



# Analytical protocols and methods for analysis of TrOCs and heavy metals

## Deliverable D5.2

First version (public)

WP5 On-site piloting and performance evaluation

Task 5.2 Identification of the boundary conditions for technology development under Indian conditions

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Deliverable type		
R	Document, report	
DEM	Demonstrator, pilot, prototype	<b>X</b>
DEC	Websites, patent fillings, videos, etc.	
OTHER	Software, technical diagram, etc.	
ETHICS	Ethics	

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**IIT Delhi**  
Indian Institute of Technology Delhi



**IIT Kanpur**



## SUMMARY

This document “**Deliverable D5.2: Analytical protocols and methods for analysis of trace organic compounds and heavy metals**” is the second deliverable of WP5 “On-site piloting and performance evaluation”. As laid down in the Description of Action (Annex 1 – Part A) Deliverable 5.2 describes the analytical protocols and methods for analysis of trace organic compounds (TrOCs) and heavy metals (HMs).

The methods for the analysis of TrOCs and HMs are used for the lab studies (WP3), water quality monitoring (WP4) and pilot studies (WP5).

It is the particular **objective to disseminate the methods** established at the labs of the Indian partners, i.e. IIT Delhi and IIT Kanpur, through this public deliverable.

- The micropollutant analysis using LC-MS is conducted at the IITD lab (in cooperation with the bilateral Dutch Indian LOTUS HR project) while the sample preparation by solid phase extraction is established at both IITs.
- The HM analysis can be conducted with a range of methods in both IITs. Besides AAS, ICP-MS and ICP-OES as well as MP-AES are available and used.



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## CHAPTER 1 DESCRIPTION AND GOAL

This document “**Deliverable D5.2: Analytical protocols and methods for analysis of trace organic compounds and heavy metals**” is the second deliverable of WP5 “On-site piloting and performance evaluation”. As laid down in the Description of Action (Annex 1 – Part A) Deliverable 5.2 describes the analytical protocols and methods for analysis of trace organic compounds (TrOCs) and heavy metals (HMs).

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- The HM analysis can be conducted with a range of methods in both IITs. Besides AAS, ICP-MS and ICP-OES as well as MP-AES are available and used.

**The present first version summarizes the actual state of the work since the LC-MS method is not yet fully implemented due to the late start of the Indian DBT funding and the impact from COVID-19 having led to a complete shutdown of the IIT D lab. It will be updated as soon as the methods are operational.**



## CHAPTER 2 WATER QUALITY PARAMETERS

India's water resources are under severe stress resulting from overexploitation, climate change and pollution. Besides the analysis of well-established standard parameters such as COD, BOD, TDS and nutrients, Pavitra Ganga investigates also contaminants less frequently studied in India, i.e. organic micropollutants and heavy metals.

### 2.1. HEAVY METALS

Heavy metals such as arsenic (As), cadmium (Cd), chromium (Cr), silver (Ag), copper (Cu), iron (Fe), nickel (Ni), mercury (Hg), lead (Pb) and zinc (Zn) are metals with high atomic mass and high density of at least  $5 \text{ g cm}^{-3}$  (Saleh 2018). Many of them may induce toxicity at low levels, causing cellular disruptions. Some heavy metals like Cr (VI) even show toxicity at  $\text{mg kg}^{-1}$  body weight (Fishbein 2018). Hexavalent chromium can lead to necrosis or nephritis by just  $10 \text{ mg kg}^{-1}$  body weight. Other HMs like Pb can cause DNA damage, lead to hypertension and brain damage, or metabolic disorders, while for example Cd can lead to damage in kidneys and the skeleton (Gautam et al. 2014). In table 1, the most prevalent toxic effects of selected heavy metals are summarized, and suggested drinking water standards are displayed.

HM are mostly charged positive, e.g. 2+ or 3+, and have a high adsorption ability (Erdem et al. 2004), which facilitates their sequestration in soils, river sediments and wastewater sludge. Further, they show high bioavailability and biomagnification (Fishbein 2018).

