

Determining impact of a dump site on the Rio Grande river, Southern Belize, using stable isotope and trace elemental analysis of aquatic species of multiple trophic levels

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Abstract:

This study aims to determine presence/ absence of leachate transfer from the primary municipal dump site in Punta Gorda, Belize to species within the nearby Rio Grande river, through contaminant analysis of stored tissue body burdens and stable isotope analysis of C and N. Particulate organic matter (POM), sediment, algae, and bivalves were collected at four sites on the Rio Grande river, POM was collected in leachate from the dump site as well. Results revealed indicators of impact at Site 2 and Site 3, in comparison to reference Site 1. Dissolved oxygen has decreased at Site 2 to levels deemed to reduce biodiversity by the world health organization and supporting authors. This is supported by diminished species abundance of three macroinvertebrate species at Site 2. In comparison to standards reported in similar studies, water quality analysis revealed high concentrations of ammonia, phosphate, nitrate, chromium and manganese in leachate at the Rio Grande dump site as well as high values of nitrate and chromium at Site 2. Trace element analysis of biota revealed elevated concentrations of zinc, chromium, copper, lead, and iron in sediment at Site 3. High concentrations of zinc, manganese, copper, iron and cadmium in algae were also discovered at Site 2, revealing trends supporting impact from dump site on the Rio Grande river; with values highest at Site 2, slightly lower at Site 3, and further decreasing in concentration at Site 4 to levels found at uncontaminated Site 1. Stable Isotope analysis of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ revealed unique signatures at impacted sites, in comparison to reference Site 1, supporting impact from the dump site on the river. $\delta^{15}\text{N}$ values in algae, bivalves and snook at Site 2 in comparison to reference Site 1 are consistently lower. $\delta^{13}\text{C}$ values also showed significant decreases at Site 2 in comparison to reference Site 1 in POM and snook as well as in bivalves. Lastly both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ at Site 2 and Site 3 show similarities to signatures found in leachate at the dump site in POM. The Rio Grande dump site is currently within a wetland ecosystem that drains into the Rio Grande river and the adjoining Port Honduras Marine reserve (PHMR). Results support the need for relocation of the dump site to an area that is deemed to be more geographically appropriate, and further, restoration of the immediate area to mitigate further impacts from leachate as the dump site inevitably grows in coming years.

Key words: dump site; leachate; contamination; carbon and nitrogen stable isotopes; methanogenic; water quality

1. Introduction:

Open dump sites, such as the Rio Grande dump site in Southern Belize, are those that are completely unregulated, with no liner or barrier between waste and soil nor any collection or treatment system to manage runoff passed through the waste mass (leachate); mixtures of dangerous chemicals from leachate can then find natural paths into the surrounding environment. Analyses of leachate impacts are most often conducted on surface and groundwater (1, 2, 3, 4, 5). However, water quality analysis provides only a snapshot of contaminant concentrations. Testing organisms, in contrast, provides information on contaminant concentrations over the entire lifespan of the tested species. Furthermore, uptake of anthropogenic pollutants in food webs, termed bioaccumulation, can cause organisms to accumulate contaminant concentrations several times greater than the water they inhabit and the prey they ingest (6, 7, 8). Analysis of tissues is therefore considered a more accurate representation of contaminant concentrations present in affected ecosystems as well as their route of travel through food webs (9, 10, 28, 29, 30).

Results from studies analyzing trace elements in tissues are often inconclusive. There are often several possible nutrient input sources and, in river systems in particular, it is difficult to prove a contaminant came from the area under investigation rather than other sources upriver. Stable isotope analysis (SIA) of carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$) is a well-established tool used to trace transfer of nutrients through food webs. In particular it has been used to determine impact from a specific anthropogenic source and to establish conclusive boundaries of influence from that source into nearby environments (9, 10, 26). This is done by tracing isotopic signatures of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ as they are enriched in their ratios of heavy to light isotopes by influence from an anthropogenic source and/or transfer through food webs (31, 32, 3, 25, 26, 21, 34).

In all landfills, the breakdown of organics within the waste mass will result in a final methanogenic phase, typically after ten years; at this stage organics are no longer actively decomposing complex molecules, instead they are fully broken down into their elemental constituents, resulting in the release of methane gas and carbon dioxide (12, 13, 14,

15). The Rio Grande dump site has been active for 45 years according to local accounts, implying the onset of methanogenesis.

2. Parameter selection justification:

Biota Analysis: Zinc, chromium, manganese, nickel, copper, lead, cadmium, and iron were selected for biota (Appendix 6: Glossary) trace element analysis based on waste composition at the dump site and comparable studies of landfills of similar age, as well as chemical mobility, contaminant persistence, bioaccumulation, and long range transport based on environmental conditions of southern Belize (9, 10, 52, 38, 12, 14, 54, 55, 56, 57). Methanogenesis can also alter and enrich the ratio of heavy to light isotopes of carbon and nitrogen (16, 17, 5, 26). This typically results in enriched signatures in leachate that can then be traced as they cycle through nearby environments and biota, which will have distinct signatures of their own (3, 5, 17). **This study therefore uses isotopic analysis of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, paired with trace elemental analysis in tissues and organs of indicator organisms of multiple trophic levels to determine the presence or absence of contaminant impact from the dump site under investigation to the second largest river system in southern Belize, the Rio Grande river.**

Water Quality Analysis: Analysis of biota is complimented by standard water quality analysis of temperature and salinity to ensure that results cannot be attributed to changes in basic environmental conditions between sites rather than impact from the dump site. pH was measured for this reason and also to confirm if leachate values are within the typical range of methanogenic waste sites of similar ages, at 7.5-9.0 (36). Conductivity is a measure of the concentration of dissolved ions in water and was measured as a result of its tendency to increase as a result of influence from nutrient inputs (37). Lastly, to determine if possible nutrient input was impacting oxygen availability to species, dissolved oxygen was measured and referenced to guideline values recommended for healthy ecosystem functionality (6.0 mg l^{-1}) and levels reported by the world health organization to negatively impact the majority of aquatic species (at 3.2 mg l^{-1}) (38). Furthermore, water was analyzed for the following trace elements: ammonia, phosphate, nitrate, chromium, manganese and zinc. As many elements as possible selected for biota analysis were tested in water as well to support the possibility of bioaccumulation in biota from their aquatic environment.

