1 TRACE ELEMENT REFERENCE INTERVALS IN THE BLOOD OF HEALTHY GREEN SEA
2 TURTLES TO EVALUATE EXPOSURE OF COASTAL POPULATIONS

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19 **Capsule:**

20 Blood reference intervals (20 elements) were calculated for healthy green turtles from a remote
21 region and were applied to 3 coastal groups whose clinical health markers correlated with elevated
22 blood metals.

23 **Keywords**

24 Metals; Trace elements; Marine Turtles; Reference Intervals; Great Barrier Reef

25

26 Abstract

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

47

1. Introduction

Tidal and subtidal seagrass meadows and algal turfs are important foraging grounds for maturing and adult green sea turtles (Limpus, 2008). Some of these habitats include shallow coastal embayments close to shore, which are subject to natural and diverse anthropogenic impacts (e.g. floods, agricultural and industrial runoff, urbanisation, coastal dredging) that can elevate concentrations of a wide range of pollutants, including metals, metalloids and their compounds. In polluted areas, trace elements are typically enriched in surrounding water and sediments, as well as in the seagrass and algae (Talavera-Saenz et al., 2007) that make up the bulk of the diet of neritic green turtles (*Chelonia mydas*). Having high fidelity to relatively small (few km²) foraging areas (Hazel et al., 2013; Shimada et al., 2016, Submitted) over several decades (Chaloupka et al., 2008), resident coastal sea turtles can experience long-term exposure to contaminant impacted areas. Both chronic and acute exposures may pose significant risks to their health and survival (Camacho et al., 2013; da Silva et al., 2016; Day et al., 2010; Faust et al., 2014; Flint et al., 2014; Labrada-Martagon et al., 2011). Their life-history traits are particularly relevant given frequent observations that chronic diseases (e.g. fibropapilloma) are more prevalent in green turtles from inshore, shallow, and polluted waters (Foley et al., 2005; Van Houtan et al., 2010). There is also evidence suggesting metal exposure is involved in the aetiology and development of fibropapillomatosis, impaired immune functions (da Silva et al., 2016; Grillitsch and Schiesari, 2010), and as a contributing factor in mass strandings (Flint et al., 2014).

The composition, concentrations, and bioaccessibility of trace elements in coastal sediments and aquatic plants can vary considerably on a regional scale depending on local sources and environmental conditions. Moreover, species-specific toxicokinetic processes (absorption, distribution, metabolism, and elimination), which are poorly understood for reptiles in general, govern the trace element differences among various tissues. Only a few descriptions of these processes exist for freshwater turtle species, and even then only for Cd, Se, and Hg (Blanvillain et al.,

2007; Dyc et al., 2016; Guirlet and Das, 2012; Schwenter, 2007). Since internal tissue concentrations reflect the integration of all trace element exposure pathways, they are more informative as biomonitoring matrices than external ones (i.e. diet, air, sediment). Given the ethical considerations of collecting the ideal biomonitoring tissues (i.e. liver, kidney, and muscle) from free-ranging protected species, blood, which acts as a transport medium and reservoir for all endogenous and exogenous chemicals in the body, can be collected using minimally invasive methods (Faust et al., 2014). At constant exposure, trace element concentrations in blood are correlated to those in organs (Grillitsch and Schiesari, 2010). With blood elimination rates of many trace elements generally on the order of weeks to months (depending on the element and its speciation), blood is considered a suitable matrix for evaluating relatively recent exposure (Angerer et al., 2007; Burger et al., 2005), 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 (Blanvillain et al., 2007; Day et al., 2010; Guirlet and Das, 2012).

Several studies have investigated trace element concentrations in blood from free-ranging green sea turtles around the world (Camacho et al., 2014a; Komoroske et al., 2011; Ley-Quin6nez et al., 2013; McFadden et al., 2014; Suzuki et al., 2012; van de Merwe et al., 2010). These data, however, are generally from regions suspected to be or known to be, environmentally impacted with ongoing or legacy mining and industrial activities, making them unsuitable as control populations. In addition, much of the data originates from stranded, moribund or recovering individuals. Since poor health and nutrition can alter toxicokinetic processes, trace element concentrations in the blood can be considerably altered in compromised individuals (Camacho et al., 2014b). In the absence of a physiological healthy or baseline range in trace elements for sea turtles, a major gap exists in our ability to interpret reported element concentrations in context to adverse health risks. Some blood and other tissue-based effect concentrations have been derived for immunotoxicity associated with Hg exposure (Day et al., 2010) in an exposed group of loggerhead turtles (*Caretta caretta*), and fibropapillomatosis associated with Fe, Cu and Pb exposure (da Silva et al., 2016) for juvenile green

turtles. However, the paucity of any tissue-based effect data and the ever-changing composition of elements to which these species are potentially exposed to further adds to the difficulty in extrapolating baselines from exposed populations. Alternatively, blood from healthy turtles that forage in areas distant to point sources allows us to determine the likely concentration limits of non-essential elements associated with low exposure and functionally healthy concentration ranges for essential elements (Aggett et al., 2015). The heterogeneous global distribution of elements in the environment and the ability of anthropogenically enriched elements to disperse via long-range transport mechanisms (water, land, air) makes it improbable that a truly pristine site might exist; nonetheless, offshore and distant foraging grounds within marine zones protected from anthropogenic activity offer us the closest alternative to sample healthy and minimally exposed turtles.

