

# Pollution Status of Incomati River Estuary Based on Meiofauna Analyses (Free-living Nematodes) in Maputo, Mozambique

Soko Mthobisi Innocent<sup>\*</sup>, Gyedu-Ababio Thomas

Inkomati-Usuthu Catchment Management Agency, Mbombela, South Africa

## Email address:

sokom@iucma.co.za (Soko M. I.), thomasga@iucma.co.za (Gyedu-Ababio T.)

<sup>\*</sup>Corresponding author

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**Abstract:** The Incomati Estuary, is located at Latitudes 25.430 S and 25.530 S and Longitudes 32.410 E and 32.44° E and discharges in the northern part of Maputo Bay. Four sites E1 (Oligohaline Zone), E2 (Euhaline Zone), E3 (Mesohaline zone), and E4 (Polyhaline Zone) were selected following the salinity gradient of the Incomati River Estuary. Sediments samples were collected in these sites for both the analyses of meiofauna communities especially free-living nematodes and environmental variables such as Heavy Metal, Chlorophyll-a, Nitrates and Total phosphorus. Multivariate statistical analyses were used to analyses the data, and nematodes were identified into genus level. Higher concentration of Heavy Metals such as Cadmium, Cobalt, Chromium, Copper, Iron, Manganese, Nickel, Vanadium, Zinc, and Aluminium were found at site E2. Nematodes such as *Terschellingia* and *Theristus* were found to be suitable indicators in identifying pollution. The Maturity Index further indicated that sites E2 followed by E1 were the polluted sites in the estuary. Further studies confirming the findings of this study must be done in the Incomati River Estuary, and other studies must be done in the African Coast in order to understand free-living nematodes and close the gap in our monitoring strategies.

**Keywords:** Nematodes, Pollution, Sediments, Chlorophyll-a, Metals, Nitrates

## 1. Introduction

Estuarine areas are the most fruitful natural ecosystem because they provide important ecological functions and services such as habitat, shoreline protection, food for migratory and resident species, harbour and recreational purpose [1-3]. Because they are situated in the lower reaches of basin, estuarine receives large amount of nutrients and pollutant derived from anthropogenic activities such as agricultural and industrial effluents [3, 4]. The Incomati River Estuary is also susceptible to this kind of pollution from different activities such as mining, industries, agricultural and afforestation which exist in the upper catchment (Swaziland and South Africa) of the basin. Pollutants from these anthropogenic activities reaches the estuary either through seepage, effluents, and run-offs, and affects the estuarine ecosystem and other goods and services

rendered by the estuary [5]. Nematodes have been used in assessing human and natural impacts in sediment because of their numerous advantages as biological indicators [6-9].

Numerous studies have used nematodes to assess soil pollution produced by heavy metal and nematodes were found to be dominant in finer sand less than 300µm other than copepods which have been found to be dominant in sediment coarser than 500µm [10-13]. The sensitivity of nematodes community to various kinds of anthropogenic disturbance have been emphasized by different studies [8, 14-18]. Meiobenthic assemblage provides good information because of their ecological features which gives them several benefits over macrofauna communities as monitoring fauna [6-7, 19, 20-21]. The assessment of benthic assemblage's composition is a valuable tool for determining and describing environmental changes of estuarine and marine system [22].

As pollution indicators, meiofauna have been thoroughly

studied in Bohai Sea [23-24], in the Huanghai Sea in China [23, 25-26] and in Changjiang Estuary areas [27-28]. In Tunisia and South Africa few studies of nematodes have been done [29-30], and only few studies used nematodes as pollution indicators [18, 31-32]. Several studies used Maturity Index to assess the impact caused by heavy metal, organic enrichment and eutrophication towards which nematodes responded positively [15, 33-38]. In a study conducted in the New York Bight [33], Maturity Index had a significant correlation with heavy metal concentration such as Chromium, Copper, Nickel, Lead, and Zinc. No studies in relation to meiofauna have been conducted in the Incomati

River Estuary to assess the pollution status of the Estuary. Thus, this study is the first to be done in this Estuary, and the main objective was to assess the pollution status of the Estuary using nematode assemblages as indicators.

## 2. Materials and Methods

### 2.1. Study Area

The Incomati Estuary, is located at Latitudes 25.430 S and 25.530 S and Longitudes 32.410 E and 32.44° E (Figure 1), and discharges in the northern part of Maputo Bay.

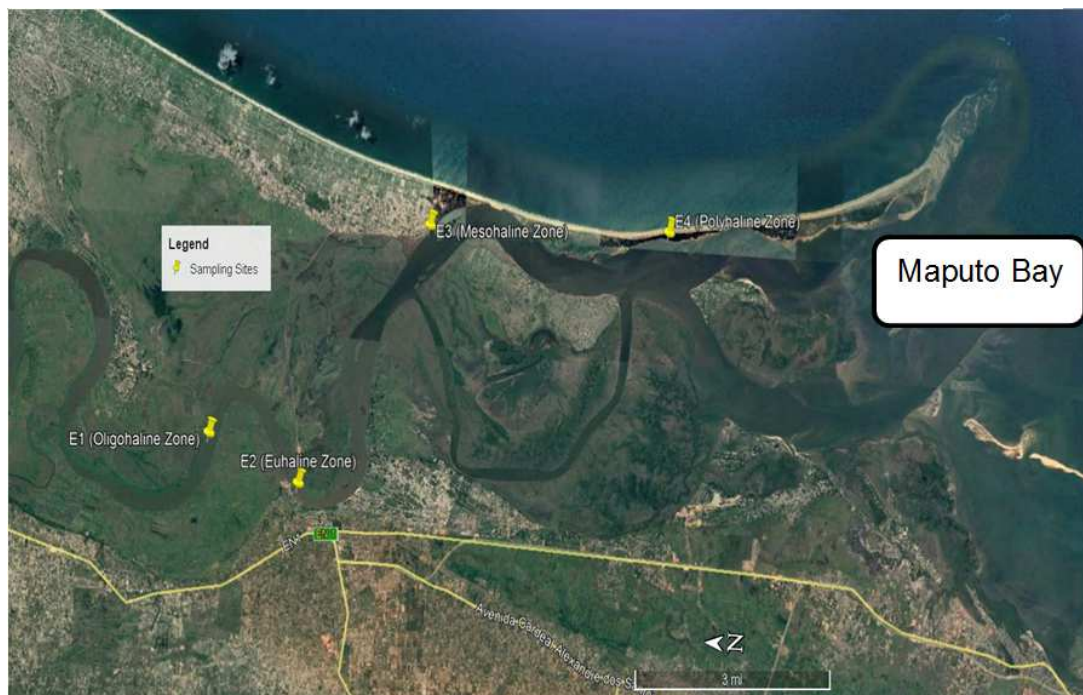


Figure 1. Map showing the monitoring sites in the study area.

Constructed dams in these rivers have changed the flow in the lower reaches, affecting the goods and services, therefore results in salt intruding in the inland [39-40]. The Incomati River Estuary is about 40-50 km long and meanders within the coastal plain. It is separated from the ocean by a narrow sand dune, a manifestation of the sluggish flow of the river. Four sites E1 (Oligohaline Zone), E2 (Euhaline Zone), E3 (Mesohaline zone), and E4 (Polyhaline Zone) were selected following the salinity gradient of the Incomati River Estuary for the purpose of this study.

