 had mean concentrations just above RI-UCIs for Na and Mg, at least 2-times RI-UCI for Mn and Sb, and as high as 5-times for Co. Turtles from nearby UPB sampled in 2014 had elevated Na, Mo, and Mn approximately 2-times RI-UCIs that returned to near RI-UCI levels in 2015. Mean Sb concentrations in 2014 UPB turtle blood were 4-times RI-UCI and remained elevated at 3-times RI-UCI in 2015. Conversely, mean Co concentrations rose from 15-times RI-UCI in 2014 to 20-times RI-UCI in 2015.

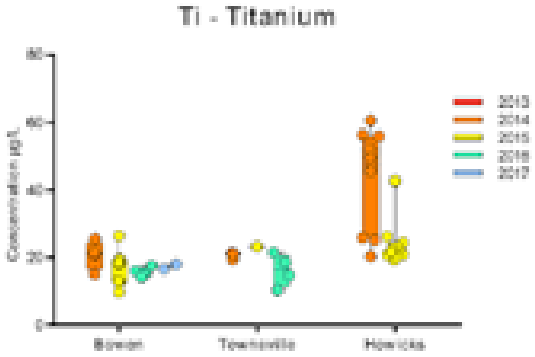
While there are no baselines developed for scute, trace element concentrations in this matrix are discussed in each of the case-studies below in the context temporal exposure trends.

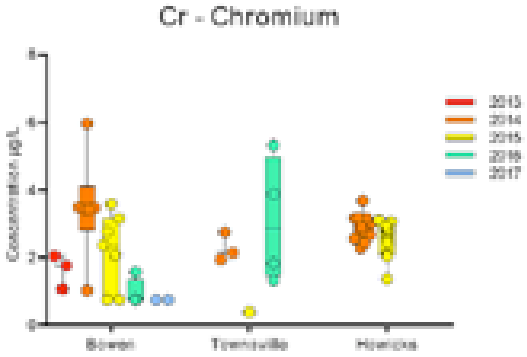
General trends in trace element concentration changes in blood over the sampling periods are presented below.

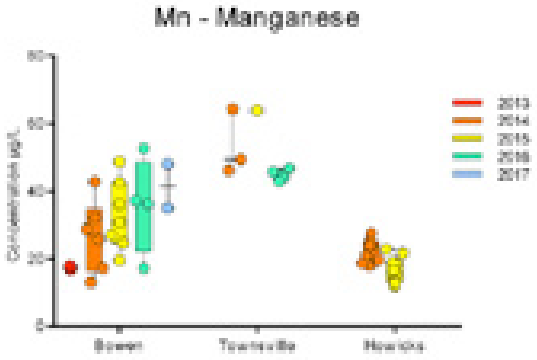


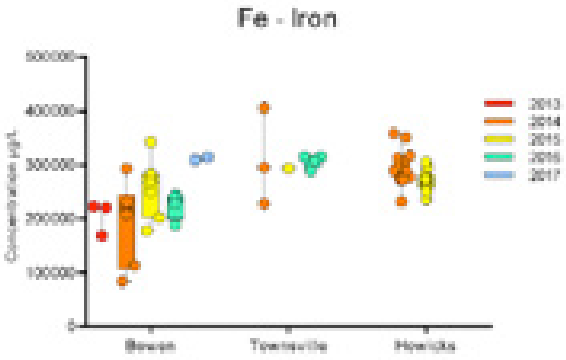

K - Potassium
Bowen
Townsville
Howicks
2013
2014
2015

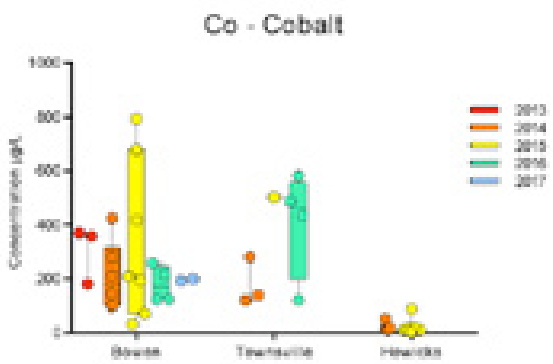

Ca - Calcium
Bowen
Townsville
Howicks
2013
2014
2015

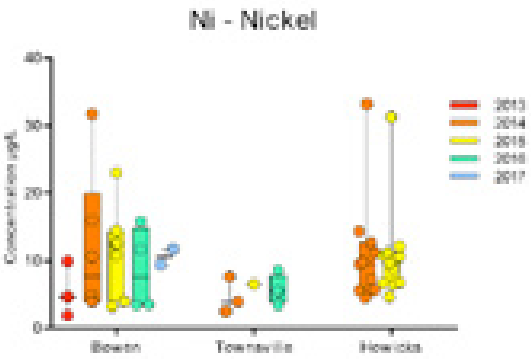

Ti - Titanium
Bowen
Townsville
Howicks
2013
2014
2015
2016
2017

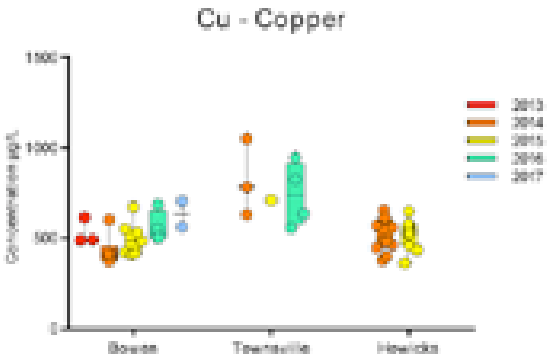

Cr - Chromium
Bowen
Townsville
Howicks
2013
2014
2015
2016
2017

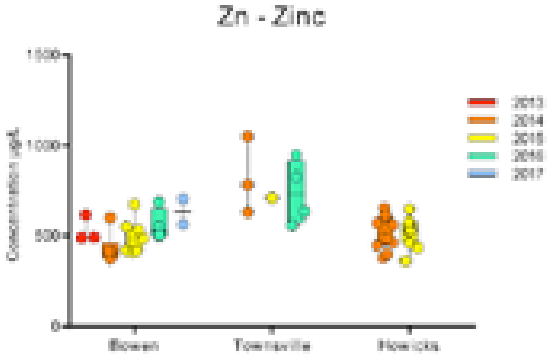

Mn - Manganese
Bowen
Townsville
Howicks
2013
2014
2015
2016
2017


Fe - Iron
Bowen
Townsville
Howicks
2013
2014
2015
2016
2017


Co - Cobalt
2013
2014
2015
2016
2017


Ni - Nickel
Bowen
Townsville
Howicks
2013
2014
2015
2016
2017


Cu - Copper
Bowen
Townsville
Howicks
2013
2014
2015
2016
2017


Zn - Zinc
Bowen
Townsville
Howicks
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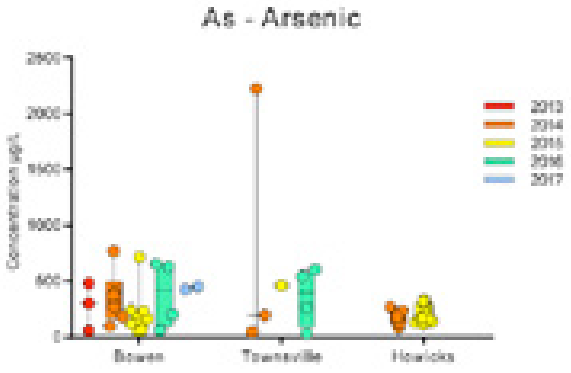
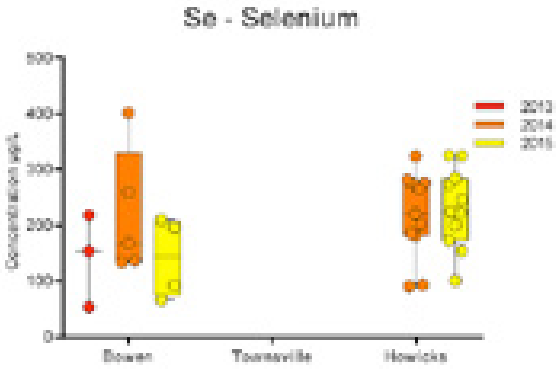
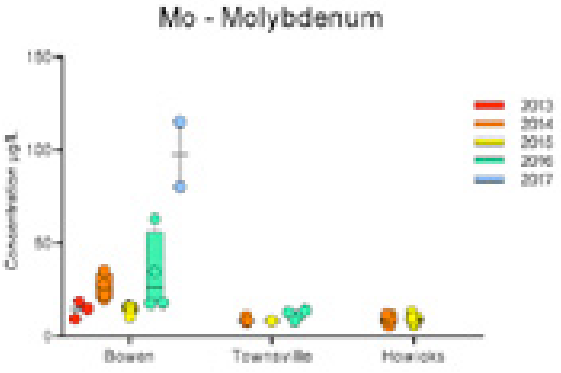
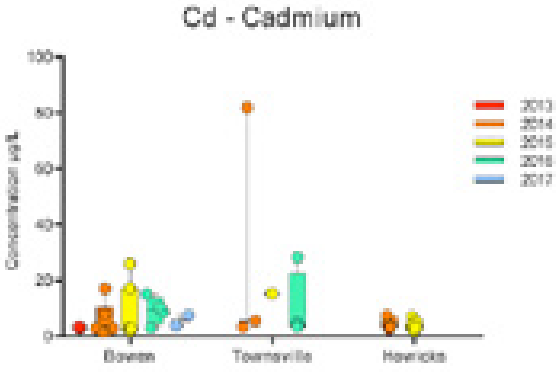
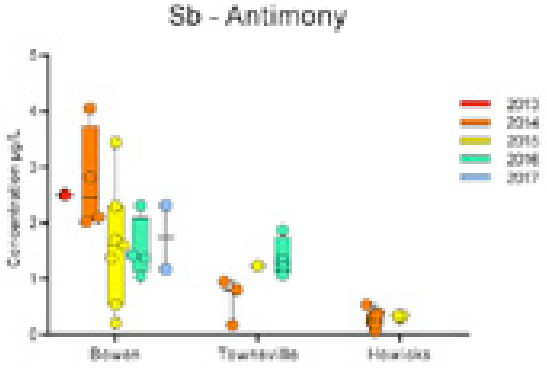
2014
2015
2016
2017

