

PCDD/F and Dioxin-Like PCB Determinations in Mtoni Estuarine Sediments (Tanzania) Using the Chemically Activated Luciferase Gene Expression (CALUX) Bioassay

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Abstract

Sediments from Mtoni estuary and 2 tributaries, Tanzania, were screened for polychlorinated-*p*-dibenzodioxins, -dibenzofurans (PCDD/Fs) and dioxin-like PCBs (dl-PCBs) using the chemically activated luciferase gene expression (CALUX) bioassay approach. PCDD/Fs expressed as bio analytical equivalence (BEQ) values ranged from 5.7 ± 1.4 to 39.9 ± 5.8 pg BEQ/g sediment in the wet season and from 14.1 ± 2.0 to 32.8 ± 4.7 pg BEQ/g sediment in the dry season, with higher levels observed in Kizinga River and stations close to the mouth of that river. Dioxin-like PCB levels ranged from 0.21 ± 0.03 to 0.53 ± 0.03 pg BEQ/g sediment in the wet season and from 0.22 ± 0.03 to 0.59 ± 0.04 pg BEQ/g sediment in the dry season. Higher PCDD/F and dl-PCB levels in sediments are probably related to open burning of plastic scraps, household burning of wood or charcoal and traffic related emissions, all of which occur in the Dar es Salaam region. The denser population and the more intense industrial activities in the Kizinga River basin may explain the enhanced PCDD/F and dl-PCB levels observed in the sediments of that river compared to the levels in the Mzingira River basin. A third sampling campaign, including also stations in the downstream estuary, confirmed the enhanced levels in the Kizinga River (maximum of 400 pg-BEQ/g) and also showed that a clear decreasing concentration gradient in the downstream direction exists. It cannot be excluded that the levels of these pollutants in the sediments of the Mtoni estuary pose a threat to the local biological community.

Keywords: PCDD/Fs, Dioxin-like PCBs, sediment, Mtoni Estuary, Tanzania, CALUX Bioassay, ecological risk

1. Introduction

Polychlorinated dibenzo-*p*-dioxins and polychlorinated dibenzofurans, which are collectively referred to as PCDD/Fs, have no commercial use and occur in the environment as unintended by-products of technological processes (Pan et al., 2010; Roots, Henkelmann, & Schramm, 2004). PCDD/Fs can be formed from natural combustion processes like bushfires and volcanoes (Birch, Harrington, Symons, & Hunt, 2007) or during incomplete anthropogenic combustion processes of chlorinated organic wastes such as for example incineration of polyvinyl chloride plastics (Birch et al., 2007; Terauchi, Takahashi, Lam, Min, & Tanabe, 2009). Formation of dioxins in such incinerators occurs due to the presence of both chlorine and catalytic metals (Manahan, 2008). Industrial activities such as metallurgy and manufacture of chlorinated chemicals, like wood preservatives and pesticides, can also produce dioxins (El-Kady et al., 2007; Ryoo et al., 2005).

Polychlorinated biphenyls (PCBs) were once produced commercially (Koistinen, Stenman, Haahti, Suonper, & Paasivirta, 1997; Srogi, 2007) and used in industrial and consumer products (Liu et al., 2006; Wang et al., 2007), such as anti-corrosion materials, coolants and insulators in heat transfer systems, electronic appliances and hydraulic fluids (Shen et al., 2009; Yang, Shen, Gao, Tang, & Niu, 2009) and as capacitors in electrical industries (Pan et al., 2010). Common PCB sources in the environment include the use as well as disposal of PCB-containing products and the formation of PCBs as by-products at low temperature (less than 800 °C) waste

incineration (Chi, Chang, & Kao, 2007). Twelve of these PCBs have a planar structure and elicit biochemical and toxic responses similar to dioxins and are therefore known as dioxin-like PCBs or dl-PCBs (Okay et al., 2009).

Both natural and anthropogenic sources can lead to increased levels of these compounds in estuaries and coastal marine ecosystems (Kumar, Sajwan, Richardson, & Kannan, 2008). Major anthropogenic activities are linked to population growth, urbanization and industrialization (Kumar et al., 2008; Müller et al., 2002) and include effluents from municipal wastewater plants (Moon, Choi, Choi, Ok, & Kannan, 2009), combustion processes from waste incinerators or cement manufacturing, power plants and automobile exhausts (Zhang et al., 2010) and industrial processes like pulp bleaching and metallurgy (Bruckmeier et al., 1997).

Mangrove sediments can act as sinks and later as sources of PCDD/F and PCB contaminants to marine environments (Chi et al., 2007; Guzzella et al., 2005; Müller et al., 1999). Hence, sediments can be used to evaluate pollutant sources, historical trends and fate processes, since the amounts of these compounds in sediments will reflect regional discharges (Lee, Kim, Chang, & Moon, 2006; Moon et al., 2009). Contaminated sediments may therefore threaten the lives of organisms in the marine environment due to the toxicity, long time persistence, bioaccumulation and biomagnification of these lipophilic organic micro-pollutants (Kumar et al., 2008; Zhao et al., 2010).

Various analytical methods have been used to characterize PCDD/Fs and dl-PCBs in a sediment matrix. Gas chromatography-high resolution mass spectrometry (GC-HRMS) offers a possibility to chemically identify and quantify individual congeners (Besselink et al., 2004; Denison et al., 2004; Denison, Pandini, Nagy, Baldwin, & Bonati, 2002; Schecter et al., 1999) in the matrix and to enable the assessment of risks associated with the congeners (Long et al., 2006). To estimate the risks from the GC-HRMS results it is assumed that the additivity principle of a pollutant's response or effect is valid, which means the absence of agonistic and antagonistic interactions, and that these effects are produced through the same mechanism of toxicity. However, it has been shown that complex mixtures of PCDD/F and dioxin-like PCB congeners elicit synergistic and/or antagonistic interactions (Joung, Chung, & Sheen, 2007; Schroyjen, Windal, Goeyens, & Baeyens, 2004). In addition, chemical analysis of individual congeners, particularly in small concentrations, can be very expensive and time consuming. Presence of compounds with aryl hydrocarbon receptor (AhR) affinity, but not commonly measured, and the absence of toxicological equivalents (TEQ) for several congeners further limit the use of this analytical method (Joung et al., 2007; Long et al., 2006). To overcome some of these drawbacks, biological assays utilizing either biomolecular techniques (e.g. immunoassays) or living materials (e.g. *in vitro* chemically activated luciferase gene expression, CALUX) have been used as rapid and cost-effective screening methods for chemicals with selective and specific biochemical interactions (Roy, Mysior, & Brzezinski, 2002). For example, the CALUX bioassay screens for chemicals with AhR potential (Denison et al., 2004, 2002; Schecter et al., 1999;) and produces a single integrated biological equivalent of the mixtures (Besselink et al., 2004). CALUX also measures a response which is a single toxicity end-point produced by AhR active compounds that cannot be measured and/or are below the detection level of chemo analysis (Joung et al., 2007). The major drawback of a bioassay such as CALUX is that there is no information about the congener pattern.

