Assessing Patterns of Trace Metal Bioaccumulation in *Crocodylus moreletii* and *Crocodylus acutus* in Belize

Félix Thirion
Department of biology, Vrije Universiteit Brussels

Contact: Felix.Thirion@vub.be

Abstract:

***Crocodylus moreletii* and *Crocodylus acutus* are generalist, apex predators of subtropical aquatic habitats in Central America. As such, the status of their population can be considered indicative of that of the entire ecosystem. As top predators, crocodiles are exposed to high levels of trace metals via bioaccumulation that enter the food web as a consequence of human activities. In this study, I report on the concentrations of trace metals found in the caudal scutes of 178 specimens of Morelet's and American crocodiles from Belize. The objectives were three-fold: 1) Investigate metal concentration differences from the scutes of Morelet's and American crocodile's populations to identify locations at risk, 2) uncover variations in metal concentrations between sexes, species and age classes within sites and 3) further assess crocodile scutes as bio-indicators for trace metal bioaccumulation from surrounding land use and sediment metal concentrations. All nine metals tested (Hg, Pb, Cd, As, Cu, Zn, Co, Cr, Ni) could be detected in at least several scutes. Higher and significant Hg concentrations were observed in adults from Chiquibul Forest (4.21 µg/g) and Ambergris Caye (1.26 µg/g). Significant differences were also reported for males displaying higher arsenic and copper concentrations than females, and a negative correlation between snout-vent length, copper and zinc, was observed. Lead concentrations in the scutes were significantly elevated near urbanized areas while cadmium and zinc concentrations in the scutes were correlated to the concentrations of these metals in the sediments. Differences of trace metal bioaccumulation between the two species could not be tested due to species repartition in the locations surveyed.**

Introduction:

Due to its long history of unregulated pest use, rapid urbanisation, overdevelopment and problematic waste disposal, Belize is subject to considerable trace metal issues (Castillo et al., 1997; Alegria, 2009). While the country is recognized for preserving most of its natural environment compared to many other Central and South American nations, the coastal zone that crocodiles typically inhabit remains a site of social and economic importance threatened by foreign investments. Clearing forests for agricultural use and illegal logging reduce the terrestrial habitats' filtering capacity, causing an enhanced release of pollutants into the aquatic networks while the

increasing use of fertilizers and pesticides, combined with improper wastewater disposal, further intensifies trace metal concentrations in the environment. In this study, I will examine metal contamination in two species of crocodiles in Belize in an attempt to highlight underlying pollution threats faced by their subpopulations. The Morelet's crocodile (*C. moreletii*) inhabits freshwater rivers, swamps, lakes and can also be found in brackish coastal lagoons. Legal protection and decreased hunting pressure helped this species to recover from near extinction and achieve status of Least Concern, yet conservation dependent in Belize (Platt and Thorbjarnarson, 2000a, IUCN, 2020). The American crocodile (*C. acutus*) is considered Critically Endangered in Belize. Monitoring and conservation actions are still required for this species as it suffers from illegal hunting and degradation of key nesting habitat exposing nursery sites to environmental pollution (Lutz and Dunbar-Cooper, 1984; Platt and Thorbjarnarson, 2000b, IUCN, 2020). Objectives of this study were three-fold. A first priority was made of analysing metal concentrations in the scutes of Morelet's and American crocodiles belonging to multiple populations to identify locations at risk. Secondly, variations in metal concentrations between sexes, species and age classes within sites were analysed to uncover underlying accumulation patterns faced by the populations and assist in implementing targeted conservation solutions. Finally, a further assessment of crocodile scutes as bio-indicators for trace metal bioaccumulation was made by relating scute metal concentrations to surrounding land use and sediment metal concentrations. Caudal scutes of both species and sediment samples were tested for the nine most prevalent trace metals mercury (Hg), arsenic (As), cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), nickel (Ni), lead (Pb) and zinc (Zn).

Methods and study sites:

A total of 178 caudal scutes corresponding to 178 different specimens, and an additional 7 sediment samples were analysed for their trace metal concentrations in this study. The country wide sampling for the 178 crocodile scutes and 7 sediment samples is indicated in Figure 1. The sites surveyed are representative of Belize's different ecosystem types from high salinity offshore developed cayes (Ambergris Caye and Caye Caulker) to coastal estuarine areas (Flower Camp) and lower salinity inland protected and non-protected watersheds. I further collected sediment samples from seven of the study sites in an attempt to link environmental trace metal accumulation to the trace metal bioaccumulation observed in the scutes.