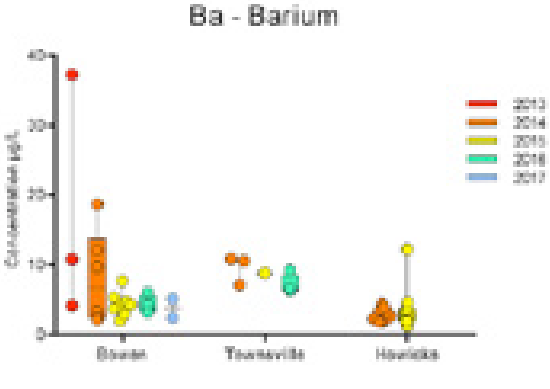

As - Arsenic
Bowen
Townsville
Howicks
2013
2014
2015
2016
2017


Se - Selenium
Bowen
Townsville
Howicks
2013
2014
2015


Mo - Molybdenum
Bowen
Townsville
Howicks
2013
2014
2015
2016
2017


Cd - Cadmium
Bowen
Townsville
Howicks
2013
2014
2015
2016
2017


Sb - Antimony
Bowen
Townsville
Howicks
2013
2014
2015
2016
2017


Ba - Barium
Bowen
Townsville
Howicks
2013
2014
2015
2016
2017

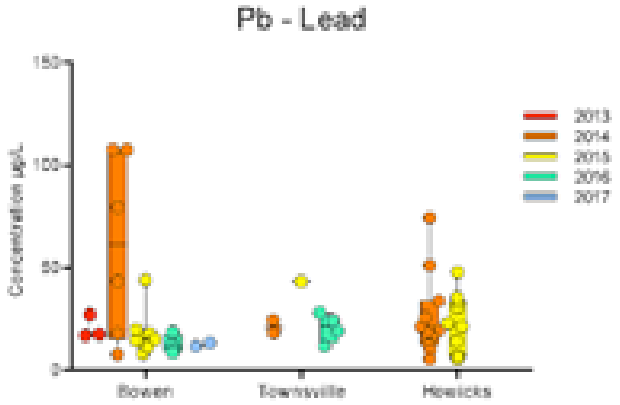


Figure 3: Blood concentrations (µg/L ww) for recaptured turtles by year and location

Constant Exposure and Steady-State Blood Scute ratios

Turtles sampled in 2014 from two sites with expected relatively stable and constant exposure regimes were used to evaluate steady-state relationships between blood and scute trace element concentrations: HWK (offshore) and SHL (coastal). Significant positive correlations, and normally distributed standardised regression residuals were obtained for Co, As, Mo, Cd, and Sb. No significant mean differences (p=0.05) for these elements were found in both scute or blood concentrations between 2014 HWK and 2015 HWK populations. This confirms that the HWK subadult population is generally subject to low and relatively stable trace element exposure for these elements. No such temporal trend data is available for the SHL turtles. The assumption of constant exposure is based on the distance of this foraging site from known major point sources, the high flushing rate for the Bay, the absence of significant riverine inputs, and a previous investigation in this region indicating low persistent organic contaminants in green turtle blood (Hermanussen, 2009). In addition, turtles from the SHL region were previously used by Flint et al. (2010a) as part of a healthy cohort to establish reference ranges for clinical health markers and haematology.

The haplotypes used to identify the genetic stocks for these two populations differ slightly (HWK = 35% northern GBR, 51% southern GBR, 18% other; SHL = over 95% southern GBR; (Jensen et al., 2016)). Nonetheless, there is insufficient evidence to indicate that these genetic differences translate to significant physiological differences in trace element kinetics. It is, therefore, reasonable to combine both data sets into a single group, which has the advantage to provide a larger range of steady-state blood-scute trace element concentrations than either could produce alone (particularly for Co, Mo, and Sb).

The linear regression obtained from the combined blood and scute concentrations for Co, As, Mo, Cd, and Sb of HWK and SHL are expected to approximate a steady-state ratio between these two matrices. The analytical measurement error for the scute (y-axis) was >3x that of blood (x-axis) for Co, As, Mo, and Sb, indicating that OLS is the preferred method to estimate regression variables (Figure 3). Normality of Cd blood and scute concentration distributions could not be achieved through transformation (Logarithmic or Box-Cox) which precluded the use of any Model II regression methods other than OLS, even though the measurement error ratio (scute to blood) was < 3. No transformation was necessary prior to OLS regression to achieve normality of the Cd residuals. Thus it is the only element plotted using untransformed concentration data (Figure 3).

Constant Exposure and Steady-State Blood Scute ratios

Turtles sampled in 2014 from two sites with expected relatively stable and constant exposure regimes were used to evaluate steady-state relationships between blood and scute trace element concentrations: HWK (offshore) and SHL (coastal). Significant positive correlations (Error! Reference source not found.), and normally distributed standardised regression residuals were obtained for Co, As, Mo, Cd,

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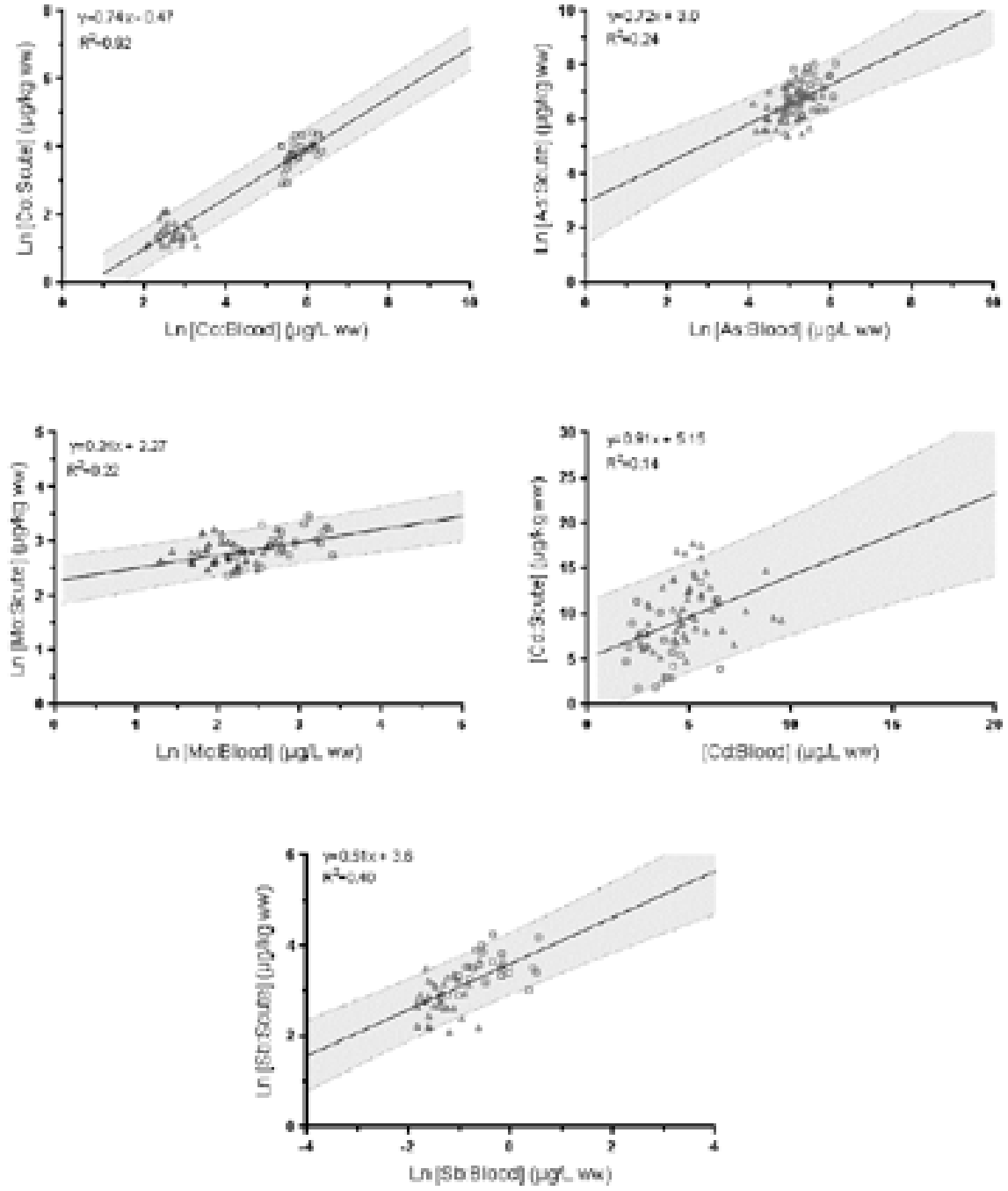

Figure 4: Regression between blood and scute (2014 HWK open triangles and SHL open squares) showing 90% prediction interval (grey area; RPI_{90}) for natural log-transformed Co, As, Mo and Sb concentrations. Concentrations and regression parameter estimates for Cd are for non-transformed data.