Due to the naturally occurring leaching of heavy metals from soil parent materials into freshwater, e.g. by geogenic washout from the rocks, a natural occurrence is given (Morais et al. 2012). Traces of some heavy metals such as Fe, Se Zn, Cr, and Mg are even essential for human metabolism if they are not exceeding homeostatic levels (Saleh 2018). Due to an increase of anthropogenic emissions from industry, transport vehicles as well as from mining activities (Ahuja 2019), natural systems are disturbed by excess amounts of some HM's. After various research related to their toxicity and harmful effects on living organisms, including human life, the WHO has recommended threshold values for drinking water quality (table 1).

Especially the non-degradability and the sequestering properties of HM in the soil lead to continuous distribution into the environment and make it challenging to remove them once they have entered the atmosphere. Therefore, measures shall be taken to avoid HM spreading into the environment, replacing them by suitable substitutes or install procedures sufficient to prevent their leakage from point sources into the water bodies. Current treatment technologies to extract or remove heavy metals from wastewater flows are mainly based on precipitation and flocculation, through particle size separation, ion exchange, and adsorption (Lenntech 2020). Depending on contaminant concentrations and aimed discharge criteria, applicable processes are applied. Adsorbents and





especially bio adsorbents, for example, have the benefit of good availability, low costs, regeneration possibility, technically feasible utilization and the affinity for heavy metals to be removed efficiently (Renu et al. 2017).

*Table 1: Effects of heavy metals and their concentration guidelines (Gautam et al. 2014)*

Metal	Effects	Drinking water standards
<b>Lead</b>	• Toxic to humans, aquatic fauna and livestock	• By the US Environmental Protection Agency maximum concentration: 0.1 mg/l
	• High doses cause metabolic poison	• By the European Commission: 0.5 mg/l
	• Tiredness, irritability anemia and behavioral changes of children	• by the Bureau of Indian standards: 0.1 mg/l
	• Hypertension and brain damage	
<b>Nickel</b>	• Phytotoxic	
	• High conc. can cause DNA damage	• By the US Environmental Protection Agency maximum concentration: 0.1 mg/l
	• Eczema of hands	• By European Commission: 0.1 mg/l
	• High phytotoxicity	• by the Bureau of Indian standards: 0.1 mg/l
<b>Chromium</b>	• Damaging fauna	
	• Necrosis nephritis and death in man (10 mg/kg of body weight as hexavalent chromium)	• By the US Environmental Protection Agency maximum concentration: (hexavalent and trivalent) total 0.1 mg/l
	• Irritation of gastrointestinal mucosa	• By European Commission: 0.5 mg/l
<b>Copper</b>	• Causes damage in a variety of aquatic fauna	• by the Bureau of Indian standards: 0.1 mg/l
	• Phytotoxic	• By the US Environmental Protection Agency maximum concentration: 1.0 mg/l
	• Mucosal irritation and corrosion	• By European Commission: 3 mg/l
	• Central nervous system irritation followed by depression	• by the Bureau of Indian standards: 0.01 mg L <sup>-1</sup>
<b>Zinc</b>	• Phytotoxic	
	• Anemia	• By the US Environmental Protection Agency maximum concentration: 5 mg/l
	• Lack of muscular coordination	• By European Commission: 5 mg/l
<b>Cadmium</b>	• Abdominal pain etc.	• by the Bureau of Indian standards: 0.1 mg/l
	• Cause serious damage to kidneys and bones in humans	• By the US Environmental Protection Agency maximum concentration: 0.005 mg/l
	• bronchitis, emphysema, anemia	• By European Commission: 0.2 mg/l
<b>Mercury</b>	• Acute effects in children	• by the Bureau of Indian standards: 0.001 mg/l
	• Poisonous	• By the Environmental Protection Agency maximum concentration: 0.002 mg/l
	• Causes mutagenic effects	• By European Commission: 0.001 mg/l
<b>Arsenic</b>	• Disturbs the cholesterol	• by the Bureau of Indian standards: 0.004 mg/l
	• Causes toxicological and carcinogenic effects	• World Health Organization guideline of 10 µg/l
	• Causes melanosis, keratosis and hyperpigmentation in humans	• By European Commission: 0.01 mg/l
	• Genotoxicity through generation of reactive oxygen species and lipid peroxidation	• by the Bureau of Indian standards: 0.05 mg/l
	• Immunotoxic	
	• Modulation of co-receptor expression	



In order to be sure that treated wastewater is at an acceptable quality level for reuse, i.e. not to degrade soil quality nor to lead to harmful effects towards organisms and human health, international water reuse guidelines had been introduced in this century. Table 2 shows international guideline values for heavy metals in irrigation water. In India, the first irrigation guideline had been set in order in 1986; updated regulations for maximum permissible concentrations have been set in charge in 2019.