Blood-based reference intervals (RIs) are standard tools for interpreting health diagnostics in humans and animals. They commonly include biomarkers of metabolic stress and disease as well as free-ion trace element biochemistry and haematology and have recently been described for Queensland green turtles by Flint et al. (2014). While green turtle RIs for health now exist, the RI approach has yet to be employed in determining population level trace element exposure. In general, RIs define an analyte upper and lower 95% prediction intervals in an asymptomatic population. Methodologies to derive RIs are well established (CLSI-IFCC, 2010; Friedrichs et al., 2012), with validated statistical approaches for handling complex data features such as excessive skew (common for many blood analytes), outliers, and small sample sizes (Horn and Pesce, 2003). The ability to develop robust RIs using between 20 and 100 carefully selected individuals, makes these approaches ideal for describing trace element baselines in difficult to catch, free-ranging, and protected species such as green turtles.

Thus, our objective for this study was to calculate blood-based biomonitoring RIs for 20 trace elements (11 essential and 9 non-essential), using a reference group (baseline cohort) of clinically

124 healthy green turtles foraging in a northern Great Barrier Reef habitat that is located off-shore and
125 presumably distant to point or diffuse anthropogenic sources. Those 20 exposure baselines were
126 then used to identify and evaluate elevated trace element exposure in the blood of three different
127 turtle populations foraging in three different coastal habitats, which were categorised based on their
128 distinct anthropogenic influences; agricultural, urban, and military activities. Finally, clinical health
129 data (biochemistry and haematology) were assessed for each coastal turtle to identify any
130 associations between elevated exposure and adverse health.

2. Materials and Methods

2.1. Turtle populations

2.1.1. *Baseline cohort*

For the development of the trace element exposure RIs, we analysed whole blood from n=49 subadult green turtles from a population foraging at the Howick Group of Islands (HWK), a collection of sheltered mid-shelf, uninhabited reefs which lie within the remote northern Great Barrier Reef Marine Park (-14.416695°S, 144.880484 °E; Figure 1). HWK is located approximately 30 km offshore from the Cape York region catchment, a remote coastal wetland with low pressures from nutrient, sediment, and pesticide loads, or water regime changes and habitat alterations (Senior et al., 2015). Therefore, this remote and offshore sea turtle foraging ground was predicted to be a suitable surrogate for a site free from anthropogenic contaminant point sources.

2.1.2. *Three coastal populations*

To evaluate trace element exposure of green turtles foraging near shore, whole blood was collected from subadults foraging at three different coastal sites (Figure 1): Cleveland Bay (CLV; n=28; -19.235428 °S, 146.938284 °E) adjacent to the city of Townsville (population >175,000) with metal processing (Zn, Cu, Ni, and Co) and other heavy industries, and home to a major port (Esslemont, 2000), Upstart Bay (UPB; n=35; -19.767554 °S, 147.702955 °E), a rural coastal area approximately 100 km south of CLV and 50 km north of an international coal terminal, with a system of mostly ephemeral creeks receiving wet-season discharges from one of Queensland's largest catchments (130,000 km²) dominated by intensive agriculture, grazing and mostly legacy mining (Bartley et al., 2014), and Triangular Island in Shoalwater Bay (SHL; n=19; -22.370076 °S, 150.515198 °E), approximately 400 km south of UPB and a military training ground for intermittent underwater demolition and explosive ordinance disposal. Triangular Island is situated within a 4,545 km² Australian Defence 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 (Limpus et al., 2005).

2.2. Sample collection

Green turtles were captured from June to October 2014 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), and laparoscopy), in accordance with our ethics approval (AEC Approval Number: NRCET/147/14/APA/WWF). Additional health metrics were calculated using body condition index ($BCI = \text{body mass (kg)} / \text{straight carapace length (SCL; cm}^3) \times 10,000$; (Bjorndal et al., 2000)) and ranked according to Flint et al. (2009). The Australian convention is to measure and report curved carapace length (CCL) rather than SCL, thus, in order to calculate BCI, CCL was converted to SCL using parameters defined for immature turtles in Limpus et al. (1994). We selected subadult turtles, several years after their pelagic phase and prior to the sexually mature adult breeding and migration phase, as this is the most suitable age class to evaluate habitat-dependent trace elements exposure. For the baseline cohort, turtle sex and maturity were confirmed via laparoscopic examination of gonadal development (Limpus et al., 2005). For the other sites, sexual maturity was estimated using CCL ranges that have previously been shown to predominantly include larger sexually immature (subadult) individuals (Limpus et al., 1994; Limpus et al., 2005). The CCL criteria for subadults was as follows: females ≥ 60 cm to ≤ 100 cm CCL and males ≥ 63 cm to ≤ 95 cm CCL based on Limpus and Chaloupka (1997), and when sex was not determined ≥ 65 cm to ≤ 90 cm CCL according to Limpus (2008). Location, sampling IDs, dates, morphometric data, and BCI are summarised in Figure 1 and detailed for all individual turtles in Table SI 1 of the Supporting Information (SI).

Blood was collected according to Owens and Ruiz (1980) and prepared as previously described in (Villa et al., 2015) with modifications. Briefly, whole blood was collected from the dorsal cervical sinus using disposable 20-50 mL 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, polyethylene tubes (1,000 IU/ml whole blood; DBL™ Hospira, Australia). For clinical blood biochemistry, an additional 5 ml of whole blood was transferred into evacuated lithium heparinized tubes (BD Vacutainer®; Becton, Dickinson and Co., NJ, USA) and centrifuged (total centrifugal force of $2.5 \times 10^6 \text{ g} \cdot \text{s}$) for plasma separation as soon as practical in the field (typically within 2-6 hours after collection). Samples were preserved on ice during transport to the laboratory, and stored frozen (-18 °C) until further processing. For haematology, whole blood smears were prepared and air-dried at the time of sampling (Flint et al., 2010) and stored at room temperature pending analysis.