### 2.2. Sample Collection and Analysis

Sediments were taken from the estuary bi-monthly from June 2017 to April 2018 using a hand held perplex corer which had a diameter of 3.6 cm which penetrated a depth of 10cm. Three replicate samples were taken for meiofaunal and environmental variables analyses on each visit to the Estuary.

### 2.3. Heavy Metal Analysis

Heavy metals analysis was done using the method

described [31]. Sediments samples were dried for 48hrs at 80°C in a petri-dishes. The dried samples were then crushed and about 2g of each sample was taken into a glass beaker with 20ml Aqua Regia (1:3 HNO<sub>3</sub>: HCL) and allowed to react overnight. The mixture was heated to near dryness and allowed to cool, before 20ml of a 5M HNO<sub>3</sub> solution was added. The samples were left to react overnight and were then filtered using a Whatman No 41 filter paper. The filtrates were transferred to a 100ml volumetric flask and made up the mark with 0.5M HNO<sub>3</sub>. Metal determinations of the solutions were performed on Shimadzu sequential plasma spectrometer (ICPS-1000II) using the calibration curve method. Concentration of the following metals: Mn, Al, V, Ti, Cu, Cr, Pb, Zn, Cr, Fe, Cd, and Co were determined using this method.

### 2.4. Sediment Particle Analysis

Sediment particle size analysis was done using the method described [41]. A 30-g portion of the sediment from each site was washed with tap water and reweighed after

drying. The samples were oven-dried at 60°C for 48 hrs. The dry samples were put on the topmost of a nest of sieves (with mesh size ranging from 0.002µm to 2mm) and sieved by a machine for 8 minutes. The fractions of each sieve were weighed. The median grain size, sorting values, mud composition and all the other sand fractions were determined using a computer programme, SANDX (SANDSTA.BAJ) by Olivetti (1984).

### 2.5. Chlorophyll-a

A 10g of each sediment sample collected from the sites was placed in a 20ml screw cap vial. About 10ml of 90% acetone was then added in the vials containing sediments and swirled once gently [42]. The vials were placed in a 5°C incubator overnight. After incubation, the solution was filtered through a Whatman GF/C and placed in a screw cap test tube. The filtered solution was adjusted to pH 9 using sodium hydroxide (NaOH) as a buffering solution. This was done to reduce the interference of pheophytin with spectrophotometric analysis of chlorophyll. A spectrophotometry with a 1nm spectral band width and optically matched 4cm cuvettes was used. 3ml of extracts from each sample was poured in to the 4cm cuvettes and the absorbance was measured at 664 and 750nm before acidifying (664<sub>b</sub> and 750<sub>b</sub>). This was done very quickly to prevent light from breaking down the chlorophyll. The absorbance was blanked at 664nm using the 90% acetone solution with a second recorded at 750nm to correct for primary pigments absorbance. After taking the initial measurements, a 0.1ml of 1N HCl was added directly to the cuvettes to estimate the amount of phaeopigments and the acid was allowed to react for 90 seconds and the absorbance was recorded at 665nm and 750nm (665<sub>a</sub> and 750<sub>a</sub>). The following equation was used to calculate Chlorophyll-a.

$$\text{Chlorophyll} - a \text{ (mg/m}^3\text{)} = \frac{26.7 (E_{664b} - E_{665a}) \times V_{\text{(extraction)}}}{V_{\text{(sample)}} \times L}$$

Where:

b: Before acidification

a: After acidification

$E_{664b} = [(Abs_{664b}(\text{sample}) - Abs_{664b}(\text{blank})) - (Abs_{750b}(\text{sample}) - Abs_{750b}(\text{blank}))]$

$E_{665a} = [(Abs_{665a}(\text{sample}) - Abs_{665a}(\text{blank})) - (Abs_{750a}(\text{sample}) - Abs_{750a}(\text{blank}))]$

$V_{\text{(extraction)}}$ : Volume of 90%acetone used in the extraction(ml)

$V_{\text{(sample)}}$ : Volume of water filtered

L: Spectral path length

### 2.6. Nitrates (NO<sub>3</sub>)

A 25ml of 1M NaCl solution was added to 1g of undried sediments and shaken on Kotterman's mechanical shaker for 30 minutes [43]. The supernatant was then pass through Whatman's no. 1 filter paper, and only 3ml of the filtrate was used for analysis in a test tube. A buffer solution (2 ml) was added to the filtrate and shaken for 10 minutes. Standard

solutions were then made in series: 0; 0.5; 1.0; 1.5; 2.0 and made up to a volume of 100 ml with distilled water. A 1ml sulphanilimide was added to 1 ml of the sample followed by 1 ml solution of diamine-hydrochloride. The mixture stayed for 5 minutes and the nutrient concentrations were then read on a Pye Unicam Spectrophotometer SP 1800 at 540 nm.

### 2.7. Total Phosphorus (TP)

Sediment was dried in an oven at 80°C, and sieved through a 4µm sieve [44]. 1g of the sieve sieved sample was heated in a 50ml conical flask on a digestion rack in a 4ml mixed acid digester for 2 hrs. The mixture was cooled down, and then diluted to 50 ml with distilled water. Ten ml colour reagent (1.06 g ascorbic acid, 1.2 g ammonium molybdate ((NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>·4H<sub>2</sub>O) and 27 mg of potassium antimony oxide tartrate in 180 ml distilled water) were added to 1 ml of the solution/mixture. This was followed by the addition of 9.5 ml concentrated H<sub>2</sub>SO<sub>4</sub>. The mixture was left to stand for an hour for the colour to develop. Standard series, 0, 0.5, 1.0, 1.5, 2.0, 2.5, was made and observed at 690nm using a spectrophotometer. A phosphorus standard curve was used to determine the total phosphorus content in the sediment sample.

### 2.8. Meiofauna Field Collection

A 6% MgCl<sub>2</sub> was used to rinse the inner diameter of the corer as to allow meiofauna to relax during sediment sampling. Sediment samples were taken from the four sites selected in the salinity gradient of the Incomati River Estuary. In each site, a duplicate of sediment samples was taken using a 1m long PVC corer with an inner diameter of 3.6 cm, corresponding to a surface area of 10 cm<sup>2</sup>.

#### 2.8.1. Meiofauna Laboratory Analysis

In the laboratory, sediment samples were transferred to centrifugal bottles and weighed. A sucrose solution of 589g prepared in a 1L bottle was added in the centrifugal bottles containing sediments to separate meiofauna from the sediments [45-47]. The sediment samples were centrifuged at 35000rpm for 5 minutes and the supernatant were decanted to another jar. After the supernatant was decanted, the bottles were weighed, and an amount of sucrose solution was added again. The samples were centrifuged for another 5 minutes. The final supernatant was decatant in to the same jar and sieve on a mesh aperture of 1mm followed by a sieve with a mesh aperture of 63µm. Detritus and macrofauna were retained in the upper sieve were discarded, and meiofauna were retained by the lower sieve. Meiofauna were then collected in 5% formalin with Rose Bengal for staining and easier identification.