The haplotypes used to identify the genetic stocks for these two populations differ slightly (HWK = 35% northern GBR, 51% southern GBR, 18% other; SHL = over 95% southern GBR; (Jensen et al., 2016)). Nonetheless, there is insufficient evidence to indicate that these genetic differences translate to significant physiological differences in trace element kinetics. It is, therefore, reasonable to combine both data sets into a single group, which has the advantage to provide a larger range of steady-state blood-scute trace element concentrations than either could produce alone (particularly for Co, Mo, and Sb).

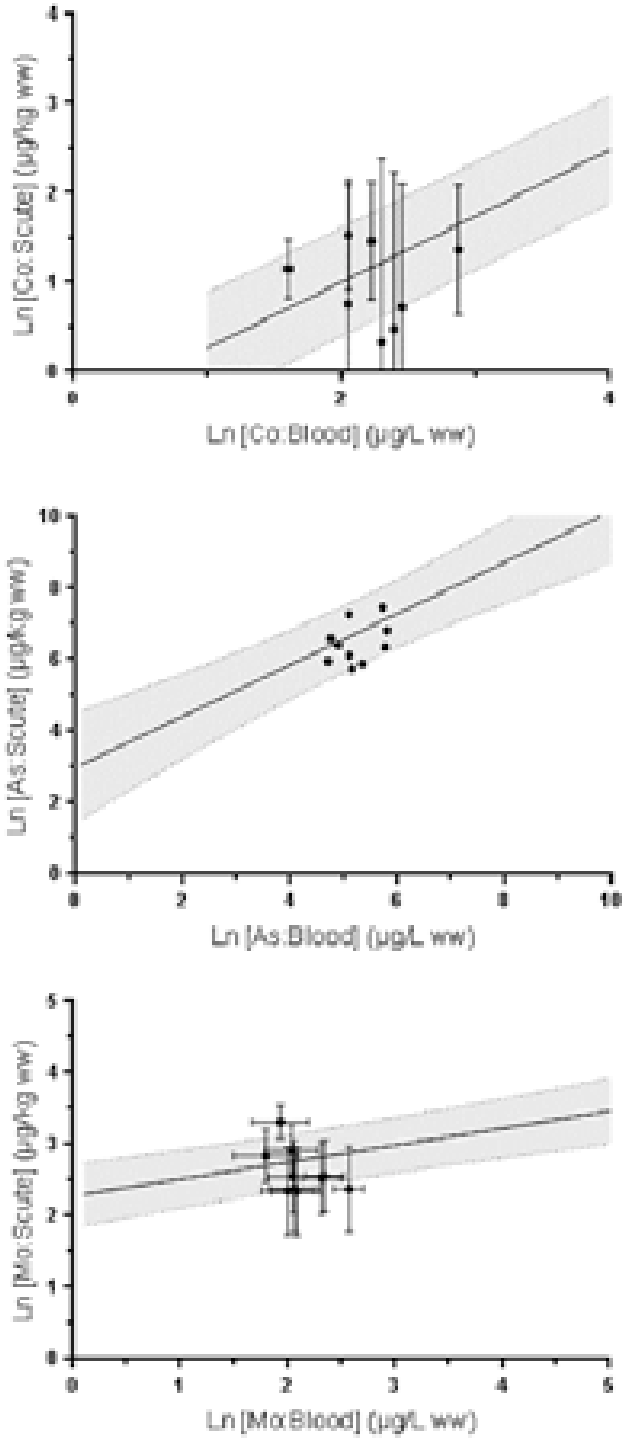

Figure 5: Paired blood and scute Co, As, and Mo concentrations for 2015 HWK turtles. The steady-state regression model (2014 HWK and SHL) is shown as a black line and the 90% prediction interval as a grey shaded area.

The linear regression obtained from the combined blood and scute concentrations for Co, As, Mo, Cd, and Sb of HWK and SHL are expected to approximate a steady-state ratio between these two matrices. The analytical measurement error for the scute (y-axis) was >3x that of blood (x-axis) for Co, As, Mo, and Sb, indicating that OLS is the preferred method to estimate regression variables (Figure 3). Normality of Cd blood and scute concentration distributions could not be achieved through transformation (Logarithmic or Box-Cox) which precluded the use of any Model II regression methods other than OLS, even though the measurement error ratio (scute to blood) was < 3. No transformation was necessary prior to OLS regression to achieve normality of the Cd residuals. Thus it is the only element plotted using untransformed concentration data (Figure 4).

Day et al. (2005) calculated blood and scute regression residuals for a group of moribund turtles that were compared to the linear regression they developed using live captured turtles. Residuals from each moribund data point (Euclidean distances) and their sign (positive or negative) were used to identify departures from their regression model, but the authors did not indicate how large a residual must be before it is no longer considered within the steady-state relationship. Since we estimated the 90% confidence limits of where future observation will fall (90% regression prediction intervals; RPI_{90}) we reason that the upper and lower bounds of these limits define a conservative threshold to identify points that do not lay within a steady-state. Hence, paired blood and scute (± analytical error) ratios can inform on the often elusive initial time of exposure where recent exposures are identified as data below the RPI_{90} and those above the RPI_{90} indicative of past exposure.

An example of this can be seen with the HWK 2015 paired blood and scute concentrations for Co, As, and Mo which all lay within their respective RPI_{90} further indicating no major alterations in exposure conditions over approximately 1-year between sampling. The HWK 2015 Cd scute samples and Sb blood samples were below their respective MDLs, supporting the notion that stable and low exposure profiles continue for the HWK turtles.

Case Studies

Individual paired blood and scute concentrations from turtles foraging at two coastal sites in Queensland, Australia (UPB and CLV) were compared against trace element steady-state relationships developed from the HWK and SHL populations. Both UPB and CLV populations were sampled over several years and recaptured turtles, some many years apart (Figure 5). The recapture data allows us to track the short-term signal (blood) and long-term signal (scute) as exposure conditions change over time, offering a unique opportunity for validating the conceptual model of this study (Figure 7).

Each of coastal sites is influenced by mixtures of trace elements that are expected to differ based on the anthropogenic actives occurring within their catchments. Thus each of these case studies offers a different perspective to temporal exposure investigations using real world data. The first, UPB, is a case of suspected acute exposure to Co, Sb, and Mo in turtles sampled at year 2 and resampled at year 3 later. The second case, CLV, only involves a single recapture year and further illustrates how even a single recaptured sampling point can help direct future investigations.

Case-Study 1: Upstart Bay

Upstart Bay (UPB), is a rural coastal area approximately 100 km south of CLV and 50 km north of an international coal terminal. It has a system of mostly ephemeral creeks receiving wet-season discharges from one of Queensland's largest catchments (130,000 km^2) dominated by intensive agriculture, grazing and mostly legacy mining (Bartley et al., 2014).

The green turtle population at UPB was subject to an unusual mass stranding and mortality event in 2012, 2-years prior to the first sampling campaign of this study, where neurological symptoms were observed closely followed by death, and with recurrent isolated episodes. Semiquantitative trace element blood analysis performed for a different study (for one of the 2012 euthanised turtles) identified V, Co, Ni, Sb, Tl, and U as potential monitoring targets. Turtles that entered rehab and subsequently died (2013 and 2014), were sampled by others and analysed for the afore mentioned study which confirmed elevated Co and Sb. Additional 2014 samples at UPB turtles for this study further corroborated high Co, Sb, and

Mo blood concentrations relative to reference interval (Villa, 2017) leading to the hypothesis that these elements may have posed an acute threat to turtles during the mass stranding event. Additional clinical findings for the 2014 UPB turtles in this study identified markers of systemic stressors including liver dysfunction, which correlated in particular with Co and Sb concentrations in blood.

Recapture data demonstrated that elevated blood concentrations of Co, Mo, and Sb for UPB turtles remained elevated through to 2017. While there are no reference limits for trace elements in scute, we can utilise the steady-state regression model to identify a range of predicted trace element concentrations in scute that would be representative of a low exposure scenario, as predicted by actual concentrations measured in blood. Thus, we observe that Co scute concentrations are significantly higher than the steady-state scute range predicted by blood concentrations of a large proportion of 2014 UPB turtles (70%) and 2015, 2016, and 2017 UPB recaptures. This suggests Co exposure was highest prior to the first sampling event in 2013.