2.3. Trace-element Analysis

Sample preparation, acid digestion, and quality control followed previously validated methods for green turtle whole blood samples (Villa et al., 2015). Briefly, blood was manually homogenised prior to subsampling using a glass tissue homogenizer and chiller unit (Sartorius Potter S; Sartorius, Germany). 1 mL concentrated HNO₃ (65% v/v, Suprapur; Merck Pty Ltd. Australia) was added to 1 mL whole blood in 10 mL polypropylene tubes. The tubes were heated in a heating block until fully digested, with subsequent addition of 0.5 mL H₂O₂ (30% v/v, Suprapur; Merck Pty Ltd. Australia). Method blanks consisting of Milli-Q water (n=6 per batch), and certified reference materials (CRMs) of human whole blood (Serionorm 1 and 2, Sero, Norway) as well as fortified lake water (TM 24.3; National Research Council, Canada) were digested alongside the turtle samples. All digests were diluted with Milli-Q water to a final volume of 10 mL, and multi-element internal standards were added; Li, Sc, Ge, Y, Rh, In, Au, and Bi (Choice Analytical, Australia).

Fully quantitative trace element analyses were carried out using an Agilent Technologies 7500cs inductively coupled plasma mass spectrometer (ICP-MS) system equipped with an Octopole Reaction System (Agilent Technologies, USA). Full quant calibration was performed by serially diluting a multi-element standard; n=26: Ag, Al, As, Ba, Be, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, Pb, Sb, Se, Tl, V, Zn, Th, and U (Environmental Calibration Standard; Agilent Technologies, 5183-4688); and Ti (Agilent Technologies, 5183-46885190-8320) in an acid concentration equivalent to the sample preparation method. Method blanks were subtracted from sample raw data. Calibration, blank and sample data were evaluated using Agilent's ChemStation software. Method detection limits (MDL) are defined as 3 times the standard deviation of the blanks and are provided in **Error! Reference source not found.** 4. Blood samples were processed in batches based on region, soon after collection, resulting in differing MDLs between the sites for the same elements. Two of the calibrated elements, Al and Ag, had to be excluded from further analysis due to elevated blank concentrations. All concentrations are reported as µg/L of sample wet weight (ww).

2.4. Clinical Pathology

For blood biochemistry, plasma samples were warmed to room temperature and run on a calibrated (using coefficients of variance) autoanalyser (Olympus AU400, Beckman Coulter Inc.) in accordance with the testing methodology outlined by the manufacturer. The following variables were measured: albumin (ALB; g/L), alkaline phosphatase (ALP; U/L), aspartate transaminase (AST; U/L), creatinine (CREAT; mmol/L), glucose (GLUC; mmol/L), total protein (TP; g/L), urea (UREA; mmol/L), uric acid (UA; µmol/L), globulin (GLOB; g/L), total bilirubin (Tbil; µmol/L), creatine kinase (CK; U/L), gamma-glutamyl transpeptidase (GGT; U/L), lactic dehydrogenase (LDH; U/L), free ion concentrations of sodium (Na; mmol/L), potassium (K; mmol/L), calcium (Ca; mmol/L), magnesium (Mg; mmol/L), and chloride (Cl; mmol/L), as well as total inorganic phosphorus (PO₄; mmol/L). It is worth noting that free-ion concentrations for clinical health interpretation differ from total element concentration analysis via ICP-MS for trace element exposure determination. Clinical results for each turtle were

compared against biochemical and haematological reference intervals (BHRIs) for this species and age class calculated by Flint et al. (2010). Clinical health was evaluated based on clinical parameters being outside of the BHRIs and how far above or below that range they sat.

For haematology, whole blood smears were stained using haematoxylin and eosin stain, sealed with a coverslip and read manually. Haematological measures (of leukocytes) were calculated and used to estimate total white cell counts (Flint et al., 2010).

2.5. Reference Intervals and Statistical Analysis

For the development of RIs, the distribution of trace element concentrations in the HWK baseline cohort was assessed for normality using histograms and boxplots and evaluated statistically using the Anderson-Darling test. Box-Cox transformations were applied as needed to achieve normality. Outliers were identified visually and through ROUT (False Discovery Rate=1%), Tukey methods and the Dixon-Reed test. Data visualisation and ROUT outlier detection were performed using GraphPad Prism, version 5.00 for Windows (GraphPad Software, San Diego California, USA). Data transformation, outlier detection by Tukey and Dixon-Reed, and determination of RIs, including the bootstrapped upper 90% confidence interval (CI) associated with the upper and lower RI limits (RI-LCI and RI-UCI, respectively), were performed using Reference Value Advisor, version 2.1 (Geffré et al., 2011). In order to investigate whether sex-specific RIs were needed, log-normalized concentrations of each element were compared by sex using Independent Sample t-test or Welch's t-test, based on the Brown-Forsythe test of equal variance using IBM SPSS Statistics for Windows, Version 22.0 (Armonk, NY). Significance was defined as $p \leq 0.05$ for all of the above tests.

Associations between clinical measures (haematology/blood biochemistry) and trace element concentrations were evaluated through Spearman Rank Correlation (r_s) using XLSTAT (version 2015.6.01.25539, Addinsoft, NY). For this, all trace element concentrations <MDL were removed, and significance was defined as $p \leq 0.0001$.

A Principal Component Analysis (PCA) was undertaken to identify priority elements for each of the nearshore populations relative to the HWK cohort. PCA factors, loadings, and biplots were generated using Multibase for Windows (Numerical Dynamics, Japan).

