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Field Report 2018

Baseline Study of Endocrine-Disrupting Compounds and Pharmaceuticals and Personal Care Products in Waterways Surrounding Chitwan National Park, Nepal

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ICIMOD gratefully acknowledges the support of its core donors:
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Baseline Study of Endocrine-Disrupting Compounds and Pharmaceuticals and Personal Care Products in Waterways Surrounding Chitwan National Park, Nepal

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International Centre for Integrated Mountain Development, Kathmandu
September 2018

Published by

International Centre for Integrated Mountain Development
GPO Box 3226, Kathmandu, Nepal

ISBN 978 92 9115 626 9 (electronic)
978 92 9115 625 2 (print)

Production team

Rachana Chettri (Editor)
Samuel Thomas (Senior editor)
Punam Pradhan (Graphic designer)

Photos: Nabin Bhattarai

This publication is available in electronic form at www.icimod.org/himaldoc

Citation: Pandey, A., Bhattarai, N., Adhikary, B., Singh Karky, B.,Pokhrel, C.P., Pathak, A. (2018) *Baseline study of endocrine-disrupting compounds and pharmaceuticals and personal care products in waterways surrounding Chitwan National Park, Nepal.* ICIMOD Field Report 2018. Kathmandu: ICIMOD

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Government of Nepal
Ministry of Forest and Environment
Department of National Parks & Wildlife Conservation

Foreword

The Government of Nepal is developing the first REDD+ programme in the Terai Arc Landscape where numerous Protected Areas are linked through corridors. Chitwan National Park is an integral part of this proposed REDD+ programme given that it is a climate relevant reserve storing carbon in aboveground vegetation and in soils.

The Chitwan National Park is a World Heritage Site that harbours floral and faunal diversity of global significance. The country's first protected area, it provides refuge to 68 species of mammals, 544 species of birds, 56 species of herpetofauna, and 126 species of fish. It is also adjacent to the country's industrial belt and there have been concerns about the impacts of industrialization on the buffer zone areas outside the Park.

This study is the first of its kind that looks at the impact of endocrine-disrupting compounds on the waterbodies of Chitwan National Park. It serves as a baseline on chemicals that severely impact aquatic biodiversity and clearly such monitoring needs to be done on a more regular basis.

I am grateful to the team of highly skilled Nepalese researchers that undertook this pioneering research in Nepal. More such studies are needed to monitor the quality of waterbodies in and around the Protected Areas of the country.

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Introduction and Background

There is very limited knowledge on the impacts of endocrine-disrupting compounds (EDCs) on the environment and aquatic animals. In fact, much remains to be done to ascertain whether a certain class of chemicals even qualifies as an EDC. Recently, there have been joint efforts by the U.S. Geological Survey and National Park Survey to pursue pilot studies on the identification and quantification of synthetic compounds with hormone mimicking properties (classified as EDCs), to assess their effects on aquatic and other organisms within national park units (Ellsworth et al. 2014).

Records of similar research, to quantify EDCs in waters, have been conducted in neighbouring India (Kumar et al. 2008). The study reported on the presence of androgenic compounds which posed a threat to the general population and animals in the region. There are no other reported studies on EDCs in South Asia. This research is the first of its kind in studying concentrations of EDCs in surface waters surrounding the Chitwan National Park (CNP) of Nepal.

Rivers flowing through mountainous countries like Nepal, based on their size and strength, transport sediments and nutrients from natural processes, along with any foreign pollutants introduced from human settlements, agricultural practices, and industrial processes. This implies that, if not properly disposed, harmful chemicals like EDCs could end up in waterways and be transported to vulnerable locations downstream (Runkel and Bencala 1995). Given the stage of development and weak enforcement of regulations in Nepal, it is highly likely that waste generated from industries, commercial, residential, and agricultural sectors could end up in waterways anywhere in Nepal. Chitwan National Park is one such susceptible region, which is likely to be a sink for pollutants brought in by large rivers such as Narayani in the northwest and Rapti in the northeast, which are open to contamination, as they flow through dense residential and industrial areas like Bharatpur and Hetauda, respectively.

Chitwan National Park is a leader in conservation efforts in Nepal. Tiger, rhino, vulture and crocodile conservation activities have gained global fame. To further strengthen conservation activities, it was necessary to investigate threats to the wellbeing of aquatic life in and around the park, as there was a dearth of information available. To ensure the wellbeing of the park's diverse population of plants and animals, a range of policies and management efforts aimed to maintain water quality are required to be implemented, which should be based on accredited data collected over a long period of time (Miller 2004). Likewise, EDCs that are known to cause a decline in populations and an increase in cancer and reproductive abnormalities even at trace amounts (Kavlock et al. 1996) and their concentrations must be considered while formulating water quality regulations for conservation sites (Ellsworth et al. 2014). Being the first of its kind in Nepal, this study also includes detailed explanations on the effects of EDCs and their potential sources in the country.

EDC and PPCP Effects

Historically, certain toxins were recognized to have prominent damaging effects on the sexual characteristics of male and female members in aquatic animals. A 1980 study associated the abnormal male-like behaviours of female mosquito fish of Eleven Mile Creek in Florida, with the discharge of paper industry effluents into the stream (Howell et al. 1980). Similarly, synthetic hormones widely used as contraceptives were also studied and showed hazardous effects of sexual disruption on fish population when present in water bodies (Bevans et al. 1992; Desbrow et al. 1998; Kramer et al. 1998; Renner 1998; Daughton and Jones-Lepp 2001; Snyder et al. 2001).

Primarily, EDCs have raised caution among conservationists and health personnel, due to their endocrine-mimicking properties that can disrupt normal hormonal pathways by inhibiting or exacerbating the effects of estrogenic, androgenic, and thyroidal hormones in animals and humans. In 2002, the World Health Organization officially defined EDCs as "exogenous substances that alter function(s) of the endocrine system and consequently cause adverse health effects in an intact organism, or its progeny, or (sub)-populations" (Damstra et al. 2002).

Water sources contaminated by endocrine disrupting (ED) pesticides can have damaging effects on the health of wild animals, among which amphibians, invertebrates, reptiles, fish, and birds are most susceptible. For example, polyaromatic hydrocarbon and organochloride pesticide exposure to common eiders (*Somateria mollissima*) of the Baltic Sea resulted in significant levels of DNA damage (Matson et al. 2004). Similarly, many species in Chitwan National Park could be vulnerable to pesticide runoff, from surrounding areas with dense agricultural fields and urban settlements, mixing into their water sources. Popularly used organophosphates such as acephate are known to cause significant damage to the process of hormone expression in the hypothalamus whereas chlorpyrifos and dichlorvos are known to compete and inhibit androgenic activities at varying strengths and are highly toxic for bees (Mnif et al. 2011). More damaging effects have been recorded for organochlorides such as endosulfan, which can inhibit the activities of androgenic hormones and enzyme aromatase, and stimulate estrogenic activities like receptor production (Mnif et al. 2011). Likewise pyrethroids such as cypermethrin and deltamethrin, which are ubiquitous in the environment due to excessive use in households, are also known to have estrogenic activities (Chattopadhyay et al. 1999). Some herbicides also fall under the EDC category; for example, atrazine is known to have severe effects such as disruption of androgenic activities and the activities of the hypothalamus including the control of luteinizing hormone (Mnif et al. 2011). Atrazine also induces aromatase to increase oestrogen production and ultimately causes disrepair to the adrenal glands and the body's capacity to digest steroidal hormones (Mnif et al. 2011).

Although most EDCs are known to be organic compounds mainly characterized by incumbent aromatic rings on their structure, inorganic molecules such as heavy metals are being added to this group due to their oestrogen, androgen, and glucocorticoid-like hormonal activities. Arsenic, a common contaminant in pesticides, showed oestrogen-agonistic behaviour and caused selective toxicity to reproductive organs in female mice that were given a dose of 4 ppm in water per day (Chattopadhyay et al. 1999). Similarly, cadmium has been proved to have severe effects on females, from premature deliveries to reduced progesterone levels and early onset of puberty. Cadmium also causes toxicity to the male testes, whereas lead can disrupt onset of puberty in females and can cause delayed spermatogenesis and reduced fertility in males resulting in skewed sex ratios with fewer male births (Dyer 2007). Exposure to mercury also results in dysfunctional reproductive systems in females, and bioaccumulation can be dangerous to entire species as shown by the aggravated numbers of cryptorchidism among panthers in Florida (Facemire et al. 1995). Similar studies have shown effects of long-term exposure to zinc on river fish that caused androgenic disruption such as reduced fast motility duration in *Salmo trutta* sperm (Giardina et al. 2009).