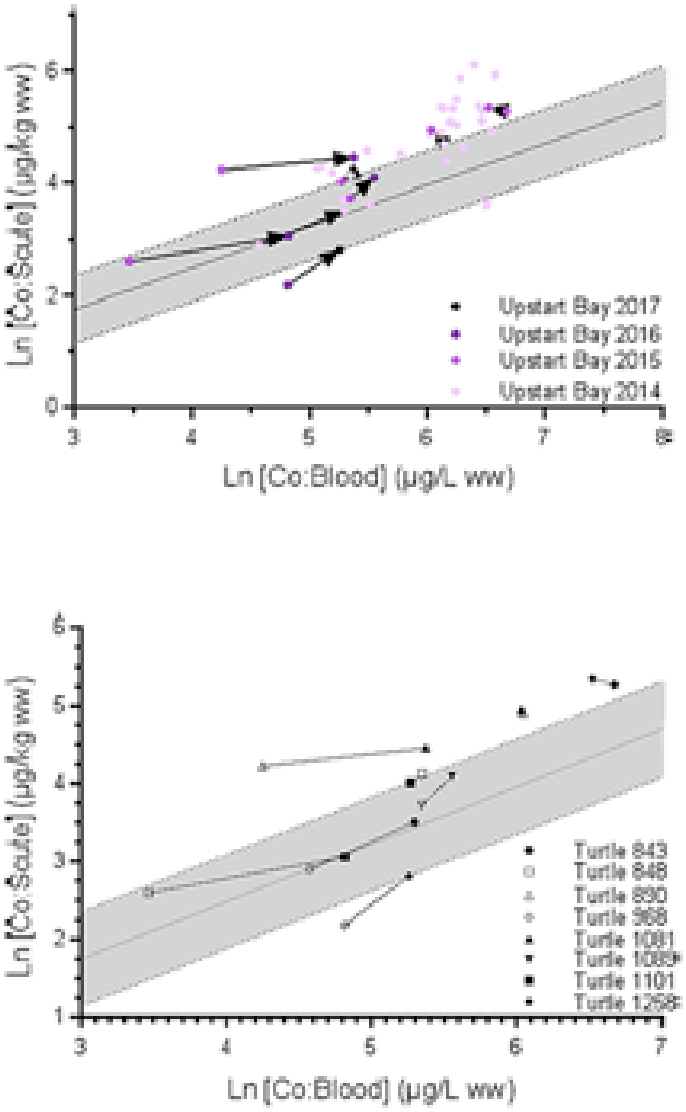

Figure 6: a) Paired blood and scute cobalt concentrations for Upstart Bay turtles (individuals = circle, colours=year, arrows=recaptures). Second plot just recaptures. Regression model (HWK and SHL) shown with 90%prediction intervals (grey area).

Although the exact time of exposure cannot be determined yet, scute is assumed to integrate a time over the past ~1.4-2.8 years (i.e. its estimated lifetime before shedding; Vander Zanden et al. (2013)). While it may be tempting to extrapolate the historical peak blood concentrations (> approx. 5,000 µg/L based

on the observed scute concentration), we do not yet know the toxicokinetic behaviours of Co in green turtles or the saturation concentration limits for any particular biological matrix. This is particularly relevant for essential trace elements like Co where various metabolic processes are believed to be responsible for maintaining Co levels within different compartments of the body (Leggett, 2008). Thus, linear relationships are unlikely to hold at either extreme of the model where we expect increased rates of detoxification (i.e. excretion or protein binding). The observation of only minor decreases in Co blood concentrations over the 1 year period since the 2015 recapture is not surprising considering the relatively slow blood elimination rates of Co in other vertebrates (Ayala-Fierro et al., 1999). Similarly, due to the relatively long time span of scute retention, scute element concentrations, after an initial increase, remain constant for some time after elevated exposure before returning to baseline levels.

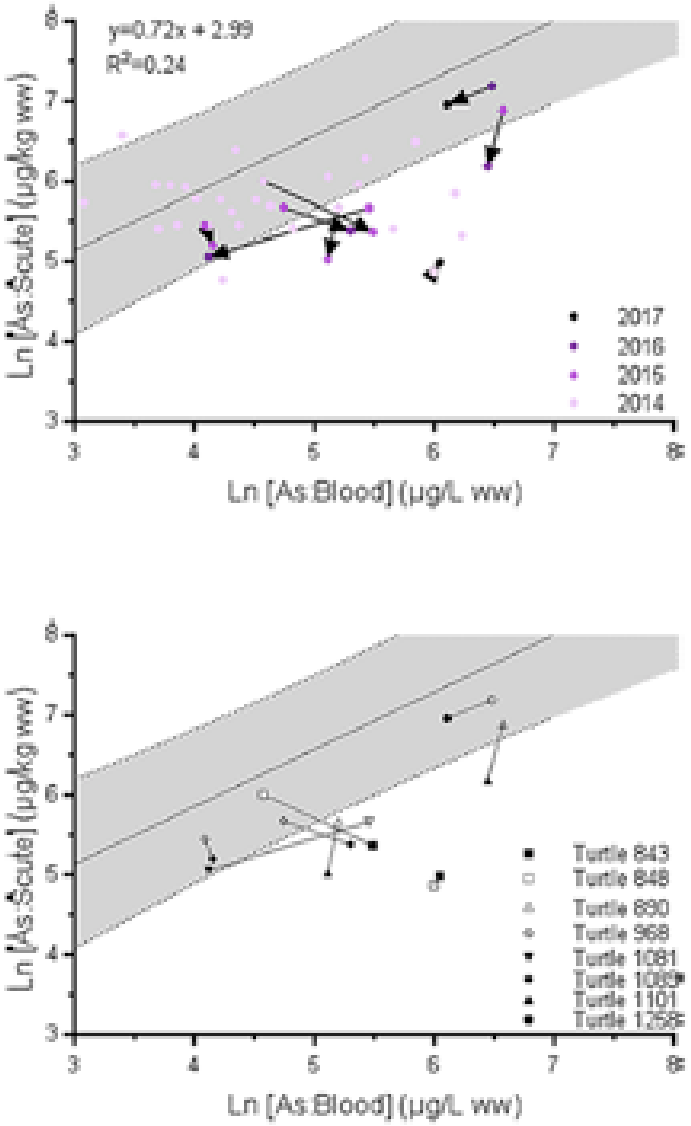

Figure 7: a) Paired blood and scute arsenic concentrations for Upstart Bay turtles (individuals = circle, colours=year, arrows=recaptures). Second plot just recaptures. Regression model (HWK and SHL) shown with 90%prediction intervals (grey area).

Blood-scute ratios for As from several UPB turtles, indicate recent exposure in each sampling year. Blood and scute As concentrations in recaptures appear to be changing in a manner that is inconsistent with the conceptual model of this study. This is likely due to the expected higher variability in As accumulation as a result of the natural abundance of As in seagrass and algae which make up the bulk of the green turtle diet. Studies on the kinetics of As have demonstrated that organoarsenicals (the dominant As form in

marine systems) are readily absorbed in the gastrointestinal tracts of both animals and humans and can be rapidly excreted in urine and faeces (Fowler et al., 2015). Considering this and the relatively low As concentrations in UPB turtles, the relatively random temporal patterns may simply reflect recent feeding behaviour or a change in diet composition favouring one source (i.e. algae) over the other.

Paired blood-scute concentrations of Mo in UPB turtles indicate historical exposure for some 2014 individuals (above the RPI_{90}) with all turtles analysed in subsequent sampling years within the RPI_{90}. Individual recaptures all fall within the RPI_{90} indicating a relatively constant exposure for those individuals. One 2014 individuals with previous elevated exposure had a blood-scute ratio within the RPI_{90} over the course of a year which is consistent with trends predicted by the conceptual model.

No toxicokinetic information is available for Mo in green turtles, but studies on its distribution in other vertebrates indicate rapid accumulation 1-24 hrs after dietary exposure with a return to near basal blood levels after 1-hr in humans and near complete excretion within 2-weeks for guinea pigs, rats, goats, and pigs (Tallkvist and Oskarsson, 2015). Thus we cannot make inferences regarding Mo exposure conditions during the 2012 mass mortality event, especially as there are only a few individuals with blood-scute ratios above the RPI_{90} that were subsequently recaptured.