3. Results and Discussion

3.1. Baseline Cohort Reference Intervals and Health Assessment

For all HWK turtles, health metrics (e.g. BCI, visual inspection) were as expected for a healthy population, and all clinical indicators (biochemistry and haematology) fell within or only slightly outside the respective BHRIs for all individuals (Table 2; individual data in **Error! Reference source not found.** 2). The lower observed concentrations of CK and UA were not low enough to be indicative of a renal compromise. Similarly, the marginally low concentrations of LDH and PO₄ were considered clinically insignificant in this case (Campbell, 2005). Overall, the clinical data indicated that this baseline cohort was clinically healthy. Reference Intervals (RIs) could be derived for 20 trace elements in green turtle whole blood (Table 1); four other elements (Be, Tl, Th, U) were analysed but their concentrations were <MDL for >30% of individuals and are therefore summarised using only basic statistical descriptors (results for all individual turtles are provided in **Error! Reference source not found.**4). Precision for those 20 elements, calculated as %RSD of each set of CRM results (n=4 runs), ranged from 2% to 5% while CRM recoveries ranged from 87% to 114%. To our knowledge, these are the first baseline RIs describing trace element exposure in sea turtles. Inter-individual variation was low for essential elements (coefficient of variation (CV) 9%-76%; mean=29%). Given their critical role in many biological functions, essential elements are generally maintained within relatively narrow optimal ranges that are governed by physiological processes. They are therefore expected to show little variation within individuals of the same species unless poor health or elevated exposure affects the body's ability to maintain homeostasis (Aggett et al., 2015). In contrast, non-essential elements (e.g. Cd, As, Hg, Pb) are not under homeostatic control and their

clearance can be slower than their intake rates, resulting in bioaccumulation in green turtles. Considering the HWK turtles forage long-term in a remote offshore environment with no known anthropogenic sources, exposure to non-essential elements (both from natural sources and via long-range transport mechanisms and atmospheric deposition) was expected to be rather low in the baseline cohort. This was reflected in the relatively low inter-individual variation of non-essential elements (mean CVs=52%) and concentrations which are among the lowest reported in green turtle blood (Camacho et al., 2014a; Komoroske et al., 2011; Labrada-Martagon et al., 2011; McFadden et al., 2014). Therefore, we consider the RIs to be within the optimum ranges of essential elements for healthy green turtles, and a natural baseline range for non-essential elements. This is further corroborated through the similarity in essential element concentrations reported from other offshore populations such the central pacific Palmyra Atoll and Boa Vista, Africa (Camacho et al., 2014a; McFadden et al., 2014).

The clinical reference interval approach, described at length elsewhere (CLSI-IFCC, 2010; Friedrichs et al., 2012; Horn and Pesce, 2003), provides robust estimates of a population's upper and lower limits even for heavily skewed data, which is often the case for trace element concentrations in blood. For the present study, non-Gaussian distributions of V, Cr, Fe, Co, Ni, As, Mo, Cd, Sb, Ba, and Pb were successfully transformed using the Box-Cox family of transformations, facilitating reliable estimates of the 95% prediction intervals (via Robust method) and the associated 90% confidence limits (via non-parametric bootstrapping). For Na, Mg, K, Ca, Ti, Mn, Cu, Se, and Zn, the distribution of untransformed data was sufficiently normal. Outliers were identified for V (44.82 µg/L and 105 µg/L) and Cr (14 µg/L); these values were excluded. We found no statistically significant differences in trace element concentrations between sexes in the subadult turtles. Our results are consistent with studies that included larger proportions of subadults (McFadden et al., 2014). After recruitment to their neritic habitats, juveniles (of both sexes) have been reported to forage more frequently in shallower protected zones compared to more mature age classes of the same population (Lopez-Mendilaharsu et al., 2005). The potential differences in foraging behaviour, as well as remaining and

variable signatures from exposure during the pelagic phase may explain the often greater variability of trace elements in juveniles; this variability is compounded when data are averaged across mixed age classes (Camacho et al., 2014a; Komoroske et al., 2011; Labrada-Martagon et al., 2011; McFadden et al., 2014). Although green turtles have a complex life cycle, which in the adult phase includes long-distance migrations, metabolic shifts and maternal trace element transfer associated with breeding, most of the key inter-individual factors known to affect trace element concentrations in organisms can be minimised by selecting subadults. Due to their long-term (up to ~35 years) localised foraging (within few km²), and high site fidelity, several years after the pelagic omnivorous life stage and prior to adult migration and breeding, subadults are expected to be the most suitable age class to evaluate foraging habitat-dependent differences in trace element exposure.

3.2. Coastal Populations Exposure Evaluation and Health Assessment

For the present study, we used our newly calculated RIs to identify whether green turtle populations from coastal habitats may experience different exposure to trace elements. Given that the three coastal sites are dominated by different types of land-use and anthropogenic influences (CLV=urban; UPB=agricultural; SHL=military) and are flushed to different extents, we expected that their blood profiles will differ for some elements, particularly non-essential elements such as As, Cd, and Pb due to fertilizer and recycled waste soil applications (de Vries and McLaughlin, 2013) and Pb, Cu, Zn, Ni, and Cr as a result of industrial pollution and urban waste discharges (Esslemont, 2000). Patterns of habitat-specific elevated concentrations were clearly apparent relative to the RIs (Figure 2). While many trace elements were above RIs in only a few individuals (e.g. Cu, V, Cd and Se particularly at CLV; Figure SI 1), some elements clearly exceeded the upper 90% confidence interval of the RI (RI-UCI) for a large proportion (>60%) of one to all three coastal subpopulations. These were the essential elements Co, Mo, Mn, Mg and Na, and the non-essential elements As, Sb, and Pb. Mean Co concentrations were particularly elevated in UPB turtles (mean: 470±190 µg/L; range 130-840 µg/L; mean-max 14-25 times >RI-UCI, respectively; Table 3). Similarly, Sb, Mo, Mn and Na showed the

greatest concentrations in UPB turtles (mean-max 3.6-9.3, 1.8-4.9, 1.8-5.4, and 1.1-1.4 times >RI-UCI, respectively), while SHL turtles contained the greatest concentrations of Pb and Mg (mean-max 1.3-2.4 and 1.3-1.7 times >RI-UCI, respectively), and CLV turtles contained elevated As concentrations (mean-max 1.6-8.1 times >RI-UCI).