Other than pesticides and heavy metals, a class of chemicals called persistent polyaromatic hydrocarbons (PAHs) can also affect behaviour in mammals by disturbing their activities and space use, in reptiles by disturbing type of diet and migration patterns, and in birds by changing parental behaviours (Montiglio and Royauté 2014). One such PAH considered in this study is pyrene. Pyrene and some of its derivatives have been associated with detrimental teratogenic effects on mice (Guo 2011) and have also induced obesity by inhibiting break-down of fat in the adipose tissue (De Coster and van Larebeke 2012). Exposure to other PAHs such as fluoranthene can also reduce the levels of androstenedione and other hormones in the tissues of ovaries. Fluoranthene derivatives are also known to behave as anti-oestrogens in the cancerous cell linings in mammary glands (Higley et al. 2012).

In addition, daily used pharmaceutical and personal care products (PPCPs) are known to have similar effects and can be hazardous if disposed into surface water systems. Caffeine, which is one of the most commonly consumed chemicals, can have significant eco-toxicological effects on smaller animals as shown by growth inhibition in rotifers (Zarrelli et al. 2014). More toxic effects are demonstrated by the commonly used mosquito repellent N,N-diethyltoluamide (DEET) such as inducing genetic diseases and spreading through generations of the specimen by modifying normal biomarkers in the sperm (Manikkam et al. 2012). Fluoroquinolone-derivative antibiotics such as norfloxacin and enrofloxacin have also shown toxic effects in crustaceans and other aquatic animals (Langdon et al. 2010). Even considerably mild analgesic drugs such as paracetamol (acetaminophen) are known to cause androgenic disruption in rats exposed to the drug (Manikkam et al. 2012).

Recent EDC-related investigations focus on identifying and quantifying very trace levels of such compounds in samples containing a matrix of diverse groups (Petrovic et al. 2002; Campbell et al. 2006; Yoon et al. 2010). It is essential to evaluate the threshold concentrations of EDCs that can disrupt an ecosystem, which is a complex

interlinked matrix of eclectic species placed in a sensitive hierarchy based on their specific niche. Aquatic animals, however small, are part of this complex matrix and are also most susceptible to the effects of the chemicals being studied. Obstruction of reproductive functions of any species can have a ripple effect throughout the food web.

The concentrations of some of the chemicals that show adverse effects on different aquatic animals are listed in Table 1.

Table 1: **Lethal dose of chemicals/EDCs studied on various species**

EDC	Species studied	Class of animal	Studied	Lethal concentration (µg/l)
Chlorpyrifos	Daphnia magna	Crustacean	Reproduction	0.09 (45)
	Neomysis integer	Mysid	Behaviour	0.038 (46)
	Xenopus laevis	Frog	Development	100 (47)
	Ceriodaphnia dubia	Crustacean	LC_{50}	0.054 (48)
Dichlorvos	Brachionus calyciflorus	Rotifer	Reproduction	10 (49)
	Tigriopus brevicornis	Crustacean	LC_{50}	0.92 (50)
2,4-dichlorophenoxy acetic acid (2,4 D)	Rattus norvegicus	Rodent	Reproduction	30,000 (51)
	Orconectes rusticus	Fish	Behaviour	7650 (52)
	Simocephalus vetulus	Crustacean	Development	0.75 (53)
Carbendazim	Aporrectodea caliginosa	Annelida	Development	400 (54)
	Daphnia magna	Crustacean	LC_{50}	35.54 (55)
Fenvalerate		Amphipod	LC_{50}	0.022 (56)
Cypermethrin	Ceriodaphnia dubia	Crustacean	Reproduction	1 (57)
	Daphnia magna	Crustacean	Behaviour	0.055 (58)
	Ceriodaphnia dubia	Crustacean	Development	0.05 (59)
	Daphnia magna	Crustacean	LC_{50}	0.00061 (60)
Deltamethrin	Ceriodaphnia dubia	Crustacean	Reproduction	0.025 (61)
	Palaemon serratus	Crustacean	Behaviour	0.0006 (62)
	Ceriodaphnia dubia	Crustacean	Development	0.005 (63)
	Oncorhynchus mykiss	Fish	LC_{50}	0.6961 (64)
Atrazine	Carassius auratus	Fish	Reproduction	100 (65)
	Oncorhynchus mykiss	Fish	Behaviour	1 (66)
	Sciaenops ocellatus	Fish	Development	80 (67)
	Tigriopus brevicornis	Crustacean	LC50	120.9 (68)
Cadmium	Lymnaea stagnalis	Gastropod	Reproduction	200 (69)
	Oncorhynchus mykiss	Fish	Behaviour	2 (70)
	Salvelinus confluentus	Fish	Development	0.786 (71)
	Macrobrachium lanchesteri	Crustacean	LC_{50}	7 (72)
Mercury	Crepidula fornicata	Mollusc	Reproduction	0.42 (73)
	Pomatoschistus microps	Fish	Behaviour	3 (74)
	Crepidula fornicate	Mollusc	Development	0.42 (75)
	Daphnia obtusa	Crustacean	LC_{50}	2.8 (76)
Arsenic	Colisa fasciatus	Fish	Reproduction	14000 (77)
	Corbicula fluminea	Bivalve	Behaviour	350 (78)
	Oncorhynchus mykiss	Fish	Development	16000 (79)
	Tigriopus brevicornis	Crustacean	LC_{50}	10.9 (80)
Lead	Ceriodaphnia dubia	Crustacean	Reproduction	5.2 (81)
	Lymnaea stagnalis L.	Mollusc	Behaviour	200 (82)
	Lymnaea stagnalis	Gastropod	Development	24 (83)
	Ceriodaphnia dubia	Crustacean	LC_{50}	18 (84)

EDC	Species studied	Class of animal	Studied	Lethal concentration (µg/l)
Zinc	Artemia parthenogenetica Mytilus galloprovincialis Farfantepenaeus paulensis Macrobrachium lanchesteri	Crustacean Bivalve Crustacean Crustacean	Reproduction Behaviour Development LC_{50}	80 (85) 100 (86) 106 (87) 525.1 (72)
Fluoranthene	Daphnia magna	Crustacean	Behaviour	8570 (88)
Pyrene	Danio rerio Sparus aurata Chanos chanos Chanos chanos	Fish Fish Fish Fish	Reproduction Behaviour Development LC_{50}	1.63 (89) 50 (90) 0.29 (91) 14 (92)
DEET	Daphnia magna	Crustacean	LC_{50}	160,000 (93)
Caffeine	Lemna gibba	Plant	Toxicity	>1000 (94)
Acetaminophen	Danio rerio	Fish	Toxicity	4000 (94)
Norfloxacin Enrofloxacin	Cyclotella meneghiniana Brachionus calyciflorus	Algae Invertebrate	Toxicity (long term)	31.2 (94) 12500 (94)

Study Parameters: EDCs and PPCPs in Nepal

In Nepal, the main sources of contamination of surface water include direct discharge of municipal wastewater, surface runoffs containing chemicals used in households and agriculture, and accidental leaks or unsupervised discharge of industrial effluent into rivers (Barnhoorn et al. 2004). Anthropogenic activities that are likely to introduce EDCs include haphazard use of pesticides and herbicides for agriculture, heavy metal leeching from poorly managed industrial and poultry wastes, unsupervised forest fires generating polyaromatic hydrocarbons through incomplete combustion, and pharmaceuticals and personal care products (PPCPs) that are improperly disposed of and collect in river basins. Based on the extent of use and the adverse effects of these chemicals on the aquatic ecosystem, this study was narrowed down to 21 EDCs and PPCPs originating from different sources to be identified and quantified in the surface waters surrounding Chitwan National Park.

Although the average amount of pesticide consumed in Nepal is relatively low at 142 g/ha, there has been a steady increase of 10–20% in annual consumption levels (Diwakar et al. 2008) used mainly in vegetable and fruit farming (Diwakar et al. 2008). Annual intake amounts of insecticides including fungicides and herbicides have increased from 183909.8 kg in 2003 (Diwakar et al. 2008) to 191416 kg in 2010 (Sharma et al. 2013), and 25% of these are used in the southern plain region of Nepal (Sharma et al. 2013). Furthermore, misuse of pesticides by farmers, such as using excess amounts at the wrong times and using wrong compounds for undesignated processes, are common issues in Nepal. Cases of use of endosulfan, a broad spectrum pesticide and a known EDC (Mnif et al. 2011) banned in many countries including India in 2011 by farmers through direct injection into rivers for fishing have been observed (Sharma 2011). Likewise, despite the Nepalese government's recent ban on the import and production of organochloride, a 2015 study showed 50µg/l concentration of this pesticide in surface waters in Kavre region. Similarly, other insecticides commonly used in Nepal that are known to show endocrine-disrupting characteristics (Mnif et al. 2011) included in this study are the registered public health pesticide deltamethrin; the registered household pesticide cypermethrin; commonly used organophosphates such as acephate, dichlorvos, and chlorpyrivos; organochlorides such as 2,4-dichlorophenoxy acetic acid (2,4 D); and herbicides such as atrazine.