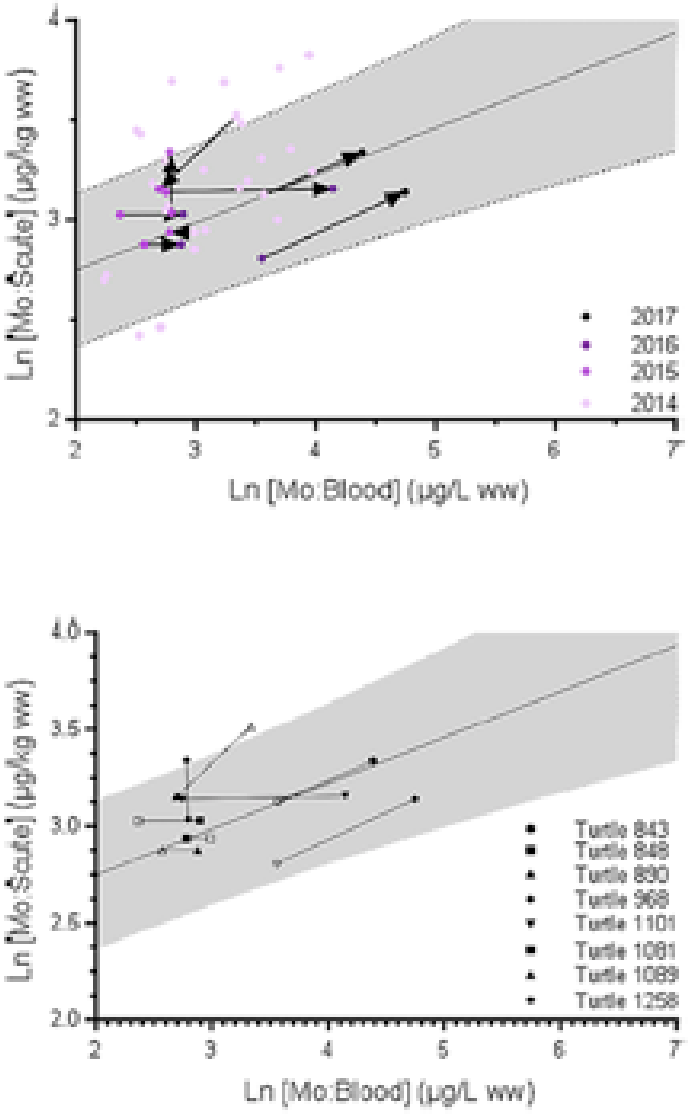

Figure 8: a) Paired blood and scute molybdenum concentrations for Upstart Bay turtles (individuals = circle, colours=year, arrows=recaptures). Second plot just recaptures. Regression model (HWK and SHL) shown with 90%prediction intervals (grey area).

Cd concentrations in blood were within reference intervals for green turtles, in 2013 and 2014, elevated concentrations were observed for each of the subsequent years through to 2017. Blood scute ratio in recaptured turtles follow patterns of decreasing concentrations which appear to follow the conceptual model predictions for a previous elevated exposure.

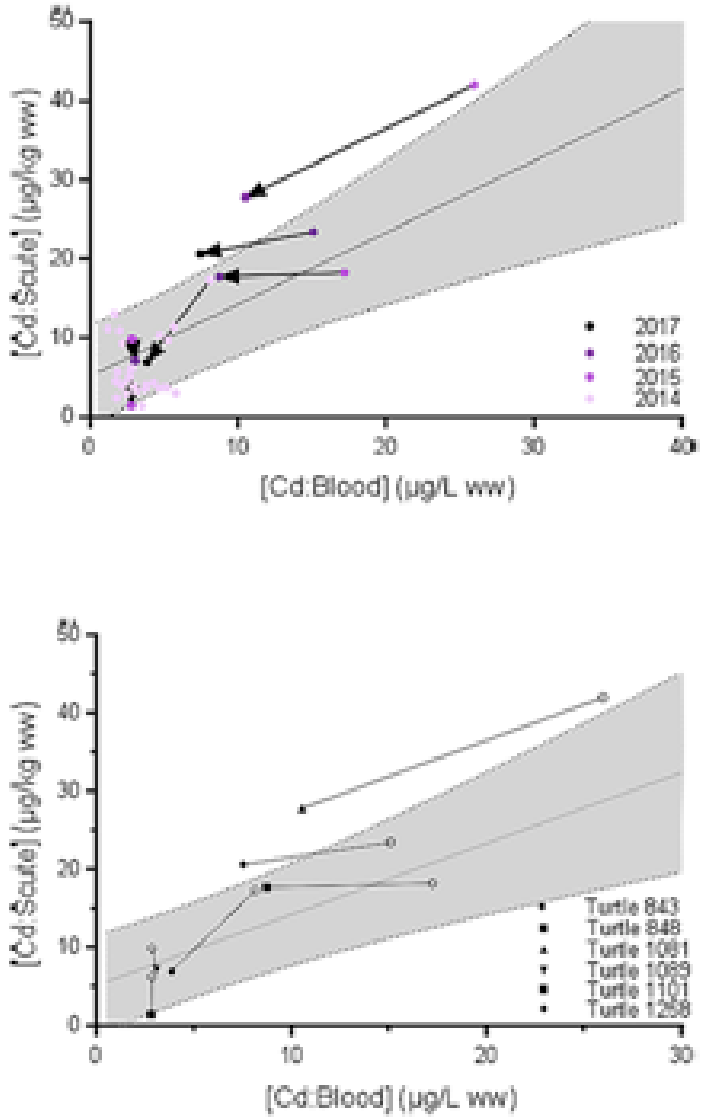

Figure 9: a) Paired blood and scute cadmium concentrations for Upstart Bay turtles (individuals = circle, colours=year, arrows=recaptures). Second plot just recaptures. Regression model (HWK and SHL) shown with 90%prediction intervals (grey area).

Among most vertebrates, Cd is reported to accumulate with age, primarily in liver and kidney tissues, with long biological half-lives (7-10 years) (ATSDR, 2008). The exception is blood, where clearance rates can be very fast due to a high binding affinity to transport proteins (primarily metallothioneins). A Cd feeding study of red-eared sliders turtles (Trachemys scripta elegans) demonstrated that even with highly contaminated diets, blood levels remained low making it a poor biomonitoring matrix for elevated exposure (Guirlet and Das, 2012). In a similar study using painted turtles (Chrysemys picta) <10% of total Cd dose remained in blood 8-days post exposure with rapid accumulation in carapace and plastron over that time period (Rie et al., 2001).

While elevated blood Sb concentrations in 2013 have been declining through 2017, all turtles sampled remain above the Sb reference interval. Blood-scute concentrations of Sb in 2014 individuals were mostly within the RPI_{90} limits with a few exceptions. In subsequent years, all blood-scute Sb concentrations were

below the RPI_{90} limits indicating a potential recent or on-going exposure. Unfortunately, the relatively large analytical error for these data points makes it difficult to interpret the results with a great degree of confidence. This is apparent in the recapture data where turtle 968, a within year recapture, is observed to have similar blood concentrations but whose scute concentrations differ by an order of magnitude. Nevertheless, the elevated blood concentration still warrants concern. Sb blood elimination kinetics in other vertebrates is very rapid (70%-90% of dose eliminated within 48-hours; (ATSDR, 1992; Sundar and Chakravarty, 2010), thus we are unable to make inferences regarding Sb exposure condition during the 2012 mass mortality event, particularly since we do not observe blood-scute ratios above the RPI_{90} that would be indicative of elevated past exposure.

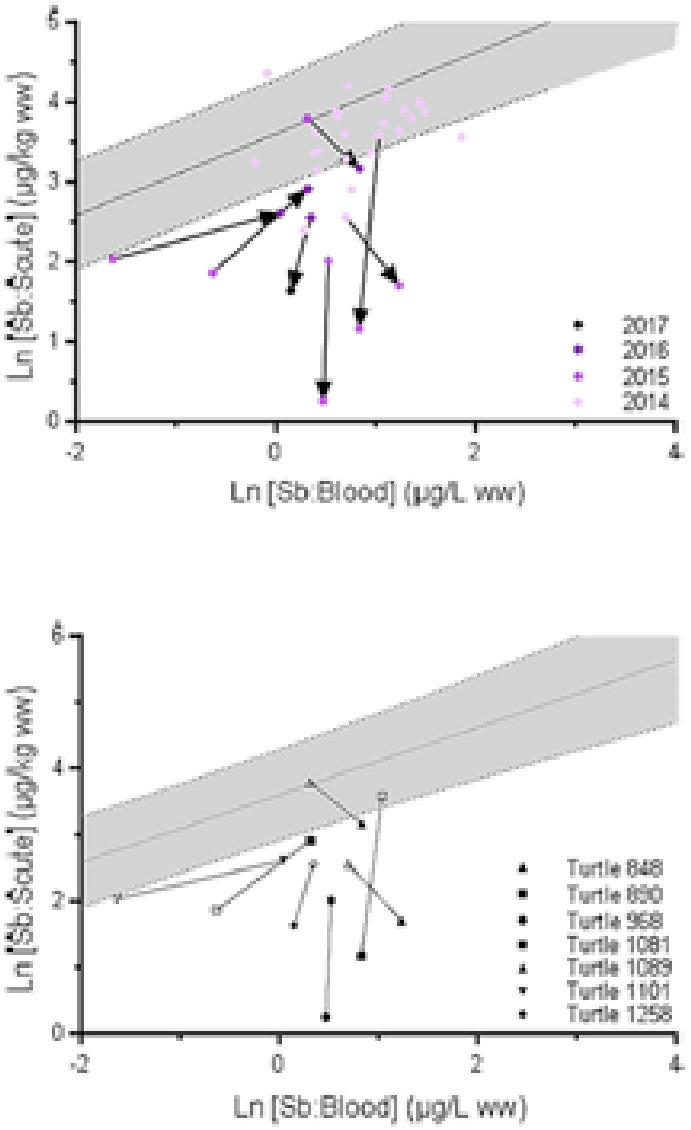

Figure 10: a) Paired blood and scute antimony concentrations for Upstart Bay turtles (individuals = circle, colours=year, arrows=recaptures). Second plot just recaptures. Regression model (HWK and SHL) shown with 90%prediction intervals (grey area).