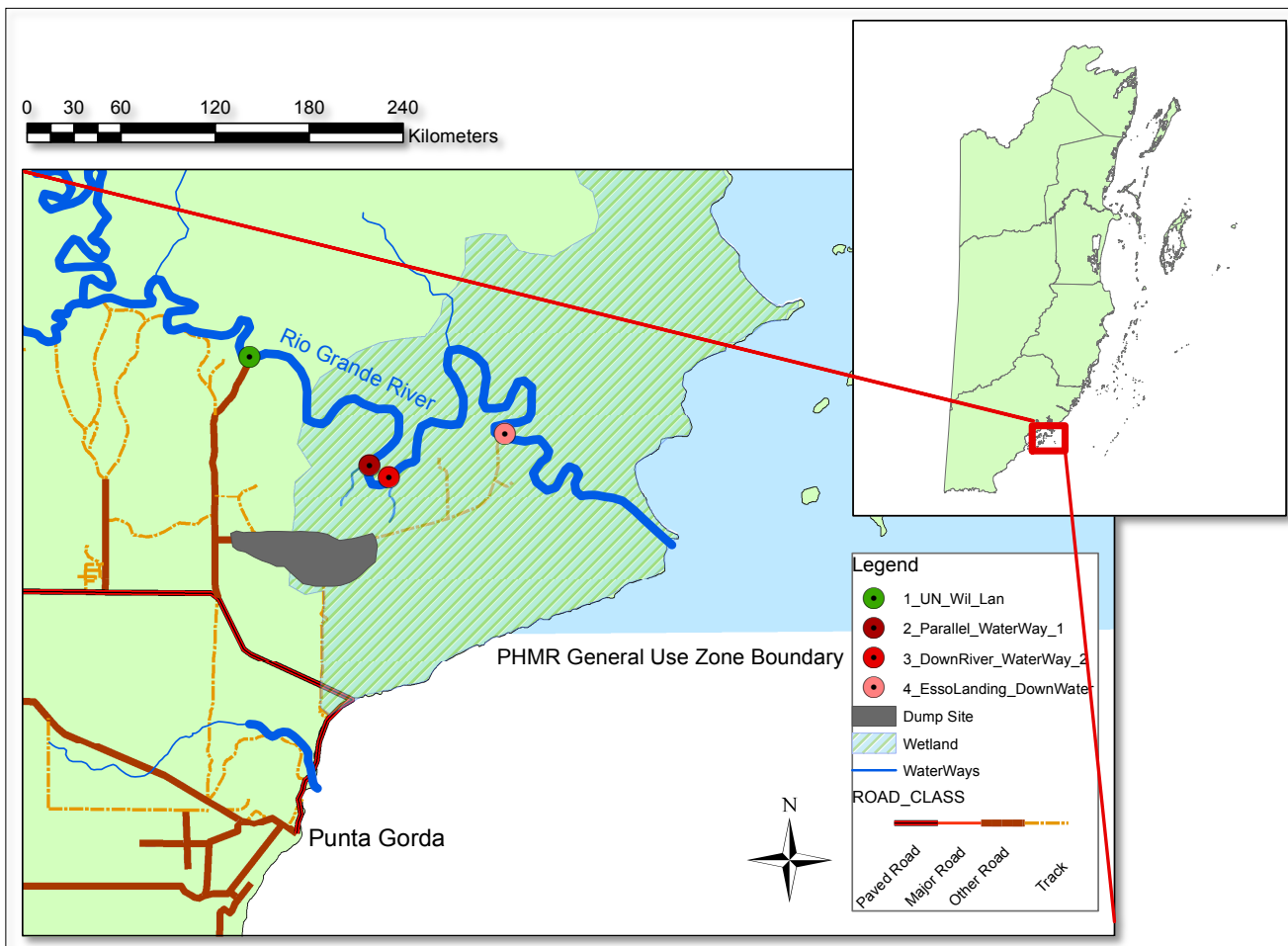
Macroinvertebrate Surveying: Finally, macroinvertebrate benthic surveying was conducted to determine if biodiversity and abundance of species were changed above and below point of potential contamination from the dump site. Combined, all forms of analysis aimed to test the null hypothesis ‘there is no impact from the dump site on $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ signatures of aquatic species of varying trophic levels in the Rio Grande river, and further, no indication of trace elemental transfer’, and to meet the following objectives:

- i) *To establish background levels of nutrients in water sources near the dump site and in the Rio Grande for reference to biota analysis.*
- ii) *To determine if the dump site is affecting species composition and biodiversity in the Rio Grande by contrasting relative species abundance between sites 1_UN_Wil_Lan and 2_Parallel_WaterWay_1.*
- iii) *To determine if leachate is reaching the Rio Grande river and influencing aquatic biota through use of carbon and nitrogen stable isotope signatures to establish point source of impact.*
- iv) *To determine the relationships between C and N stable isotope signatures at upstream control site (1_UN_Wil_Lan) and downstream from the dump site (2_Parallel_WaterWay_1) and furthermore, to determine relationships between signatures at (2_Parallel_WaterWay_1) and leachate signatures at the dump site, as established through analysis of particulate organic matter (POM).*
- v) *To test ecologically and economically important species, for bioaccumulation of pollutants and to determine if trophic transfer of pollutants is occurring between organisms in Rio Grande with increasing trophic position.*

3. Materials and Methods:

Area Description: The dump site under investigation is located within the wetland ecosystem that encompasses all the lower reaches of the Rio Grande River, which in turn, drains into the Port Honduras Marine Reserve (PHMR), co-managed by the Toledo Institute for Development and Environment (TIDE) and the Belize Fisheries Department. The dump site has been active and uncontrolled since 1970 and is predominantly used for municipal waste, though there have also been contributions of commercial waste and chemical toxins (18). Waste quantity is dramatically reduced by open burning. In this study, four sites were established on the Rio Grande river, each chosen based on relative distance from the dump site, assuming an inverse relationship between distance and impact (45) and geographical influences, namely topography, to determine varying levels of impact in comparison to reference Site 1.

Fig. 1: Site map



Sampling Methodologies: Leachate and benthic water samples from all sites were collected in 250ml hydrochloric acid washed plastic bottles on seven occasions (Sept 13th-Nov.13th). Samples were immediately put on ice during transport to the TIDE laboratory. Samples were filtered using a 0.45 μm microfiber filter and analyzed same day to minimize microbial activity within samples that could alter readings. Concentrations of ammonia, zinc, nitrate, phosphate, manganese and chromium were determined using a Hach DR2800 spectrophotometer following standard procedures (19). pH and conductivity were measured before filtration using digital probes. Complimentary standard water quality was conducted on-site on 26 occasions (June 12th- Nov. 13th) for temperature ($^{\circ}\text{C}$), dissolved oxygen concentration ([DO]) (mg l^{-1} and %) and salinity (ppt) using a YSI 550A probe at the surface and just above the river floor.

Macroinvertebrate benthic surveying took place on eight occasions (May 29th 2013- July 05th 2013), at sites (1_UN_Wil_Lan) and (2_Parallel_WaterWay_1). Three sub-sites were sampled within each site's 50 m parameter. Two different trap designs and light sources were used to diversify in attempt to attract a wider range of species. One translucent fluorescent light trap (appendix 1) and two LED light traps (appendix 2) were deployed before sunset and retrieved before sunrise the following day to ensure that natural light sources did not entice species out of the traps. All traps were active for 8-10 hours on all occasions and the same combination of traps was used for the same time span each survey.

Trap contents were sifted twice through a 1.1 mm sieve to separate the organisms from water and were preserved immediately in 40% ethanol alcohol. Species were sorted under a microscope on the same day into groups and counted at TIDE. Each species was then photographed, given a coded name, and incorporated into an identification key. Species relative abundance (individuals attracted per hour) was then calculated at both sites for each species by compiling data from all trips and applying the following equation:

$$\frac{\text{Total number (n) of captures}}{\text{Total time (t) of trap deployment}}$$

Species and tissue selection: A representative trophic pyramid was constructed in the Rio Grande and included particulate organic matter, sediment, algae, bivalves, and snook. All species were selected based on trophic position, accessibility at all sites, reported likeliness to bioaccumulate, and diet. Species tissues with the slowest turnover rate were selected for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ analysis as these tissues represent dietary information over the longest period of time, allowing exclusion of short term variability (40, 31, 21, 26). Bivalves were then dissected for adductor muscles (49) while the left dorsal fin tissue was dissected in snook (9, 34, 39). For trace element analysis snook were dissected for their livers as these contain the highest concentrations of most elements (47, 48). Whole body excluding the shell was used for trace element analysis of bivalves due to a large sample weight needed and a small average mass of the bivalves in the Rio Grande (0.25 g).

Sediment was used only in trace elemental analysis, as there is not enough organic content to obtain accurate $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ isotopic signatures. However sediment is commonly tested in trace elemental analysis studies as a result of high quantities of stored contamination that tend to be found in sediment, particularly heavy metals (42, 44, 45, 46, 34), which are then available for uptake by higher trophic levels. This is particularly prevalent among silts and clay sediments, at $>0.63 \mu\text{m}$ (50, 43), which is the case at all sites on the Rio Grande. $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ signatures in algae can be affected by influence from landfill leachate (3). Algae is also an important food source to many upper trophic level species, including snook (confirmed via gut content analysis), therefore algae was used in conjunction with POM in both forms of biota analysis to provide foundational information on trace elemental and isotopic alteration availability for transfer from foundational trophic species to higher levels. POM is composed of decomposing fauna material and waterborne algae and can become enriched in ^{13}C and ^{15}N as a result of landfill leachate and can therefore allow impact to be traced great distances (37). POM therefore provided foundational food web data on availability of suspended pollutants in the Rio Grande for uptake by higher trophic level bivalves. Bivalves are omnivores and feed on waterborne debris and POM, making them effective secondary trophic level representatives. The environmental rate of uptake for bivalves greatly exceeds their rate of metabolism, causing bioaccumulation of pollutants and increasing the chance of isotope detection (6, 7). Snook acted as a highest trophic level representative in this study. Snook were selected due to their high fat content favoring bioaccumulation, and their tendency to eat a wide range of species, increasing possibility of exposure from different channels of contamination (41). All species for stable isotope and elemental analysis were collected within each site's 50 m parameter (Table 1).