#### 2.8.2. Nematodes Counting and Identification

Nematodes were then counted under a stereo microscope at 40x magnification using a counting petri-dish or a sorting tray [48]. They were then place in to solution of 5 parts glycerine, 5 parts ethanol and 90 parts distilled water. Finally, nematodes were mounted on a glass slides and identify to

genus level using the pictorial keys of [49]. The functional feeding groups designated [50] was used to investigate the trophic composition of nematodes assemblages.

## 2.9. Statistical Analyses

### 2.9.1. Environmental Variables

To find the pattern of multidimensional data of the environmental variables, a Principal Component Analyses (PCA) was performed to reduce the number of dimensions with a minimal loss of information. Environmental variables and each of the granulometric classes were transformed in to a square root. The calculation of environmental variables similarity matrix was based on Euclidean distance. A one-way ANOSIM was used to determine the significant difference between sites based on their environmental variables.

### 2.9.2. Meiofauna Assemblages

To investigate the trophic composition of nematodes assemblage, the features of buccal cavity morphology was followed [50]. According to this approach, four groups of feeders were defined: Selective deposit feeders (1A), non-selective deposit feeders (1B), epigrowth feeders (2A), and predator or omnivores (2B). The Index of Trophic Diversity was calculated as  $ITD = \sum \theta^2$  where  $\theta$  was the density contribution of each trophic diversity [19]. The ITD was used to compare the sites in terms of sediment contamination by heavy metals. To analyse nematodes life strategy, the Maturity Index was used [34], The Maturity Index formular

$$MI = \sum_{i=1}^n v(i) \cdot f(i)$$

was used to calculate the weighted average of the individual colonizer-persisters (c-p) values. The following symbols in the formular:  $v(i)$  represented the c-p value of the taxon, then  $i$  and  $f(i)$  was the frequency of that taxon. To determine the ecological quality status of the Incomati River Estuary the Maturity Index thresholds proposed by Moreno et al. 2011 were used to analyse the results.

A PRIMER VERSION 6 software was used to perform a univariate and multivariate analyses for both meiofauna and nematodes data. A PERMANOVA analyses using a Bray-Curtis similarity matrix to test the notion that meiofauna and nematodes community changes over a space and time with sites and month as fixed factors. Nematode groups were square root transformed to scale down their densities and to increase the importance of less abundance group analysis. A Redundancy Triplot analyses (XLSTATS software) was used to determine the relationships of nematodes and environmental factors. A non-parametric correlation spearman analyses were used to test the relationship between

nematodes and environmental factors. The one-way ANONISM permutation test was further used to test the difference between monthly sampling, and sites for the relative abundance of the four feeding types. A K-dominance curve was plotted for the comparison of species composition at the sampling sites. A Distance Linear Model (DistLM) was used to determine the relationship between environmental variables and the structure of nematodes community [51-52]. The DistLM routine was based on the AIC model selection criterion using the step-wise selection procedure [53]. The above analyses were conducted using PRIMER 6.0 which is a multivariate statistical package developed by Plymouth Marine Laboratory [54].

## 3. Results

### 3.1. Sediments

Variation of sediment particle sizes was found in the four sites sampled in the Incomati river estuary and the percentage of fine sand particle was higher towards fresh water where deposition was higher. Site E1 was characterised mostly by fine sand with 46.32%, followed by granules (>2.0mm), and mud and fine sand (<212µm) with 21.31% and 19.6% respectively. Site E2 was mostly characterised by granules sand (>2.0mm) with 32.28%, followed by medium grain size, and mud and fine sand with 30.48% and 14.7% respectively. At site E3, variation of particles sites was found at this site, but the most dominant particle size was coarse to very coarse sand (600µm) with 35.47%, followed by granules (>2.0mm), very coarse sand (1.4mm) with 22.64% and 16.91% respectively. Fine and medium sand particles (212-355µm) contributed 12.56% of sediment particle sizes. Site E4 was mostly characterised by coarse to very coarse sand particles with 47.79%. A one-way ANOVA indicated that there was no significant difference ( $p > 0.05$ ) of sediments particle size between the sites sampled.

### 3.2. Heavy Metals

Ten metal concentrations (Cadmium, Cobalt, Chromium, Copper, Iron, Manganese, Nickel, Vanadium, Zinc, and Aluminium) were found (Table 1) in the estuary. The highest concentration of heavy metals was found at sites E2 (Euhaline Zone) and E1 (Oligohaline Zone). The PERMANOVA results indicated that there was a significant difference ( $p < 0.05$ ) between sites sampled, but significant difference ( $p > 0.05$ ) did not exist between months sampling which indicated that the concentration of metals changes spatial, but not temporal.

Table 1. Environment factors analyse in the Incomati River Estuary from June 2017 to April 2018.

Environmental Factors	E1						E2					
	Jun-17	Aug-17	Oct-17	Dec-17	Feb-18	Apr-18	Jun-17	Aug-17	Oct-17	Dec-17	Feb-18	Apr-18
Cd (ppm)	0.27	0.12	0.1	0.1	0.1	0.1	0.3	0.29	0.13	0.12	0.09	0.09
Co (ppm)	6	2.7	2.8	2.7	2.7	2.7	8.6	8.7	1.9	1.8	0.82	1.5
Cr (ppm)	22	12	9.4	6.1	6.1	6.1	20	30	11	14	8.3	6.2

Environmental Factors	E1						E2					
	Jun-17	Aug-17	Oct-17	Dec-17	Feb-18	Apr-18	Jun-17	Aug-17	Oct-17	Dec-17	Feb-18	Apr-18
Cu (ppm)	9.5	5.6	5	3.8	3.8	3.8	12	13	6.5	6.3	4.4	4.9
Fe (ppm)	9549	4667	4191	2574	2574	2574	20595	21130	3938	3892	1951	3245
Mn (ppm)	242	164	132	68	68	68	320	391	116	101	55	181
Ni (ppm)	18	7.3	6.7	6.1	6.1	6.1	22	24	7.6	8	4.4	5.8
V (ppm)	16	6.8	6.1	4.1	4.1	4.1	27	27	6	6.1	2.9	4.8
Zn (ppm)	15	18	7.1	14	14	14	24	25	5.9	6.1	3.6	11
Al (ppm)	11648	4974	3898	2764	2764	2764	16945	15945	4415	4150	2236	3921
TP	110	79	35	27	27	27	273	300	68	68	36	59
NO <sub>3</sub> (mg/l)	0.05	0.05	0.05	0.05	0.05	0.05	0.01	0.01	0.01	0.01	0.01	0.01
Chl-a (mg/m <sup>3</sup> )	0.8	0.6	1.2	0.9	0.7	1.5	0.6	1.6	0.9	0.5	0.9	0.7

Table 1. Continued.