The CALUX method has been explained by various authors (Denison et al., 2004, 2002; Murk, 1996;). It uses genetically modified cells (hepatoma cells stably transfected with a luciferase reporter gene) which respond to chemicals that activate the cytosolar AhR by induction of luciferase (Denison et al., 2004). By this method, the toxicity of pollutants such as PCDD/Fs and dioxin like-PCBs is produced either as a change in gene expression mediated through the AhR or by interference with other pathways (Hurst, Balaam, Chan-Man, Thain, & Thomas, 2004). Estimation of relative potency and toxic potential can therefore be done by measuring the activation level of AhR gene expression (United States Environmental protection Agency [USEPA], 2008). However, even when a rigorous clean-up and separation procedure of the sample extract is performed, interferences by PCDD/Fs on dl-PCBs and vice versa or by other AhR ligands are still possible (Sanctorum, Elskens, & Baeyens, 2007).

The literature regarding CALUX analyses in marine sediments is very limited. Most of the PCDD/F analyses in the world have been performed with GC-HRMS. In those studies where the CALUX technique was used, the focus was more on method development, on the comparison with the GC-HRMS method and on the screening of food and feed (Hoogenboom et al., 2006; Van Overmeire, Van Loco, Roos, Carbonnelle, & Goeyens, 2004). In addition, very little data on dl-PCBs are available in literature. The fact is that, PCDD/F BEQ levels are in general by far higher than those of the dl-PCBs. Both chemo-analysis and CALUX analysis research on marine sediments in Africa are scarcely documented (Nieuwoudt et al., 2009; Pieters, 2007). Regarding Tanzania, only total PCBs in sediment (Machiwa, 1992) and PCDD/Fs and dl-PCBs in free range chicken have been reported (International POPs Elimination Network, 2005).

Although there exist no data about the presence of PCDD/Fs and dl-PCBs in the environment (more specifically in the sediment) of Tanzania, the applications of PCBs in electrical transformers and in other equipment is known (Loomis, Browning, Schenck, Gregory, & Savitz, 1997). In coastal Tanzania, there are a lot of municipal, chemical and even hospital wastes that are discharged into the Indian Ocean after incineration and open burning of mixed wastes (Machiwa, 1992). Wood burning is a common source of fuel as most households use either charcoal or firewood for cooking. In many local households, there is uncontrolled burning of plastics. Vehicle emissions are abundant due to increased traffic and importation of old, second-hand cars (Mbuligwe & Kassenga, 1997). The current major outcry of the country has been on the vandalism of electrical transformers (Maleko, 2005) in search of their coolant for unspecified domestic or commercial use. Since Dar es Salaam is by far the largest city in Tanzania, the Mtoni estuary, being the main aquatic system in that area, was selected to study PCDD/F and dl-like PCB levels in the aquatic environment for the first time. The first objective was to assess PCDD/F and dl-PCB levels in sediments of the mixing zone of the Mtoni estuary, including the Kizinga and Mzinga River mouths, during wet and dry seasons. A second objective was to eventually link the observed levels to local sources.

2. Method

2.1 Study Area

The Mtoni estuary (Figure 1) is located at approximately 3 km south of Dar es Salaam (Tanzania) and receives fresh water input from the Kizinga and Mzinga Rivers. Numerous creeks in the estuary and the river mouths host mangrove trees such as *Avicennia marina*, *Bruguiera gymnorrhiza*, *Ceriops tagal*, *Rhizophora mucronata* and *Sonneratia alba* species.

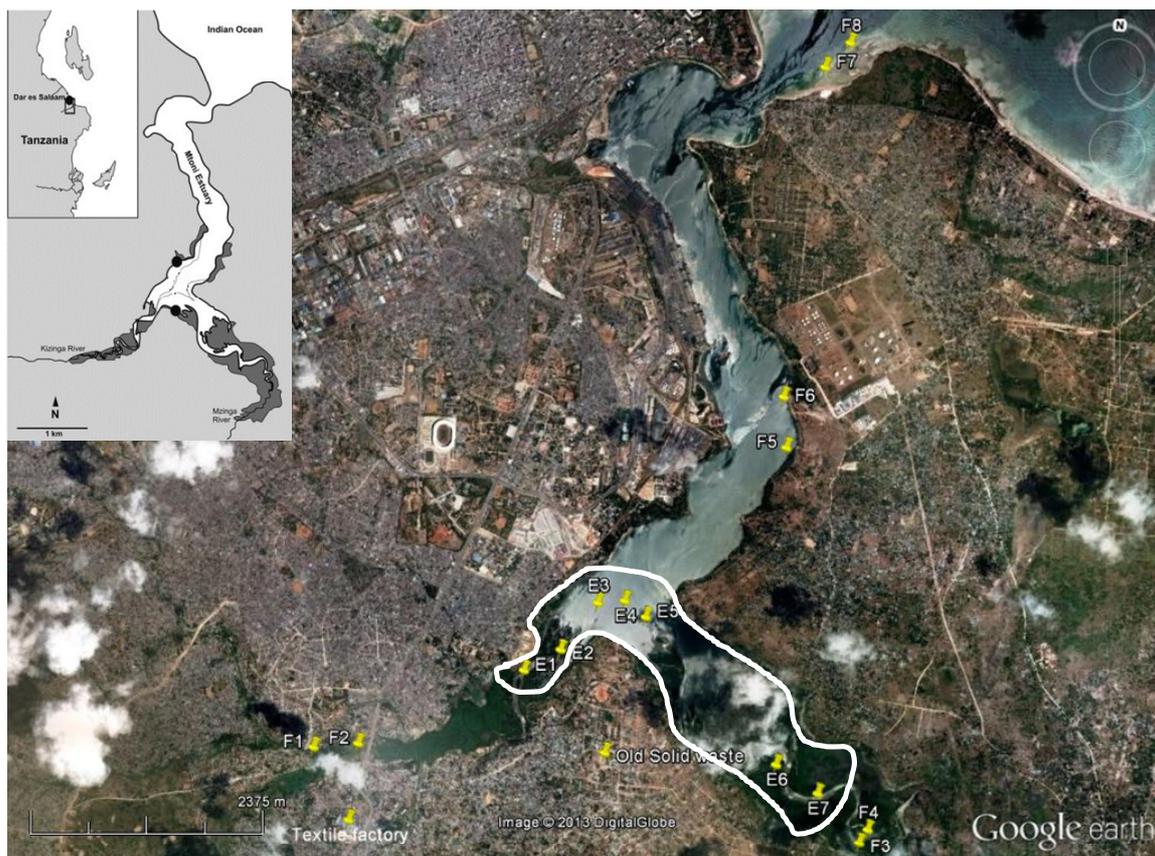


Figure 1. Sampling points in the Mtoni estuary, Dar es Salaam: E1 and E2 in the Kizinga River, E3-E5 at the confluence and E6 and E7 in the Mzinga River. Stations F1-F2 in the Kizinga River, F3-F4 in the Mzinga River, F5-F6 at the Navy shore and F7-F8 at Kigamboni Seaway are additional sampling points. White line delimits the estuarine mixing zone. The solid waste dumping site and textile factory are also indicated

The fresh water input from both rivers is low. An average base-line flow rate of $1 \text{ m}^3/\text{s}$ is observed in Kizinga river with an increase to $8 \text{ m}^3/\text{s}$ in the rainy season while the water-flow rate of the Mzinga River is unstable and lower than in Kizinga River (Van Camp, Mjemah, Al Farrah, & Walraevens, 2013). Hence, the effect of the river discharges on the hydrodynamics of the Mtoni estuary is very limited. The seven sampling stations (E1 to E7) are located in the mixing zone and their salinities vary from almost fresh to brackish water with somewhat higher salinities in the dry season (Kristensen et al., 2010). Downstream this mixing zone (stations F5 to F8) the water becomes rapidly sea water while the stations F1 to F4 more upstream are fresh water. This estuarine mixing zone was selected because it integrates influences of natural and anthropogenic sources in the riverine and marine systems.