Table 1: Species selection parameters

Species	Common name	Target organ or material	# of replicates per site	Collection method	Diet composition	Length (cm)
<i>Particulate Organic Matter (a)</i>	POM	All	5	water grab at benthic layers	none	none
<i>Sediment (b)</i>	Sediment	All	5	metal sediment grab at benthic layers	none	<63 μ m
<i>Alga (a,b)</i>	Algae	All	5	retrieved from submerged mangrove roots at surface	none	none
<i>Unionoida (a,b)</i>	Bivalves	Adductor Muscle (a) Whole body (minus.shell) (b)	5	Scuba Diving	Debris, POM, zooplankton, algae and bacteria	1.37 +- 0.85
<i>Centropomus undecimalis (a,b)</i>	Snook	Muscle Tissue (left.dorsal.fin) (a) Liver(b)	1	Gill netting	Shrimp, algae, small fish, invertebrates	36.5+- 2.5

a: Stable Isotope analysis

b: Elemental Analysis

Sample preparation: Species representing five trophic levels were collected from all four sites on the Rio Grande (Oct. 8th- Dec. 5th). Particulate organic matter (POM) was also collected at an additional site, in leachate at the dump site. Samples were collected by hand or by scuba diving and were immediately put in labeled plastic bags on ice at <3°C for a maximum of 5 hours during transport, until storage in a -30°C industrial freezer to preserve the samples and prevent changes in isotopic signatures. In the laboratory, samples were controlled for size (1.18 cm \pm 0.03 SE) to eliminate bias from increasing $\delta^{15}\text{N}$:size ratio. Prior to dissection samples were weighed, measured and washed with deionized water to prevent contamination from outer tissues to targeted internal organs. Tissue wet weight was individually measured and samples were then put in a glass petri dish to be dried.

POM samples were strained on site with a nitric acid washed Buchner funnel and hand pump through a GF/F 0.7 μ m Whatman microfiber filter; separating suspended particles from water. Filter papers were immediately put on ice (<3°C) in sealed plastic bags. All samples, including tissues, organics, and POM were dried in a laboratory grade oven for at least 48 hours at 60°C (40). Dried tissue and organic samples were ground into a fine powder (<100 μ m) to achieve homogeneity of the sample using a laboratory grade pestle & mortar with the exception of POM, which was left on the filter paper to ensure that an accurate reading was not obstructed by only analyzing the scraped off top layer. All samples were transferred to nitric acid washed, labeled, plastic sample vials for storage at -30°C in a moisture proof container lined with silica beads to prevent moisture uptake. All equipment in contact with exposed tissues or water samples was cleaned with phosphate free soap, 1:10 nitric acid and deionized water prior to use.

Samples for C and N stable isotope analysis were then weighed on a microbalance for 0.2-0.3 μ g aliquots of material, sealed into tin capsules, and stored in a desiccation cabinet until shipping. Samples for trace element analysis were digested according to standard procedures in concentrated nitric and hydrochloric acid at 85-90°C for 2 hours to bring all elements within the sample into solution (20). Samples were shipped in solution in 6ml polyethane vials. All samples were analyzed at Trent University's Dr. Hintelman laboratory.

Analysis: $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ were tested using a dual input isotope ratio mass spectrometer (IRMS). Throughout analysis, every 12-15 samples, certified reference material USGS 40 was run through the mass spectrometer for calibration and then with casein to normalize the data. All results are expressed in standard delta notation (δ) expressed as deviations in ‰ from the PDB standard & atmospheric N_2 respectively. This is the raw ratio of the heavy to light isotope as calculated in an internationally accepted standard according to the following formula: $\delta R = [(X_{\text{SAMPLE}}/X_{\text{STANDARD}} - 1)] \times 10^3$ (where $R = {}^{13}\text{C}$ or ${}^{15}\text{N}$ and $X = {}^{13}\text{C}/{}^{12}\text{C}$ or ${}^{15}\text{N}/{}^{14}\text{N}$).

Concentrations of copper, chromium, manganese, lead, cadmium, iron, nickel and zinc were tested using an inductively coupled plasma mass spectrometer (ICP-MS) and reported in parts per million. A blank was run every 16 samples and was then subtracted from all sample results to account for background sources of elemental contamination acquired during preparation and analysis. To ensure accuracy further, three duplicated samples were run during both stable isotope and trace element analysis and results compared for consistency; no significant discrepancies were detected.

4. Results:

Benthic Surveying: Throughout all trips, 2,651 specimens were collected, of 34 unique taxa (Appendix 3 for complete key). Of these, species 2, 3, 5 and 8 (Appendix 4) were captured enough times to provide a reliable sample size, having an abundance (mean number caught per hour) of 6.8, 14.7, 5.8 and 3.8 respectively. These four species have shown marked differences in abundance between areas expected to be non-influenced and highly influenced by the Rio Grande dump site (Appendix 5: relative abundance of all species, Sites 1 and 2 comparison).

Standard Water Quality: Dissolved oxygen continues to support impact from the dump site on the Rio Grande. Values are highest at Site 1, with little variance between surface ($4.4 \text{ mg l}^{-1} \pm 0.22 \text{ SE}$) and floor ($4.3 \text{ mg l}^{-1} \pm 0.27 \text{ SE}$) readings, then decrease significantly at Site 2 to $2.9 \text{ mg l}^{-1} \pm 0.3 \text{ SE}$ at the surface and to anoxic levels, at $1.6 \text{ mg l}^{-1} \pm 0.31 \text{ SE}$ at the floor. Site 3 shows increases to values just below Site 1 at $4.0 \text{ mg l}^{-1} \pm 0.27 \text{ SE}$ at the surface and $3.6 \text{ mg l}^{-1} \pm 0.48 \text{ SE}$ at the floor. Site 4 has similar values to site 3 at $3.9 \text{ mg l}^{-1} \pm 0.22 \text{ SE}$ on the surface and $3.1 \text{ mg l}^{-1} \pm 0.37 \text{ SE}$ on the floor.

	pH	Conductivity (us/l)	Temperature (°C)		Dissolved Oxygen (mg/l)		Salinity
Site 1	7.49 ± 0.11	352.56 ± 44.64	Surface:	25.0 ± 0.09	Surface:	4.4 ± 0.22	0
			Floor:	25.04 ± 0.10	Floor:	4.3 ± 0.27	
Site 2	7.34 ± 0.15	246.44 ± 37.67	Surface:	25.2 ± 0.13	Surface:	2.9 ± 0.30	0
			Floor:	25.25 ± 0.11	Floor:	1.6 ± 0.31	
Site 3	7.34 ± 0.15	282.42 ± 31.51	Surface:	25.2 ± 0.11	Surface:	4.0 ± 0.27	0
			Floor:	25.4 ± 0.22	Floor:	3.6 ± 0.48	
Site 4	7.37 ± 0.13	583.62 ± 167.01	Surface:	25.6 ± 0.13	Surface:	3.9 ± 0.22	0
			Floor:	26.3 ± 0.33	Floor:	3.1 ± 0.37	
Leachate	7.04 ± 0.43	590.49 ± 212.80	~		~		~

Table 2: Mean pH, Conductivity, Temperature, Dissolved oxygen and Salinity, all sites

Temperature is relatively uniform across all sites within 0.60 °C on the surface and 1.26 °C on the floor. pH values in the leachate (7.04± 0.43 SE) support methanogenic conditions within the waste mass, though a higher standard error in leachate compared to all other sites (0.11-0.15) can be seen as incidences of high rainfall altered conditions within the waste mass.