Environmental Factors	E3						E4					
	Jun-17	Aug-17	Oct-17	Dec-17	Feb-18	Apr-18	Jun-17	Aug-17	Oct-17	Dec-17	Feb-18	Apr-18
Cd (ppm)	0.09	0.09	0.09	0.09	0.17	0.1	0.09	0.09	0.09	0.09	0.09	0.09
Co (ppm)	1.3	1.3	1.3	0.95	3.6	1.2	0.34	0.77	0.62	0.38	0.52	0.36
Cr (ppm)	17	17	17	16	44	9.3	6	13	7.6	6.3	7.2	7.1
Cu (ppm)	3.8	3.8	3.8	3.1	7.2	4.5	3.8	5.1	3.6	3.5	4	4.6
Fe (ppm)	1952	1952	1952	1799	6336	2676	1648	1531	1761	1472	1495	1315
Mn (ppm)	44	44	44	45	101	51	26	172	50	33	41	36
Ni (ppm)	2.3	2.3	2.3	2.1	8.3	3.4	2.1	7.5	3	2.8	2.9	3.1
V (ppm)	2.9	2.9	2.9	2.9	10	4.1	1.1	1.1	1.8	1.3	2	1.3
Zn (ppm)	4	4	4	5.3	14	9.2	13	25	2.7	3.7	3.8	5.1
Al (ppm)	1328	1328	1328	1316	5976	2312	748	551	1101	1003	1150	872
TP	34	34	34	34	83	46	25	15	30	24	25	24
NO <sub>3</sub> (mg/l)	0.07	0.07	0.07	0.07	0.1	0.1	0.14	0.14	0.14	0.1	0.06	0.1
Chl-a (mg/m <sup>3</sup> )	0.8	0.7	4	4.3	4.6	4.9	0.04	2.2	1.8	0.7	2	0.7

### 3.3. Chlorophyll-a

The highest Chlorophyll-*a* concentration from sediments was found at site E3 with mean concentration value of 3.2 mg/m<sup>3</sup> followed by site E4 with a mean concentration of 1.24 mg/m<sup>3</sup>. There was a significant difference ( $p < 0.05$ ) between the sites pertaining the chlorophyll-a concentration between the sites.

### 3.4. Nitrates (NO<sub>3</sub>)

A variation of nitrates concentration was observed at site E3 with a range of 0.07 (mg/l) to 0.1 (mg/l) and a mean concentration of 0.08 (mg/l) (Table 1). Similarly, at site E4 a variation of nitrate concentration was observed with a range of 0.06 (mg/l) to 0.14 (mg/l), with a mean concentration value of 0.11 (mg/l). Significant difference ( $p < 0.05$ ) was observed between the sites sampled pertaining to nitrates concentration.

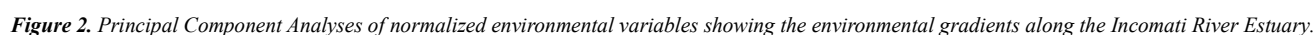
### 3.5. Total Phosphorus

The highest concentration of total phosphate was observed as site E2 with a mean value of 134 (ppm) (Table 1). Site E1 had the second highest concentration of total phosphate with a mean value of 50.8 (ppm). Sites E3 and E4 had a mean concentration of total phosphate of 44.2 (ppm) and 27 (ppm)

respectively. There was a significant difference ( $p < 0.05$ ) of total phosphate between the sites.

### 3.6. Relationship Between Environmental Factors

The first two PCA axes were accounted for a total of 92.6% of the total variation with the PCA1 and PCA2 accounted for 70% and 22.6% respectively (Figure 2). The high percentage of variations was also seen in the eigenvectors which gave consistent large values for the environmental factors except for Nitrates, Gravel, Fine Medium Sand, Zinc, Fine Sand and Chromium on the PCA1 axis. The PCA shows the eigenvector numbers graphically, with most environmental factors increasing towards site E2 (Euhaline Zone). In contrast, Nitrates, Gravel, Fine Medium Sand, Zinc, Fine Sand and Chromium all had the large eigenvector on the PCA2 axis, thus their vectors increased on the PCA2. Fine Sand, Fine Medium Sand and Zinc increased on the direction of the Oligohaline Zone at site E1. Nitrates and Coarse Very Coarse sand increased towards the Polyhaline Zone (E4) and Chlorophyll-a and Very Coarse Sand increased towards the Mesohaline Zone (E3). The PCA analyses lastly indicated the separation of sampling sites or zones within the Estuary and the ANOSIM formally confirms this with an overall ANOSIM R of 0.501, reflecting the pairwise R values of the sites.



Meiofauna density and their composition followed a clear pattern along the estuarine salinity gradient in the Incomati River Estuary. Nine meiofaunal taxa (Nematoda, Copepoda, Turbellaria, Amphipoda, Polychaeta, Kinorhyncha, Oligochaeta, Gastrotricha, Ostracoda) and other insecta were

**Table 2.** Meiofauna community identified along a salinity gradient in the Incomati River Estuary from June 2017 to April 2018.

Table 2. Continued.

MEIOFAUNA	E3						E4					
	Salinity Range											
	5-18NST						18-26NST					
	Jun-17	Aug-17	Oct-17	Dec-17	Feb-18	Apr-18	Jun-17	Aug-17	Oct-17	Dec-17	Feb-18	Apr-18
Nematoda	200	200	350	680	252	180	321	250	400	890	422	322
Copepoda	8	10	7	32	21	1	7	3	16	13	10	0
Turbellaria	5	0	2	0	0	4	0	3	9	6	8	3
Amphipoda	6	2	2	5	0	1	0	0	3	0	0	0
Halacarida	0	0	0	0	0	0	0	0	0	0	0	0
Polychaeta	0	0	2	0	0	0	0	0	0	2	0	0



MEIOFAUNA	E3						E4					
	Salinity Range											
	5-18NST						18-26NST					
	Jun-17	Aug-17	Oct-17	Dec-17	Feb-18	Apr-18	Jun-17	Aug-17	Oct-17	Dec-17	Feb-18	Apr-18
Kinorhyncha	2	0	0	0	2	1	7	0	2	2	4	3
Oligochaeta	0	0	0	2	0	0	0	0	0	0	0	0
Gastrotricha	4	5	0	0	3	0	4	2	0	3	0	0
Ostracoda	3	11	21	8	8	9	1	2	5	19	6	4
Ciliophora	0	0	0	0	0	0	0	0	0	0	0	0
Cladocera	0	0	0	0	0	0	0	0	0	0	0	0
Insecta	5	0	0	1	3	0	0	3	0	0	2	0

In all sites nematodes were the most dominant meiofauna taxa with a density of 6311 individual/10cm<sup>2</sup> and contributed about 92% of the total meiofaunal abundance. Copepods were found to be the second dominant meiofaunal group in the estuary. They had a density of 177 individuals/10cm<sup>2</sup> contributing 2.7% of the total meiofauna group. *Ostracoda* and *Turbellaria* contributed 2% and 0.9% of the total meiofauna group respectively.

### 3.7.1. Nematodes Density

A total of 5989 nematodes were found with variation of density between the months sampled. The highest density of nematodes at sites E1 (200 individual/10cm<sup>2</sup>), E2 (160 individual/10cm<sup>2</sup>), E3 (680 individual/10cm<sup>2</sup>), and E4 (890 individual/10cm<sup>2</sup>) were found in the months of February 2018, August 2017 and December 2017 respectively (Table 3).