*Table 2: Guideline values of heavy metals for irrigations  
(WHO 2006, NRMCC& AHMC 2006, US EPA 2012)*

Metal concentration	WHO (2006)	NRMCC& AHMC (2006)		US EPA (2012)	MoEFCC (2019)	
	root crops	Landscape irrigation	Commercial food crops	Food crops	Inland surface water	On land irrigation
Aluminium (mg/L)	5.00	5.00	20.00	5.00		
Arsenic (mg/L)	0.10	0.10	2.00	0.10	0.2	
Beryllium (mg/L)	0.10	0.10	0.50	0.10		
Boron (mg/L)		0.50	0.5-15	0.75		
Cadmium (mg/L)	0.01	0.01	0.05	0.01	0.05	
Cobalt (mg/L)	0.05	0.05	0.10			
Chromium (VI) (mg/L)		0.10	1.00			
Chromium (mg/L)	0.10			0.10	2	2
Copper (mg/L)	0.20	0.20	5.00	0.20	3	3
Fluoride (mg/L)	1.00	1.00	2.00	1.00		
Iron (mg/L)	5.00	0.20	10.00	5.00	3	3
Lithium (mg/L)	2.50	2.50	2.50	2.50		
Manganese (mg/L)	0.20	0.20	10.00	0.20	2	
Mercury (inorganic) (mg/L)		0.00	0.00		0.01	
Molybdenum (mg/L)	0.01	0.01	0.05	0.01		
Nickel (mg/L)	0.20	0.20	2.00	0.20	3	
Lead (mg/L)	5.00	2.00	5.00	5.00	0.1	
Selenium (mg/L)	0.02	0.02	0.05	0.02	0.05	
Uranium (mg/L)		0.01	0.10			
Vanadium (mg/L)	0.10	0.10	0.50	0.10	0.2	
Zinc (mg/L)	2.00	2.00	5.00	2.00	5	15



## 2.2. MICROCONTAMINANTS

Organic microcontaminants, or also called organic micropollutants (OMPs) or trace organic compounds (TrOCs), are those substances, which occur at low nano- to microgram level per litre in natural water bodies and other points along the water cycle. Micropollutants are mostly from organic origin, but also inorganic compounds are accounted for within this group (Umweltbundesamt 2018).

### 2.2.1. PHARMACEUTICALS AND PERSONAL CARE PRODUCTS

Most of the compounds are from anthropogenic sources, while just a few of them are occurring naturally at small amounts. Among MPs are pharmaceuticals and personal care products (PPCPs), which include a wide range from antibiotics over antipyretic to active hormone substances. Many of those substances do not totally metabolize during medical treatment and are therefore released into the environment through different pathways (Ternes and Joss 2006, Figure 1). The most prevalent entry path is through the sewer system/sewage treatment plant into natural water bodies, as only a small percentage of these MPs are removed effectively during wastewater treatment (Umweltbundesamt 2018).