The Mtoni estuary is highly impacted (Peri-urban mangrove forests as filters and potential phytoremediators of domestic sewage in east Africa [PUMPSEA], 2007) by discharges of various origin: (1) the Kizinga and Mzinga Rivers that drain the mangrove forest (Kruitwagen, Pratap, Covaci, & Wendelaar-Bonga, 2008), (2) the wastewater drainage systems from industrial and residential areas (of a population of around 500,000 inhabitants (National Bureau for Statistics [NBS], 2003)), (3) charcoal burning, (4) mangrove harvesting for residential places, (5) salt mining, (6) tourism and (7) agriculture (Taylor, Ravilious, & Green, 2003).

The Kizinga River that drains the urbanized areas of Keko, Chang'ombe, Kurasini and Temeke (approximately 400,000 inhabitants (NBS, 2003)) is suspected to carry a variety of wastes and discharges originating from agricultural, industrial as well as residential sources (Taylor et al., 2003). The Mzinga River, on the other hand, drains the rural areas of Vijibweni, Tuangoma and Mji Mwema with a population of around 90,000 (NBS, 2003). Due to rapid growth of settlements along the Mzinga creek resulting from increased human population, the river is suspected to carry agricultural and residential wastes and discharges presumed to be emptied into the creek. The estuary further receives inputs from the Dar es Salaam harbor which is located near the mouth of the estuary during diurnal tides (up to 5 m amplitude) and from the Mtoni solid waste dumping site located in between the two rivers.

2.2 Sampling

Sampling of sediments was conducted in the mangrove forests during low tides at Kizinga and Mzinga creeks (Figure 1) of Mtoni estuary. Two sampling campaigns were conducted: one during the wet season (19th-20th January, 2011) and a second during the dry season (15th-16th August, 2011). December and January have an average precipitation rate of 194 and 89 mm respectively, while these rates in July and August are much lower with 48 and 47 mm respectively. River flows in the Dar es Salaam area are mainly controlled by the precipitation rate in the previous period. The flows of Kizinga and Mzinga Rivers are highest in the wet season (the highest discharge rates can go up to $15 \text{ m}^3/\text{s}$ for the Kizinga River and $7 \text{ m}^3/\text{s}$ for the Mzinga River) while in the dry season, base-line flows of $1 \text{ m}^3/\text{s}$ in the Kizinga River and even lower in the Mzinga River are observed (Van Camp et al., 2013). The impact of both rivers on the pollutant levels in the mixing zone can thus best be estimated by sampling in this mixing zone at high (wet season) and at low (dry season) river flow and comparison of these results.

Samples were collected from exactly the same locations during both campaigns. Seven sampling stations were identified using a hand-held global positioning system (GPS): two in the Kizinga River (E1 and E2), two in the Mzinga River (E6 and E7) and three at the confluence of the two rivers (E3, E4 and E5).

From the results obtained during the wet and dry seasons in the mixing zone of the estuary, it appeared that the sampling stations in Kizinga River and close to its mouth showed higher PCDD/F values than the stations in and close to Mzinga River. It was thus interesting to investigate PCDD/F levels more upstream in both rivers. In addition, the salinity gradient in the mixing zone is also small and real marine water samples were not included in the previous samplings. We were thus not able to appreciate any evolution of the PCDD/F levels from the estuarine mixing zone towards the marine environment. Therefore, an additional sampling campaign was organized at 3 end-members in October 2012: (1) one site in the Kizinga River (fresh water stations F1-F2) much more upstream than stations E1 and E2, (2) one site in Mzinga River (fresh water stations F3-F4 are slightly more upstream than stations E6 and E7, but these latter stations were yet, compared to the stations E1 and E2 in Kizinga River, much more upstream) and (3) two sites in the marine area, close to and at the mouth of the estuary (respectively marine water stations F5-F6 and F7-F8) (Figure 1).

All those samples were taken from two sub-sites within a distance of 20 m, except in the Kizinga River. The first subsample was taken at the junction of the river and the textile wastewater stream (Figure 1) and the second was taken 200 m upstream of the river very close to unauthorized human settlements.

Sediment sampling was done as described by EPA (United States Environmental protection Agency [USEPA],

2001) using a hand corer (30 cm height, 6 cm internal diameter). The corer was gently pushed in the mangrove sediments, closed at its upper end with a lid and smoothly removed by twisting and pulling. The sediments were then pushed out of the corer tube using a piston and sectioned into three segments corresponding to depth intervals of 0-3, 3-6 and 6-9 cm. All sediment samples were packed in prior labeled and zipped polyethylene bags, stored in iceboxes and later frozen to -20 °C. Sediment samples were then air-transported (frozen) to the laboratory of the Department of Analytical and Environmental Chemistry, Vrije Universiteit Brussel (VUB) in Belgium for dioxins and dioxin-like compounds analyses.

2.3 Chemical Reagents and Standards

Acetone (Pesti-S grade, minimum 99.9%), n-hexane (minimum 96% assay) and toluene (minimum 99.8% assay) both dioxins and PCB grade, were purchased from Biosolve (The Netherlands). Ethyl acetate (Pestanal, 99.8% assay) was purchased from Sigma-Aldrich (Germany). Sulphuric acid (95-97% w/w, ACS reagent) and Dimethylsulfoxide (DMSO) were obtained from Merck (Germany). Glass fibre filters were purchased from Whatman (UK). Alpha-minimal essential medium (α -MEM), fetal bovine serum (FBS) and trypsin (0.25%) were obtained from Gibco, UK. Phosphate buffered saline (PBS) was obtained from Ambion (UK). Luciferase assay substrate and buffer were purchased from Promega (The Netherlands). Anhydrous sodium sulphate was purchased from Boom (The Netherlands). The X-CARB was purchased from Xenobiotic Diagnostics Systems, XDS Inc, USA and the solution of 2,3,7,8-TCDD standard (50 $\mu\text{g/mL}$, purity 99%) was purchased from Campro Scientific (The Netherlands).

2.4 Determination of Particle Size and Total Organic Carbon (TOC)

The concentration of TOC was determined in all 50 sediment samples with a CHN analyzer (Carlo Erba) on a known amount (about 12 mg) of sediment sub-sample placed in a silver capsule and pre-treated by acidification with 5% HCl. The grain size distribution was determined by an external laboratory at 3 stations with low, medium and high organic matter (OM) content in their sediments (stations E1, E2 and E7) to test the relation between both variables. Approximately 10 g lyophilized and homogenized sediment sample was prepared by removing salts, OM and carbonates using hydrogen peroxide and hydrochloric acid respectively. A stable suspension was obtained after rinsing and adding 5 ml of a peptizing agent. The coarse fraction (above 75 μm) was separated by wet sieving on a 75 μm sieve, then dried at 105 °C, and finally dry sieved. The grain-size distribution of the fine fractions 2-75 μm and <2 μm was obtained using the Sedigraph 5100 coupled to a Mastertech 51. The precision for 10 consecutive measurements on aliquots of the same sample was around 1% for each grain-size fraction.