Data and scute collection:

Caudal scutes for the different sites were collected between 2016 to 2019 by the Crocodile Research Coalition, and in August and September 2019 by myself. The sampling permit for scutes was obtained through Belize Forest department. Crocodiles were spotted through eye-shining methods and captured following IUCN/SSC-Crocodile Specialist Group protocol. Upon catching of an individual, coordinates, air and water temperatures, salinity and pH were recorded. When possible, sex was determined by lubricating and inserting a finger in the cloaca to feel for male penis or female hemi-clitores. Weight and morphometric measurements were also recorded, while species determination was made following methods reviewed by CONABIO (2011). One scute per animal was clipped following a method allowing for specimens identification. I furthermore estimated the body condition of each crocodile sampled based on the ratio between snout-vent length and tail girth, as I did not have body mass for all individuals sampled. 10-20 grams of sediments corresponding to 5 centimetres of the sediment layer were collected close to river banks in the seven locations shown in Fig 1. GPS coordinates of the sampling point were taken to compare sediment metal concentrations to metal concentrations in scutes of captured crocodiles in a 3km radius. GIS analyses were performed on all samples in an attempt to determine if any specific land-use parameter was related to trace metal contamination levels shown in the crocodile scutes. Analyses were performed using the free QGIS software version 3.8.3 and the 2017 version of the Belize Ecosystem Map available from the Biodiversity and Environmental Resource Data System of Belize.

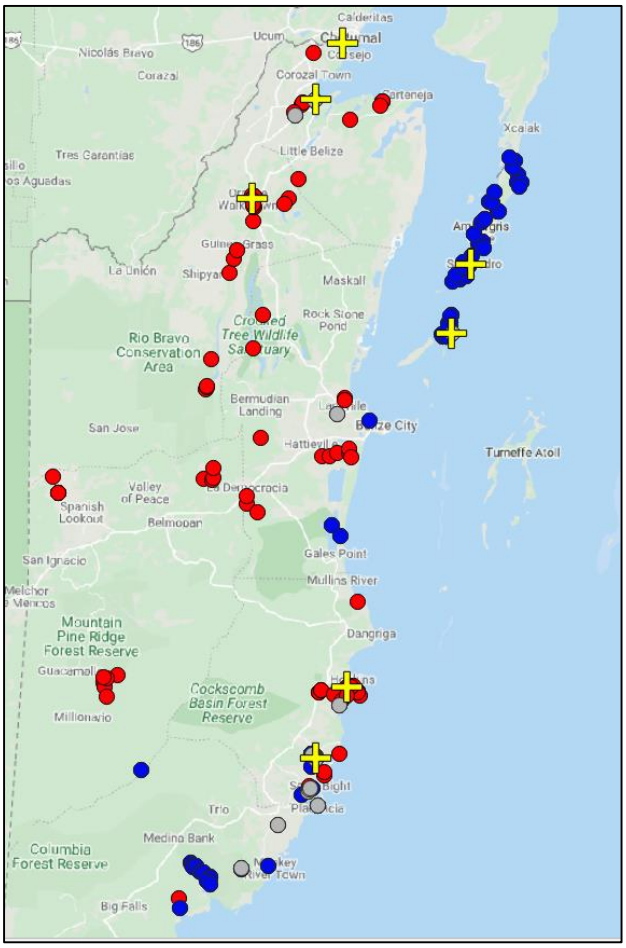


Fig 1. Map of Belize displaying the 178 locations where scutes for *Crocodylus acutus* (blue), *Crocodylus moreletii* (red) and hybrids between the two species (grey) were sampled, as well as the 7 sediment samples (Yellow cross).

The scutes were processed for their trace metal concentrations at the Systemic Physiological and Ecotoxicological Research laboratory from the University of Antwerp. The samples were dried at -70°C for 48 hours using a Heto PowerDry LL3000 Freeze dryer, weighed with a precision balance (Mettler AT261 DeltaRange; ±0.1 mg) and left overnight in a 500 µl nitric acid HNO3 and 1500 µl chlorhydric acid HCL solution. The samples were then processed through an automated microwave digestion system Discover® SP-D 80 to account for the high volatility of mercury and tested for Hg, As, Cd, Co, Cr, Cu, Ni, Pb and Zn through high resolution inductively coupled plasma mass spectrometry. The accuracy for the methodology was assessed using certified reference materials and blanks. Recoveries for As, Co, Cu and Pb differed maximally by 15 % form the certified values so we did not correct the measured values. For Hg and Zn the concentrations of the samples were corrected respectively by 1.21 and 1.4 since recoveries differed by 21 and 40 % from the certified values. The measurements of Ni and Cr could not be used since recoveries deviated > 500 % from the