Nematode density decreased towards site E1 which was situated in the Oligohaline zone. A significant difference ( $p < 0.05$ ) of nematodes density between the sites sampled during the study period was observed.

### 3.7.2. Nematode Diversity

A total of 2363 nematode were identified using a compound microscope, and a total of 39 nematode genera were found in the Incomati River Estuary (Table 4). The highest diversity of nematode was found at site E4 (Polyhaline zone) and the

lowest was found at sites E2 (Euhaline Zone) and E1 (Oligohaline Zone). The diversity of nematodes at site E1 (Oligohaline Zone) ranged from 4 to 13 and the genus dominated this site was *Haliplectus* which dominated the community with 41%, followed by *Axonolaimus* with 13.2%. At site E2 (Euhaline Zone), the number of nematodes genera ranged from 4 to 12, and nematode genera dominated this site were *Terschellingia* with 47.5%, followed by *Theristus* with 20.8% of the total community. Other nematode genera that dominated the community at site E2 were *Axonolaimus*, *Sabatiera*, *Daptonema*, and *Parodontophora*. At sites E3 and E4, nematode genera ranged from 11 to 18 and 13 to 21 respectively.

**Table 3.** Mean monthly density (Individuals/10cm<sup>2</sup>) at the study sites in the Incomati River Estuary from June 2017 to April 2018.

Site Names	E1	E2	E3	E4
June 2017	61	70	200	321
August 2017	110	160	200	250
October 2017	100	150	350	400
December 2017	150	123	680	890
February 2018	200	157	252	422
April 2018	100	141	180	322
Total	721	801	1862	2605
MEAN (SD)	120.2 (48.3)	133.5 (33.8)	310.3 (191.3)	434.2 (231.7)

**Table 4.** Feeding types, c-p values, salinity ranged, and Nematodes Genera identified in the Incomati River Estuary from June 2017 to April 2018.

NEMATODE GENUS	c-p values	Feeding types	E1						E2					
			Salinity range amongst the sites											
			0-3NST						3-5NST					
			Jun- 17	Aug- 17	Oct- 17	Dec- 17	Feb- 18	Apr- 18	Jun- 17	Aug- 17	Oct- 17	Dec- 17	Feb- 18	Apr- 18
<i>Adoncholaimus</i>	3	2B	13	12	15	3	0	0	0	0	1	0	0	0
<i>Aegialolaimus</i>	4	1A	3	0	0	4	2	0	0	0	2	0	0	0
<i>Anoplostoma</i>	2	1B	10	15	0	9	13	11	0	0	2	0	0	0
<i>Axonolaimus</i>	2	1B	3	15	16	12	3	26	0	10	9	10	10	20
<i>Batylaiumus</i>	2	1B	0	0	0	0	0	0	0	0	0	0	0	0
<i>Camacolaimus</i>	3	2A	0	0	0	0	0	8	3	2	0	1	0	0
<i>Cephalainticoma</i>	2	2A	0	0	0	0	0	0	0	0	0	0	0	0
<i>Daptonema</i>	3	1B	0	3	1	2	0	0	1	1	1	10	3	0
<i>Dichromadora</i>	2	2A	0	0	0	0	0	0	0	0	0	0	0	0
<i>Dolicholaimus</i>	2	2B	0	0	0	0	0	0	0	0	0	0	0	0
<i>Enoplus</i>	5	2B	0	0	0	0	0	0	0	0	0	2	0	0
<i>Filoncholaimus</i>	4	2B	0	0	0	0	0	0	0	0	0	4	0	0
<i>Halalaimus</i>	4	1A	0	0	0	0	0	0	0	0	0	0	0	0
<i>Haliplectus</i>	2	1A	23	29	35	34	54	55	0	3	0	5	0	0
<i>Leptolaimus</i>	2	1A	0	0	0	0	0	0	0	0	0	0	0	0
<i>Metachromadora</i>	3	2A	0	0	0	0	0	0	0	0	0	0	0	0
<i>Metacyatholaimus</i>	3	2A	0	0	0	0	0	0	0	0	2	1	0	0
<i>Microilaimus</i>	2	2A	0	0	0	0	0	0	0	0	2	0	0	0

NEMATODE GENUS	c-p values	Feeding types	E1						E2					
			Salinity range amongst the sites											
			0-3NST						3-5NST					
			Jun- 17	Aug- 17	Oct- 17	Dec- 17	Feb- 18	Apr- 18	Jun- 17	Aug- 17	Oct- 17	Dec- 17	Feb- 18	Apr- 18
<i>Monhystera</i>	2	1B	0	0	0	0	0	0	0	0	6	0	0	0
<i>Neochomadora</i>	3	2A	0	0	0	0	0	0	0	0	0	0	0	0
<i>Oncholaimellus</i>	3	2B	0	0	0	0	0	0	0	0	0	0	0	0
<i>Oxystomina</i>	4	1A	0	0	6	0	0	0	0	0	0	0	0	0
<i>Paracyatholaimus</i>	2	2A	0	0	0	0	0	0	0	0	0	0	0	0
<i>Paramonohystera</i>	4	1B	0	4	3	4	13	0	14	4	9	6	8	0
<i>Pomponema</i>	3	2B	0	0	0	0	0	0	0	0	0	0	0	0
<i>Pseudochromadora</i>	3	2A	0	0	0	0	0	0	0	0	0	1	0	0
<i>Rhabditis</i>	1	1A	1	3	4	8	3	0	0	0	0	0	0	0
<i>Sabatiera</i>	2	1B	0	0	0	4	1	0	8	3	0	0	0	0
<i>Scaptrella</i>	2	2B	0	0	0	0	0	0	0	0	0	0	0	0
<i>Spirinia</i>	3	2A	0	0	0	0	0	0	0	0	0	0	0	0
<i>Synonchium</i>	3	2B	0	8	12	2	4	0	6	6	0	0	0	0
<i>Terschellingia</i>	3	1B	2	5	2	8	4	0	56	56	50	30	52	41
<i>Theristus</i>	2	1B	6	3	3	4	0	0	12	10	13	31	25	34
<i>Viscacia</i>	3	2B	1	3	3	6	5	0	0	5	3	0	2	5
<i>Xyala</i>	3	1B	0	0	0	0	0	0	0	0	0	0	0	0

Table 4. Continued.