Brick, plastic, metal, wood, and paper are some of the most recurrent medium-scale industries in Nepal and Chitwan District, along with large-scale cement factories. Some of the compounds with endocrine-disrupting properties and that are known wastes from these manufacturing processes were also chosen for this study. A case of contamination of Narayani River through direct discharge of effluents from the Bhrikuti Paper Factory – which was laden with potent EDCs such as dioxin and its perilous effects on water-dependent species such as the Ganges river dolphin, gharial, and grey-headed fish eagle – were recorded in studies dating back to the 1990s (Poudel 1996). Moreover, Nepal also has a natural abundance of arsenic in areas close to Chitwan District (Henke 2009),

and leaching due to erosion of mineral rocks, sediments, and soils can cause contamination of both surface water and groundwater. Other sources of arsenic include arsenical pesticides, wood preservatives, smelting of glass and copper, and combustion of coal (Dyer 2007). Similarly other metals that are case and endocrine include cadmium, which is released from pigments, plastic production and tobacco production and consumption; lead, which is released from fuels, pipes, paints, and soldered food cans; and mercury from metal smelting, coal combustion, and urban pollution (Dyer 2007).

Most commonly used pharmaceuticals like acetaminophen and popular veterinar fluoroquinolone antibiotics like norfloxacin and enrofloxacin have also been studied in this paper on the basis of literature review (Daughton and Ternes 1999). These products are available over the counter and are sold in large volumes and improperly disposed of once they expire. The hypothesis is that the source of these compounds is mostly household and poultry farming wastes. Similarly, personal care products such as N,N-diethyltoluamide (DEET) and the consumable chemical caffeine have also been chosen for this study. DEET is a frequently used insecticide in mosquito repellents (Manikkam et al. 2012) and caffeine is the primary alkaloid present in widely consumed coffee and tea beverages (Zarrelli et al. 2014). Since the national park is largely isolated from human habitation, and given the scope of this project, fewer PPCPs have been studied in this project. Table 2 lists the chemicals studied in this project.

Table 2: **Types of pollutants, chemical compounds and their use**

Type of pollutants	Target compounds	Use
Agricultural pesticides, herbicide and fungicide	Chlorpyrifos	Pesticide
	Dichlorvos	Pesticide
	2,4-dichlorophenoxy acetic acid (2,4 D)	Pesticide
	Carbendazim	Pesticide
	Fenvalerate	Pesticide
	Cypermethrin	Pesticide
	Deltamethrin	Pesticide
	Atrazine	Herbicide
Industrial metals and combustion by-products	Cadmium	
	Mercury	
	Arsenic	
	Lead	
	Zinc	
	Fluoranthene	
	Pyrene	
Pharmaceutical and personal care products	N,N-diethyltoluamide	Mosquito repellent
	Caffeine	Beverages
	Acetaminophen	Pharmaceuticals
	Norfloxacin	Pharmaceuticals
	Enrofloxacin	Pharmaceuticals

Sampling Site and Area Description

Established in 1973 as Nepal's first national park, Chitwan National Park has pioneered the paradigm for ecosystem conservation in Nepal. It is one of the few surviving examples of the natural ecosystems of the Terai region and covers subtropical lowland, wedged between two east-west river valleys at the base of the Siwalik Range of the central Himalaya. As shown in Figure 1, the national park core area lies between the Narayani (Gandak) River and Rapti River to the north. Reu River and the Nepal-India international border mark the southern border. Daunne hills marks the western border while Parsa Wildlife Reserve lies at the eastern edge. The geographical location of the park is between N 270 20′ 19′′ to 270 43′ 16′′ longitude and E 830 44′ 50′′ to 840 45′ 03′′ latitude, whereas the geographical location of the buffer zone is between N 270 28′ 23″ and 270 70′ 38″ longitude and E 830 83′ 98″ and 840 77′ 38′′ latitude (MoFSC 2015). The park is home to a total of 68 species of mammals, 126 species of fish, and 544 species of birds.

The diverse aquatic and terrestrial wildlife populations of the park depend mostly on the tributaries of the Rapti and Narayani rivers for their existence and sustenance. Water sources in the southern section include Reu Khola along with several other lakes and ponds. The park receives a mean annual rainfall of 2,150 mm and maintains a healthy supply of water for the fauna and the fecund aquatic and amphibian population of the region.

A total of 45 water samples were collected upstream from Narayani, Rapti, and Reu Rivers, of which 19 were from Rapti, 17 from Narayani, three from Reu, and six from various core regions inside the park (Figure 1). Based on the composition of water sources, sampling sites have been divided into five zones as outlined in Table 3 and shown in Figure 2. Separation into specific zones will help to create narrower spatial correlations between sources of contamination and to compare and contrast the samples from individual rivers with each other as well as with the region where the three merge.