certified values, variation among the different replicated reference samples was very high as were blanks concentrations. Although the recoveries for Cd were very low (51 %) they were very consistent among the replicates. We therefore decided to include Cd data by multiplying values by 0.51 however the results have to be interpreted with caution. The detection limits for metals were 0.005 µg/L for Hg, Cu, Zn and As, and 0.001 µg/L for Cd, Pb, Cr, Co and Ni.

All statistical analyses were performed using R, version 3.5.1. As metal concentrations often remained under the level of detection resulting in highly skewed distributions with an excess of zeros, I resorted to non-parametric analyses. Wilcoxon rank sums test was used to assess differences in trace metal concentrations between sexes. Kruskal-Wallis were performed to assess differences between locations, age classes and interactions between sexes, locations and age classes. Significant results were further explored through pairwise Wilcox tests with a Bonferroni correction. A two way Anova explored body condition between sexes and locations. The relationship between snout-vent length, body condition and trace metal concentrations in the scutes were determined through Pearson product-moment correlations and multiple regressions. The relationship between trace metal concentrations in the scutes and percentage of land-use cover from the GIS analyses was evaluated with canonical correlation and Pearson Product-moment correlations. Spearman rank and Pearson Product-moment correlations explored associations between metal concentrations in sediments and mean trace metal concentrations in scutes.

Results and discussion:

A positive correlation was found between snout-vent-length and body condition, which might be resulting from juveniles allocating their resources for rapid growth (Thorbjarnarson, 1986). Complex combination of both biotic and abiotic factors are furthermore likely influencing the body condition of individuals, as seen in *C. moreletii* to which a decrease in BMI was associated with an interaction between high water levels and high water temperatures (Mazzotti et al., 2012). Such interactions could not be highlighted with this dataset as sample size in some areas studied were small with high disparity of measures, and certain environmental variables such as water levels were not recorded. Although no correlation between a decrease in body condition and non-essential trace elements could be assessed, the argument can be made that increasing sampling efforts for scutes and sediments in these specific areas, associated to a different metric better reflecting health status for crocodile populations, might yield results that could have been overlooked from a disparity of measures in this study. The following metal concentrations are displayed as arithmetic mean ± standard deviation.

Mercury

Mercury levels found in this study (1.13 ± 1.36 µg/g) were higher than those previously reported for Belize's populations from Rainwater et al (2007) (0.38 µg/g, *C. moreletii* and *C. acutus*) and Buenfil-Rojas et al. (2015) (0.326 µg/g, *C. moreletii*). The highest mean concentration of Hg was detected in scutes from Chiquibul Forest (n = 6 *C. moreletii*; 4.2 ± 2.67 µg/g), highlighting a growing concern from an otherwise considered healthy population (Tellez et al., 2017). High mercury levels in fish previously reported from the Macal River crossing the Chiquibul Forest (Belize ministry of Health, 2011) could echo with concentrations found in these populations. Such mercury accumulation could be due to the controversial Challilo dam construction upstream of the Chiquibul Forest (Barton et al., 2004) since hydroelectric dams are believed to impact mercury dynamics in aquatic ecosystems if not managed properly (Pestana et al., 2019). High mercury concentrations were also found in Southern

Toledo (n=14 *C. moreletii;* 3.59± 11.9 µg/g) and Flower Camp (n=3 *C. acutus*, n= 2 hybrids and n=1 *C. moreletii*; 1.18± 0.12 µg/g). These could be attributed to agrochemical and nutrient runoff originating from large-scale agricultural bananas and citrus enterprises, aquaculture farms and open dump sites in Southern Belize district locations (Alegria, 2009). Mercury concentrations were also of concern in Ambergris Caye (n = 27 *C. acutus*; 1.26 ± 1.21 µg/g) and might result from the ongoing illegal dumping of noxious garbage around the island (Chenot-Rose, 2013). No sediment comparison for mercury could be made since this metal remained below detection limits in sediment samples. Fauna inhabiting swamps and wetlands with low pH and dissolved oxygen levels are believed to be particularly at risks to bioavailable methylmercury (Grieb et al., 1990), which was associated in crocodilians to a decrease in body condition (Nilsen et al. 2017). Although such relationship could not be highlighted from this study, a larger scute sampling effort involving a different health metric for the animals could further elucidate potential underlying threats faced by the populations.