2.5 Analysis of Dioxin and Dioxin-Like Compounds in Sediments

2.5.1 Sample Preparation

In total, 50 sediment samples were analyzed for PCDD/Fs and dl-PCBs. Lyophilized sediment (5 g) was extracted using pressurized liquid extraction in an Accelerated Solvent Extractor, ASE[®], (Dionex, USA) with a toluene:methanol (4:1 v/v) solvent system (Baston & Denison, 2011) and 33 mL extraction cells. The ASE extraction conditions were: 125 °C oven temperature; 1500 psi (100 MPa) pressure; 10 min static time; 6 min oven heating time; 60 s purge time; 60% of extraction cell volume as flush volume and 2 static cycles. The extracts were then concentrated in a vacuum centrifuge to near dryness and later re-suspended in n-hexane (5 mL).

2.5.2 Column Preparations for Clean-Up and Fractionation

The clean up and fractionation is based on the EPA Method 4435 (United States Environmental protection Agency [USEPA], 2008) from which we use the same piggybacked setup of columns. A sequential setup of columns is used to remove PAHs and break down undesired compounds (acid silica gel) and differentially elute PCDD/Fs and dl-PCBs (X-CARB affinity chromatography column). An additional, third, column was added for sulphur removal. Column preparation is described below and all columns are prepared daily.

An activated copper column (for elemental sulphur removal) was prepared by filling a Pasteur pipette from bottom to top with glass wool and 1 cm of activated (with a 20% hydrochloric acid solution) copper. The activated copper column was first rinsed with de-ionized water (Milli-Q): 3 x 1 mL and then with acetone, toluene and n-hexane (each 3 x 1 mL) in that order. The activated copper columns were stored submerged in n-hexane to avoid oxidation.

An acidified silica column was prepared by filling a 10-mL disposable column (ID 0.8mm), from bottom to top, with glass wool, sodium sulphate (0.5 cm^3), sulphuric acid-silica gel (33% H_2SO_4 on silica gel w/w; 4.3 cm^3) and sodium sulphate (0.5 cm^3). The acid silica column was then rinsed with n-hexane (3 x 10 mL).

Similarly, an X-CARB column was prepared by using an open ended tube (ID 0.8 mm), but this was filled (bottom to top) with glass wool, sodium sulphate (0.5 cm^3), 1% X-CARB (1 cm^3 packed), sodium sulphate (0.5 cm^3) and glass wool. The column was inverted and rinsed sequentially with acetone (5 ml), toluene (20 ml) and n-hexane (10 ml).

The acid silica gel column is placed on top, the copper column in the middle and the X-CARB column at the bottom end prior to sample loading. The individual columns are connected to each other and rinsed with n-hexane ensuring that the columns do not run dry.

2.5.3 PCDD/Fs and dl-PCBs Clean up and Fractionation

The sediment extract in n-hexane was first sonicated for 5 minutes followed by vigorous vortexing. An aliquot (2 mL from the original 5 mL) was quantitatively loaded on the acid silica gel column, followed by elution of the column with n-hexane (total 21 mL). The acid silica gel and activated copper columns were removed once the solvent had passed through. The remaining X-CARB column was further rinsed with extra n-hexane (5 mL), followed by elution with a mixture of 8:1:1 of n-hexane:toluene:ethylacetate ($3 \times 5 \text{ mL}$) to collect the fraction containing coplanar PCBs (i.e. PCB fraction). The fraction containing the PCDD/Fs (dioxin fraction) was afterwards eluted (back-flushed) with toluene ($3 \times 5 \text{ mL}$) after inverting the X-CARB column. The PCB and dioxin fractions were later concentrated to dryness in a vacuum centrifuge and re-suspended in n-hexane (4 mL) for CALUX analysis.

2.5.4 CALUX Analysis

CALUX analysis was performed as described elsewhere (Van Langenhove et al., 2011). Briefly, mouse hepatoma cells (H1L7.5c1 cell line) were cultured in α -MEM with 10% FBS at 37°C , 80% relative humidity and 5% CO_2 . The cells were seeded (at an approximate density of 7.5×10^5 cells/mL in a clear bottom 96-well plate (Greiner Bio-One, Germany) and incubated for 24 hrs to reach a monolayer of cells.

TCDD standard solutions (from 125 nM down to 30 pM) were made in DMSO and 4 μL of this solution was transferred to 2 mL of n-hexane. Samples were serially diluted in n-hexane and 4 μL of DMSO was added to each vial as a carrier solvent. For both the TCDD and sample solutions, the remaining n-hexane was evaporated using a vacuum centrifuge, leaving behind either 4 μL DMSO with TCDD standard or 4 μL DMSO containing either PCDD/Fs or dl-PCBs from the sample extract.

Prior to dosing, these standards and sample dilutions were diluted 100 times with cell culture media (4 μL standard solution or sample in 396 μL α -MEM with 10% FBS). Final solutions were vortexed vigorously and dosed in triplicate ($3 \times 100 \mu\text{L}$ per well).

After 24 hrs of incubation, cells were rinsed and visually inspected for abnormalities. Then, cells were lysed and shaken for 5 min. Luciferin treatment was performed in the Glomax 96-well microplate luminometer (Promega, USA), where the light output in relative light units (RLUs) was measured. Sample responses were expressed as percentage maximum induction to 2,3,7,8-TCDD (%TCDD_{max}).

2.6 Statistical Analysis of Data

TCDD standards were used to generate the calibration curve. A four-variable Hill equation fitting the calibration curve (Elskens et al., 2011) was used to produce a sigmoid curve of the standard solutions. The calibration equation was then used to convert the measured RLU values of the samples into a biological equivalency (CALUX-BEQ) value (Goeyens et al., 2010) by comparing the sample response curve with the sigmoid dose-response curve (Elskens et al., 2011). Further data treatment was done using slope ratio and Box-Cox transformation methods by linearization of the non-linear Hill regression equation as described previously (Elskens et al., 2011). Effective concentrations at 50% TCDD_{max} (EC₅₀) of standard and samples were used to determine the potency or bio analytical equivalency (BEQ) of the samples. Statistical analysis was performed using Microsoft Spreadsheet for Windows 2007 and graphical representations were performed using Spreadsheet and SigmaPlot programs (SigmaPlot 10.0). Pearson Correlation and Principal component analysis (PCA) were performed using Predictive Analytic Software (PASW, version 16.0 for Windows) with the PCDD/F and dl-PCB values and the geochemical properties (% total nitrogen (TN), % total carbon (TC) and %TOC) as variables (total 5), and using the mean concentrations of both seasons.

2.7 Quality Control (QC) and Quality Assurance (QA)

For each batch of samples, a blank sample was introduced through the complete treatment procedure (procedural blank) to monitor the activity contributed by solvents and column matrices used in the sample treatment. Moreover, DMSO and media blanks were added during dosing to detect contamination and to determine the

experimental background level. All blank samples were measured in triplicate and were treated in a similar way as real samples. The results with $p < 0.05$ (Student T-test) were considered statistically significant.