Figure 1: **Sampling sites distribution in zones**

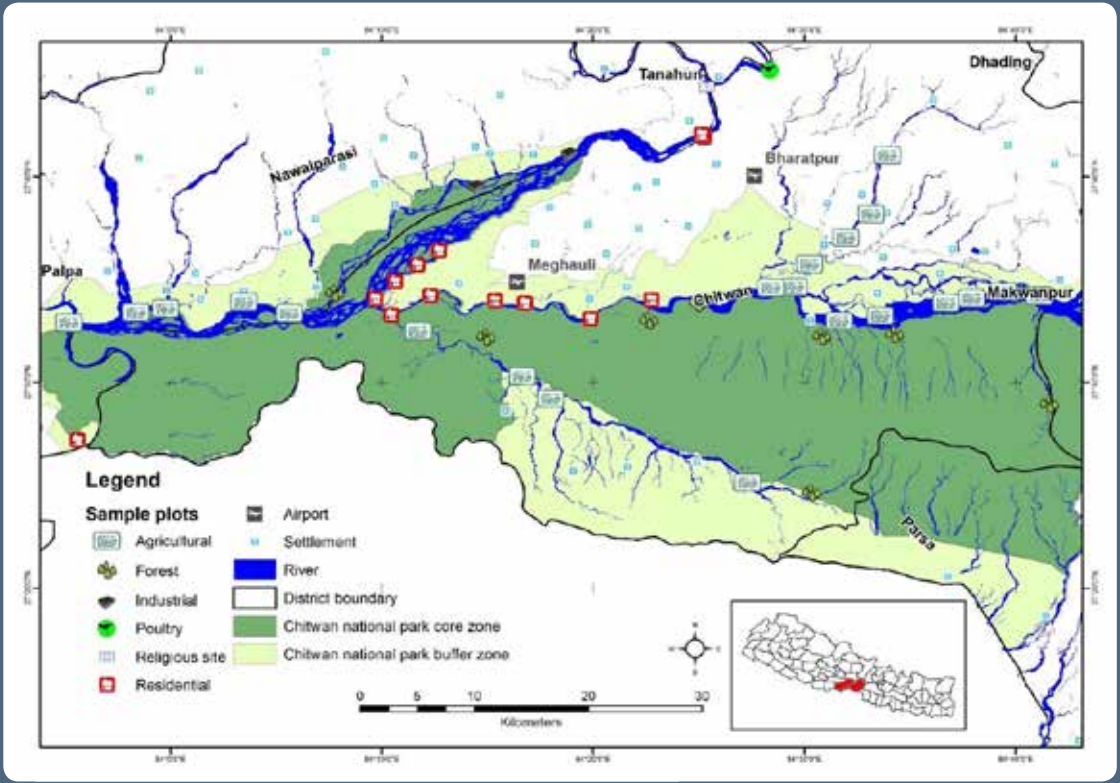


Figure 2: **Sampling site details and zone allocations**

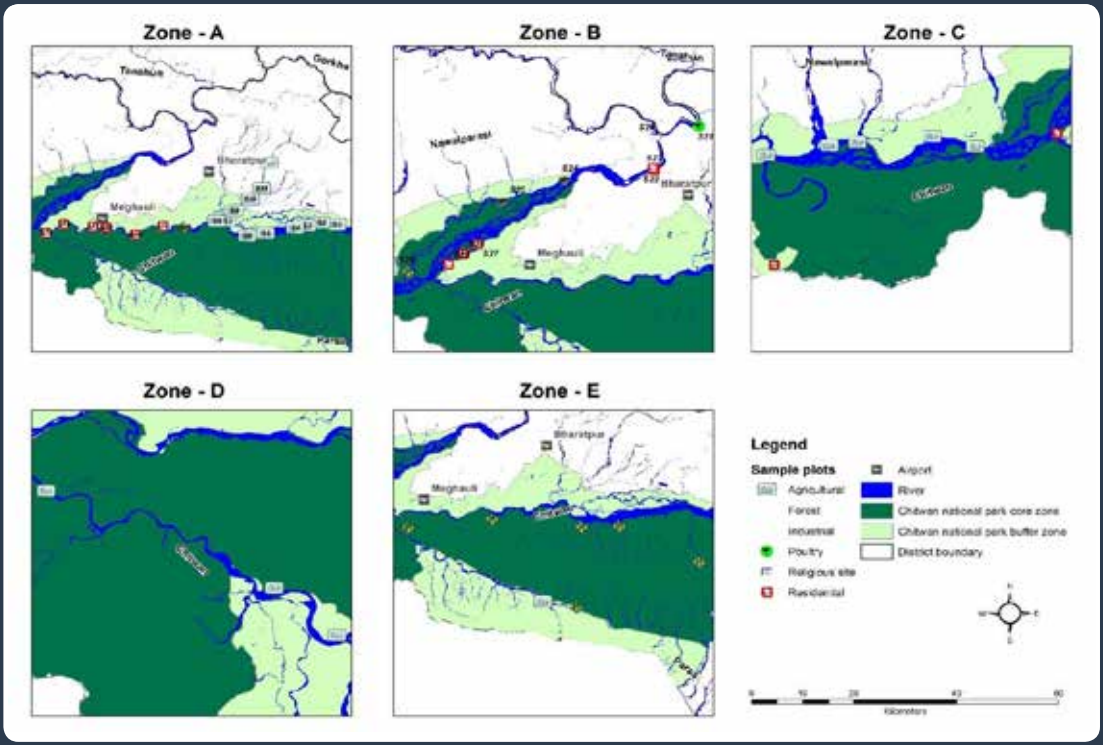


Table 3: **Allocation of sampling sites by zones**

Zone	Sample numbers	Water composition
A	S1 to S19	Rapti and tributary
B	S20 to S29	Narayani
C	S30 to S36	Narayani, Rapti, and Reu
D	S37 to S39	Reu
E	S43 to S49	Core runoffs and streams
	S40, S41 and S42	This was planned but not sampled due to terrain difficulty

Table 4: **Site description and river location**

Sampling site number	Site description	River
S1, S2, S3, S4, S5, S6, S7, S8, S9, S10, S11, S12, S14, S15, S16, S18, S19, S20, S21, S31, S32, S33, S34, S35, S37, S38, S39	Agricultural lands and farming community	Rapti, Narayani, Reu
S22, S23, S27, S28, S29, S30, S36	Residential and urban settlements	Narayani
S13, S26, S43, S44, S45, S46, S47, S48, S49	Core region of Chitwan National Park (CNP), jungle ecosystems	Core streams
S24, S25	Industrial locations with manufacturing factories	Narayani
S17, S21	Poultry farming: chicken coops	Rapti, Narayani

Along Narayani, 10 samples were collected upstream as shown in Zone B (Table 3). Further samples from Narayani were taken after the point of mixture of Rapti and Reu in Zone C. Bharatpur Municipality is the densest area of Chitwan with its major city, Narayanghat, and Bharatpur housing 280,502 people within 433 km^2 (CBS 2014). Field observation showed household wastes are still being dumped directly into Narayani which is also a holy site for cremation ash disposal. On the other side of Narayani opposite to Narayanghat, the river bank is occupied by the country's largest brewery, Gorkha Brewery, among other brick and metal factories.

Rapti flows east to west within the district and passes through one of Nepal's densest cities, Hetauda – with a population of 84,671 in 187.7 km^2 – before entering Chitwan. However, in this district it falls under the protection of the park and no direct disposal of wastes was observed. Rapti also passes through both Kasara and Sauraha, which are popular among tourists, and is a site for river rafting and other river recreational activities. Eighteen locations along the Rapti were sampled, all listed as Zone A (Table 3). In addition, a tributary running through a significant industrial region, Ratnanagar Municipality, from 27.6842N to 27.5768N latitude, has been sampled.

The last river studied in this project, Reu, flows east-west into Narayani and marks the southern border of the park. It flows through Madi, a farming community located in a clearing within the park. Reu does not pass through large settlements or industrial areas making it prone only to agricultural runoffs. The location of each sampling site and the type of area surrounding it is shown in Figure 1.

Sampling Technique

In order to avoid dilution, samples were collected before the months of heavy rainfall during the pre-monsoon season, specifically from 24 to 30 May 2016. Figure 3 illustrates the 10-year average discharge data from Narayani and Rapit Rivers. The data were obtained from the Department of Hydrology and Meteorology.

The above figure shows May to be the ideal sampling time in the year since it is just before the onset of heavy monsoon rain and after the more arid months. Occasional showers will further increase the probability of the contaminants to be displaced all the way to the main rivers. Therefore samples collected in this time would be representative of majority of the months in a year.

Figure 3: **Average monthly discharge Narayani (left) and Rapti (right)**

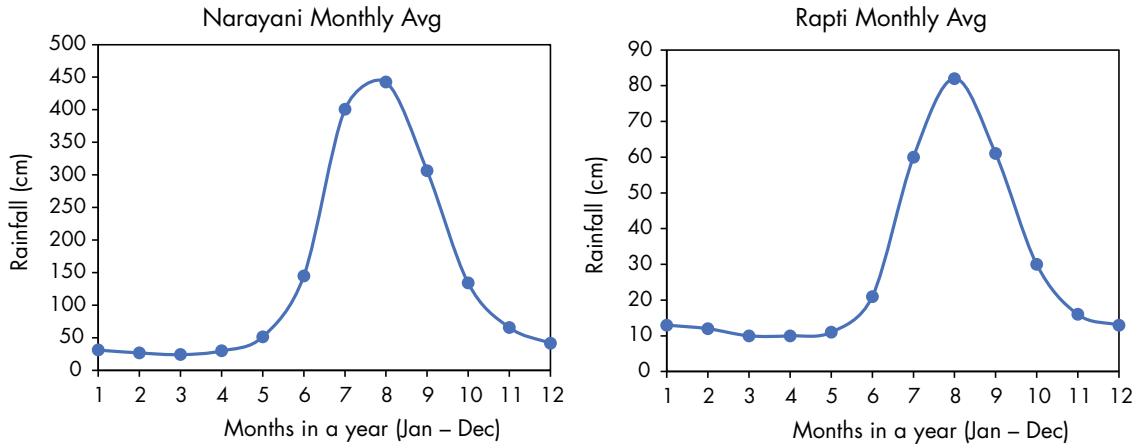


Table 5: **Details of sampling containers used**

Container	Type of material	Preservative	Volume
1	Polyethylene glycol	HNO3 (68%)	500 ml
2	Polyethylene glycol	HCl (33%)	250 ml
3	Polyethylene glycol	HCl (33%)	250 ml
4	Glass	HCl (33%)	250 ml

Samples have been taken using the grab sample technique commonly utilized in analysing wastewater treatment effluents. This technique has allowed the samples to represent the chemistry of the river or stream at a specific location at a given point in time (Nilsson and Rosenberg 2000). For every site a total of 1.15 L of water was collected (500 ml for EDC/PPCPs and 650 ml for metal analysis) using four different containers. Each container was treated differently and the details of preservatives and types of containers used are listed in Table 5.

Water samples were collected from about 100 cm away from an accessible site along the river bank and approximately 30 cm deep from the surface. Water was collected using a standard plastic bucket attached to the end of a retrieving device and immediately transferred to the four containers. Samples were transported to the laboratory in cold storage containers at temperature maintained at 4°C until extraction. All samples were extracted within three weeks of collection.