Lead

Lead concentrations were high in Flower Camp (6.4 ± 13.28 µg/g), New River (2.93 ± 10.83 µg/g), Northern (2.42 ± 0.46 µg/g) and Southern Toledo watersheds (1.3 ± 2.98 µg/g). Lower lead levels were previously reported in scutes of *C. moreletii* by Rainwater et al (2007) in the New River watershed (0.416± 0.29 µg/g) and in *C. acutus* by Newberger (2014) in Ambergris Caye (1.109 µg/g). Our results suggest several areas in Belize in which high lead concentrations are increasing that could imply detrimental health issues for local biodiversity and communities. Although no relationship was found with snout-vent length or body condition, a significant positive correlation of 25% between urban land-use and lead concentrations in scutes was highlighted in the present study. Furthermore, a close to significant correlation (p-value = 0.07) of 71% was found between lead levels from the sediment samples and lead levels found in the scutes, which might have been strengthened if this study was provided with additional sediment sampling for the different locations examined. These two relationships attest of the utility of crocodilians and scute sampling as bio-indicators for lead contamination in the environment. Lead concentrations in sediment sampling were rather high for all locations, particularly for Caye Caulker, New River and Sittee River. Since lead is considered analogous to calcium, it is hypothesized that a lack of calcium uptake might induce an altered development for bones where lead would become sequestered instead (Pemmer et al., 2013). Throughout 2019, high crocodile casualties were reported in the New River watershed with necropsies done by the Crocodile Research Coalition highlighting symptoms including whitening of the skin, soft bones, and glassy teeth (a sign of lack of calcium uptake). High lead concentrations in individuals could potentially concur with such symptoms. Trace metal accumulation is also worsened in stagnating watersheds with low elevation differences, as in the current case of New River (The San Pedro Sun, 2019).

Cadmium

Due to only a 50% recovery from reference materials, concentration values in µg/g provided in this study are to be interpreted with caution. Cadmium levels found in the present study appeared to be higher than levels in the New River watershed found by Rainwater et al. (2007) (0.07 ± 0.05 µg/g and those found by Buenfil-Rojas et al. (2015) (0.032± 0.04 µg/g) from Rio Hondo) in scutes of *C. moreletii*. Flower camp (0.524± 0.378 µg/g) and Southern Toledo (0.669± 0.99 µg/g) displayed the highest concentrations, and a significant positive correlation was seen between cadmium concentrations found in the sediment samples and mean cadmium concentrations in scutes collected from crocodiles near the area of sediment sampling. Overall, 128 out of the 178 individuals studied displayed cadmium levels above detection limit, illustrating a negative correlation with snout-vent length while no significant differences could be observed between sexes, age classes or locations for

this metal. Although to be interpreted with caution, our results suggests that cadmium contamination is a prevalent issue in many of Belize's surveyed locations.

Arsenic

Arsenic concentrations were significantly higher in male crocodiles, and primarily detected in *C. acutus* in saline environments (Flower Camp, Ambergris Caye, Caye Caulker), with mean concentrations of Caye Caulker being the highest (n = 22; 0.82± 1.64 μg/g). Sediment samples for Caye Calker and Ambergris Caye further reflected high arsenic concentrations, and a close to significant relationship between sediment and scute arsenic concentrations was observed. Biomethylation of inorganic arsenic in organisms is of concern as it could result in dimethylarsinic acid causing genotoxic and tumorigenesis (Yamanaka et al., 2004), which could concur with the deteriorating skin condition reported from populations of *C. acutus* in Ambergris Caye (Chenot-Rose, 2013). In Caye Caulker, dredging around docking pier to ease Water-taxi access could be investigated as a source for anthropogenic arsenic release in the suspended sediments.