The aim of the UPB case study was to test the hypothesis that acute exposures of trace elements (particularly Co, As, Cd, Mo, and Sb) played a role in the 2012 mass stranding event. The key limitation was that no paired blood and scute samples were collected during or immediately after the mass stranding. A resulting 2-year gap between suspected exposure and first sampling was far too long for some elements to have remained within the available biomonitoring matrices to provide insight into potential elevated exposures. This is especially true for those elements suspected of having rapid blood

elimination half-lives (i.e. Mo and Sb) in green turtles. In contrast, 2-years may have been long enough for the peak concentration in scute to have been shed since each new layer is expected to be retained for 2-6 years. Nevertheless, elements with very long blood elimination rates in most vertebrates (i.e. Co or Cd) could persist, but the kinetic information required to make this inference is lacking for green turtles.

The blood-scute ratios observed for Co are consistent with the hypothesis of an acute cobalt exposure in 2012. The continued increase in blood-scute Co concentrations in the recaptured turtles are troubling as it appears that elevated Co exposures are persisting in 2015 and 2016.

While blood-scute Mo temporal patterns appear consistent with the 2012 acute exposure hypothesis for a few individuals, the recapture data indicates a more recent exposure occurring in 2016 and 2017.

Temporal patterns in blood-scute concentration for Cd follow the conceptual model of an acute exposure. Interestingly, the toxicological information available for Cd in other vertebrates indicates that an elevated concentration in blood would be an unlikely observation given the exceedingly fast blood half-life. In 2015 and 2016 we observed elevated blood Cd concentrations with one case of an accompanying scute concentration indicative of an elevated past exposure. The recapture data consistently shows a large decrease in blood concentrations with some evidence of decreasing scute concentrations, becoming more pronounced over greater time intervals. This suggests an elevated past exposure as well as a persisting exposure occurring at the time of sampling in 2015 and 2016.

Case-Study 2: Cleveland Bay

Cleveland Bay (CLV) is adjacent to the city of Townsville (population >175,000), which is home to a major port and major industries including metal processing and refining (Zn, Cu, Ni, and Co) (Esslemont, 2000). Mean trace-element concentrations in blood of 2014 CLV turtles for Co, Sb, and As were previously identified as elevated in 25% of individuals observed to have elevated Cd. Additional haematological findings suggested poor health in the 2014 CLV population due to a yet to be identified systemic stressor. The correlations described between trace elements and the haematology suggest that turtles from CLV face chronic trace element exposure conditions. As with the previous case-study, the aim here is to utilise the blood-scute trace element ratios to help elucidate temporal trends in a wild population of green turtles. But in the case of CLV turtles, blood and scute concentrations from only three recaptured turtles is currently available from subsequent years thereby limiting our ability to interpret temporal trends. The graphs presented below should therefore be interpreted with caution.

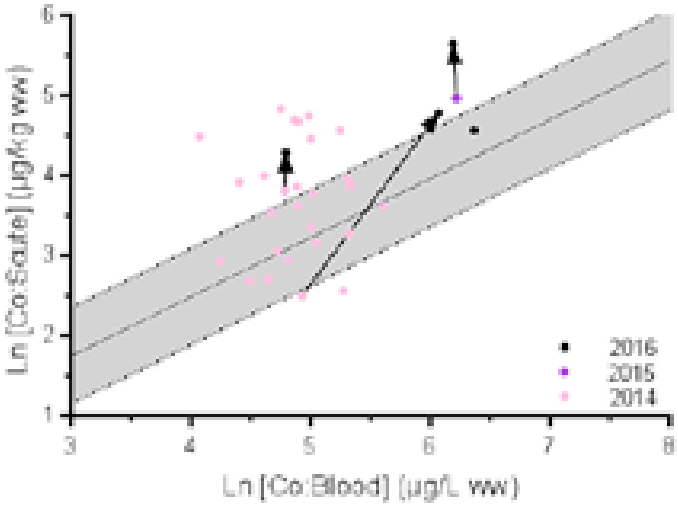

Figure 11: Paired blood and scute cobalt concentrations for Upstart Bay turtles (individuals = circle, colours=year, arrows=recaptures). Regression model (HWK and SHL) shown with 90%prediction intervals (grey area).

Blood Co concentrations in CLV were elevated above reference interval limits for each of the three sampling years. Blood-scute concentrations from the 2014 sampling event indicate that 44% of those turtles are above the RPI_{90}. The recaptured turtles analysed in subsequent years all showed an increase in scute cobalt levels. Each of these lines of evidence point corresponds with our conceptual model expectations for a past elevated exposure.

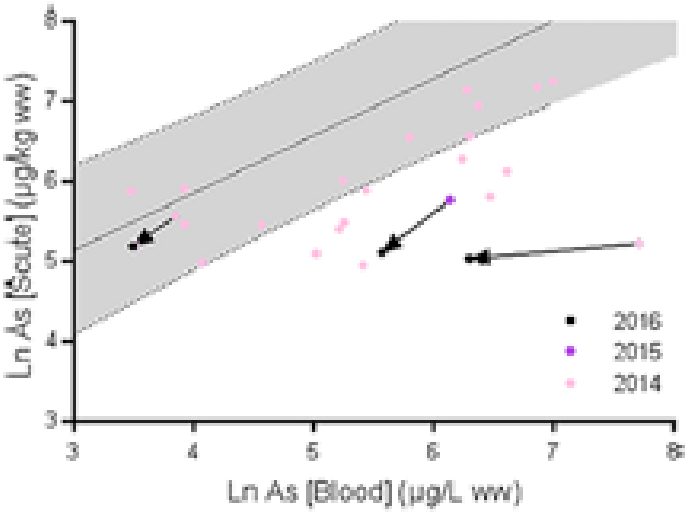

Figure 12: Paired blood and scute arsenic concentrations for Upstart Bay turtles (individuals = circle, colours=year, arrows=recaptures). Regression model (HWK and SHL) shown with 90%prediction intervals (grey area).

In 2014, 40% of CLV turtles had blood As concentrations above the reference interval limits with an equal number of turtles with blood-scute ratios below the RPI_{90} indicating that the exposure was recent. There is insufficient data from subsequent years to interpret a trend with any confidence, but blood levels in subsequent years have fallen back to reference interval levels for the three recaptures. A corresponding increase in scute concentrations of As was not observed for these recaptures. As described in the UPB case-study, concentrations of As can fluctuate with dietary habit and therefore difficult to interpret without corresponding dietary information.

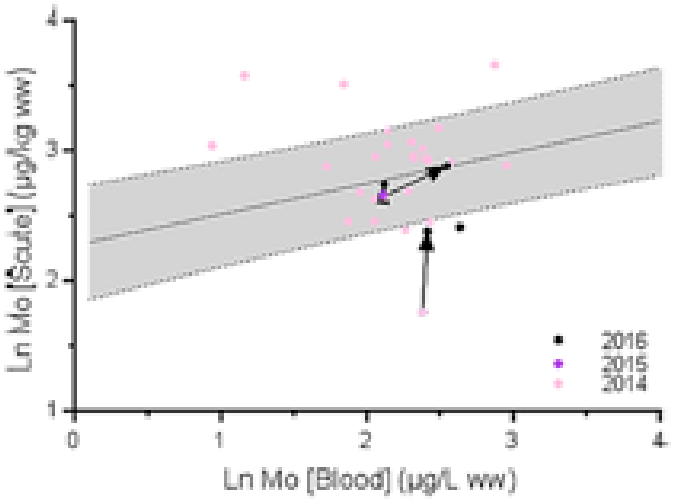

Figure 13: Paired blood and scute molybdenum concentrations for Upstart Bay turtles (individuals = circle, colours=year, arrows=recaptures). Regression model (HWK and SHL) shown with 90%prediction intervals (grey area).

Blood concentrations of Mo for the majority of 2014 CLV turtles fell within the reference intervals. Likewise, the majority of blood-scute concentrations fell within the RPI_{90}. Although one turtle appears well below the RPI_{90} for Mo, the blood concentration is within the Mo reference interval, indicating that this is likely not an elevated exposure of concern. Two of the three recaptured turtles remain within the RPI_{90} 2-years later. Additional recapture data for each of the years past 2014 are needed to provide an better indication of exposure trend.

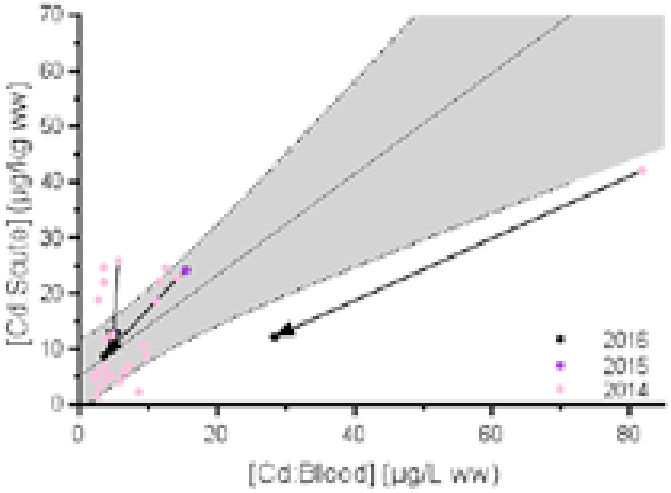

Figure 14: Paired blood and scute cadmium concentrations for Upstart Bay turtles (individuals = circle, colours=year, arrows=recaptures). Regression model (HWK and SHL) shown with 90%prediction intervals (grey area).