Methods of Analysis

Due the varied nature of parameters being studied, a range of known analysis methods were used including combining a high performance liquid chromatography (HPLC) with UV detector and gas chromatography (GC) with flame ionization detector (FID). Instrument parameters were set based on standardized methods of analysis for each compound using direct injection or liquid-liquid solvent extraction, extracted from the Indian Standard Method of Analysis and the United States Pharmacopoeia as no standard analytical test is prescribed by the Government of Nepal. Extraction and analysis of EDCs were carried out at Zest Laboratory, the only ISO/IEC 17025:2005

Table 6: **Summary of analytical methodology for each compound**

Target compound	Method source and number	Solvent system of extraction	Method details
Chlorpyrifos	Indian Standard Method of Analysis	100 ml sample by 25 ml dichloromethane extracted in three batches 10 ml, 10 ml, 5 ml Evaporated and re-extracted with 10 ml acetone	GCFID injection temp: 270C Flow rate: 2 ml/min Carrier gas: N_2 Detector temp: 280C
Dichlorvos	Indian Standard Method of Analysis	100 ml sample by 25 ml dichloromethane extracted in three batches 10 ml, 10 ml, 5 ml Evaporated and re-extracted with 10 ml acetone	GCFID injection temp: 270C Flow rate: 2 ml/min Carrier gas: N_2 Detector temp: 280C
2,4-dichlorophenoxy acetic acid (2,4 D)	Indian Standard Method of Analysis	50 ml sample by 20 ml chloroform extracted in two batches 10 ml, 10 ml Evaporated and re-extracted with 10 ml methanol	HPLC column temp: 40C Flow rate: 1 ml/min Column: C18 MP: acetonitrile:water (pH 3) 50:50 UV detection wavelength: 230 nm
Carbendazim	Zest Laboratory	50 ml sample by 20 ml chloroform extracted in two batches 10 ml, 10 ml Evaporated and re-extracted with 10 ml methanol	HPLC column temp: 40C Flow rate: 1 ml/min Column: C18 MP: acetonitrile:water (pH 3) 50:50 UV detection wavelength: 286 nm
Fenvalerate	Indian Standard Method of Analysis	100 ml sample by 25 ml dichloromethane extracted in three batches 10 ml, 10 ml, 5 ml Evaporated and re-extracted with 10 ml acetone	GCFID injection temp: 270C Flow rate: 2 ml/min Carrier gas: N_2 Detector temp: 280C
Cypermethrin	Indian Standard Method of Analysis (with modification)	100 ml sample by 10 ml solution of isooctane and 1,4 dioxane (8:2) Dried with sodium sulphate	HPLC column temp:40C Ambient flow rate: 1.5 ml/min Column: silica MP: isooctane:1,4 dioxane (95:5) UV detection wavelength: 236 nm

Target compound	Method source and number	Solvent system of extraction	Method details
Deltamethrin	Indian Standard Method of Analysis (with modification)	10 ml solution of isooctane and 1,4 dioxane (8:2) Dried with sodium sulphate	HPLC column temp: Ambient flow rate: 1.5 ml/min Column: silica MP: isooctane:1,4 dioxane (95:5) UV detection wavelength: 236 nm
Atrazine	Indian Standard Method of Analysis (with modification)	50 ml sample by 20 ml chloroform extracted in two batches 10 ml, 10 ml Evaporated and re-extracted with 10 ml methanol	HPLC column temp: 15C Flow rate: 0.8 ml/min Column: C18 MP: methanol UV detection wavelength: 254 nm
Cadmium	Standard	Direct injection	AAS
Mercury	Standard	Direct injection	AAS
Arsenic	Standard	Direct injection	AAS
Lead	Standard	Direct injection	AAS
Zinc	Standard	Direct injection	AAS
Fluoranthene	Indian Standard Method of Analysis (with modification)	50 ml sample by 20 ml chloroform extracted in two batches 10 ml, 10 ml Evaporated and re-extracted with 10 ml methanol	HPLC column temp: 15C Flow rate: 0.8 ml/min Column: C18 MP: methanol UV detection wavelength: 254 nm
Pyrene	Indian Standard Method of Analysis (with modification)	50 ml sample by 20 ml chloroform extracted in two batches 10 ml, 10 ml Evaporated and re-extracted with 10 ml methanol	HPLC column temp: 15C Flow rate: 0.8ml/min Column: C18 MP: methanol UV detection wavelength: 254 nm
N,N-diethyltoluamide	Indian Standard Method of Analysis	100 ml sample by 25 ml dichloromethane extracted in three batches 10 ml, 10 ml, 5 ml Evaporated and re-extracted with 10 ml acetone	Injection temp: 270C Flow rate: 2ml/min Carrier gas: N_2 Detector temp: 280C
Caffeine	United States Pharmacopoeia	Direct injection	HPLC column temp: 45C Flow rate: 2ml/min Column: C18 MP: water, methanol, acetic acid (69:28:3) UV detection wavelength: 275 nm
Acetaminophen	United States Pharmacopoeia	Direct injection	HPLC column temp: 45C Flow rate: 2ml/min Column: C18 MP: water, methanol, acetic acid (69:28:3) UV detection wavelength: 275 nm
Norfloxacin	Zest Laboratory	20 ml sample treated with 1 ml 5% acetic acid and placed in ultrasonic bath for 10 minutes	HPLC column temp: 30C Flow rate: 1ml/min Column: C18 MP: water (pH 2.1):acetonitrile 70:30 UV detection wavelength: 275 nm
Enrofloxacin	Zest Laboratory	20 ml sample treated with 1 ml 5% acetic acid and placed in ultrasonic bath for 10 minutes	HPLC column temp: 30C Flow rate: 1 ml/min Column: C18 MP: water (pH 2.1):acetonitrile 70:30 UV detection wavelength: 275 nm

accredited and Global Fund approved private testing laboratory in Nepal. Zest Laboratory archives all of its previously used test methods so the documentation was easily available for this study. The details of solvent and methodology used for each of the compounds are summarized in Table 6.

Among the several available methods of determining limit of detection and limit of quantification, we have used 'Based on the Standard Deviations of the Response and the Slope,' using the following formula:

*Limit of Detection (LOD) = $3.3\sigma/S$

*Limit of Quantification (LOQ) = $10\sigma/S$

Where σ is standard deviation and S is slope.

These statistical tools are used to understand the confidence of the values calculated through HPLC and GCFID analysis. LOD defines the lowest trace level of compounds present in the samples that can be detected by the instruments as compared to a blank sample. Most instruments often show readings, called a noise, even in a blank sample. LOD defines the threshold above the noise from the analyte. LOQ defines the lowest level above which the concentration measurement can be interpreted with high confidence. Trace amounts below LOD will be recorded as BDL (Below Detection Limit) and concentrations below LOQ will be recorded as BQL (Below Quantification Limit). However, it must be noted that BQL results are still evidence for the presence of compound in the sample as compared to a blank sample but the amount calculated is uncertain.

Table 7: **Target compounds with calculated LOD and LOQ**

Target compound	CAS #	LOD (ppm)	LOQ (ppm)
Chlorpyrifos	2921-88-2	0.231	0.7000
Dichlorvos	62-73-7	0.2110	0.6420
2,4-dichlorophenoxy acetic acid (2,4 D)	95-75-7	0.0960	0.2910
Carbendazim	10605-21-7	0.0100	0.0310
Fenvalerate	51630-58-1	0.6990	2.1200
Cypermethrin	52315-07-8	0.2100	0.7000
Deltamethrin	52918-63-5	0.1065	0.3227
Atrazine	1912-24-9	0.1640	0.4971
Cadmium	7440-43-9	0.0030	
Mercury	7439-97-6	0.0010	
Arsenic	7440-38-2	0.0050	
Lead	7439-92-1	0.0100	
Zinc	7440-66-6	0.0500	
Fluoranthene	206-44-0	0.3360	1.0190
Pyrene	129-00-0	0.2140	0.6470
N,N-diethyltoluamide	134-62-3	0.527	1.597
Caffeine	58-08-2	0.0011	0.0035
Acetaminophen	103-90-2	0.0020	0.0061
Norfloxacin	70458-96-7	0.005	0.016
Enrofloxacin	93106-60-6	0.031	0.092

Results and Discussion

Results

Figure 4 shows the map of Zone A cosisting of water from Rapti River and one of its major tributaries.

Of the total parameters analysed in this study, Zone A showed positive results for arsenic, mercury, cypermethrin, deltamethrin, pyrene, and 2,4 D only. Isolated cases of cypermethrin and deltamethrin were measured at sampling sites S2 and S3 with concentrations of 6.461 ppm and 0.9681 ppm, respectively. Similar incidents of arsenic and mercury were observed at S18 and S12 with concentrations of 6 ppb and 10 ppb, respectively. Trace amounts, all below the LOQ, of pyrene and 2,4 D, were observed in 8 and 14 different locations respectively. The data from laboratory analysis of samples from Zone A are listed in Table 8 and plotted in Figure 9a.

Figure 5 demonstrates the map with sampling sites in Zone B consisting of water from Narayani River only. The results of sample analysis from this zone are listed in Table 8 and plotted in Figure 9b. Of the total parameters analysed in this study, Zone B showed positive results for arsenic, cypermethrin, deltamethrin, pyrene, and 2,4 D only. A single isolated case of deltamethrin was observed in sampling site S23 at 17.455 ppm concentration. Arsenic has been detected in several sites – S21, S24, S28, and S29 – with concentrations varying from 5 ppb to 50 ppb. Cypermethrin was observed in two locations, S20 and S22, with concentrations of 0.569 ppm and 2.0018 ppb, respectively. Pyrene and 2,4 D were found in concentrations below quantification level in all points of Zone B except for S24, S25 and S27 respectively.