The limit of detection (LOD) was calculated according to the IUPAC definition. The blank value was taken as the average background of the model fit represented in %RLU relative to the maximum TCDD-induced RLU (Elskens et al., 2011).

QC experiments were conducted on each 96-well plate, using an in-house QC solution (0.250 pg TCDD/ μ L), to assess precision of the CALUX method and detect bias. Procedural blank fractions were also spiked with the same QC solution prior to dosing to detect agonists and/or potential synergetic or antagonistic effects.

3. Results and Discussion

3.1 Grain Size Distribution

Sandy particles dominated the mangrove sediments in the study area, with sand ($x > 75 \mu\text{m}$) contributing for more than 60% of the weight. The correlation between TOC and the fine grain size fraction ($\% < 2 \mu\text{m}$) was good ($r^2 = 0.92$). This was still the case ($r^2 = 0.82$) between TOC and the mud+silt fraction ($\% < 75 \mu\text{m}$). It is well-known that muddy sediments having a high TOC content but also a high amount of fine grain size fraction ($< 2 \mu\text{m}$), accumulate by far higher amounts of pollutants than sandy sediments (Baeyens et al., 1991). This means that TOC values can eventually be used to normalize the pollutant concentrations in the sediment versus the mud fraction ($\% < 2 \mu\text{m}$).

In the Mtoni estuary, the high sand proportion implies that the capacity of the mangrove sediments to adsorb the dioxins and dl-PCBs is medium to low. On the other hand, a high sand fraction favors abiotic processes such as enhanced diffusion of oxygen in the sediment making pollutants more bioavailable due to faster oxidation of organic matter and simultaneous release of associated persistent organic micro-pollutants such as dioxins (Davies & Tawari, 2010; Holmer, 2003).

3.2 The CALUX HIL7.5c1 Bioassay

The CALUX bioassay integrates the responses of every AhR ligand available in the analyzed sample and because of this; it provides only an indication of the possible overall toxicity (Van Langenhove et al., 2011). The results presented in this study show the importance of full-dose curves for environmental samples because the additivity principle is not upheld, despite (1) the use of a sulphuric acid silica gel column in the sample clean up step aimed at eliminating interferences caused by poly-aromatic hydrocarbons (PAHs) and (2) separation of PCDD/Fs and dl-PCBs avoiding known antagonistic effects between these two compound groups (Van Langenhove et al., 2011).

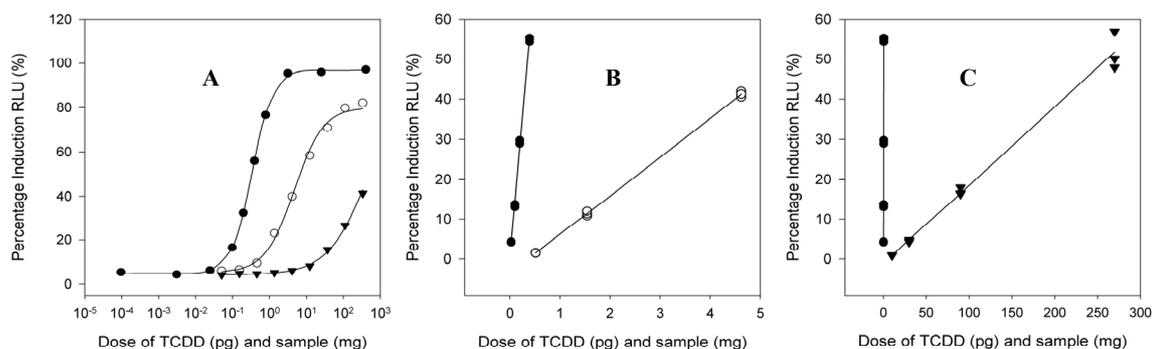


Figure 2. (A) Typical TCDD-standard curve (●), sample with full dose response (○) and sample not attaining an upper plateau (▼) using Hill regression. (B) Same data portrayed using the slope ratio method for TCDD (●) and full dose sample (○). (C) Slope ratio for TCDD (●) and sample without upper plateau (▼)

Luciferase induction was reproducible with coefficients of variation (CV) less than 15% for a given standard or sample extract measured in triplicate. Mean values of luciferase response measured in three replicate wells were used to generate the dose-response curves. The dose-response curve of TCDD standards was sigmoidal in appearance as shown in Figure 2.

3.3 Quality Control

Blank samples ($n = 6$) spiked with the in-house QC solution ($0.250 \text{ pg TCDD}/\mu\text{L}$) ranged in recoveries from 85 to 120% for the PCB fraction and from 91 to 115% for the PCDD fraction, well in accordance with an acceptable relative standard deviation of 20% (80-120% recovery of the TCDD spike). DMSO controls showed no marked difference in response to fitted background values ($p = 0.45$ for a two-tailed Student T-test). Media controls were generally lower in response, but borderline not statistically significantly different ($p = 0.06$ for a two-tailed Student T-test).

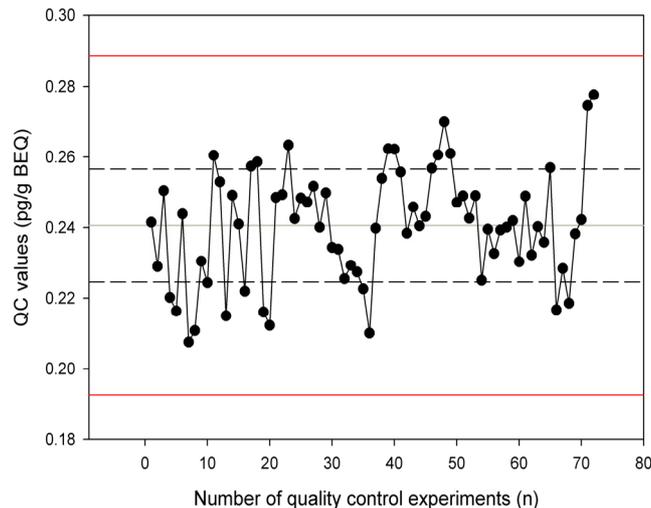


Figure 3. Scatter chart of QC analyses during the study. The dotted lines above and below the mean (0.24) equal the mean \pm 1SD values. The 2 additional lines above and below the dotted lines equal the mean \pm 3SD values

A total of 72 QC experiments were performed in this study. When these experiments are plotted on a control chart (Figure 3), a mean of $0.24 \pm 0.02 \text{ pg/g}$ indicated that the analysis procedures were reliable with a coefficient of variation (CV) at 7.2%. The LOD based on the averaged fitted background value and on the DMSO blank was $0.05 \text{ pg TCDD/well}$. Recalculating with the starting volume used in the serial dilution, an LOD of about 0.22 pg TCDD/g sediment is found.

3.4 Bio Analytical Equivalents (BEQs) in Mtoni Sediment Samples

CALUX-BEQ values of PCDD/Fs in Mtoni estuary sediments were assessed using three methods: the Hill regression equation, Box-Cox transformation (both providing an EC_{50} in pg/g BEQ) as well as the slope ratio method (providing a single BEQ value). A detailed description of the methods can be obtained elsewhere (Elskens et al., 2011). The relationships between these methods indicate that they correlate well ($r^2 > 0.8$, Figure 4), implying that either of these methods will provide reliable results in this study. Model precision for BEQ determination based on CVs ranged from 6.7-17.4% (Hill model), 5.2-14.3% (Box-Cox transformation) and 5.5-15.5% (Slope ratio method).