For the present study, we used PCA to better illustrate the habitat-specific differences in trace element patterns relative to the HWK cohort and to identify which elements are governing these differences. The original dataset (all 24 elements from all 132 turtles) was reduced to three principal components (PCs) which explained 77% of the total variance. The correlation of each element to the three PCs (i.e. its factor loading score) is provided in **Error! Reference source not found.3**; for interpretative purposes a loading score with an absolute value ≥ 0.4 was considered a strong correlation. The first PC explained 49% of the total variance across all sites and was strongly correlated with Co and Sb (and to a lesser degree also Mo). The second and third PCs accounted for the remaining 28% of the variance (PC₂ 17% and PC₃ 11%), and were strongly correlated with Pb and V (PC₂) and As and Ni (PC₃). PCA biplots separated turtles away from the HWK cohort (centred at plot origin) into three distinct groups, whereby each group coincided with a particular coastal foraging site (Figure 3). The furthest separation along PC₁ (elevated Co, Sb, and Mo) was observed for turtles from UPB which forage near the large agricultural catchment with legacy mining and adjacent coal terminal activities. SHL turtles, foraging near the military training area, separated largely along PC₂ (elevated Pb and V), and CLV turtles, which forage closest to the urban centre with port activities, metal refineries and other heavy industries, separated most along PC₃ (elevated As and low Ni). Mn, Mg and Na, although >RI-UCI for >60% of the population at all three sites, had low PCA loading factors and significant ($p < 0.0001$) correlations with several other trace elements, including Co and Sb (**Error! Reference source not found.6**). On the other hand, despite relatively high loading factors, V and Ni are less likely relevant as they were within the RIs for >95% of individuals at SHL and CLV. Thus, RI benchmarking and the PCA together suggest that the majority of individuals from coastal foraging grounds experience elevated exposure to particular elements specific to their habitat,

governed mainly by sources of Co, Sb and Mo (for UPB), Pb (for SHL) and As (for CLV). Apart from Pb and As, many of these elements (particularly Co, Sb, and Mo) are rarely analysed in biomonitoring studies, notwithstanding their numerous sources and hazard potential. Those studies that have documented impacts of trace elements on marine turtle health are dominated by Hg, Se, As, and Pb (Day et al., 2007; Lam et al., 2006; Perrault et al., 2013; van de Merwe et al., 2009). Our results emphasise the importance of broad-based screening to identify regional specific priorities, rather than targeting a common set of few *a priori* selected trace elements (Villa et al., 2015). This is particularly relevant for biomonitoring in areas with diverse and poorly characterised sources, and in species such as sea turtles for which the potential impacts of elevated exposure to most trace elements is still virtually unknown.

For haematology and blood biochemistry, plasma was analysed from the baseline HWK cohort (n=31) and from two of the three coastal sites: CLV (n=26) and UPB (n=32). No plasma samples were available for SHL. All clinical measures were compared and interpreted against previously established clinical biochemical and haematological BHRIs established in green turtles by Flint et al. (2010). Clinical data for some animals foraging at the UPB and CLV coastal sites suggests that their health was compromised (Table 2 and **Error! Reference source not found. 2**). Blood biochemistry showed that a portion of the UPB population of turtles had moderately elevated TBil and ALP (16 and 6%, respectively), relative to the BHRIs, which in reptiles may be indicative of liver dysfunction (Hernandez-Divers and Cooper, 2005). A portion of the CLV population (12% and 8%) had moderately elevated CK concentrations and abnormally elevated LDH, which may be indicative of muscle catabolism and damage. Considering all clinical data, there was a suggestion of an insipid systemic stressor active at a population level. Although no clinical data were available for SHL, previous studies at a nearby foraging ground (~30 km from the present study) indicated some disease processes (affecting cardiovascular, renal, hepatic and gastrointestinal systems) may also be occurring in turtles from this region, however, with much lower prevalence (1%) in the subadults compared to other age classes (Flint et al., 2010). The majority of anomalous haematologic findings

in the present study (Table 2) were low-grade heterophilia and monocytosis with UPB being the most impacted. Heterophilia is a result of phagocytosis and acute inflammatory responses whereas monocytosis occurs in reptiles in response to phagocytosis, debris, and bacteria (Campbell, 2005). This population also demonstrated the greatest total white cell counts (TWCC; a commonly used marker for inflammation), with 44% of the examined turtles showing signs of moderate elevation. Turtles from CLV also had some elevations in those two white cell types but at lower prevalence (19%), similar to that reported for green turtles from other urbanised/industrial habitats (Flint et al., 2014; Foley et al., 2005).