Figure 4: **Sampling sites in Zone A**

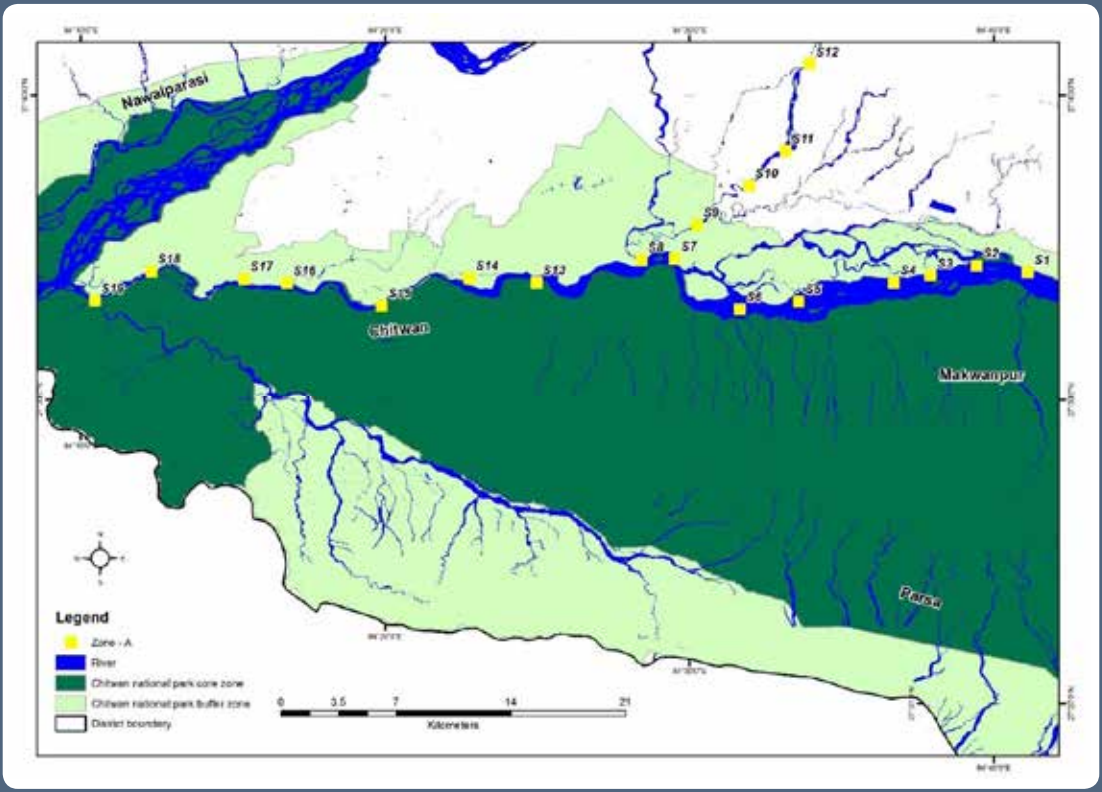
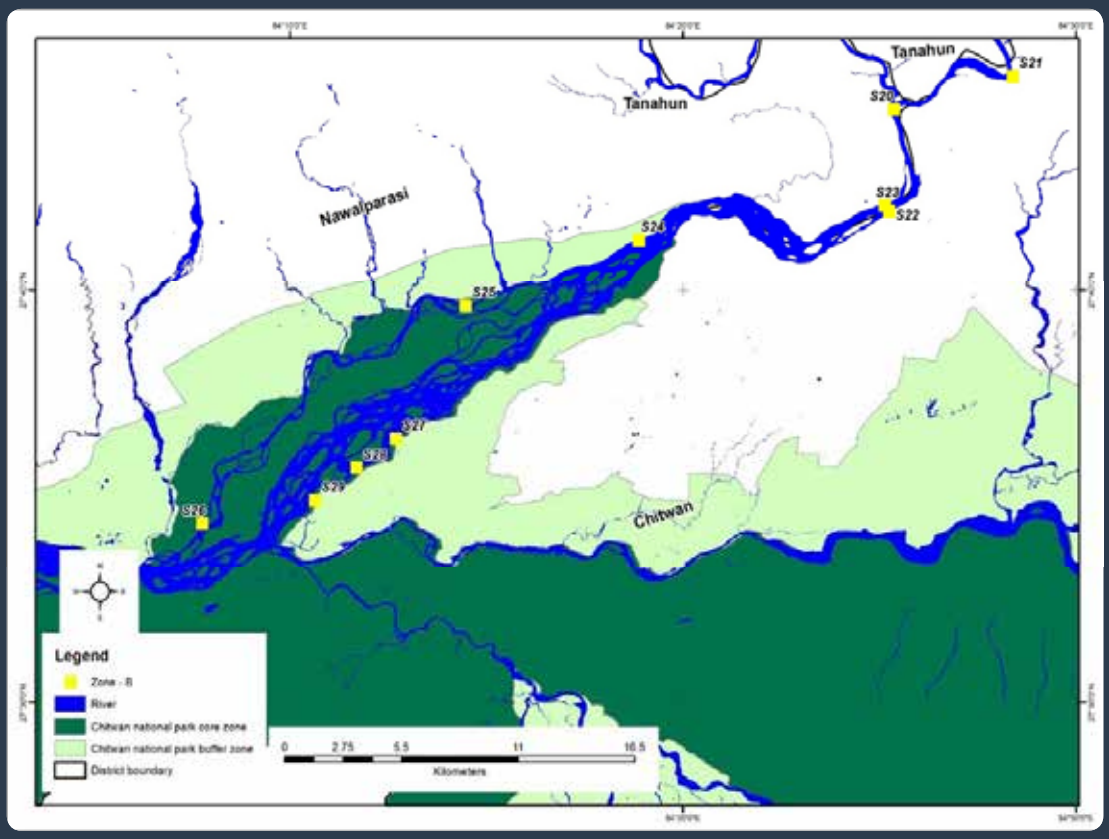


Figure 5: **Sampling sites in Zone B**



Similarly, Figure 6 shows the sampling sites in Zone C, consisting of water from Narayani River mixed with Rapti and Reu Rivers at sampling point S30. The results of the sample analysis from Zone C are listed in Table 8 and plotted in Figure 9c. Of the total parameters analysed in this study, Zone C showed positive results for zinc, caffeine, pyrene, and 2,4 D only. A single isolated case of caffeine was observed in sampling site S34 with concentrations of 35.356 ppm. Pyrene and 2,4 D have been observed in trace levels in four and three different occasions, respectively. This region has consistently seen traces of zinc ranging from 50 to 130 ppb as seen in Table 8.

Figure 7 shows the map with the sampling sites in Zone D consisting of water from Reu River only. The results of the sample analysis from Zone D are listed in Table 8 and plotted in Figure 9d. Samples S40 to S43 were not collected due to an accessibility problem and a high density of sample sites in the same area. Of the total parameters analysed in this study, Zone D showed positive results for only pyrene and 2,4 D at sites S37, S38, and S39.

Figure 8 shows the sampling sites in Zone E consisting of water from the core areas of the national park. The results of the sample analysis from Zone E are listed in Table 8 and plotted in Figure 9e. Of the total parameters analysed in this study, Zone E showed positive results for arsenic, cypermethrin, deltamethrin, pyrene, and 2,4 D. An isolated case of arsenic was observed in sampling site S49 with concentrations of 5 ppb. Cypermethrin and deltamethrin were also observed in individual spots S43 and S47, respectively. Pyrene was observed in sites S45, S46, S48, and S49 at trace levels all BQL. Similarly, 2,4 D was observed in all sites except at S43 and S46 at trace levels (BQL).