Concentrations of Cd in green turtle blood are predicted to be low based on toxicological data from most other vertebrates which demonstrate a very fast blood elimination half-life. Interestingly, we observe a CLV turtle with Cd in blood 8-time above the upper reference interval limit. This individual also had a blood and scute concentration below the RPI_{90} indicating a recent exposure in 2014. Upon being recaptured in 2017, blood and scute concentrations decreased with blood still remaining over 2-times the upper reference interval limit. Given that nearly all other 2014 turtles had blood-scute concentrations within the RPI_{90}. In order to adequately interpret these data, additional analyses would be required for turtles after the 2014 campaign.

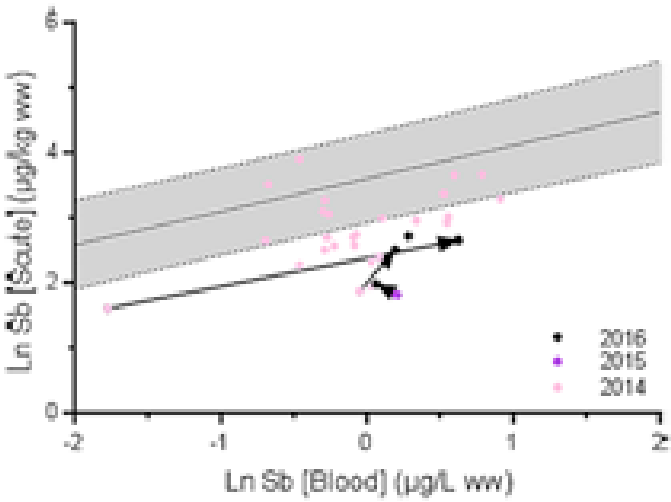

Figure 15: Paired blood and scute antimony concentrations for Upstart Bay turtles (individuals = circle, colours=year, arrows=recaptures). Regression model (HWK and SHL) shown with 90%prediction intervals (grey area).

Nearly half of CLV turtles had Sb blood concentrations above reference interval limits in 2014. Nevertheless, blood-scute concentrations for 40% of 2014 CLV turtles were within the RPI_{90}. The analytical error for Sb was large enough to warrant caution in interpreting the blood-scute results. The most conservative interpretation for 2014 would place all the blood-scute ratios within the RPI_{90}. In combination with the small number of datapoints available for the years after 2014, there is little that can be interpreted in the recaptured results.

While blood only data primarily identified Co, Sb, As, and Cd as being elevated in this population, the blood-scute ratio data has provided additional temporal insights; As and possibly Sb are recent exposures while Co, Mo, and Cd exposures likely occurred in the past. Even though the temporal exposure for Co indicates past exposure, 100% of the 2014 CLV turtles have blood concentrations above its reference interval potentially posing an on-going influence on their health. It is evident that the CLV green turtle population should continue to be monitored for changes in health and trace element accumulation, particularly for Co, Sb, and As. The blood-scute ratios presented here suggest that previous exposure to Cd and Mo should not be ignored simply because their concentrations in blood are mostly within reference interval limits.

Conclusions

Trace element concentrations found in green turtle blood can provide valuable information on recent environmental exposures, whereas scute data can reveal signals of elevated past exposures long after they have been eliminated from other tissues. We show here how these two tissues (available for biomonitoring without sacrificing turtles) can be modelled through simple linear regression to elucidate temporal exposure patterns. Linear regressions between blood and scute were developed for five elements (Co, As, Mo, Sb, and Cd) using two populations of turtles that are presumed to experience stable exposure conditions. These trace element steady-state relationships were used to help visualise blood-scute trace element concentrations in turtles from two coastal sites in Queensland, Australia (UPB and CLV). Our ability to see the changes illustrated in the conceptual model were strongly influenced by blood elimination rates, which can differ for each element. Conversely, elimination from the scute is largely a physical process (shedding of layers) and we would, therefore, expect it indiscriminately affects all elements equally. Although the overall investigation period was too short and the number of recaptured turtles too low to validate the entire timeframe of the conceptual model, each of the case-studies nevertheless allowed us to visualise changes that were generally in-line with the conceptual model predictions. Although recent exposures were evident from a blood relative to reference intervals, numerous examples are of a past exposure, one where rapid elimination from blood had already occurred, was evident through comparing blood-scute ratios against the steady-state regressions. Unlike (Day et al., 2005) who developed this technique for Hg exposure in loggerhead turtles and focused on correlations been recent exposure and proximity to a known Hg source, we demonstrate that past exposure can be identified in free-ranging green turtles without a-priori identification of the contaminants, and where rapid elimination from blood had already occurred. This method of evaluating biomonitoring data for marine turtles is, therefore, a powerful tool for investigating suspected trace element exposures (i.e. mass strandings) as well as a proactive tool for monitoring changes in accumulation in response to forecasted environmental disruptions (i.e. inclement weather, climate change, or coastal development). Green turtle specific toxicokinetic models could expand the number of elements and improve the existing Co, As, Mo, Sb, and Cd relationships between blood and scute. Until they become available, we promote the incorporation of scute sampling in marine turtle biomonitoring programs. Furthermore, we recommend that biomonitoring programs incorporate as many sampling intervals as possible (minimum annually) to capture the elements with rapid eliminations rates, for a duration spanning at least 6 years to account for the full scute shedding cycle.

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Chapter 11

Assessing the toxicity of priority elements using a green turtle cell viability bioassay

11. Assessing the toxicity of priority elements using a green turtle cell viability bioassay

Jason P. van de Merwe[1] and Kimberly Finlayson[1]

[1] School of Environment and Science, and Australian Rivers Institute, Griffith University, Queensland, 4222, Australia

Abstract

The Rivers to Reef to Turtles (RRT) project identified elevated concentrations of antimony (Sb), arsenic (As), cobalt (Co), copper (Cu), manganese (Mn) and molybdenum (Mo) in green turtles and their foraging habitats, and identified an urgent need to examine the toxicity of these elements to green turtles. In the absence of suitable whole animal tests for assessing toxicity in large threatened animals such as sea turtles, cell-based bioassays are increasingly used in animal toxicology, and are considered ethical, high throughput and indicative of the effects at the organism level. Here we used a green turtle skin cell viability bioassay to assess the acute cytotoxicity of Mn, Co, Mo, As, Sb and Cu. Green turtle cells were exposed to these elements and cell viability was measured using resazurin. The order of toxicity (from most to least toxic) was Cu2+ > Co2+ > As_{5+}+ > Mo6+. The toxicity of these elements to green turtle skin cells was 80-10,000 times lower than the toxicity observed for other previously tested elements (e.g. Cr, Hg, Cd). The elements Sb5+ and Mn2+ were not cytotoxic up to their water solubility limits (17.6 µM and 2,776 µM, respectively). Due to species-specificity of cellular responses to contaminant exposure, this green turtle specific cell-based bioassay provides a novel tool for understanding the effects of toxic chemicals in sea turtles. However, more work is required before cell-based bioassays can be used to assess the risks of chemical contamination in sea turtles. For example, the development of sea turtle-specific bioassays that can measure other endpoints such as endocrine disruption, neurotoxicity, genotoxicity and oxidative stress would provide a wider range of effects that can be investigated in sea turtles. In addition, development of toxicokinetic models would provide clearer links between the observed cellular effects and adverse outcomes at the organism level, improving our ability to conduct chemical risk assessments using cell-based bioassay data. Methods for assessing the effects of complex chemical mixtures accumulating in sea turtles (e.g. by assessing the toxicity of blood extracts) would further enhance our ability to assess chemical risk in sea turtles. Overall, this study has provided species-specific information about the toxicity of priority elements in green sea turtles, and a basis for the development of further cell- and computer-based tools for assessing the impacts of chemicals in sea turtles.

Keywords:

Cytotoxicity, in vitro bioassays, *Chelonia mydas*, metals, Queensland

Introduction

The Rivers to Reef to Turtles (RRT) project has identified a number of elements accumulating in green turtles foraging in three coastal areas of northern Queensland - Cleveland Bay, Upstart Bay and Howick Islands (Villa et al. 2017). Elements that were particularly elevated (usually in Upstart Bay) included manganese (Mn), cobalt (Co), molybdenum (Mo), arsenic (As) and antimony (Sb), and are considered priority elements for investigation into potential toxic effects. In addition, copper (Cu) was often elevated in the impacted habitats (Cleveland and Upstart Bays), and is generally toxic to aquatic organisms.

We currently know very little about the effects of elements on sea turtles, due to the ethical and logistical constraints of conducting traditional whole animal toxicity tests on these large, long lived and threatened animals. Cell-based bioassays are increasingly used in human and animal toxicology and are considered high throughput and representative of the effects at the organism level (Lillicrap et al. 2016). The aim of this project was therefore to use a recently developed green turtle skin cell viability bioassay to assess the acute cytotoxicity of the priority elements Mn, Co, Mo, As, Sb and Cu.