Trace elemental analysis of water (Figs. 2-7): In comparison to standards reported in similar studies (1-5, 12, 35-36), results from water quality nutrient analysis revealed high concentrations of ammonia, phosphate, nitrate, chromium and manganese in the leachate at the Rio Grande dump site, where zinc proved to be in low quantities in leachate compared to all sites on the Rio Grande. Nitrate and chromium had highest values at Site 2 (at 0.83 mg l⁻¹±0.13 SE and 0.032 mg l⁻¹ ± .0002 SE respectively), levels even higher than that found in the leachate (0.746 mg l⁻¹ ± 0.01 SE and 0.001 mg l⁻¹± 0.002 SE respectively). Upstream Site 1 revealed relatively high concentrations of many of the tested nutrients; in the case of ammonia (0.035 mg l⁻¹± 0.02 SE), zinc (0.124 mg l⁻¹± 0.05 SE), phosphate (0.141 mg l⁻¹± 0.04 SE) and manganese (0.563 mg l⁻¹± 0.05 SE) concentrations were higher than Site 2 (at 0.016 mg l⁻¹± 0.01 SE, 0.067 mg l⁻¹± 0.01 SE, 0.115 mg l⁻¹± 0.03 SE, and 0.478 mg l⁻¹± 0.09 SE respectively) and in the case of ammonia, phosphate and zinc, were higher or equal to values found in leachate (0.035 mg l⁻¹± 0.01 SE, 0.141 mg l⁻¹± 0.02 SE, 0.124 mg l⁻¹± 0.01 SE respectively).

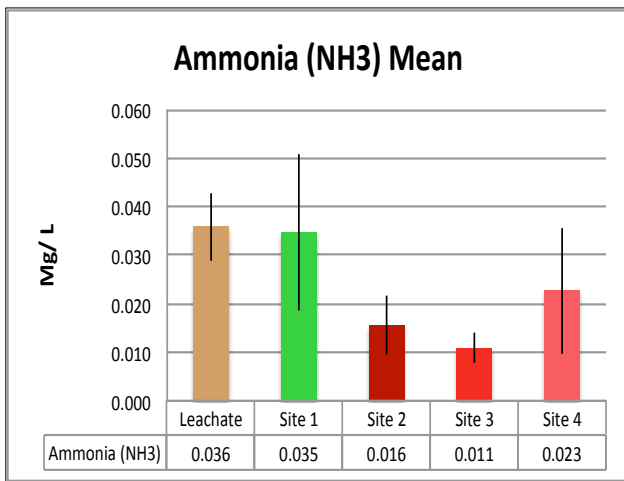


Fig 2: [Ammonia] in water, all sites

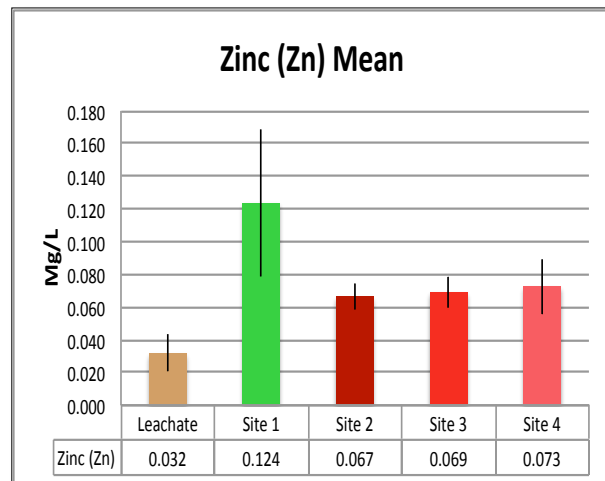


Fig 3: [Zinc] in water, all sites

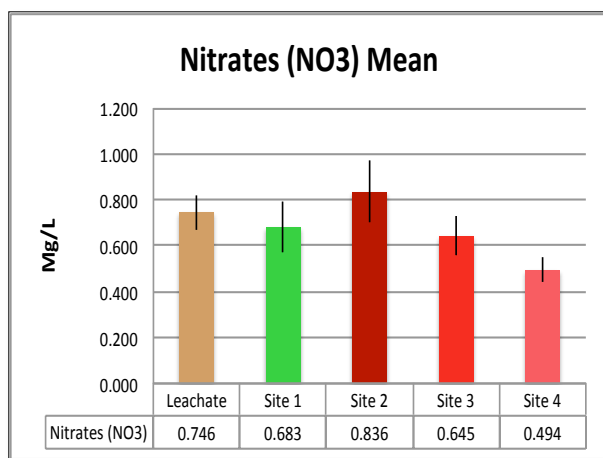


Fig 4: [Nitrate] in water, all sites

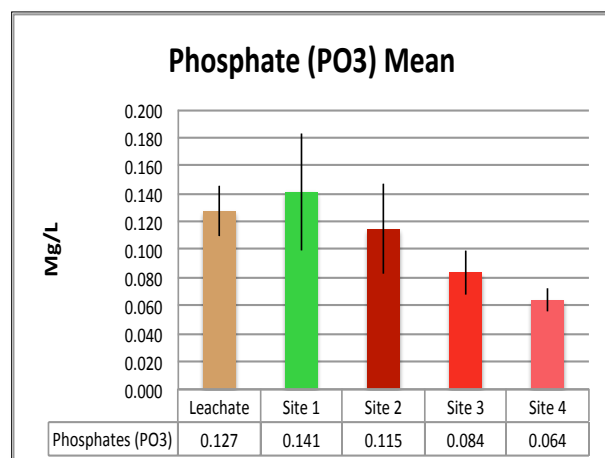


Fig 5: [Phosphate] in water, all sites

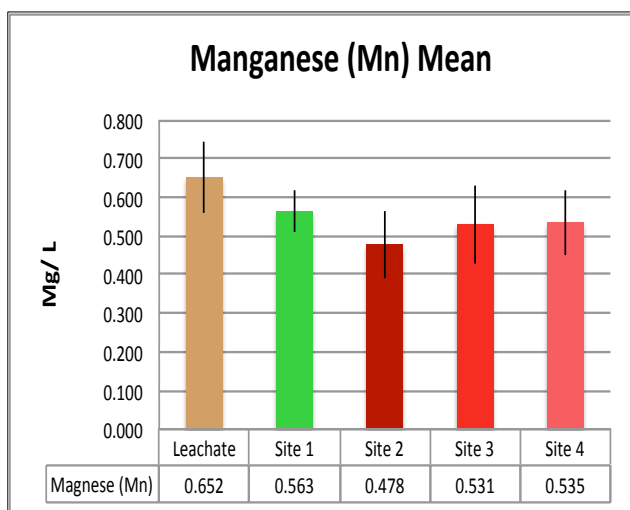


Fig 6: [Manganese] in water, all sites

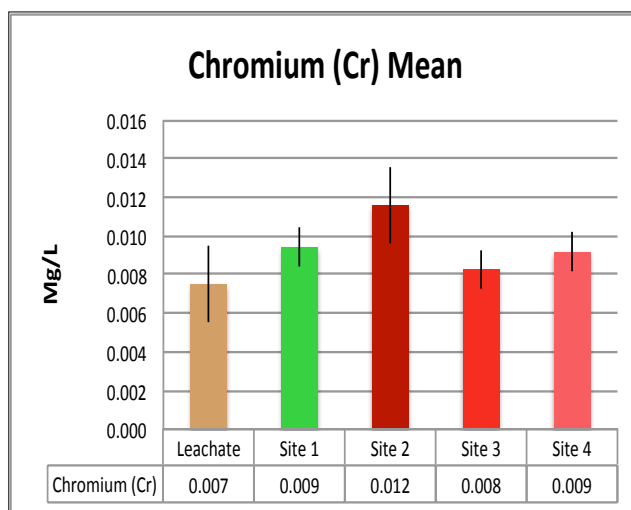


Fig 7: [chromium] in water, all sites

Trace Element Tissue Analysis (Figs. 8-15): Biological Material: In sediment, Site 3 revealed highest concentrations of zinc, chromium, copper, lead, and iron (30.749 ± 2.21 SE, 19.358 ± 0.59 SE, $11.405 \pm .46$ SE, 13.789 ± 0.01 SE, 12301.64 ± 554.13 ppm respectively). None of the tested parameters were highest at Site 2 in sediment. Site 1 (control) revealed highest values of manganese, nickel, and cadmium (289.0 ± 35.5 SE, 23.742 ± 6.04 SE, 0.189 ± 0.01 SE ppm respectively). In algae however, zinc, manganese, copper and iron levels were highest at Site 2 (28.726 ± 1.87 SE, 537.14 ± 65.18 SE, 9.568 ± 0.35 SE, 13078.4 ± 707.68 SE ppm respectively), and cadmium levels at Site 2 were equal to Site 3 (0.172 ± 0.01 SE ppm). Site 1 was highest only in chromium (22.295 ± 9.33 SE ppm). Elemental concentrations found in bivalves include no standard error due to only one sample tested per site, with the exception of Site 3, where still only 2 samples were analyzed. Results reveal high concentrations at Site 1, above all tested samples at all sites, of the following elements: zinc, chromium, nickel, copper, lead, cadmium and iron (172.93, 48.687, 71.965, 32.584, 107.131, 2.521, 12736.99 ppm respectively). Among this minute sample size, none of the parameters were highest at Site 2. Site 4 followed in concentration of zinc, chromium, nickel, copper, lead, and cadmium (120.545, 25.253, 50.595, 24.524, 82.551, 1.237 ppm respectively).