Figure 6: **Sampling sites in Zone C**

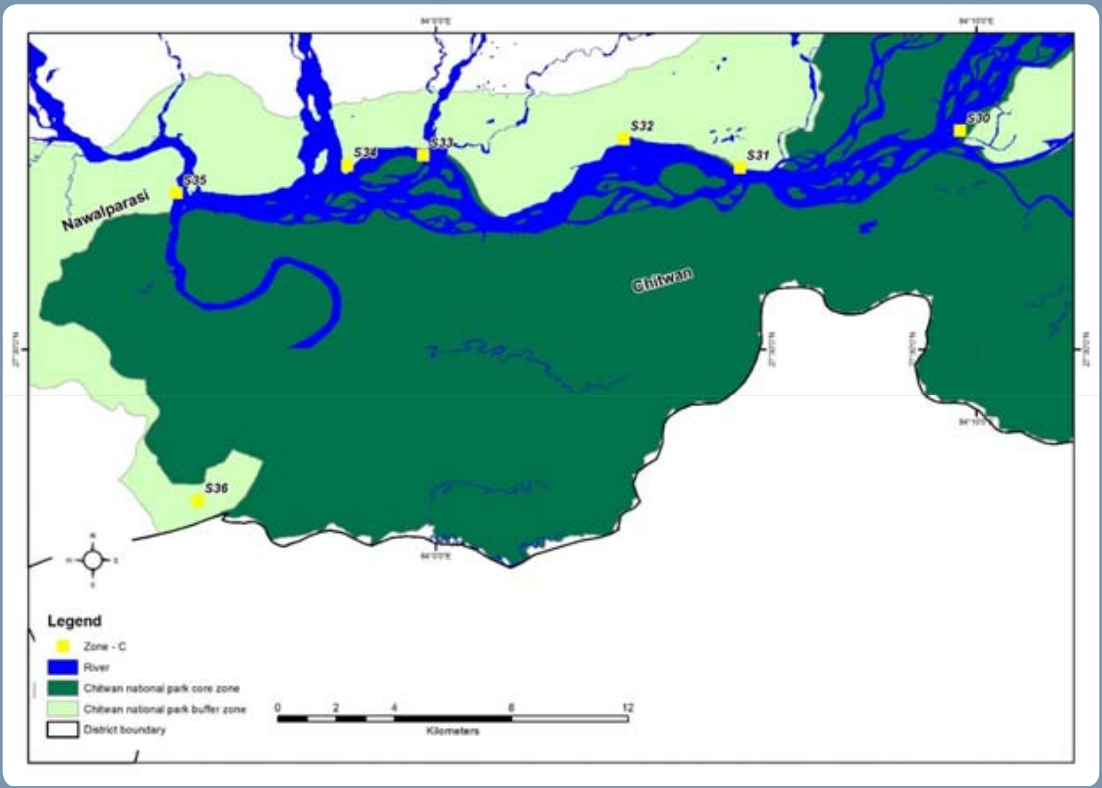


Figure 7: **Sampling sites in Zone D**

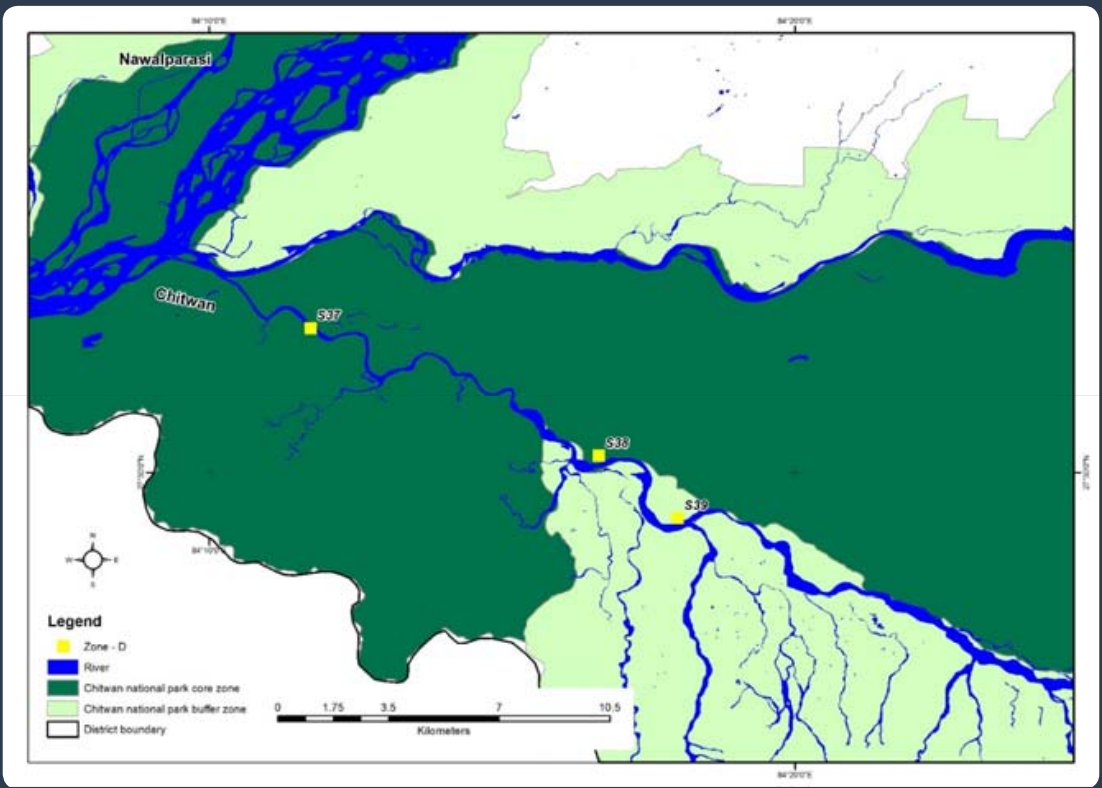


Figure 8: **Sampling sites in Zone E**

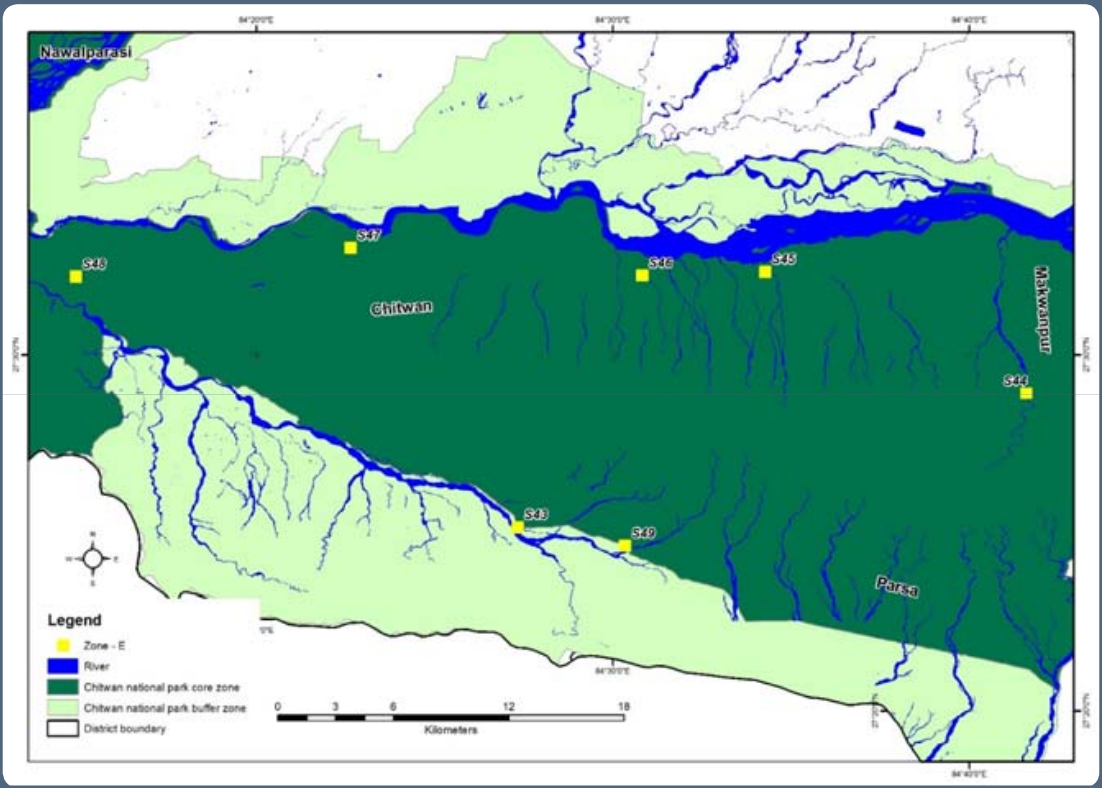
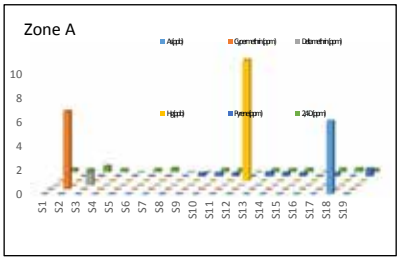
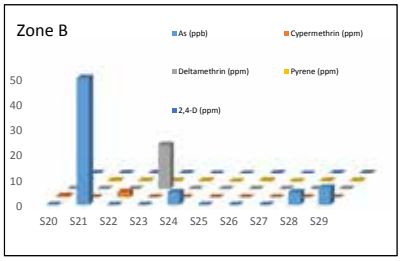