Copper and Zinc

Copper and zinc concentrations, considered as essential micronutrients, were found in all locations. Individuals with higher Zn concentrations also displayed higher Cu concentrations, and both concentrations were found increasing with a decrease in snout-vent length, complementing results from a previous study (Rainwater et al. 2007). Concentrations of copper and zinc were found to be higher in juveniles than adults, the highest concentrations displayed in three hatchlings, thus extrapolating that high concentrations of copper and zinc are beneficial in the early stages of crocodilians. This assumption could confound the high zinc concentrations found in Flower Camp (89.8± 63.3 μg/g) and the Southern Toledo watershed (88.7± 95.9 μg/g) given the overrepresentation of juveniles in these two particular areas. However, a significant positive correlation of zinc between sediment sampling and scute metal concentrations was also found from the seven other locations sampled for sediments. Future sediment sampling effort in Flower Camp and Southern Toledo could further supplement information in identifying anthropogenic sources contributing to higher zinc and copper concentrations. Excess concentrations of both elements may present cytotoxic properties for organisms, as impairing monocyte functions (Ibs and Rink, 2003) or decreasing cells viability through lipid peroxidation (Eisler, 1998), and might come from fungicides of copper sulphate composition being introduced in large amounts for rice and banana industries. No correlation was found between copper concentrations in the scutes and agricultural land-use percentage cover, however such relationship might be underestimated from the GIS model used in this study not taking into account complex hydrological systems for nutrient run-off. High copper and zinc concentrations displayed in Caye Caulker could also be artificially released from important anti-fouling paints in areas with high boat density (Ytreberg et al., 2010). Further supporting such hypothesis, both Ambergris Caye and Caye Caulker sediment samples contained among the highest copper and zinc concentrations for the seven sampled locations. Across all age classes, copper concentrations were significantly higher in males than females, which was also almost the case for zinc concentrations.

Conclusion:

- This study highlights trace metal bioaccumulation in both *C. acutus* and *C. moreletii* from Belize and the use of crocodilian's scutes as bio-indicators for non-essential trace metal contaminant exposure (mercury, cadmium and lead) and potential over-exposure to other metals (arsenic, zinc, copper).

- High levels of arsenic were seen in Ambergris Caye and Caye Caulker, potentially causing the poor skin condition observed in crocodiles on Ambergris Caye for the last several years.
- High lead levels are likely contributing to the skin necrosis observed amongst crocodiles of the New River watershed, where the community of Orange Walk recorded a rise of crocodile mortality, lethargic behaviour and whitening of crocodile skin.
- Crocodiles and communities in less-urbanized areas or near protected areas (i.e., Chiquibul Forest, Southern Toledo watershed, Sittee River) are also exposed to higher levels of non-essential trace metals.
- Assessing sources of contamination and hydrological models near reserved areas is critical as agricultural run-off might have a greater impact in high trace metal concentrations.

Acknowledgment:

I would like to express my sincere gratitude to the IUCN/SSC-Crocodile Specialist Group for believing in and according their most appreciated grant to this project, providing me with the wonderful opportunity of studying wild crocodiles in their natural habitat.

References:

Alegria, V. E. (2009). Land–Based Sources of Pollutants to Coastal Waters of Southern Belize–Comparison of Predictive Model with Empirical Data (Doctoral dissertation, University of South Florida, 109.

Barton, B., Redgwell, C., Rønne, A., & Zillman, D. N. (Eds.). (2004). Energy security: managing risk in a dynamic legal and regulatory environment. Oxford University, 442.

Belize's Ministry of Health: Mercury in Fish - Macal River (2011). Retrieved May 31, 2020, from http://www.health.gov.bz/www/index.php/health-projects/health-information-system/bhis-expansion/91-health-information-system/plugins/content/avreloaded/past-a-upcoming-events/73-past-and-upcoming-events/619-mercury-in-fish-macal-river

Buenfil-Rojas, A. M., Álvarez-Legorreta, T., & Cedeño-Vázquez, J. R. (2015). Metals and metallothioneins in Morelet's crocodile (*Crocodylus moreletii*) from a transboundary river between Mexico and Belize. *Archives of Environmental Contamination and Toxicology*, **68(2),** 265-273.

Castillo, L. E., de la Cruz, E., & Ruepert, C. (1997). Ecotoxicology and pesticides in tropical aquatic ecosystems of Central America. *Environmental Toxicology and Chemistry*, **16(1),** 41-51.

Chenot-Rose, C. R. (2013). American crocodile, Crocodylus acutus, population census in Ambergris Caye, Belize, Central America. The Rufford foundation, 8.

CONABIO (2011). Programa de monitoreo del cocodrilo de pantano - Sitio De Comisión Nacional Para El Conocimiento y Uso De La Biodiversidad, Retrieved May 15, 2020, from www.gob.mx/conabio.

Eisler, R. (1998). Copper hazards to fish, wildlife, and invertebrates: a synoptic review (No. 33). US Department of the Interior, US Geological Survey.