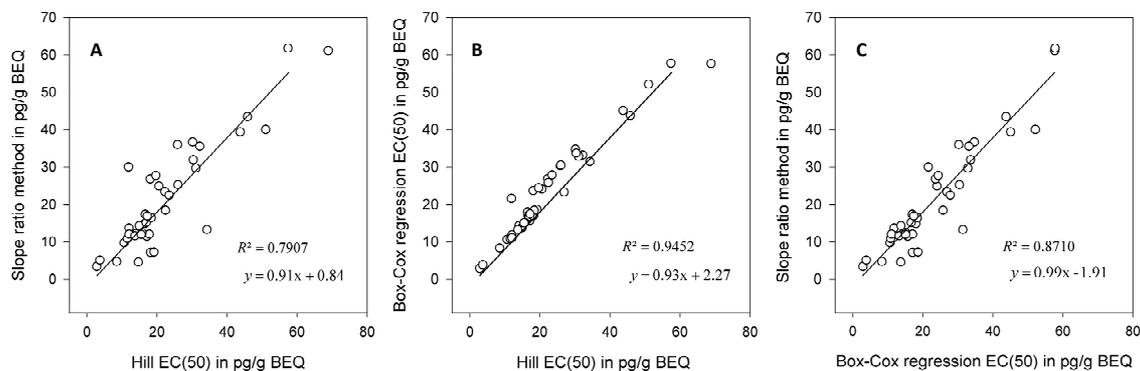


Figure 4. Relationship between PCDD/Fs concentrations (EC_{50}) in Mtoni estuary sediment samples; (A) estimated by Hill equation and slope ratio method, (B) Hill regression and Box-Cox transformation and (C) Box-Cox transformation and Slope ratio

Since the Hill equation is the most used method, and for simplicity of comparison, Hill regression BEQs will be continuously employed in this study and are presented in Table 1. To account for non-parallelism that usually exists between dose-response curves of the reference standards and the sample, the BEQ PCDD/F values in a sample were determined based on the EC_{20} , EC_{50} and EC_{80} of the maximum TCDD result ($TCDD_{max}$).

DI-PCBs responses are lower than those of PCDD/Fs owing to their lower toxicity equivalent factors (TEFs). Their potency was therefore determined using inverse prediction, assuming a sample behaves like a diluted TCDD standard solution (Elskens et al., 2011).

In order to eliminate concentration differences emanating from variations in the sand fraction of the sediments, pollutant concentrations in the Mtoni estuary sediments were normalised to TOC. Despite the normalisation, PCDD/F and dioxin-like PCB profiles showed no obvious vertical trends. The rather steady depth profiles indicate that the estuarine sediments are fairly well-mixed making the profiles more or less uniform. It is possible that the hydrodynamics of the estuary (tidal amplitude of up to 5 m at the mouth) provide frequent sediment mixing, smoothing out possible vertical concentration gradients. Perhaps, a depth of 9-cm is not sufficient enough to observe any difference and therefore it may be interesting in the future to study pollutant profiles in deeper sediment layers.

Table 1. Mtoni estuary sediment properties and the CALUX-BEQ values for PCDD/Fs and PCBs

Code and Location	Section depth (cm)	Season	TOC (%)	CALUX-BEQ (pg TCDD/g sediment)		
				PCDD/Fs BEQ ₅₀	PCDD/Fs BEQ ₂₀ -BEQ ₈₀ range	Dioxin-like PCBs*
E1: S 06°52.443 E 039°17.014	0–3 cm	wet	0.62	31.07	44.6 – 21.6	0.35
		dry	1.86	51.02	63.3 – 41.1	0.47
	3–6 cm	wet	0.63	34.28	45.4 – 25.9	0.33
		dry	1.77	30.37	53.0 – 17.4	1.03
	6–9 cm	wet	0.50	22.45	28.0 – 18.0	0.34
		dry	2.23	17.13	24.0 – 12.2	0.26
E2: S 06°52.357 E 039°17.099	0–3 cm	wet	1.02	18.20	22.3 – 14.9	0.38
		dry	1.46	45.84	55.7 – 37.7	0.63
	3–6 cm	wet	1.02	23.50	31.4 – 17.6	0.34
		dry	0.56	11.58	15.4 – 8.7	0.26
	6–9 cm	wet	0.56	14.93	21.5 – 10.4	0.33
		dry	0.24	14.02	20.0 – 10.0	0.24
E3: S 06°52.058 E 39°17.355	0–3 cm	wet	2.55	68.87	85.7 – 55.4	0.47
		dry	1.35	57.42	72.6 – 45.4	0.41
	3–6 cm	wet	3.57	30.18	38.2 – 23.9	0.15
		dry	0.68	22.25	43.6 – 11.4	0.40
	6–9 cm	wet	3.83	20.54	32.2 – 13.1	0.14
		dry	0.66	16.48	19.4 – 14.0	0.11
E4: S 06°52.090 E 039°17.501	0–3 cm	wet	1.04	32.22	42.7 – 24.3	0.48
		dry	0.98	26.93	81.2 – 8.90	0.27
	3–6 cm	wet	1.15	18.10	31.5 – 10.4	0.28
		dry	1.71	43.73	49.9 – 38.4	0.55
	6–9 cm	wet	1.23	16.66	20.0 – 13.9	0.39
		dry	0.96	25.86	42.8 – 15.6	0.10
E5: S 06°52.164 E 039°17.658	0–3 cm	wet	2.38	10.63	12.9 – 8.75	0.25
		dry	0.70	11.88	15.9 – 8.89	0.55
	3–6 cm	wet	0.90	2.82	3.73 – 2.31	0.18
		dry	0.80	19.66	35.3 – 11.0	0.47
	6–9 cm	wet	1.40	3.70	5.29 – 2.59	0.23
		dry	0.46	12.02	16.0 – 9.00	0.36
E6: S 06°52.882 E 039°18.391	0–3 cm	wet	3.12	26.00	36.9 – 18.3	0.28
		dry	3.57	18.38	24.1 – 14.0	0.39
	3–6 cm	wet	4.42	13.72	19.2 – 9.81	0.19
		dry	3.01	17.22	25.4 – 11.7	0.26
	6–9 cm	wet	2.54	15.73	22.9 – 10.8	0.18
		dry	4.30	15.51	22.7 – 10.6	0.24
E7: S 06°52.952 E 039°18.454	0–3 cm	wet	7.50	16.91	24.5 – 11.7	0.54
		dry	1.35	14.70	19.4 – 11.7	0.19
	3–6 cm	wet	2.41	17.80	25.6 – 12.4	0.44
		dry	1.72	19.17	25.4 – 14.4	0.23
	6–9 cm	wet	7.07	12.04	17.1 – 8.45	0.60
		dry	1.66	8.52	12.4 – 5.85	0.24

*Results were determined by the inverse prediction method.