NEMATODE GENUS	c-p values	Feeding types	E3						E4					
			Salinity range amongst the sites											
			5-18NST						18-26NST					
			Jun- 17	Aug- 17	Oct- 17	Dec- 17	Feb- 18	Apr- 18	Jun- 17	Aug- 17	Oct- 17	Dec- 17	Feb- 18	Apr- 18
<i>Adoncholaimus</i>	3	2B	0	1	2	10	11	0	0	6	0	1	2	0
<i>Aegialolaimus</i>	4	1A	0	0	3	0	10	0	0	11	12	6	0	8
<i>Anoplostoma</i>	2	1B	2	0	0	6	0	0	0	9	3	6	4	3
<i>Axonolaimus</i>	2	1B	0	0	0	0	0	0	0	11	0	10	4	4
<i>Batylaiumus</i>	2	1B	10	0	0	11	0	50	0	0	0	0	0	4
<i>Camacolaimus</i>	3	2A	0	0	0	0	0	2	0	0	0	0	0	0
<i>Cephalainticoma</i>	2	2A	2	0	0	0	0	0	0	0	0	2	0	0
<i>Daptonema</i>	3	1B	10	0	0	9	5	5	10	0	0	6	12	2
<i>Dichromadora</i>	2	2A	3	0	0	0	20	0	12	2	0	3	10	8
<i>Dolicholaimus</i>	2	2B	4	0	1	0	0	0	0	0	0	2	10	0
<i>Enoplus</i>	5	2B	6	0	0	0	0	0	0	3	0	0	0	0
<i>Filoncholaimus</i>	4	2B	6	20	5	0	9	0	2	0	0	0	0	0
<i>Halalaimus</i>	4	1A	5	0	0	2	3	0	0	3	0	0	2	0
<i>Haliplectus</i>	2	1A	0	0	0	1	0	0	0	0	0	0	0	0
<i>Leptolaimus</i>	2	1A	3	0	1	3	0	0	1	5	3	3	0	0
<i>Metachromadora</i>	3	2A	0	8	0	0	0	5	5	7	10	4	8	0
<i>Metacyatholaimus</i>	3	2A	0	0	0	0	0	0	0	0	0	0	0	0
<i>Microlaimus</i>	2	2A	3	2	1	1	3	0	1	6	5	7	2	0
<i>Monhystera</i>	2	1B	0	2	2	0	0	0	5	2	0	7	3	0
<i>Neochomadora</i>	3	2A	0	3	7	6	9	4	0	0	0	13	0	0
<i>Oncholaimellus</i>	3	2B	0	1	2	1	0	0	0	2	0	0	0	0
<i>Oxystomina</i>	4	1A	0	20	10	9	0	8	3	0	0	0	0	0
<i>Paracyatholaimus</i>	2	2A	0	0	0	0	2	0	4	10	0	0	13	0
<i>Paramonohystera</i>	4	1B	1	19	12	5	2	3	2	1	0	3	4	1
<i>Pomponema</i>	3	2B	0	0	3	2	5	0	6	0	0	2	4	32
<i>Pseudochromadora</i>	3	2A	4	0	10	7	5	2	2	1	12	0	0	0
<i>Rhabditis</i>	1	1A	0	0	0	3	0	5	6	0	2	0	0	4
<i>Sabatiera</i>	2	1B	5	12	14	20	0	0	10	0	5	3	0	19
<i>Scaptrella</i>	2	2B	0	0	0	0	0	0	3	4	6	5	0	0
<i>Spirinia</i>	3	2A	0	0	0	0	0	0	3	1	0	2	3	5
<i>Synonchium</i>	3	2B	0	0	0	0	0	0	0	0	0	0	0	0
<i>Terschellingia</i>	3	1B	0	4	9	0	0	0	10	6	10	10	8	0
<i>Theristus</i>	2	1B	36	3	5	4	0	1	0	5	10	0	0	1
<i>Viscacia</i>	3	2B	0	5	12	0	16	15	16	3	12	6	11	9
<i>Xyala</i>	3	1B	0	0	1	0	0	0	0	2	10	1	0	0

A K-dominance curve (Figure 3) further indicated that at cumulative dominance of 40% a single genus (*Haliplectus*) dominated the nematodes communities at site E1. The dominance of a single genus (*Terschellingia*) at a cumulative dominance



above 40% was also observed at site E2.

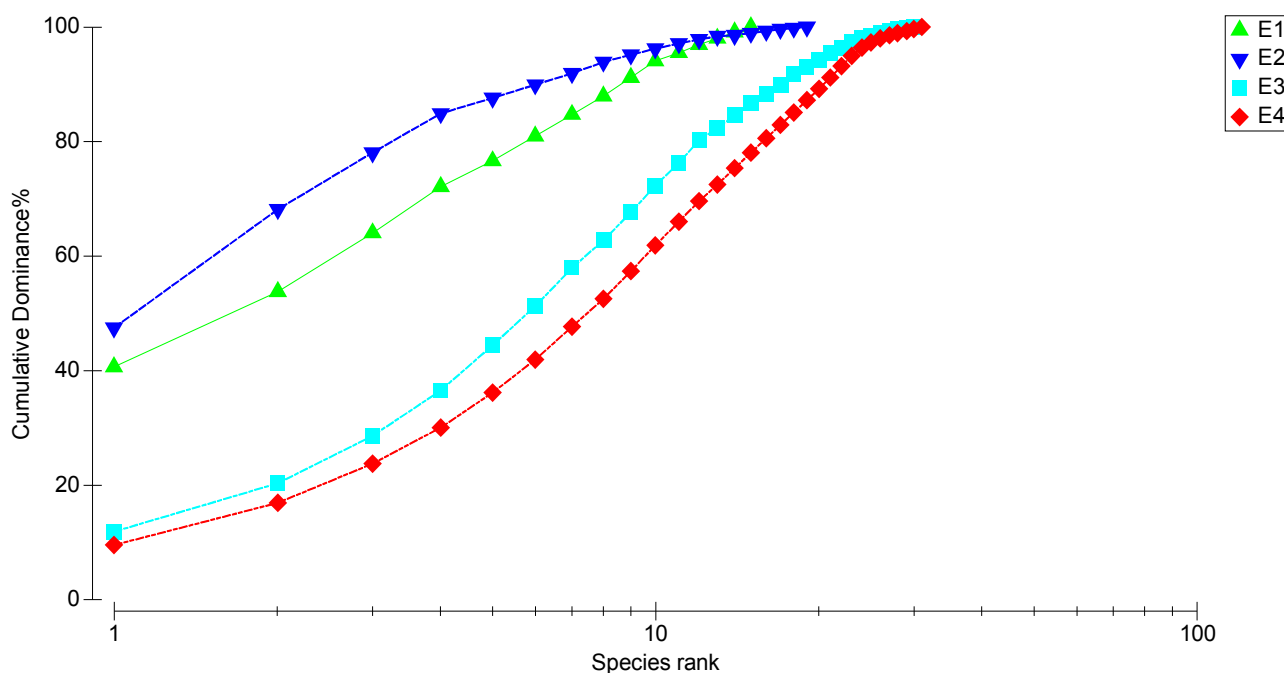


Figure 3. Ranked species K-dominance curves for the free-living nematode genera identified at the Incomati River Estuary.

### 3.7.3. Nematode Feeding Types

All four nematode feeding types were found, and their trophic diversity percentage were calculated for their dominance between the sites (Figure 4). The Trophic Diversity Index percentage indicated that throughout the study, nematodes feeding type 1B (non-selective deposit feeders) were dominant in all sites sampled. The highest mean percentage contributions of feeding type 1B was

observed at site E2 with 88.9%. Sites E2 (Euhaline Zone) and E3 (Mesohaline Zone) had similar percentage contribution of feeding type 1B. The mean percentage of the feeding types indicated that feeding type 1B was the dominant group, followed by feeding type 2B. A two-way ANONISM permutation test indicated that significant difference exists between sites ( $Rho=0.221$ ;  $p=0.043$ ), and between month sampled ( $Rho=0.688$ ;  $p=0.001$ ).