Materials and methods

Cell culture establishment

Green turtle (*Chelonia mydas*) primary skin fibroblast cell cultures were established using the explant method described by Webb et al. (2014). Briefly, a small (2 x 2 cm) tissue sample (collected from a live animal) was cut into 1-3 mm pieces in RPMI-1640 media supplemented with 10% FBS, and antibiotics. The tissue pieces were transferred into a 25 cm^2 culture flask and incubated in the same media at 30°C and 5% CO2 until cells began to propagate. Once flasks were ~70% confluent with cells, tissue pieces were removed from the flask, allowing the growth of pure cell cultures, which were passaged each time they became >80% confluent. Cells were cryopreserved at a concentration of 1 x 106 cells/mL in the media described above with 10% dimethyl sulfoxide (DMSO) as a preservative until ready for use in the bioassay.

Cytotoxicity analysis

Cytotoxicity was investigated using the resazurin assay of cell viability. Cells were seeded into a 96-well flat-bottom microtiter plate at ~30,000 cells per well and incubated (30°C, 5% CO_2) for 24 h. In a separate microtiter plate, five inorganic contaminants, antimony (as $KSb(OH)_6$), arsenic (as $Na_2HAsO_4 \cdot 7H_2O$), molybdenum (as $Na_2MoO_4 \cdot 2H_2O$), manganese (as $MnCl_2 \cdot 4H_2O$), cobalt (as $CoCl_2 \cdot 6H_2O$), copper (as $CuCl_2 \cdot 2H_20$), were serially diluted (2-fold, 8-point) in test media. Each concentration was tested in duplicate in each assay run. Stock solutions were prepared in ultrapure water, which was used as a negative control and Triton X-100 was used a positive control. Chromium (as Na_2CrO_4) was included as a control in each trial due to its high reproducibility in this assay. Following the initial 24 h incubation period (for plate seeding), the toxicants (and controls) were added to the cells at various concentrations. Following 24 h of exposure, the media in each well was replaced with 80 µL of media and 20 µL of 0.15 mg/mL resazurin dye in phosphate-buffered saline (PBS), and incubated for a further 24 h. The fluorescence in each well was then measured at λex = 544 nm and λem = 590 nm using a FLUOstar Omega plate reader (BMG Labtech). Each compound was tested on at least two separate occasions. All cells used throughout the experiment were between passage 11 and 13.

Data analysis

In each assay run, the percent of non-viable cells in each contaminant concentration was calculated using the equation: non-viable cells (%) = $(x - C_N)/(C_P - C_N) \times 100$, where x is the mean fluorescence of the sample duplicates, CN is the mean fluorescence of the solvent controls, and C_P is the mean fluorescence of the positive controls. For each contaminant and cell culture, the mean non-viable cells (%) values were plotted against the log concentration of the elements, and the EC_{50} values for each element were calculated in GraphPad Prism 5 using the Hill slope equation.

Results and discussion

The full dose response curves and EC_{50} values are presented in Figure 2 and Table 1, respectively. The order of cytotoxicity (from most to least toxic) was $Cr^{6+} > Cu^{2+} > Co^{2}+ > As^{5+}+ > Mo^{6+}$.

Compound	EC_{50} value (µM)	EC_{50} value (mg/L)
Cr^{6+}	9.8	0.51
Cu^{2+}	373	23.7
Co^{2+}	3,820	225
As^{5+}	7,070	530
Mo^{6+}	95,800	9,190

Table 1: EC_{50} concentrations for cytotoxicity of five inorganic compounds to green turtle primary skin fibroblasts following 24 h exposure.

Due to low solubility of $KSb(OH)_6$ in water, the maximum concentration of Sb5+ that could be tested was 17.6 µM, and was not cytotoxic up to this concentration. Similarly, Mn^{2+} was tested at the limits of solubility in water (2,776 µM), and was not cytotoxic up to this concentration.

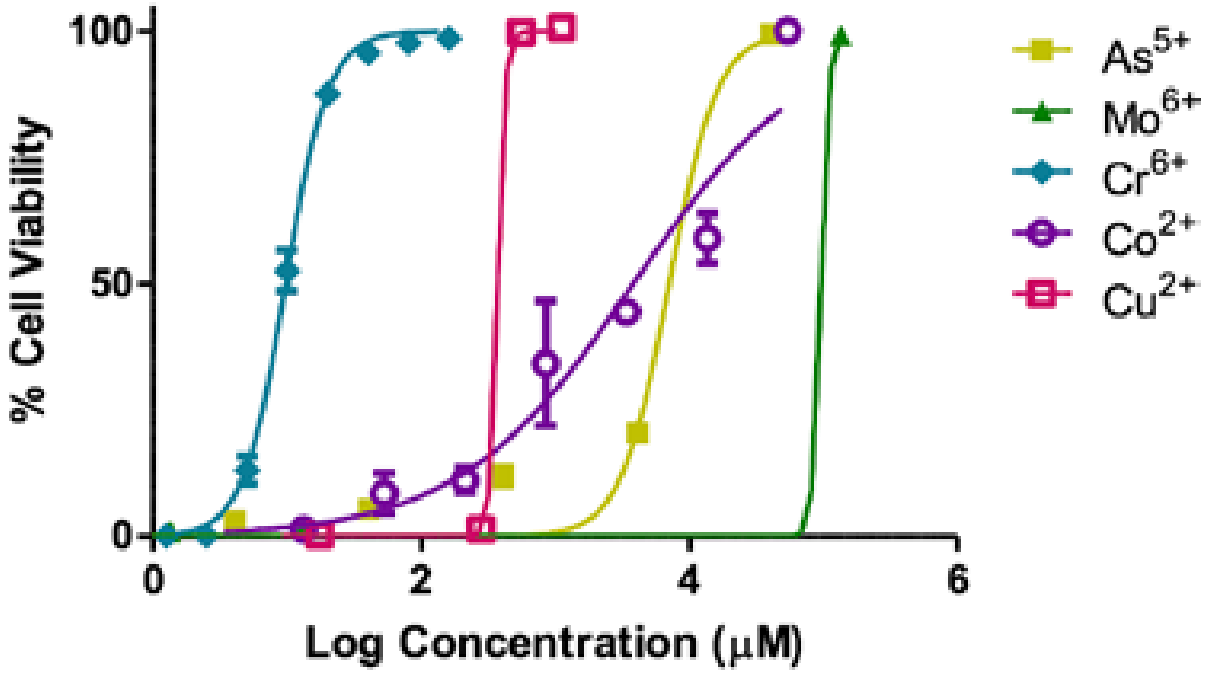

Figure 2: Dose-response curves for five elements in green turtle primary skin fibroblasts following 24 h exposure. Cell viability was measured using the resazurin assay.

The elements identified within the RRT to be priority contaminants were not very cytotoxic to green turtle skin cells. While assessment of Sb and Mn were hampered by solubility issues, Co, As and Mo, were ~400 to 10,000 times less toxic than the reference compound (Cr). In addition, these three elements were ~80 to 6,000 times less toxic than cadmium and mercury, previously assessed in this green turtle skin cell viability bioassay (Finlayson, unpublished data).

This is the first time in Australia that a sea turtle-specific cell-based bioassay has been developed for assessing toxicity in sea turtles. Given that cells from different species can respond differently to the same contaminants (e.g. Tong et al. 2016), the development of sea turtle-specific bioassays is a significant advancement for assessing toxicity in sea turtles. However, there is still considerable work to do before these types of assays and data can be used for assessing the risks of chemicals that are accumulating in sea turtles. Firstly, additional bioassays measuring endpoints such as endocrine disruption, neurotoxicity, genotoxicity and oxidative stress would provide a wider range of effects that could be assessed. The green turtle cells and cell viability bioassay presented here can provide the basis for developing these additional bioassays. In addition, the development of physiologically based toxicokinetic (PBTK) models, which incorporate in vivo processes (absorption, distribution, metabolism and elimination), can be used to predict internal tissue accumulation of contaminants under different exposure scenarios. These PBTK models can therefore provide clearer links between the measured in vitro effects and adverse outcomes at the organism level (Wetmore et al. 2013), providing improved ability to conduct chemical risk assessments using cell-based bioassay data.

It is also important to note that each of these elements has been assessed in isolation, and the potential additive or synergistic effects of contaminant mixtures in sea turtles remain untested. Due to their high throughput nature, cell-based bioassays provide a useful tool for assessing large numbers of contaminants and mixtures rapidly. In addition, cell-based bioassays can also be adapted to testing the toxicity of chemical mixtures accumulating in sea turtles (e.g. by assessing the toxicity of blood extracts), providing the opportunity for further advancements in the field of sea turtle toxicology.

Conclusions

Overall, this study indicates that the priority elements identified by the RRT project (e.g., Co, As and Mo) have low cytotoxicity to green turtle cells. Future research focussing on the development of in vitro bioassays that measure additional endpoints, inclusion of toxicokinetic modelling, and assessments of contaminant mixtures, will provide a more comprehensive set of tools for assessing the effects of these elements (and other priority contaminants) in sea turtles.

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