The PCDD/F results from the CALUX screening for the Mtoni estuary sediment extracts are shown in Figure 5A. The PCDD/F concentrations in figure 5A were based on the Hill BEQ₅₀ and ranged from 5.7 ± 1.4 to 39.9 ± 5.8 pg BEQ/g sediment in wet season and were between 14.1 ± 2.0 and 32.8 ± 4.7 pg BEQ/g sediment in dry season. Higher levels of PCDD/Fs in both seasons were found in the Kizinga River and in confluence stations close to it. Wet season samples contained less PCDD/Fs than dry season samples in the Kizinga River, however at the Mzinga site it was the opposite. Higher PCDD/F levels at confluence station E3 in both seasons could be due to a

local emission source in proximity to the Kizinga River. This is confirmed by the subsequent higher levels at the nearby station E4 in both seasons and lower levels at E5, a station closer to the Mzinga River. No significant difference ($t = 0.55$ two tailed; $p = 0.59$) in PCDD/F levels was observed between the two seasons ($n = 42$).

Response levels of dl-PCBs (Figure 5B) ranged from 0.21 ± 0.03 to 0.53 ± 0.03 pg BEQ/g sediment in wet season and from 0.22 ± 0.03 to 0.59 ± 0.04 pg BEQ/g sediment in dry season. These BEQ values are much lower (about 2 orders of magnitude) than those of the PCDD/Fs. At the 2 Kizinga stations, confluence station E5 and Mzinga station E6, lower PCBs values were observed in wet compared to dry season, while the opposite was true for confluence stations E3 and E4 as well as Mzinga station E7. High dioxin-like PCB levels were generally correlated to high PCDD/Fs values. The PCB levels detected in the estuary showed no significant variations ($t = 0.27$ two tailed; $p = 0.79$) between the seasons ($n = 42$).

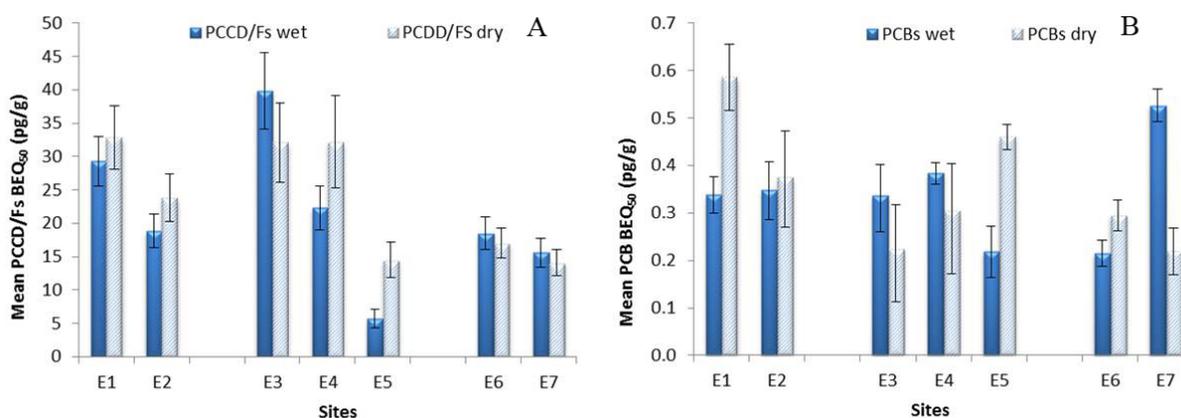


Figure 5. BEQ (with uncertainty error bars) for PCDD/Fs (A) and PCBs (B) of the Mtoni sediments presented as averaged values of the three depth layers in dry and wet seasons

The Kizinga River drains the peri-urban environments of Temeke, Mbagala, Ukonga, Charambe, Kijichi, Vituka, Keko, Kitunda, Mtoni, and Kurasini which are highly populated and have many small-scale to medium-scale industries. On the other hand, the Mzinga stream drains the rural environments of Mji Mwema, Vijibweni and Tuangoma that are less populated and have very few industries. As a result of various socio-economic activities, polymeric materials (household scraps, plastics, vehicle tires and electronic wastes) are abundantly present in most domestic and industrial wastes. Due to lack of appropriate infrastructures, most of these wastes are collected in open dumping sites and then burnt. Polymeric materials in wastes, when subjected to open burning, may lead to formation of PCDD/Fs and dl-PCBs (Estrellan & Iino, 2010). In addition, 2 other sources of PCDD/Fs and dl-PCBs have to be considered in the study area: (1) charcoal and/ or wood burning is the main source of energy for domestic purposes; (2) privately owned second hand commuter buses that need frequent services are the main means of transport for most residents. It was observed (Mkoma, 2008) that biomass burning (wood and charcoal burning) and traffic-related emissions (leaded gasoline exhausts and spills, tire wear) are the major sources of particulate matter in the Dar es Salaam atmosphere. The detected PCDD/Fs and dl-PCBs levels in the estuary can thus reasonably be associated with the open burning of polymeric waste, domestic wood/charcoal burning and with traffic-related emissions.

The presence of sources in the vicinity of the sampling stations must also be taken into account to explain the detected levels of PCDD/Fs and dl-PCBs. For example, upstream station E1 in the Kizinga River is downstream to an untreated wastewater discharge point of a textile factory. Higher levels of both PCDD/F and dl-PCBs in Kizinga stations compared to Mzinga stations may reflect the combined effects of the denser population and the more industrial activities in the Kizinga River basin because both are directly related to the various sources of PCDD/Fs and dl-PCBs mentioned above.

A third sampling campaign was organised to verify the higher PCDD/F and dl-PCB levels in the Kizinga River and to assess the change of the dioxin levels downstream the previous sampling stations in the estuary (E1 to E5). The most upstream sampling station in the Kizinga River (F1) which is 200 m upstream of a sewage factory pipe and near unauthorised human settlements, is very rich in organic matter (Table 2). Station F1 shows a high

PCDD/F level of 400 pg-BEQ/g and a dl-PCB level of 0.91 pg-BEQ/g. The second upstream Kizinga River sample (F2) is also high but not exceptional with 21 pg-BEQ/g PCDD/Fs and 0.63 pg-BEQ/g dl-PCBs. The levels in the upstream Mzingira River samples are close to those of the Kizinga River sample F2 (Table 2). A negative concentration gradient of PCDD/Fs and dl-PCBs in sediments is observed in the downstream direction. The levels at the mouth of the Mtoni estuary are very low: 1 to 2 pg-BEQ/g for PCDD/Fs and below LOD for dl-PCBs. These additional results confirm the previous conclusions that the impact of dioxins on the Kizinga River basin is higher than on that of the Mzingira River and that a dilution effect is clearly noticeable in the downstream direction.

Table 2. CALUX-BEQ values for PCDD/Fs and dl-PCBs in additional end-member samples

	Code and Location	TOC (%)	CALUX-BEQ (pg TCDD/g sediment)		
			PCDD/Fs BEQ ₅₀	PCDD/Fs BEQ ₂₀ -BEQ ₈₀ range	Dioxin-like PCBs*
F1	S 06°53'01.29'' E 039°15'48.33''	8.58	397	652-242	0.91
F2	S 06°52'58.07'' E 039°16'06.81''	2.08	20.7	26.7-16.0	0.68
F3	S 06°53'17.67'' E 039°18'52.06''	1.05	17.2	21.0-14.2	0.60
F4	S 06°53'13.60'' E 039°18'52.69''	0.56	12.5	15.8-9.86	0.74
F5	S 06°51'06.95'' E 039°18'22.82''	0.85	6.71	7.70-5.85	0.25
F6	S 06°50'57.46'' E 39°18'19.16"	0.72	6.82	7.47-6.23	0.23
F7	S 06°49'03.71'' E 39°18'24.11"	0.28	1.66	2.43-1.14	< LOD
F8	S 06°48'57.62'' E 39°18'34.60"	0.99	1.03	1.74-0.61	< LOD

*Results were determined by the inverse prediction method.