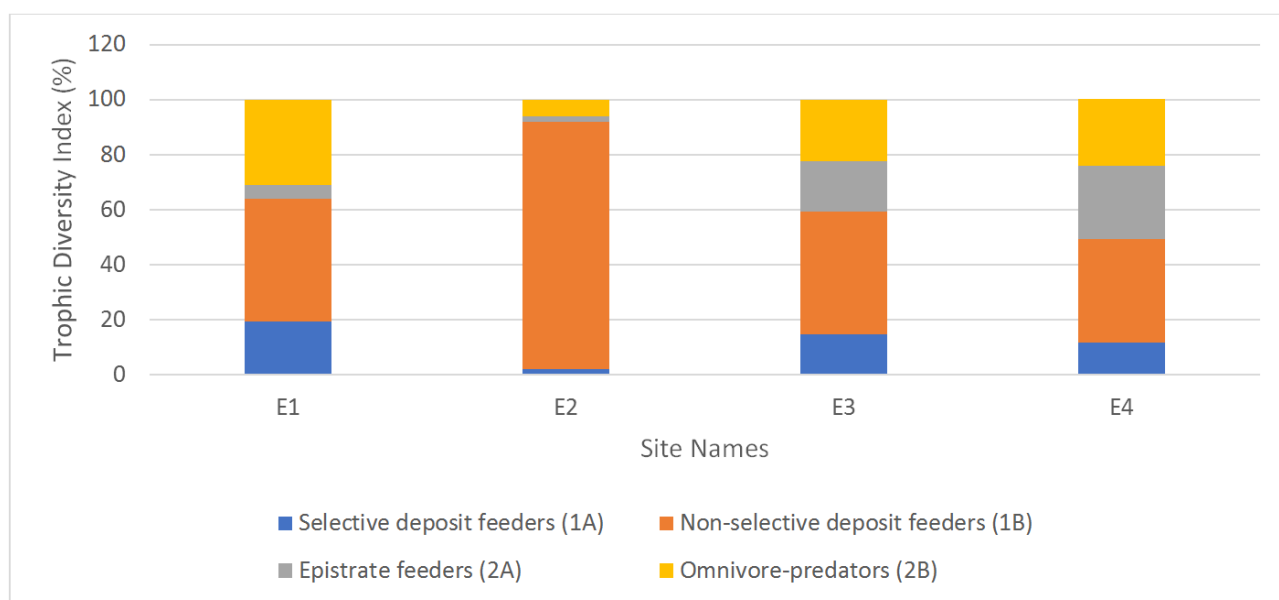


Figure 4. Trophic Diversity Index percentage of nematode feeding types in the Incomati River Estuary.

### 3.7.4. Relationships Between Free-living Nematodes and Environmental Factors

The first two axes of the RDA accounted for 92.51% of the variation in free-living nematodes and environmental factors (figure 5). The first axes accounted for 68.94% and the second axes accounted 23.57% of the variance in the data.

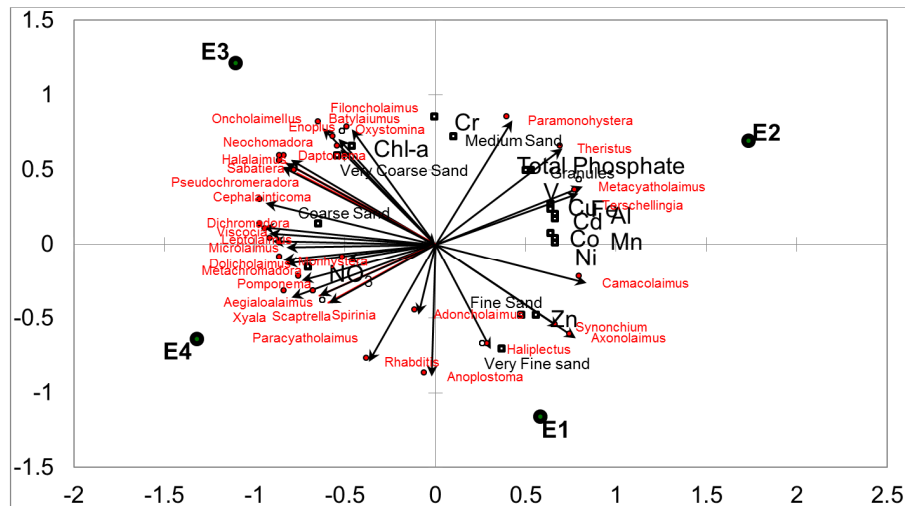


Figure 5. RDA tri-plot illustrating the relationship between nematodes diversity and environmental factors found in the Incomati River Estuary.

A spearman analysis showed that there was a significant correlation ( $Rho=0.381$ ;  $p=0.001$ ) between environmental factors and free-living nematodes. A relationship between environmental factors such as Coarse sand, Very Coarse Sand, Chlorophyll-*a*, Nitrates and free-living nematodes was found at sites E3 and E4. Another relationship between heavy metals such as Copper, Iron, Vanadium, Manganese, Cadmium, Aluminium, Nickel and Cobalt, Total phosphate with free-living nematodes such as *Paramonohystera*, *Theristus*, *Terschellingia* and *Metacyntholaimus* were found as site E2. Fine sand, very fine sand and Zinc were found to relate with free-living nematodes such as *Camacolaimus*, *Adoncholaimus*, *Synonchium*, *Axonolaimus* and *Haliplectus* at site E1. A marginal test in the DistLM procedure (Table 5) confirmed that heavy metals (Cobalt, Manganese, Nickel, Vanadium and Aluminium), Nutrients (Total Phosphate and Nitrates), and sediment particle size (Granules, Very Coarse Sand, Coarse, Very Coarse Sand, Medium Coarse Sand, Fine Medium Sand and Fine sand) were strongly associated with free-living nematodes composition in the Estuary when considered independently.

Table 5. Relationship between environmental variables and free-living nematodes based on AIC DISTLM. Marginal test considers the importance of each variable in the absence of the other variables. Sequential tests consider the importance of variables in conjunction with the other variables starting with the variable with the variable explaining the greatest variance.

Variables	SS	Pseudo-F	P values
Marginal test			
Cadmium	2697.4	1.2451	0.278
Chromium	2251.9	1.0298	0.426
Copper	3744	1.7669	0.085
Zinc	3001	1.3941	0.193
Chlorophyll-a	3898.4	1.846	0.065
Cobalt	6000.2	2.9758	0.007*
Iron	4366.5	2.0887	0.055
Manganese	6324.8	3.1599	0.006*
Nickel	6227.7	3.1046	0.008*
Vanadium	5048.8	2.4514	0.023*
Aluminium	6073.4	3.0171	0.011*
Total Phosphate	4505.5	2.1617	0.034*
Nitrate	11937	6.837	0.001*

Variables	SS	Pseudo-F	P values
Granules	8815.4	4.6683	0.001*
Very Coarse Sand	13534	8.0855	0.001*
Coarse Very Coarse Sand	14517	8.9108	0.001*
Medium Coarse Sand	9528.9	5.1343	0.001*
Fine Medium Sand	14764	9.1252	0.001*
Fine Sand	10970	6.1272	0.001*
Sequential test			
Fine Medium Sand	14764	9.1252	0.001*
Medium Coarse Sand	9321.6	7.4506	0.001*
Granules	4002.3	3.5941	0.001*
Very Coarse Sand	2.868 <sup>-11</sup>	0	1

Overall best solution			
AIC	R <sup>2</sup>	RSS	Number of Variables
171.99	0.55775	22271	4

\*indicate significant.