Overall, these clinical data indicate that turtles at both coastal sites, and particularly at UPB, have an active low-grade inflammatory response, and show signs of systemic disease or generalised processes associated with liver dysfunction (UPB) and muscle inflammation (CLV). The UPB population had experienced an unusual mass mortality event 2 years prior to sampling with turtles showing neurological symptoms, followed by recurring, albeit more isolated events. Although acute toxin exposure was initially suspected, any causative agent(s) remained unidentified. We observed associations between blood concentrations of trace elements and several clinical measures (**Error! Reference source not found.**5). Of particular interest is Co, which showed a significant and moderately strong correlations with clinical markers of inflammation (TWCC; r_s 0.41; $p < 0.0001$). Clinical markers of liver dysfunction were also significantly and moderately correlated with Co, Sb, and Mn (ALP; r_s 0.40 (Co), 0.44 (Sb) and 0.51 (Mn) and Tbil; r_s 0.43 (Co) and 0.61 (Mn); $p < 0.0001$). These relationships between elevated Co, Sb, Mn exposure with 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.

It is noteworthy that the elevated Co concentrations we observed, particularly at UPB, are to our knowledge among the greatest observed in sea turtles or any vertebrate species globally. This

includes elevated concentrations (mean±standard error) reported previously from debilitated green turtles that died in a rehabilitation centre in southeast Queensland (36 ± 6.7 µg/L; range: 3.2–88 µg/L; n=16 (van de Merwe et al., 2010); and from moribund specimens from an industrial port in central Queensland (150 ± 15 range: 28–440 µg/L; n=40; (Gaus et al., 2012). Information on Co toxicokinetics or toxicity thresholds are, however, lacking for sea turtles; the only available data identified for other reptiles (box turtles; *Terrapene ornata*) suggest that acute effects are elicited in reptiles at similar doses (in the same order of magnitude) compared to most other vertebrate species (Faroon et al., 2004). Across different vertebrates, acute adverse effects (including damage to endocrine, haematological, cardiovascular, and neurological systems) occur when blood concentrations exceed approximately 300 µg/L (Tvermoes et al., 2015). Elevated serum Co concentrations are toxic to mammalian heart and liver, with increases in AST, ALT and CK observed in mice (Liu et al., 2010). The concentrations of Co in most UPB turtles were above these effect thresholds in other vertebrates (Table 3; **Error! Reference source not found.**4). 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-1α), and oxidative stress through the generation of reactive oxygen species (Permenter et al., 2013; Pulido and Parrish, 2003). Recent investigations of green sea turtle fibropapillomatosis implicated metal induced oxidative stress (Cu, Pb, and Fe possibly via reactive oxygen species-mediated viral infections) in the aetiology of this disease (da Silva et al., 2016). 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.

It is also noteworthy that the elevated Pb concentrations observed in SHL are well below the concentrations associated with high incidence of green turtle fibropapillomatosis (approx. 1,400 µg/L; (da Silva et al., 2016)), although information on Pb effect concentrations for other endpoints is lacking for reptiles in general. In contrast, even though only two CLV turtles exceeded Cu concentrations associated with increased risk of fibropapillomatosis (approx. 1,300 µg/L blood;(da

Silva et al., 2016)), we did not observe overt signs of this disease in the respective CLV individuals, although the development of fibropapillomatosis likely has a long latency (Jones et al., 2016).

3.3. Anthropogenic and Natural Trace Element Sources

The observed habitat-specific differences in trace element exposure of coastal turtle populations suggests localised influences; however, the source(s) are unknown and could be related to geogenic or anthropogenic origins or processes. For the present study, the distinct anthropogenic activities and spatially close (<100 km) locations of CLV (urban) and UPB (agricultural) provided an opportunity to explore this further. Historical reports of the region reveal similar mineral properties in the local geology across these two sites (Bainbridge et al., 2016; Levingston, 1981), which was confirmed with Australian Government maps showing both sites overlay similar geological formations (Figure SI 2). Previous multi-element sediment analysis performed for these regions (Doherty, 2001) also provides evidence of similar historical abundances of trace elements at these two sites. Therefore, the natural abundance of trace elements is likely similar at both sites. Independent-sample t-tests identified, however, significant differences in green turtle whole blood with significantly greater Co (relative mean difference 107%, $t_{(60)}=8.64$, $p < 0.0001$), Sb (relative mean difference 71%; $t_{(59)}=6.00$, $p < 0.001$), and Mo concentrations (relative mean difference 88%; $t_{(60)}=5.54$, $p < 0.0001$) at UPB compared to CLV turtles. These differences in trace element blood profiles in geologically similar areas suggest that the elevated exposure to Co, Sb, and Mo at UPB occurs as a result of the area's anthropogenic activities. While plausible origins at UPB include mining related activities (transport, refinement, and processing of ores, coal terminals), as well as agricultural runoff, the source of these elements, and their flux and cycling in the turtles' habitat remains unknown.

3.4. Global applicability of RIs

In general, trace element concentrations in blood are governed by physiology and in areas of relatively constant exposure, are expected to be at relatively stable levels. Therefore, we expect our RIs to be applicable as benchmarks for green turtle trace element levels worldwide. Studies, using