$\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ Stable Isotope Analysis (Fig 16): Biota: Results from $\delta^{15}\text{N}$ revealed $\delta^{15}\text{N}$ enrichment with average trophic fractionation factor of 3.58 ‰ between species. Algae, bivalve and snook mean $\delta^{15}\text{N}$ values were notably lower at Site 2 (1.68 ± 0.94 ‰ SE, 5.48 ± 0.14 ‰ SE, 10.26 ‰ respectively) compared to all other sites (1.82-2.56‰, 6.02-6.37‰, and 10.41‰ respectively). POM was analyzed for twice as many replicates as all other tested species and yet $\delta^{15}\text{N}$ standard error was higher at all sites. POM demonstrated a reversed trend in comparison to other tested biota; sites expected to be most influenced, being Site 2 and leachate, demonstrated increases in $\delta^{15}\text{N}$ and are graphically grouped together, where Sites 1 and 4 are grouped together at a lower $\delta^{15}\text{N}$ ‰.

Among all tested biota within the same site, there are increases in $\delta^{13}\text{C}$ with increasing trophic level, with the exception of POM, which tends to have values similar to tested bivalves. Ratios revealed decreases in $\delta^{13}\text{C}$ in all species at Site 2. Site 4 followed in concentration of zinc, chromium, nickel, copper, lead, and cadmium (120.545, 25.253, 50.595, 24.524, 82.551, 1.237 ppm respectively).