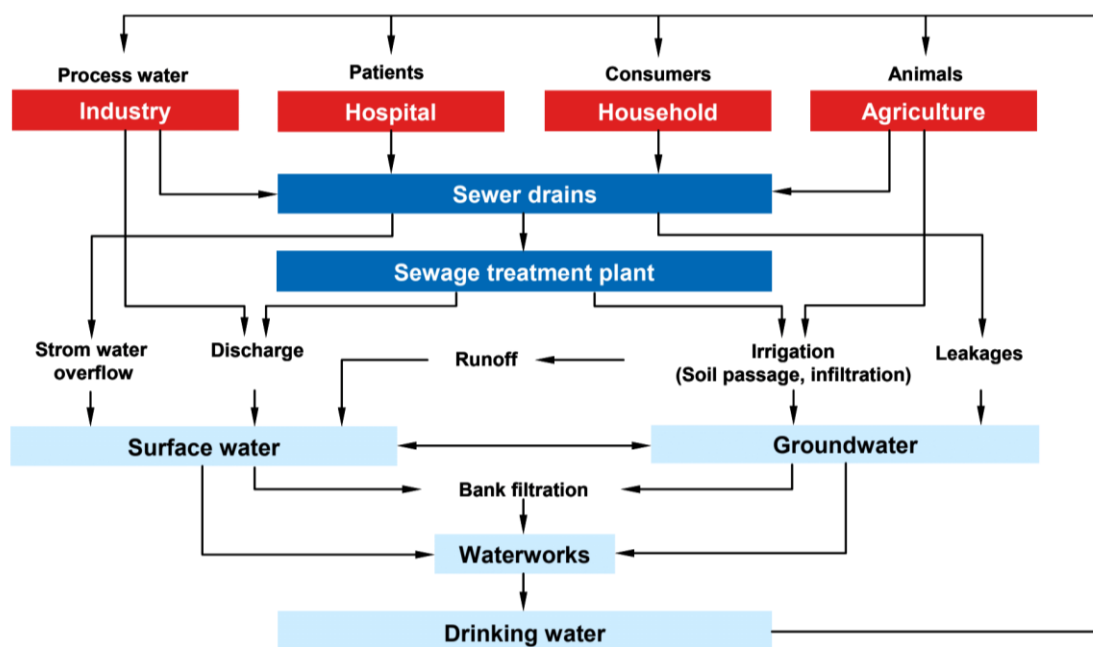


Figure 1: Microcontaminants - pathways into the water cycle (Ternes and Joss 2006)

Through leakages in pipes, overflow, or untreated discharge, which unfavourably is the case in many developing countries, MP's further enter into the environment and lead harmful impact to aquatic ecosystems. Microorganism, macrozoobenthos and fish, and humans have shown significant changes in their metabolism after exposure to certain substances (Fent 2013). Some frequent disorders can be observed in fish through endocrine-disrupting chemicals, changing the hormone

system and causing infertility of individuals or a drift in sex of populations (Fent 2013). Other MPs induce specific diseases or show an increase of different enzymes in the organisms, indicating a stress factor. The occurrence of chronic diseases from most MP's is not yet verified for human beings, as research of the mixture is very demanding (Fent 2013). Another problem is a rising resistance against antibiotics, which is caused through a steady abundance of antibiotics in the environment that leads target organisms to adapt towards these drugs and lead to their immunity (Gandra et al. 2016).

Nowadays in most parts of the world, a tertiary and quaternary treatment step of the effluent following a two-stage water treatment plant is not obligatory. Lately, the European Union is debating about new standards and Switzerland introduced regulations to remove up to 80 % of the MP's until 2030 (Swiss Confederation 2020). So far, there are only a few WWTP's with a quaternary treatment step, as additional protection for sensitive areas, like endangered wildlife in rivers and lakes. Or when wastewater has to be fit for irrigation and recreational purposes (Gerba & Pepper 2019).

On the other side, most of the drinking water treatment plants are equipped with sufficient measures to remove MPs making use of soil passage, chemical oxidation, or adsorption (Bixio et al. 2006).

### 2.2.2. PESTICIDES

The intensive use of pesticides in India has led to widespread contamination of the biotic as well as the abiotic environment in India (Yadav *et al.* 2015). Pesticides were found in surface water and groundwater (Lari *et al.* 2014; Mutiyar, Mittal & Pekdeger 2011; Sankararamakrishnan, Sharam & Sanghi 2005; Skiwar *et al.* 2014). A multitude of pesticides have been identified as endocrine disrupting chemicals (EDC). Mnif *et al.* (2011) has compiled an overview of 105 substances and their effect on the hormone system of humans. The combined effects of pesticides are a major health risk to humans. In addition, wildlife is particularly vulnerable to the toxic and endocrine effects of pesticides.