3.5 Correlation Between Pollutants and Sediment Geochemical Characteristics

Calculations of the Pearson correlation coefficients were performed on pollutant levels as well as sediment geochemical characteristics of all samples (n = 42) in the mixing zone in both seasons (Table 3). A significant correlation was observed between PCDD/Fs and dioxin-like PCB levels ($r^2 = 0.40$, $p \ll 0.05$), however, no significant correlations ($p > 0.05$) were found with % TOC although it is well-known that lipophilic compounds such as PCDD/Fs and dl-PCBs tend to preferably associate with organic fractions (Koh et al., 2004, 2006; Pieters, 2007).

Table 3. Correlation coefficients between pollutants and various geochemical parameters

	PCDD/Fs	PCBs	%TOC	%TN	%TC
PCDDs	1.000				
PCBs	0.395*	1.000			
%TOC	0.070	0.208	1.000		
%TN	-0.049	0.120	0.913*	1.000	
%TC	-0.065	0.172	0.823*	0.893*	1.000

* Significant at $\alpha = 0.05$.

The weak correlation between the pollutants and %TOC is contrary to the theoretical expectation explained elsewhere (Hilscherova et al., 2003), but the sources of TOC and PCDD/Fs or dl-PCBs are quite different in the Mtoni study area. TOC in the estuary and rivers is mainly originating from 2 sources: (1) mangrove degradation compounds (Marchand, Lallier-Vergès, & Baltzer, 2003) and (2) untreated domestic sewage water (Mihale, Kishimba, Baeyens, & Brion, 2013), while PCDD/Fs and dl-PCBs originate from open burning processes (Lee, Coleman, J. Jones, K. Jones, & Lohmann, 2005) and traffic related emissions (Coleman, Lee, Alcock, & Jones, 1997). A mixing of the PCDD/Fs or dl-PCBs with the organic matter in the estuary can occur in all kind of ratios. It is thus not surprising that there exists no correlation between both variables. Good and significant correlations ($p < 0.05$) were observed between % TOC, % TC and % TN.

3.6 Pollutant Source Analysis by PCA

Multivariate analysis can be used to identify similarities and differences between pollutants in samples as a means to detect possible sources. As we could expect, the PCA results indicated that the variables can be represented by two principal components that account for 83.7% of the total variance in the original data sets. Based on the loading distribution of the variables, % TC, % TN and % TOC constituted one related group (PC1), while the pollutants PCDD/Fs and dl-PCBs formed the other group (PC2).

3.7 Comparison with Other Studies and with Sediment Quality Guidelines

Little information is available in literature regarding CALUX analysis of PCDD/Fs and dl-PCBs in marine, estuarine and river sediments. No such research has been conducted in Tanzania as a whole. Research on dioxin-like compounds in such sediments in the world used chemo-analysis or GC-HRMS, because this method is considered as the golden standard (Baeyens et al., 2004). Furthermore, most research focused on PCDD/Fs while no data on dl-PCBs were available for comparison. Comparison with literature data indicated that the observed PCDD/F levels were covering the ranges observed in the literature (see Table 4). Only at the Belgian coast where lower values were observed (Sanctorum et al., 2007).

An attempt was made to compare the PCDD/F and dioxin-like PCB levels with the sediment quality guidelines. Since Tanzania lacks these guidelines, National Oceanic and Atmospheric Administration (NOAA), USA and Canadian Sediment quality guidelines were applied to assess the toxicity and risk of the dioxin-like pollutants in the Mtoni estuary ecosystem. Mangrove sediments from Mtoni estuary have higher PCDD/F levels than the NOAA apparent effects threshold (AET) of 3.6 pg-TEQ/g. The levels were also higher than both the threshold effect level (TEL) for Canadian sediment quality guideline (0.85 pg-TEQ/g) and the probable effect level (PEL) of 21.5 pg-TEQ/g. There were no specific guidelines for dl-PCB levels.

Table 4. Comparison of PCDD/F and PCB levels (pg-BEQ/g dw) determined by CALUX bioassay in different marine sedimentary environments

Study site	PCDD/Fs ¹	PCBs ²	Reference
Mtoni estuary, Tanzania ³	1.0–397	<LOD–0.91	This study
UK estuaries	1.0–88	-	(Hurst et al., 2004)
Hong Kong mudflats	3–68	-	(Wong, Giesy, Siu, & Lam, 2005)
North coast of Bohai Sea, China	3.4–28	-	(Hong et al., 2012)
Masan Bay, Korea	17–275	-	(Yoo, Khim, & Giesy, 2006)
Belgian coast	0.08–42.4	-	(Sanctorum et al., 2007)
West coast, South Korea	3.4–11	-	(Hong et al., 2012)

¹ Values presented are BEQ₅₀; ² values are inversely predicted; ³ Values indicate the range of mean BEQ of both seasons (n = 6).

The comparisons have shown that there could currently be a risk regarding PCDD/Fs, while the risk associated with dl-PCBs is much lower. Because the toxicity of these chemicals is assumed additive, increasing levels in line with the increasing anthropogenic activities can be alarming to the local biological community (Kruitwagen, Hecht, Pratap, & Wendelaar-Bonga, 2005) such as the barred mudskippers and soft bottom mollusks that inhabit the muddy areas. Presence of these pollutants in mangrove sediments may cause impairments, like abnormal growth and malformations. The effects can reach other organisms higher in the trophic level due to their

bio-accumulation, bio-concentration and persistent properties. The levels of pollutants observed in the frequently exchanged upper (0-3 cm) layer can have impacts on the distribution and fate of pollutants to mangrove ecosystems and to higher organisms that use mangrove sediment organisms as their food.

4. Conclusion

This study is the first of its kind that used *in vitro* bioassay analysis (CALUX) to determine dioxin and dioxin-like compounds in the environment of East Africa. Sediment samples were collected in the Mtoni estuary and its tributaries, the Kizinga and the Mzinga Rivers, in the vicinity of Dar es Salaam. While it is well known that anthropogenic activities as open burning of plastic scrap, household burning of wood or charcoal and traffic related emissions, which all frequently occur in the Dar es Salaam region, can lead to PCDD/F and dioxin-like PCB production, it was totally unknown if some or all of those sources resulted in the contamination of the nearby aquatic systems. Our CALUX analyses of the sediments in the Mtoni estuary and its 2 tributaries demonstrated that the range of PCDD/F values (1.0 to 400 pg-BEQ/g-sediment) covers the ranges observed in sediments from Western Europe and Eastern Asia. Neither significant seasonal variations nor vertical gradients in the sediments could be observed. For dl-PCBs we could not find sediment results obtained by CALUX in the literature. Sediments in the Kizinga River, which flows through a denser populated and more industrialised area than the Mzinga River, showed also higher PCDD/F and dioxin-like PCB levels. Finally, the overall BEQ value of dioxins (PCDD/F and dioxin-like PCB levels) observed in sediments of the Dar es Salaam region, indicate possible ecological and human risks that may emanate from these contaminants.

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