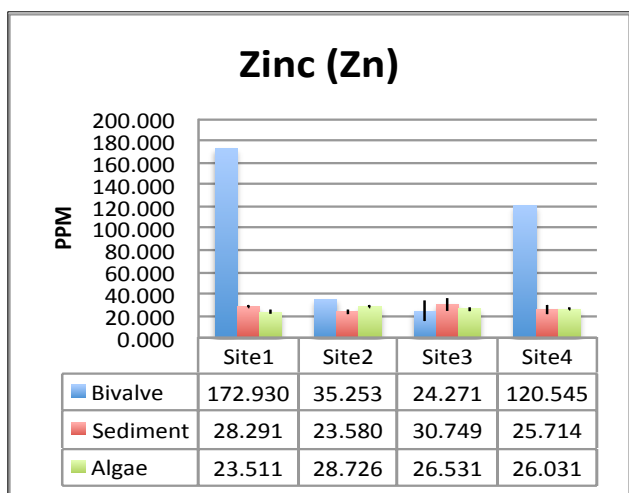


Fig 8: [Zinc] in biota, all sites

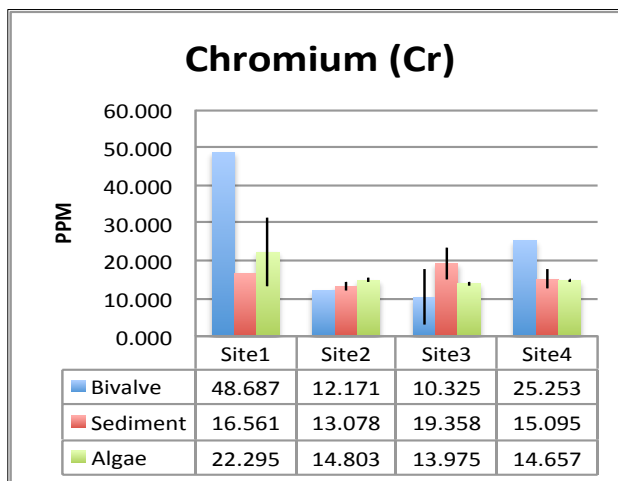


Fig 9: [Chromium] in biota, all sites

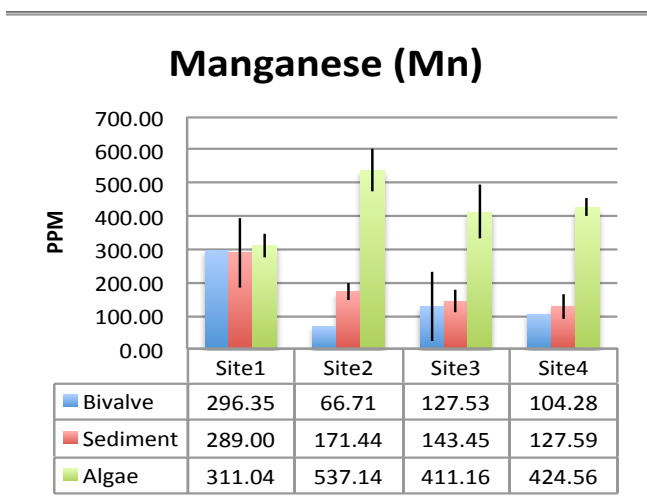


Fig 10: [Manganese] in biota, all sites

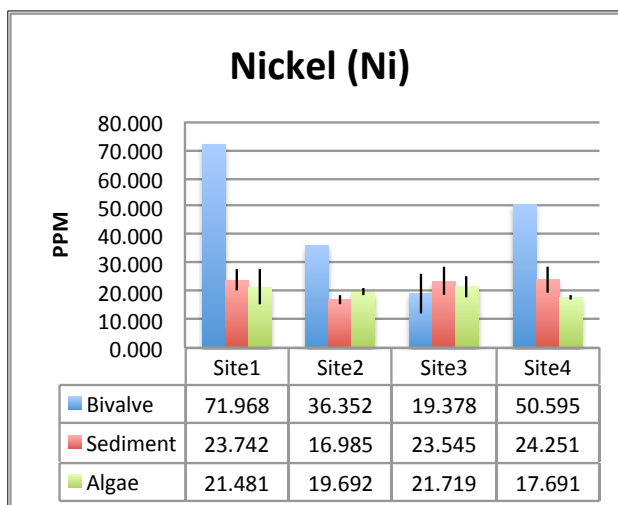


Fig 11: [Nickel] in biota, all sites

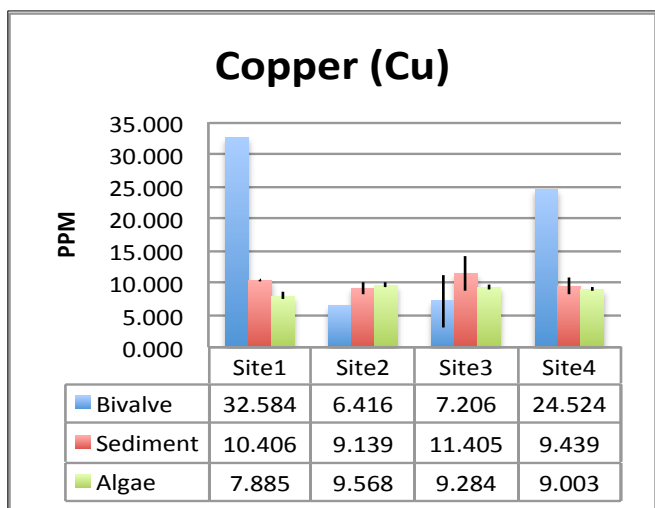


Fig 12: [Copper] in biota, all sites

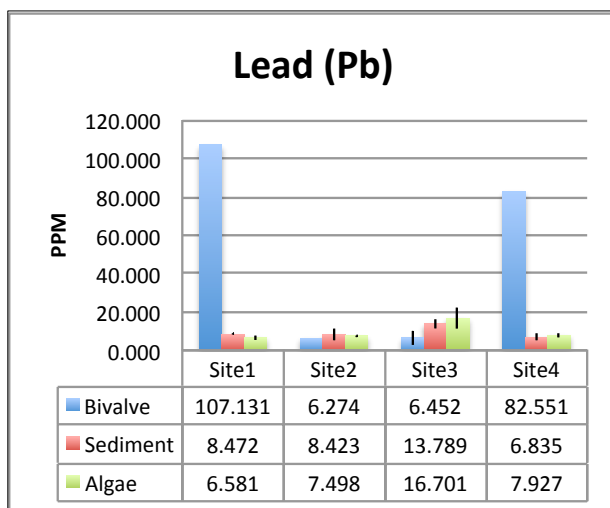


Fig 13: [Lead] in biota, all sites

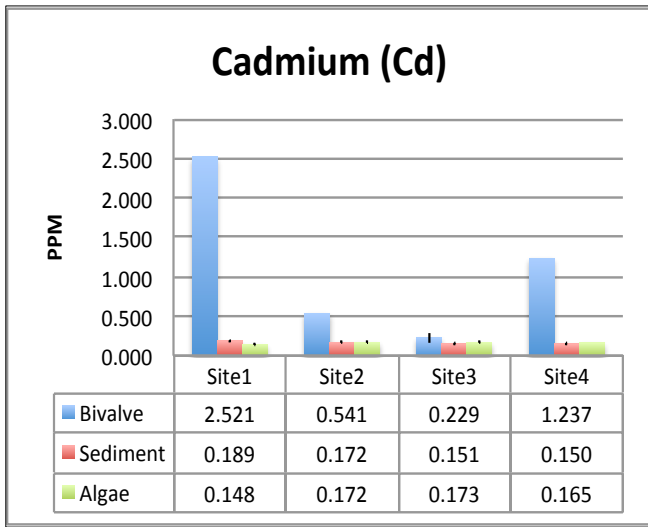


Fig 14: [Cadmium] in biota, all sites

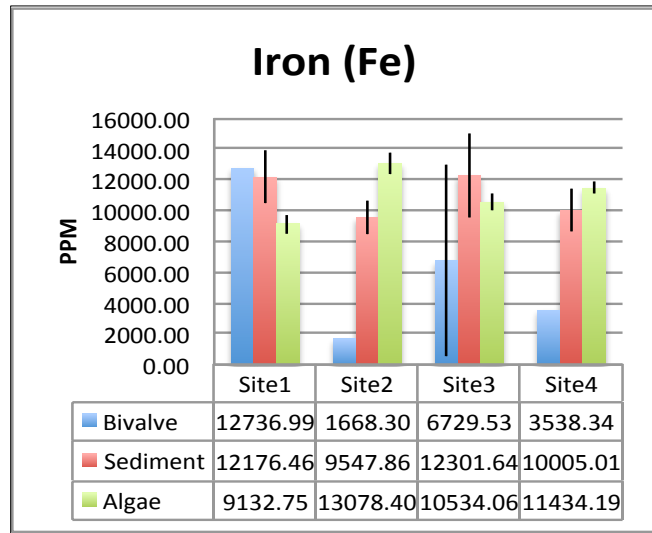
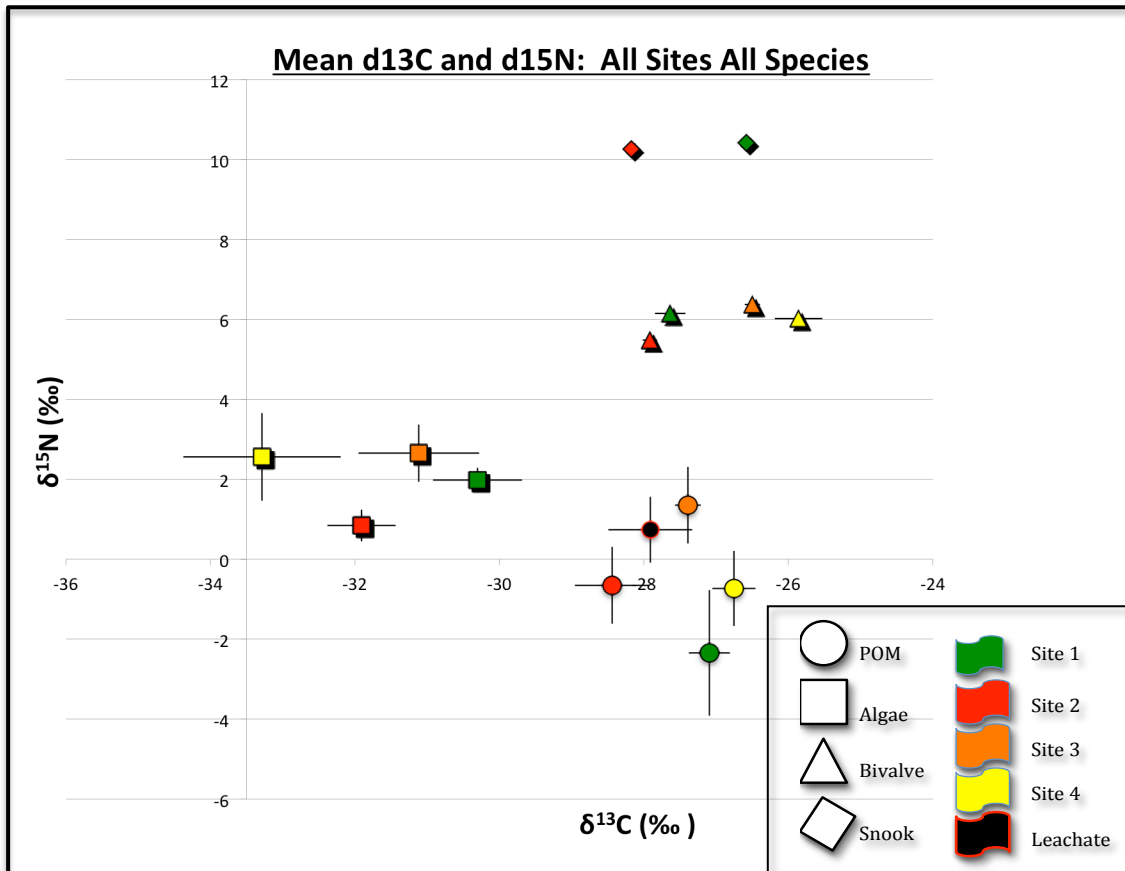


Fig 15: [Iron] in biota, all sites

Taking into account standard error, all of these decreases were significant with the exception of bivalves, which had only slightly decreased values. Among apparently impacted biota, being POM at Site 2 and in leachate, bivalves, and snook, $\delta^{13}\text{C}$ signatures are all similar, ranging from -28.44% to -27.92% . In particular, bivalve $\delta^{13}\text{C}$ signatures are nearly identical to signatures in leachate, at -27.92% to -27.91% respectively. Leachate values in POM were not significantly different to values at Site 2 and Site 3, as demonstrated with standard error. $\delta^{13}\text{C}$ in POM is highest at Site 4 ($\delta -26.75\%$ \pm 0.29 SE), followed by Site 1 ($\delta -27.09\%$ \pm 0.28 SE) Site 3 ($\delta -27.39\%$ \pm 0.17 SE), leachate ($\delta -27.91\%$ \pm 0.58 SE) and Site 2 ($\delta -28.44\%$ \pm 0.51 SE).