In India, 76% of the used pesticides are accounted for as insecticides, 13% as fungicides and 10% as herbicides. In cotton and paddy cultivation, more than 50% of the pesticides are used. Depending on the climatic conditions and the agriculture in different states in India, the pesticide consumption can vary strongly from one state to another. Uttar Pradesh, Maharashtra and Andhra Pradesh are the states with the highest pesticide consumption (Yadav *et al.* 2015). Based on the precautionary principle, the European Drinking Water Directive (98/83/EC) has set a drinking water limit of 0.1 µg/L for single pesticides (with exception of aldrin, dieldrin, heptachlor and heptachlor epoxide where the limit for the single compound is 0.03 µg/L) and 0.5 µg/L for the sum of all active pesticides detected. India has set individual values for 18 substances in the Indian Standard Drinking Water Specification as shown in the Table below (Table 3). It can be seen that the European standards are much stricter and less compound specific than the Indian threshold values.

In general, the mobility of pesticides and thus their risk of leachability into the groundwater have been correlated with a weak adsorption of the soil matrix quantified in terms of a small soil organic carbon-water partitioning coefficient ( $K_{oc}$ ) (Arias-Estévez *et al.* 2008). Generally, pesticides with  $K_{oc} \leq 1,000$  are potentially leaching compounds whereas pesticides with  $K_{oc} \geq 1,000$  have also been found in the groundwater.



*Table 3: Pesticide residue limits according to the Indian drinking water guidelines (Indian Standard Drinking Water - Specification (second revision) 2012)*

Pesticide	Limit [ $\mu\text{g/L}$ ]
Alachlor	20
Atrazine	2
Aldrin/Dieldrin	0.03
Alpha HCH	0.01
Beta HCH	0.04
Butachlor	125
Chlorpyrifos	30
Delta HCH	0.04
2,4- Dichlorophenoxyacetic acid	30
DDT ( <i>o, p</i> and <i>p, p</i> – Isomers of DDT, DDE and DDD)	1
Endosulfan (alpha, beta, and sulphate)	0.4
Ethion	3
Gamma - HCH (Lindane)	2
Isoproturon	9
Malathion	190
Methyl parathion	0.3
Monocrotophos	1
Phorate	2



## CHAPTER 3 ANALYSIS OF TRACE ORGANIC CONTAMINANTS

### 3.1. SELECTED COMPOUNDS

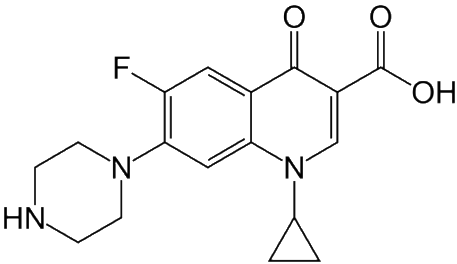
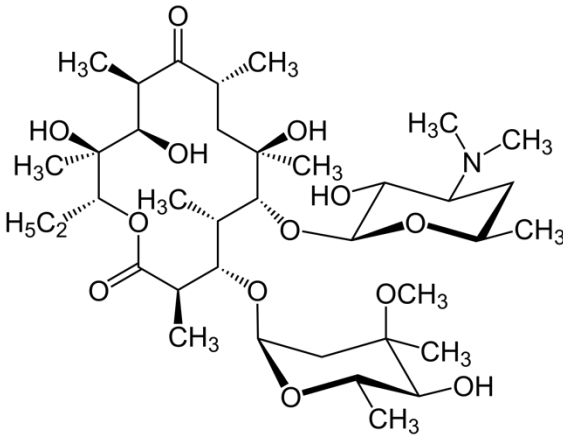
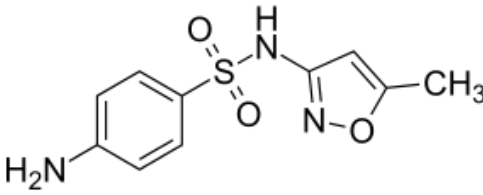
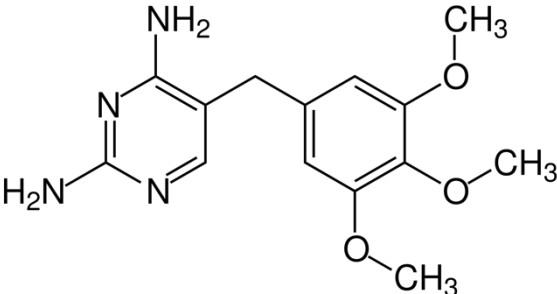
To assess the adsorption capacity of applicable adsorbents, a set of indicator substances was selected (Table 4). MP's detected in Indian water bodies were evaluated and substances with elevated concentration, distinct polarities and dissociation constants were chosen to be measured with the LC-MS for further adsorbents evaluation. For the ease of the measurement, the used chemicals show distinct dissociation constants, which indicates different retention times on the column and therefore offer proper separation within the chromatogram (Bade et al. 2015). The set of chemicals also links to the LOTUS<sup>HR</sup> subproject 1b, led by Prof. G. Medema (TU Delft) and Dr. Nagarnaik (NEERI, Nagpur). It is further planned to synchronize the methods used in "Pavitra Ganga" with the parallel EU-India H2020 Project "PANI WATER" where the micropollutant measurement is supervised by Prof. Despo Fatta-Kassinou from the University of Cyprus and links further to the EU NORMAN network.