subadults from several other minimally impacted foraging grounds should be used to test this. To date, only two studies have reported trace elements in green turtle blood from other offshore sites: a healthy population from Palmyra Atoll (PA) in the Central Pacific (McFadden et al., 2014) and a population from Boa Vista, the Republic of Cape Verde (RCV), of unknown health status (Camacho et al., 2014a). Both sites included either mixed age classes (mean SCL 67 ± 15 cm; PA) or focused on juvenile specimens (mean CCL 45 ± 10 cm; RCV), and the inter-individual variation in trace element concentrations (CV 4% - 244% PA and 23% - 128% RCV) was generally greater than in the HWK baseline cohort for both essential and non-essential elements. Despite this, the majority (70%) of analysed trace elements (K, Fe, Zn, Cu, As, Mn, Co, V, Pb, Ni, and Mo) from PA, and nearly half of the reported elements (Zn, Cu, Mn, and Pb) from RCV either fell within or just below our RIs (Figures 2 and SI 1). Elements considerably above the RIs at these two sites include Ni, Cd and Cr (mean 81, 36 and 8 times >RI-UCI, respectively) at RCV and Ba, Cr, Se and Cd (101, 7, 4 and 3 times >RI-UCI, respectively) at PA. Due to the inconsistency of turtle age classes sampled in these studies, compared to our baseline cohort, further evaluations would need to take into account possible contributions from the known bioaccumulation potential for some of these elements (particularly Cd and Se), as well as the omnivorous diet of pelagic juveniles compared to other neritic life stages. In addition, some regions may be naturally enriched with particular elements, although human activities can considerably modify the natural abundance of metals not only on a local scale but are a significant factor in their mobilisation globally (Rauch and Pacyna, 2009). In this respect, it is noteworthy that neither of the two offshore sites in these other studies can be classified as remote from potential anthropogenic impacts, which include agriculture, raw sewage, and desalination discharges at RCV and historical military naval activities at the PA.

In contrast to offshore sites, studies on green turtle exposure to trace elements are more common in coastal areas with suspected or known point sources. Previous studies have for example identified Mn and Cu as elevated in the blood of green turtles from San Diego Bay relative to a local reference site (Komoroske et al., 2011). The elevated status of these elements is confirmed when the San

Diego data is interpreted against our RIs (Figures 2 and SI 1), with Mn 13 times above the RI-UCI, and just above RI-UCI for Cu. Interestingly, reinterpretation of the San Diego data against our RIs highlights Pb (17 times >RI-UCI) as possibly of concern for these turtles, which appears justified considering the average Pb concentration in these animals ($1,260 \pm 1,216 \mu\text{g/L}$) is near the recently proposed Pb thresholds for fibropapillomatosis (da Silva et al., 2016). Similarly, Ni, Se and Cd concentrations, which were previously hypothesised as elevated in green turtles from Punta Abreojos (Labrada-Martagon et al., 2011), considerably exceeded their respective RI-UCIs by 2249, 4 and 7 times. Conversely, the hypothesised elevated Zn concentrations in these animals were found to be within our Zn RI. Overall, these comparisons suggest that our RIs have a potentially wide spatial applicability and can be used retrospectively to evaluate green turtle trace element exposure.

Acknowledgements

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500 Table 1. Upper and lower reference intervals (RIs; grey shaded) for essential and non-essential trace
501 element concentrations (µg/L ww) and their corresponding 90% confidence intervals (CI) in whole
502 blood of green turtles from the Howick island Group.

Element	n	90% CI of Lower Limit		Lower RI Limit	Upper RI Limit	90% CI of Upper Limit	
Co	49	6.3	8.2	7.1	33	27	40
Mn	49	5.5	10	7.7	35	32	38
Mo	49	3.5	4.5	4.0	14	12	15
Na	49	1,500,000	1,600,000	1,500,000	2,300,000	2,200,000	2,400,000
Mg	49	52,000	59,000	55,000	85,000	82,000	88,000
Cu	49	260	330	290	690	650	740
Se	49	10	75	40	380	340	420
K	49	790,000	890,000	840,000	1,400,000	1,400,000	1,500,000
Fe	49	190,000	220,000	210,000	410,000	380,000	460,000
Ca	49	31,000	41,000	35,000	81,000	76,000	85,000
Zn	49	6,900	7,700	7,300	14,000	13,000	15,000
Sb	36	0.071	0.12	0.094	0.69	0.51	0.94
Pb	49	4.4	7.8	5.7	75	62	89
As	49	63	84	72	350	300	420
V	49	0.87	1.3	1.0	37	26	50
Cd	48	2.0	2.9	2.4	8.3	7.6	9.1
Ni	49	4.3	6.0	4.9	34	28	41
Cr	49	1.5	1.8	1.6	5.2	4.5	6.1
Ba	49	1.1	1.4	1.3	5.5	4.2	8.2
Ti	49	15	20	18	40	38	43

503

504 Table 2. Percent of turtles with abnormal clinical health markers in the baseline cohort (HWK) and two coastal populations (CLV and UPB) relative to
 505 biochemical and haematological reference intervals (BHRI; (Flint et al., 2010)): Units for each analyte are listed in the materials and methods.

	ALB	ALP	AST	CREAT	GLUC	TP	UREA	UA	GLOB	Tbil	Ca	CK	GGT	LDH	Mg	PO4	Na	K	Cl	TWCC
HWK <BHRI			6%	26%				26%					nd	39%	6%	84%			3%	
CLV <BHRI	8%			35%				4%				31%	nd	38%	15%	19%		4%		
UPB <BHRI	3%			81%	3%						3%	13%	nd	31%	6%	22%				

	ALB	ALP	AST	CREAT	GLUC	TP	UREA	UA	GLOB	Tbil	Ca	CK	GGT	LDH	Mg	PO4	Na	K	Cl	TWCC
HWK >BHRI											6%	3%	nd				19%			
CLV >BHRI		4%			12%			8%		12%		12%	nd	8%			15%		8%	19%
UPB >BHRI	9%	6%	3%			3%		6%		16%	6%	6%	nd				19%		3%	44%

507

508 Table 3. Average, standard deviations and range concentrations ($\mu\text{g/L ww}$) of trace elements in blood of turtles foraging at three different coastal sites (SHL,
509 CVL, UPB). Values below MDL are included as half the MDL value (see **Error! Reference source not found.4**).