Fig. 16



5. Discussion

Phase 1: Water Quality and Biodiversity Analysis

Water quality analysis revealed significantly decreased values of dissolved oxygen at Site 2 in comparison to reference Site 1. With no known additional anthropogenic inputs into the Rio Grande river between these two sites (22), it is plausible that the dump site is responsible for downstream decreases in this parameter. Values at Site 2 ($2.9 \text{ mg l}^{-1} \pm 0.3 \text{ SE}$ on the surface and $1.6 \text{ mg l}^{-1} \pm 0.31 \text{ SE}$ at the floor) (Table 2) are well below levels reported to negatively impact aquatic species and biodiversity (at 3.2 mg l^{-1}) (23). This is supported with results from macroinvertebrate community surveying revealing changes in community structure and biodiversity between Site 1 and Site 2 of species 2, 5 and 8. Analysis of pH in leachate at the dump site, averaging at $7.04 \text{ mg l}^{-1} \pm 0.43 \text{ SE}$ supported methanogenesis as being within the waste mass of the Rio Grande dump site and were similar to values reported in studies of waste sites of similar age (9,10).

Trace elemental water quality analysis revealed high concentrations of many of the tested parameters expected to be produced by a methanogenic waste mass in leachate and at Site 2. Leachate contained high ammonia, phosphate, nitrate, chromium and manganese; zinc was the only exception and had relatively low values compared to tested sites on the Rio Grande. This could indicate naturally high levels of zinc in the Rio Grande, or an alternative anthropogenic source upriver, or at, Site 1. Phosphates had values at Site 2 ($0.115 \text{ mg l}^{-1} \pm 0.03 \text{ SE}$) comparable to leachate values ($0.127 \text{ mg l}^{-1} \pm 0.02 \text{ SE}$). Nitrates, zinc and chromium had values at Site 2 ($0.836 \text{ mg l}^{-1} \pm 0.13 \text{ SE}$, $0.07 \text{ mg l}^{-1} \pm 0.01 \text{ SE}$, $0.012 \text{ mg l}^{-1} \pm 0.002 \text{ SE}$ respectively) that exceeded leachate values ($0.746 \text{ mg l}^{-1} \pm 0.08 \text{ SE}$, $0.03 \text{ mg l}^{-1} \pm 0.01 \text{ SE}$, $0.0007 \text{ mg l}^{-1} \pm 0.002 \text{ SE}$ respectively). It is possible that these values exceed leachate as a result of Site 2 acting as a sink for trace elemental accumulation or as a result of leachate inputs mixing with other anthropogenic inputs and some background levels as well, creating increased values to leachate.

Phase 2: Biota Analysis:

Trace Element Biota Analysis: Of the elements tested both in water and biota, all values, among all samples were significantly higher in biota. Results then reveal trends demonstrating the possibility of increased contaminant concentration with increased trophic position, or bioaccumulation. Sites 2 and 3, being within 200m of one another and both expected to be receiving impact from the dump site, show elevated levels of many of the tested elements. In sediment, Site 3 had elevated concentrations of zinc, chromium, copper, lead, and iron. Algae analysis revealed more of an impact at Site 2, with high concentrations of zinc, manganese, copper, iron and cadmium; in algae all of these elements follow trends supporting impact from dump site on the Rio Grande river; with values highest at Site 2, slightly lower at Site 3, and further decreasing in concentration at Site 4 to levels found at uncontaminated Site 1. All of the above listed transition metals are not typically in high concentrations in methanogenic landfills and further, do not typically travel distances as far as what can be seen in data from this study (5, 13, 2, 27, 35). Many of these elements could then have been accumulated in biota from natural or other anthropogenic sources.

Possible Trace Elemental Inputs at Site 1: Ammonia, zinc, phosphate and manganese in water analysis and sediment [manganese], algae [chromium], and all tested elements in bivalves, are highest at Site 1. These results could indicate the possibility of an unexpected anthropogenic influence upstream, or at Site 1 that could be increasing the concentrations of these parameters in the Rio Grande River. Agriculture has been shown to input these elements into river systems (33). Belcampo Belize Lodge and organic farm is just 50m upstream from Site 1 and is built on land that was historically used for cane and cattle production (51); soil could then be retaining historical levels of elements inputted from these practices. The Rio Grande river was also used for industrial shipping 70-100 years ago according to numerous local accounts, at

Site 1 lie the remains of rusted metal structures, these historical factors could have an impact outside the scope of this study at Site 1. It is also possible that influence from the dump site could be reaching Site 1 via a presently unknown dominant water pathway or groundwater leaching. Lastly, these elements could simply be naturally occurring in high quantities as a result of the geology of the river.

Nitrogen and Carbon Stable Isotope Analysis: $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$: $\delta^{15}\text{N}$ values for each species increased by an average of 3.58 ‰ with trophic position, each species then shows higher fractionation than the trophic level below with algae and POM having similar fractionation, and snook and bivalves having clear increases in fractionation compared to the lower trophic levels sampled. $\delta^{13}\text{C}$ values in contrast show similar reduced fractionation between trophic levels with the exception of algae, which shows increased fractionation and/or the uptake of a more negative source of $\delta^{13}\text{C}$. Primary producers like aquatic algae have been shown to have highly variable $\delta^{13}\text{C}$ signatures as a result of diffusional resistance of $\delta^{13}\text{C}$ in water (58), this could explain the significant differentiation of $\delta^{13}\text{C}$ in algae in comparison to other tested biota.

Methanogenesis typically results in fractioning and enrichment of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ with heavy isotopes within landfill leachate (3, 15, 25). $\delta^{15}\text{N}$ values did not reveal enrichment in algae, bivalves and snook at Site 2; instead more negative $\delta^{15}\text{N}$ values were discovered at (1.68 ‰ \pm 0.94 SE, 5.48 ‰ \pm 0.11 SE, 10.26 ‰ respectively) compared to other sites (1.82-2.56‰, 6.02-6.37‰, and 10.41‰ respectively).

$\delta^{13}\text{C}$ analysis also revealed more negative values at Site 2 in all species compared to reference Site 1. Analysis of POM did however demonstrate trends of $\delta^{15}\text{N}$ enrichment in leachate at 0.736 ‰ \pm 0.82 SE, compared to reference Site 1 at -2.34 ‰ \pm 1.57 SE, and enrichment just above leachate values at Site 3 at 1.35 ‰ \pm 0.95 SE. Though, in contrast, $\delta^{13}\text{C}$ values in POM decreased, POM values at Sites 2 and 3 demonstrated similarities to leachate signatures in both parameters.

Comparable studies report similar trends, being changes in signatures at impacted sites, but instead report enrichment of tested isotopes as a byproduct of methanogenic bacteria up taking lighter $\delta^{12}\text{C}$ and $\delta^{14}\text{N}$ to produce methane and carbon dioxide (27). $\delta^{13}\text{C}$ values have been reported to range from -27‰ (5) to +20‰ in leachate (3, 5, 17) depending on whether organic or inorganic carbon was analyzed. $\delta^{15}\text{N}$ reports enrichment of +27.42 ‰ (3). Studies reporting such results are all conducted on water and soil, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ signatures in biota may not follow trends seen in comparable studies. Signatures are inevitably changed by geographical and biochemical conditions as leachate is transferred through an environment (25). Furthermore uptake of leachate affected $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ by primary producers and consumers, and eventually to upper trophic level species via bioaccumulation, will inevitably affect the final signature. Therefore, in contrast to enrichment seen in sediment and water analysis, stable isotopes in biota could decrease in heavier $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ isotopes and instead increase in lighter $\delta^{12}\text{C}$ and $\delta^{14}\text{N}$ with input from anthropogenic solid waste.

Changes in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ signatures in the Rio Grande's biota at impacted Site 2 and 3, in comparison to reference Site 1, are likely a result of leachate impact on the river based on the following;

1. Site 2 and Site 3 $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ POM values show similarities to signatures found in leachate at the dump site.
2. Consistently more negative $\delta^{15}\text{N}$ values in algae, bivalves and snook at Site 2 in comparison to reference Site 1.
3. Significantly decreased $\delta^{13}\text{C}$ in POM and snook at Site 2 in comparison to reference Site 1, as well as notable decreases in $\delta^{13}\text{C}$ in bivalves.