Table 4: Physicochemical properties of the selected compounds

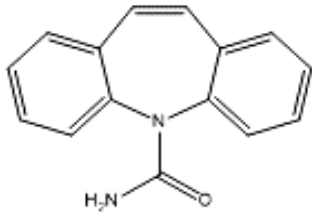
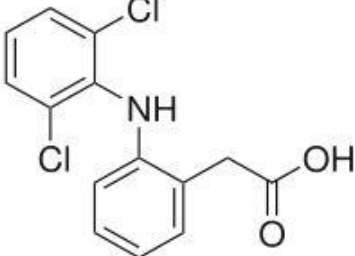
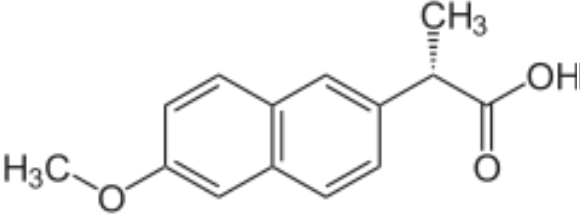
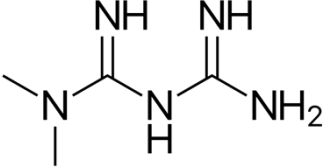
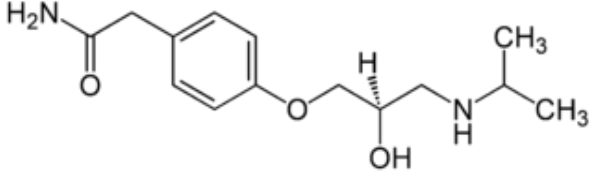
Category	Core parameter
Antibiotics	<ul style="list-style-type: none"> <li>• Ciprofloxacin</li> <li>• Erythromycin</li> <li>• Sulfamethoxazole</li> <li>• Trimethoprim</li> </ul>
Antiepileptic	<ul style="list-style-type: none"> <li>• Carbamazepine</li> </ul>
Analgesics	<ul style="list-style-type: none"> <li>• Diclofenac</li> <li>• Naproxen</li> </ul>
Antidiabetics	<ul style="list-style-type: none"> <li>• Metformin (with internal standard)</li> </ul>
Betabockers	<ul style="list-style-type: none"> <li>• Atenolol</li> </ul>
Personal care products	<ul style="list-style-type: none"> <li>• Methylparaben</li> <li>• Triclosan</li> </ul>
Pesticides (preliminary selection)	<ul style="list-style-type: none"> <li>• Atrazine</li> <li>• Butachlor</li> <li>• Dichlorvos</li> <li>• Phorate</li> </ul>