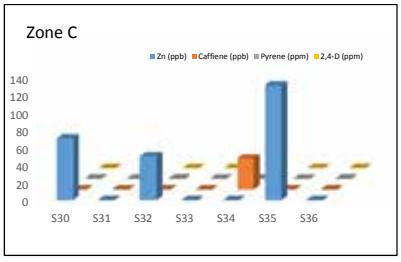
Figure 9: **Graphical representations of the results from laboratory analysis: 9a. Zone A; 9b. Zone B; 9c. Zone C; 9d. Zone D; 9e. Zone E** (x-axis represents concentration in ppm and y-axis represents sample sites)



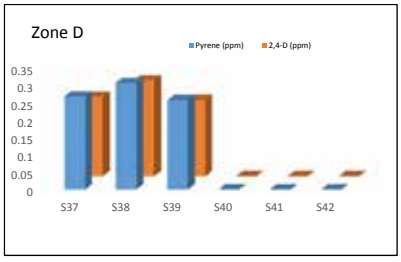
9a



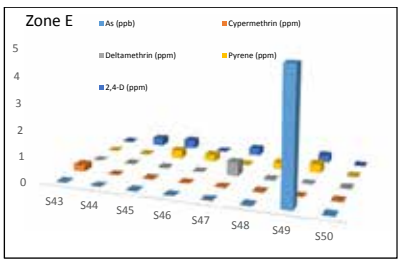
9b



9c



9d



9e

Table 8: **Results of samples combined for all zones**

Sample site	As (ppb)	Hg (ppb)	Zn (ppb)	Cypermethrin (ppm)	Deltamethrin (ppm)	Pyrene (ppm)	2,4-D (ppm)	Caffeine (ppb)
S1							0.2662	
S2				6.461			0.2159	
S3					0.9681		0.4853	
S4							0.2301	
S5								
S6							0.2337	
S7							0.3097	
S8								
S9						0.2677		
S10						0.2885	0.2553	
S11						0.2668	0.2503	
S12		10						
S13						0.3497	0.2355	
S14						0.3229	0.2315	
S15						0.2602	0.2494	
S16								
S17						0.2674	0.2686	
S18	6						0.2764	
S19						0.6345	0.2279	
S20				0.569		0.6345	0.2279	
S21	50					0.3923	0.2339	
S22				2.0018		0.2752	0.2524	
S23					17.455	0.2628	0.2282	
S24	5					0.2721		
S25								
S26						0.3534	0.2192	
S27								
S28	5					0.2987	0.268	
S29	7					0.2755	0.2745	
S30			70			0.2375		
S31							0.2523	
S32			50			0.2291		
S33								
S34						0.2346	0.2444	35.356
S35			130					
S36						0.2425	0.2283	
S37						0.2681	0.2288	
S38						0.3069	0.2762	
S39						0.2578	0.2197	
S43				0.24				
S44							0.2772	
S45						0.2962	0.3235	
S46						0.257		
S47					0.5424		0.2728	
S48						0.231	0.2278	
S49	5					0.3295	0.2745	

Discussion

Zone A consisted mainly of agricultural fields and grasslands towards the eastern half, up to site S5. Site S3 had spots where the river water was being diverted for irrigation purposes along with ongoing construction of other smaller canals. Nepal's East-West Highway is also located parallel to the river and closes in with the river at sites S1 to S3. Consistent siting of 2,4 D can be caused by the mixing of agricultural field surface runoffs or through irrigation channels. Similarly prevalent practices of burning agricultural fields, falsely perceived to improve soil quality, can contribute to the presence of pyrene in the water (Edwards 1983). Consequently, isolated incidents of finding common household pesticides and heavy metals in the water samples need to be investigated for reoccurrence in the future.

Higher levels of arsenic in Zone B could be explained by the natural abundance of the metal in the area as mentioned earlier. Narayani is likely to be contaminated by arsenic from natural erosion processes. Contamination from large-scale poultry farms could also be a potential source of arsenic in sampling site S21 which shows the highest concentration of arsenic measured. Organic arsenicals present in poultry feeds are excreted as inorganic arsenicals and if not properly disposed of can lead to contamination of waterways (Price et al. 2005). Such contamination is a matter of concern for both residents of the cities and the animal population of CNP.

The observation of pyrene in this region is difficult to explain since the area is surrounded mostly by residential settlements and has no direct source of contaminants from large field combustions. S24, the location of Gorkha Brewery, showed presence of traces of arsenic and pyrene, but neither in alarming levels. Consistent traces of organochloride 2,4 D have been recorded and can be attributed to contaminated agricultural runoffs from locations surrounding the river. The very high concentration of Deltamethrin recorded at S23 raises a flag, since this compound is a potent EDC and calls for further investigation in both S23 and S22 since they are located under the Narayani bridge connecting Gaidakot Municipality to western Nepal.

Similarly in Zone C no large metal shops were recorded in the region, so other sources of zinc contamination must also be considered. Zinc is commonly found in poultry waste and the region is also a popular site for elephant feeding and bathing, which might introduce faeces into the river water (Edwards 1983). Other sources could be illicit dumping of chemical wastes like batteries in open fields that can cause leeching of such contaminants into the river. Caffeine concentrations found in S34 is treated as an isolated incident and must be investigated further. Identification of pyrene and 2,4 D in the samples is consistent with Zones A and B. More detailed studies on the frequency of organochloride pesticide use and burning of fossil fuels, forests, and fields in the area must be established.

Zone D had sparse settlements and agriculture-based land use. The presence of pesticide and polyaromatic hydrocarbon at trace levels can be related to surface runoffs from this region. An exception is S37 where there were no settlements but it is a common site for driving safari vehicles which could be a source of the PAHs.

Zone E lies inside the core region of the national park itself, so ideally the samples from this zone should have been clean of all parameters studied. Siting of arsenic could be attributed to natural sources of the metal, but we cannot be certain and must push for further investigation. Presence of pesticides in the jungle ecosystem excluding any contamination from anthropogenic activities could be explained from biodegradation of plants that naturally produce pyrethroid and organochlorine compounds known as biopesticides (Cantrell et al. 2012). However, the exact chemicals observed as contaminants in the samples have not been reported as naturally produced by common biopesticide plants (Cantrell et al. 2012).

The presence of pyrene in several samples can be explained by considering forest fires. Forest fires, both natural and human induced, are a common phenomenon in the buffer regions of the park and could be a contributing factor for the presence of PAHs in the water inside the park.

This study has demonstrated that the level of EDC contamination in the waterways surrounding CNP is not alarmingly high. However, it is also not completely negligible. Besides, this study is limited to the temporal characterization of the chemical studied. One sample from a point location could be an outlier and not a normal

condition. At the same time, the same could be true for parameters which were below the detection limit during the course of the field study. Several factors, including the season (for EDC sources, the time of fertilizer usage, agricultural burning, etc.) and the time of sampling or the relevance of parameters chosen to be studied in each area and the large scale of sampling site, could contribute to understanding of the impacts of EDCs in and around Chitwan National Park. Similar studies conducted in South Korea and Spain showed much higher levels of EDC concentration in surface waters (Yoon et al. 2010; Esteban et al. 2014). However, these studies were conducted on wastewater treatment plant effluents and streams from urban areas, Seoul and Madrid, which is quite different from our sampling site and cannot be truly compared (Yoon et al. 2010; Esteban et al. 2014).

Due to limited or no public access, the core areas of PAs in Nepal are still clean and unpolluted. Water pollution can be seen in those areas where there are settlements or in and around the buffer zone of National Parks. So, it is worthwhile to concentrate water sample collection and analysis in and around buffer zones or near settlement areas of PAs.

Conclusion

This study has successfully pioneered a scientific baseline concentration analysis of 20 internationally recognized endocrine-disrupting organic and inorganic compounds. It thus fulfils the objective of archiving present concentration levels to assist any future comparative studies.

This study, a first of its kind in Nepal, does leave room for improvement by future researchers to conduct similar research but also refine it by limiting their area of study and by analysing specific compounds and their effects on species with known sensitivity. This research can be reproduced in lake ecosystems as well and can be further validated by combining biological analysis of species to isolate specific characteristic modifications that are generally caused by EDC contamination. In addition, this study has also shown that skilled human resources and highly sensitive instrumentation and technology exist within the country to carry out studies of this nature and scale.

It is recommended that samples should be collected from different water bodies across different seasons to arrive at more accurate estamitaitons of pollution levels in the waters of CNP.

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