6. Conclusions:

Stable isotope analysis of nitrogen ($\delta^{15}\text{N}$) and carbon ($\delta^{13}\text{C}$) paired with toxicity testing of stored tissue body burdens in species of various trophic levels, complimented by water quality analysis has resulted in quantitative

assessment of potential bioaccumulation and contaminant transfer from the dump site under investigation to the Rio Grande river. Results from water analysis indicate that the dump site may be expelling relatively high levels of ammonia, phosphate, nitrate, chromium and manganese into the Rio Grande river and surrounding environment. Further, notable changes in both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in POM support the notion that the dump site is influencing the immediate area, and further, is impacting surface and groundwater that is reaching the Rio Grande, accumulating in inhabiting biota, as established via trace element and $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ analysis of biota and tissue. Further contaminant and statistical analysis is needed to determine unequivocally that this association is a negative one.

Plans for Relocation and restoration: The Solid Waste Management Authority (SWMA) has established the Rio Grande dump site as a priority for relocation. Currently the organization is relocating the central dump site in Belize City. The Rio Grande dump site will become priority soon after this existing project is complete, (estimated three years) (30). Results from this study will inform negotiations over the coming years involving the SWMA, TIDE, Punta Gorda Town Council, the public, and the Belize Government in regards to relocating the site and reforming solid waste management in the southern districts.

Recommendations for further analysis/ Limitations of study: It is important to determine the reason for high values of many of the tested trace elements in both water and biota at Site 1. This could involve testing additional sites even further upriver and comparing results with an additional river deemed to be similar enough in environmental conditions and inputs to the Rio Grande. Selection of additional sites must be deemed appropriate after a thorough hydrological and topographic survey. Lack of confidence in reference Site 1 being uninfluenced by the dumpsite via groundwater leaching is the greatest limitation in this study and analysis must be done to provide additional confidence in comparing all results to a more certain reference site. Additional replicates must also be tested to support the conclusions in this paper, particularly of bivalves and snook. Furthermore an additional species, Mayan cichlids (*Cichlasoma urophthalmus*) need to be analyzed as well to provide more location specific isotopic information than what can be achieved through analysis of higher trophic level snook. Analysis of additional trophic levels will also allow for increased confidence in expected bioaccumulation of trace elements in. Furthermore, analysis in this project excluded soil analysis in leachate; this must be included as a reference to discovered trace elemental values in the Rio Grande.

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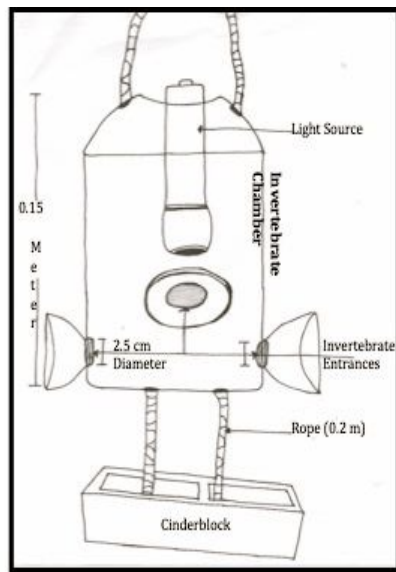
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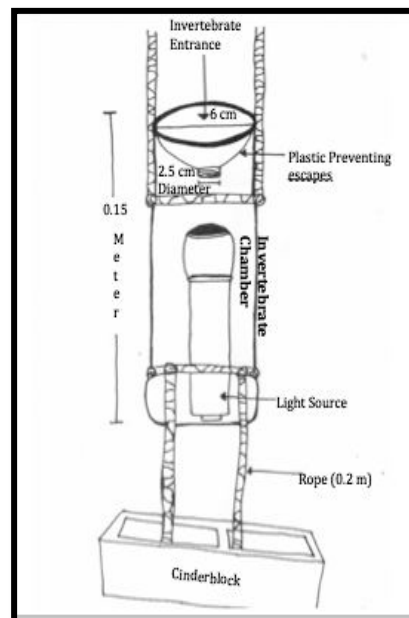
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Appendix:

Appendix 1: Quatrefoil Light trap design



Appendix 2: Sediment trap design



Full Macroinvertebrate Key: Results from Benthic Surveying

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In Collaboration with:

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Species 1



Species 2



Species 3



Species 4



Species 5



Species 6



Species 7



Species 8



Species 9



Species 10



Species 11



Species 12



Species 13



Species 14



Species 15



Species 16



Species 17



Species 18



Species 19



Species 20



Species 21



Species 22



Species 23



Species 24



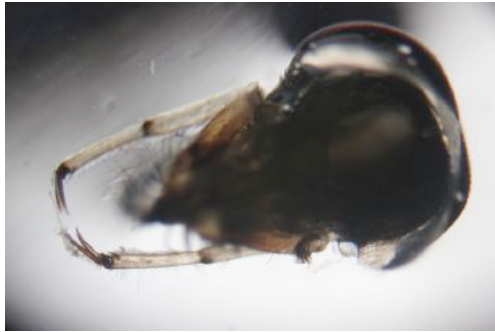
Species 25



Species 26



Species 27



Species 28



Species 29



Species 30



Species 31



Species 32



Species 33



Species 34

Appendix 4:
Key invertebrate species



Fig. 4: Species 2 (Family *Atyidae*)

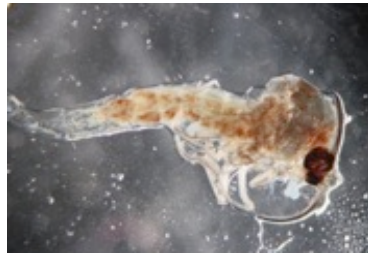


Fig. 5: Species 3 (Family *Tanaisidae*)

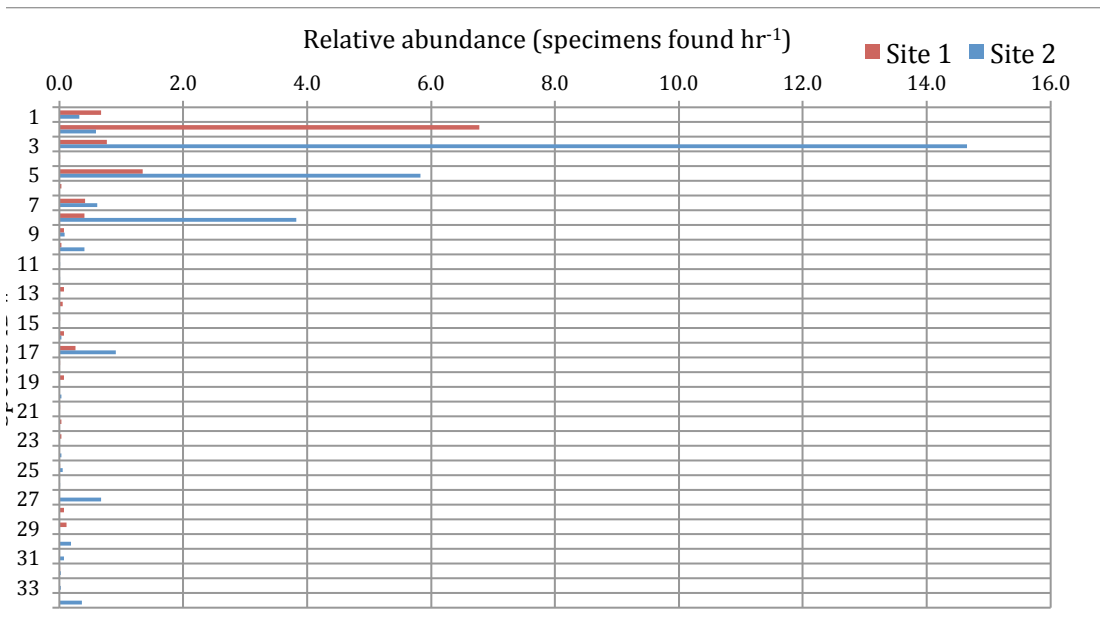


Fig. 6: Species 5 (Family unknown)



Fig. 7: Species 8 (Family unknown)

Appendix 5:
Relative abundance of all species per hour on the Rio Grande River, Sites 1 and 2 (29/05/2013) - (07/05/2013)



Appendix 6:
Glossary:

- Biota: The total collection of organisms that make up the biosphere.