Grieb, T. M., Bowie, G. L., Driscoll, C. T., Gloss, S. P., Schofield, C. L., & Porcella, D. B. (1990). Factors affecting mercury accumulation in fish in the upper Michigan peninsula. *Environmental Toxicology and Chemistry*, **(7),** 919-930.

Ibs, K. H., & Rink, L. (2003). Zinc-altered immune function. *The Journal of Nutrition,* **133(5)**, 1452S-1456S.

IUCN 2020. The IUCN Red List of Threatened Species. Version 2020-1. https://www.iucnredlist.org. Retrieved on 19 March 2020.

Lutz, P. L., & Dunbar-Cooper, A. (1984). The nest environment of the American crocodile (*Crocodylus acutus*). *Copeia*, 153-161.

Mazzotti, F. J., Cherkiss, M. S., Brandt, L. A., Fujisaki, I., Hart, K., Jeffery, B., ... & Vinci, J. (2012). Body condition of Morelet's crocodiles (*Crocodylus moreletii*) from Northern Belizea. *Journal of Herpetology,* **46(3),** 356-362.

Newberger, D. (2014). Transition Metal Accumulation in Caudal Scutes of American Crocodiles (*Crocodylus acutus*) from Belize (Doctoral dissertation, Baylor University), 61.

Nilsen, F. M., Dorsey, J. E., Lowers, R. H., Guillette Jr, L. J., Long, S. E., Bowden, J. A., & Schock, T. B. (2017). Evaluating mercury concentrations and body condition in American Alligators (*Alligator mississippiensis*) at Merritt Island National Wildlife Refuge (MINWR), Florida. *Science of the Total Environment*, **607**, 1056-1064.

Pemmer, B.; Roschger, A.; Wastl, A.; Hofstaetter, J. G.; Wobrauschek, P.; Simon, R.; Thaler, H. W.; Roschger, P.; Klaushofer, K.; Streli, C. (2013). Spatial distribution of the trace elements zinc, strontium and lead in human bone tissue. *BONE*, **57**, 184-193.

Pestana, I. A., Azevedo, L. S., Bastos, W. R., & de Souza, C. M. M. (2019). The impact of hydroelectric dams on mercury dynamics in South America: A review. *Chemosphere*, **219**, 546-556.

Platt, S. G., & Thorbjarnarson, J. B. (2000). Status and conservation of the American crocodile, *Crocodylus acutus*, in Belize. *Biological Conservation,* **96(1),** 13-20.

Platt, S. G., & Thorbjarnarson, J. B. (2000). Population status and conservation of Morelet's crocodile, *Crocodylus moreletii*, in northern Belize. *Biological Conservation*, **96(1)**, 21-29.

Rainwater, T. R., Wu, T. H., Finger, A. G., Cañas, J. E., Yu, L., Reynolds, K. D., ... & Anderson, T. A. (2007). Metals and organochlorine pesticides in caudal scutes of crocodiles from Belize and Costa Rica. *Science of the Total Environment*, **373(1),** 146-156.

Tellez, M., Arevalo, B., Paquet-Durand, I., & Heflick, S. (2017). Population status of Morelet's Crocodile (*Crocodylus moreletii*) in Chirquibul Forest, Belize. *Mesoamerican Herpetology*, **4(1),** 8-21.

The San Pedro Sun (2019, September 20). New River and Macal River contaminated. Retrieved May 15, 2020, from https://www.sanpedrosun.com/environment/2019/09/20/new-river-and-macal-river-contaminated/

Thorbjarnarson, J. B. (1986). Ecology of the American crocodile, *Crocodylus acutus*. In Crocodiles: Proceedings of the 7th Working Meeting of the Crocodile Specialist Group of the Species Survival Commission of the International Union for Conservation of Nature and Natural Resources, Caracas, Venezuela (p. 228).

Yamanaka, K., Kato, K., Mizoi, M., An, Y., Takabayashi, F., Nakano, M., ... & Okada, S. (2004). The role of active arsenic species produced by metabolic reduction of dimethylarsinic acid in genotoxicity and tumorigenesis. *Toxicology and Applied Pharmacology,* **198(3),** 385-393.

Ytreberg, E., Karlsson, J., & Eklund, B. (2010). Comparison of toxicity and release rates of Cu and Zn from anti-fouling paints leached in natural and artificial brackish seawater. *Science of the Total Environment,* **408(12),** 2459-2466.