The sequential test in the DistLM analysis also showed that sediment particle size such as Granules, Medium Coarse Sand, Fine Medium Sand were the best subset of explanatory environmental factors to account for the differences in free-living nematodes community structure.

### 3.7.5. Maturity Index

A Maturity Index (MI) which is a potential indicator of nematode assemblage under stress were calculated for the four sites sampled (Table 6).

Table 6. Monthly and Mean Maturity Index values from June 2017 to April 2018 and the Maturity Threshold proposed by Moreno et al. 2011.

Site Names	E1	E2	E3	E4
June 2017	2.40	2.85	2.81	2.67
August 2017	1.71	1.70	2.50	2.80
October 2017	2.60	2.75	2.84	2.46
December 2017	2.63	2.53	2.75	2.62
February 2018	2.80	2.50	3.07	2.88
April 2018	2.53	2.00	2.08	2.54
Mean	2.44	2.38	2.67	2.66

Maturity Index threshold (Moreno et al. 2011)				
High	Good	Moderate	Poor	Bad
>2.8	2.8≤MI<2.6	2.6≤MI<2.4	2.4≤MI<2.2	≤2.2

The results of the MI varied monthly which indicated that there was a change in nematodes diversity and density in the estuary. The lowest MI values at sites E1 (Oligohaline Zone) and E2 (Euhaline Zone) were found in August 2017 with MI values of 1.71 and 1.72, while the highest MI values were found in February 2019 and June 2017 with MI values of 2.8 and 2.85 respectively. The mean MI values for sites E1 situated in Oligohaline Zone and E2 situated in Euhaline Zone were 2.44 and 2.38 respectively. At sites E3 (Mesohaline Zone) and E4 (Oligohaline Zone) the highest MI values were observed in February 2018 with a MI values of 3.07 and 2.88, while the lowest was observed in April 2018 and October 2017 with MI values of 2.08 and 2.46 respectively. The mean MI values for sites E3 situated in Mesohaline Zone and E4 situated in Polyhaline Zone were 2.67 and 2.66 respectively.

## 4. Discussions

Sites E1 and E2 were mostly characterised by fine sand and granules respectively, while both sites E3 and E4 were characterised by very coarse particle size which were believed to be attributed to tidal action that washes the sand from small particles. There was no significant difference ( $p > 0.05$ ) of sediment particles size between the sites sampled which indicated that sediments composition in terms of particle size was not very heterogeneous. A higher concentration of heavy metals such as Nickel, Cobalt, Chromium, Copper, Iron, Manganese, Cadmium, Vanadium, Zinc and Aluminium found at sites E1 and E2 were attributed to anthropogenic activities along the Incomati River which discharges in to the Incomati River Estuary in Mozambique (Table 1). Heavy metals have been found to be introduced in the marine environment through natural and anthropogenic process [55-56]. These anthropogenic activities in the study include mining, agriculture, and industries in South Africa and Mozambique [40]. The concentration of heavy metals in the study were found to change spatially but not temporal, which indicated that within the Estuary different sites received different amount of pollution concentration. The sources of the higher concentration of total phosphate found at sites E1 and E2 and nitrates found at sites E3 and E4 were believed to be due to agricultural activities. A positive correlation (Figure 2) between heavy metals and sediments particle size such as granule and fine sand indicated that heavy metals were distributed base on sediment particles size. In a similar study fine sediment were found to contribute to the distribution of higher metal concentration by giving enough specific areas for metal attachments [57]. Free-living nematodes were found to be the dominate meiofauna in the study, and their density and diversity decreased towards the Oligohaline Zone (Site E1). Several authors also found that nematodes decrease towards the Oligohaline Zone and they were dominant meiofauna in an estuarine environment [3, 58-62]. Nematode feeding type 1B (non-selective deposit feeders) were dominant at all the sites but mostly at site E2. The dominance of this feeding type 1B

explained the food availability of nematodes at the sites [50, 63-64]. A positive correlation of heavy metals such as Copper, Iron, Vanadium, Manganese, Cadmium, Aluminium, Nickel, Cobalt, Total Phosphate with free-living nematode such as *Paramonohystera*, *Theristus*, *Terschellingia* and *Metacytholaimus* at site E2 (Euhaline Zone); and another positive correlation of fine sand, very fine sand and Zinc with free-living nematodes such as *Camacolaimus*, *Adoncholaimus*, *Synonchium*, *Axonolaimus* and *Haliplectus* at site E1 (Oligohaline Zone) indicated that these genera are tolerant to pollution [17, 34, 65-66]. The marginal test in the DistLM further confirmed that heavy metals, nutrients and sediment particle size were strongly associated with free-living nematodes composition when considered independently, while the sequential test DistLM indicated that sediment particle size such as granules, fine medium sand and medium coarse sand were the best subset accountable for the structuring of free-living nematode. Within an area of uniform salinity; grain size sediments are the dominant factor in determining the composition of nematode communities [67-69]. Several authors found that nematodes composition is mostly structured by sediment grain size [32, 70-72]. Sites E2 and E1 which were under stress were mostly dominated by single genus (Figure 3). The Maturity Index (Table 5) also indicated that site E2 (Euhaline Zone) was in a poor condition while site E1 was in a moderate condition [73]. The poor condition at site E2 (Euhaline Zone) was attributed to the higher concentration of heavy metals and total phosphates. The lower values of MI indicate disturbed/polluted environments [73]. Sites E1 and E2 had average MI of 2.44 and 2.38 respectively. Nematode genera such as *Terschellingia* and *Theristus* that are tolerant to pollution were found at sites E1 and E2. Similar observations were also made in other studies [18, 33].

## 5. Conclusion

Free-living nematodes such as *Terschellingia* and *Theristus* were dominant genera at site E2 (Euhaline Zone) and site E1 (Oligohaline Zone). The two sites were found to be polluted. The two genera (*Terschellingia* and *Theristus*) were found to be good indicators of pollution associated with heavy metals and nutrients in Incomati River Estuary. A Maturity Index threshold used indicated that the Incomati River Estuary pollution status was poor at site E2 (Euhaline Zone), moderate at site E1 (Oligohaline Zone) and good at both sites E3 (Mesohaline Zone) and E4 (Polyhaline Zone). The source of pollution in the Incomati River Estuary was believed to be anthropogenic activities from upper catchments.

## 6. Recommendation

Further studies confirming the findings of this study must be done in the Incomati River Estuary and other regions on the African Coast to understand free-living nematodes in relation to pollution and use the results to update in our

monitoring strategies.

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