	UPB			CLV			SHL			HWK		
	Mean	Stdev	Range	Mean	Stdev	Range	Mean	Stdev	Range	Mean	Stdev	Range
Co	470	± 190	130-840	150	± 70	59-360	350	± 120	210-570	16	± 6.4	6.8-36
Sb	2.5	± 1.2	0.81-6.4	1.2	± 0.65	0.50-3.3	1.0	± 0.69	0.35-3	0.25	± 0.19	0.072-0.89
Mn	65	± 43	21-190	50	± 19	23-120	38	± 10	20-57	22	± 6.8	9.2-39
Mo	25	± 15	3.3-68	9.4	± 3.9	2.6-19	19	± 9.3	9.1-48	7.9	± 2.9	3.6-19
Na	2,500,000	$\pm 380,000$	1,200,000-3,100,000	2,500,000	$\pm 170,000$	2,100,000-2,800,000	2,400,000	$\pm 140,000$	2,100,000-2,600,000	1,900,000	$\pm 180,000$	1,400,000-2,200,000
Pb	23	± 11	8.0-55	21	± 11	6.9-52	94	± 36	30-180	29	± 17	4.4-70
Mg	99,000	$\pm 11,000$	57,000-120,000	91,000	$\pm 11,000$	65,000-110,000	110,000	$\pm 18,000$	87,000-150,000	69,000	$\pm 7,500$	50,000-82,000
As	180	± 250	21-1,400	580	± 700	32-2,800	360	± 260	120-1,000	170	± 70	60-360
Cu	640	± 170	350-1,200	680	± 240	390-1,400	540	± 73	410-670	490	± 99	240-710
V	6.8	± 4.2	2.0-24	17	± 21	2.5-100	15	± 9.0	3.0-35	11	± 16	1.3-100
Cd	3.7	± 2.0	1.2-11	6.7	± 5.7	1.4-26	3.2	± 1.3	1.8-6.3	5.1	± 1.7	2.5-9.5
Se	150	± 130	28-760	340	± 320	72-1,400	400	± 210	120-840	220	± 85	74-440
Ni	14	± 7.5	2.4-32	4.4	± 3.7	1.1-19	12	± 6.6	3.2-30	14	± 7.3	5.7-38
Cr	1.7	± 1.7	1.0-10	3.7	± 1.5	1.8-9.9	4	± 0.92	2.6-6.1	3.2	± 1.8	1.3-14
Ba	3.1	± 1.5	1.1-9	4.4	± 1.3	2.5-9.3	2.4	± 2.5	1.0-11	2.5	± 1.2	1.0-8.1
Ti	26	± 7.7	14-41	24	± 14	15-94	29	± 3.1	23-33	29	± 5.6	15-44
K	1,300,000	$\pm 300,000$	560,000-1,800,000	1,200,000	$\pm 240,000$	580,000-1,600,000	1,400,000	$\pm 130,000$	1,100,000-1,700,000	1,100,000	$\pm 160,000$	710,000-1,500,000
Fe	300,000	$\pm 85,000$	100,000-420,000	260,000	$\pm 68,000$	110,000-380,000	340,000	$\pm 39,000$	280,000-430,000	290,000	$\pm 52,000$	170,000-440,000
Ca	58,000	$\pm 9,400$	37,000-75,000	52,000	$\pm 6,800$	42,000-68,000	59,000	$\pm 8,200$	47,000-85,000	57,000	$\pm 11,000$	31,000-88,000
Zn	11,000	$\pm 2,500$	5,700-16,000	9,600	$\pm 2,700$	4,100-17,000	11,000	$\pm 1,500$	8,100-14,000	10,000	$\pm 1,700$	7,200-14,000
Tl	0.11	± 0.13	0.052-0.4	0.19	± 0.20	0.038-0.72	0.15	± 0.084	0.026-0.39	0.11	$\pm 4.2\text{E-}17$	0.11-0.11
Be	0.80	± 0.51	0.57-1.9	0.58	± 0.54	0.37-1.9	0.27	± 0.11	0.24-0.7	0.34	± 0.13	0.3-1
Th	4.7	± 8.5	0.89-23	3.50	± 8.1	0.13-23	0.090	± 0.13	0.052-0.62	1.0	± 0.43	0.95-4
510 U	0.13	± 0.16	0.055-0.48	0.089	± 0.16	0.024-0.48	0.029	± 0.019	0.0073-0.072	0.050	$\pm 3.5\text{E-}17$	0.05-0.05

511

Figure 1. Map of sampling locations for the turtle populations sampled offshore (HWK; baseline) and at three coastal sites (CLV, UPB and SHL) along the Great Barrier Reef, Queensland, Australia. Inserts: sample numbers (N) for trace element analysis/haematology and blood biochemistry, sampling date, curved carapace length (CCL; cm), weight (kg), and body condition index (BCI); $\pm$ Standard deviation.

Figure 2. Blood Reference Intervals (solid grey with dotted 90% CI) for trace elements comparing significantly different (*) mean blood concentrations ($\mu\text{g/L w.w.}$) in turtles from SHL=blue, CLV=black and UPB=red (lines=geometric mean and 95%CI) and where $\geq 60\%$ exceed upper RIs. ea Mean concentrations previously reported from Palmyra¹ (McFadden et al., 2014); Cape Verde² (Camacho et al., 2014a); Baja³ (Labrada-Martagon et al., 2011); San Diego⁴ (Komoroske et al., 2011) included.

524 Figure 3. Loading scores (left) and bi-plots (right) for PCAs 1 (49%), 2 (17%) and 3 (11%), centred on
 525 the offshore baseline cohort (HWK; green), for populations foraging in coastal areas dominated by
 526 agricultural (UPB; red), urban (CLV; grey) and military (SHL; blue) activities.

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Graphical Abstract