Table 5: Chemical properties of investigated Substances

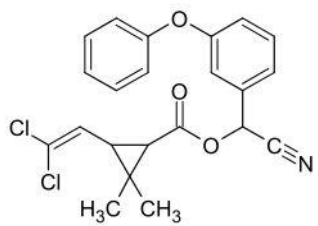
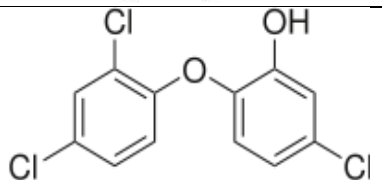
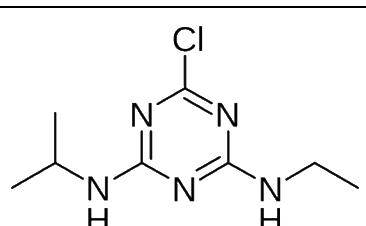
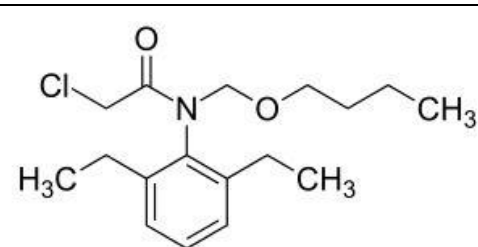
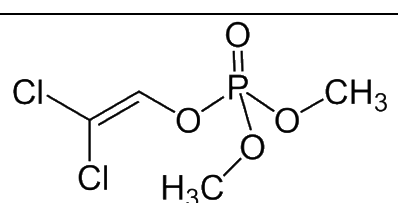
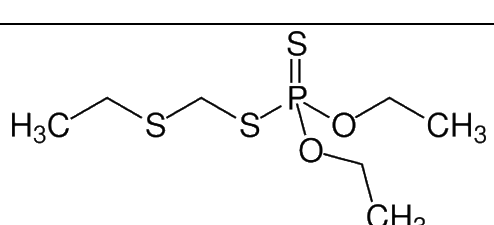
Substances Formula Application Distribution coefficient	Typ. Conc in Indian wastewater effluent [ng/l]	Mass [g/mol]	Chemical Structure
Ciprofloxacin $C_{10}H_{11}N_3O_3S$  Antibiotic $\log K_{ow} = 0.28$	N/A	331.34	
Erythromycin $C_{10}H_{11}N_3O_3S$  Antibiotic $\log K_{ow} = 3.06$	N/A	733.93	
Sulfamethoxazole $C_{10}H_{11}N_3O_3S$  Antibiotic $\log K_{ow} = 0.89$	400	253.8	
Trimethoprim $C_{10}H_{11}N_3O_3S$  Antibiotic $\log K_{ow} = 0.91$	N/A	290.32	



Substances Formula Application Distribution coefficient	Typ. Conc in Indian wastewater effluent [ng/l]	Mass [g/mol]	Chemical Structure
Carbamazepine $C_{15}H_{12}N_2O$ Anticonvulsant $\log K_{ow} = 1.51$	500	236.27	
Diclofenac $C_{14}H_{11}Cl_2NO_2$ anti-inflammatory drug $\log K_{ow} = 4.51$	500	296.15	
Naproxen $C_{14}H_{14}NaO_3$ nonsteroidal anti-inflammatory drug $\log K_{ow} = 3.18$	400	252.25	
Metformin $C_4H_{11}N_5$ antidiabetic $\log K_{ow} = -2.645$	N/A	129.167	
Atenolol $C_{14}H_{22}N_2O_3$ betablocker $\log K_{ow} = 0.16$	1400-2900	266.34	





<p>Cypermethrin C<sub>22</sub>H<sub>19</sub>Cl<sub>2</sub>NO<sub>3</sub></p> <p>insecticide log K<sub>ow</sub> = 6.6</p>	N/A	416.3	
<p>Triclosan C<sub>12</sub>H<sub>7</sub>Cl<sub>3</sub>O<sub>2</sub></p> <p>Antibacterial agent log K<sub>ow</sub> = 4.76</p>	500	289.53	
<p>Atrazine C<sub>8</sub>H<sub>14</sub>ClN<sub>5</sub></p> <p>herbicide log K<sub>ow</sub> = 2.61</p>	N/A	215.68	
<p>Butachlor C<sub>17</sub>H<sub>26</sub>ClNO<sub>2</sub></p> <p>herbicide log K<sub>ow</sub> = 4.5</p>	N/A	311.85	
<p>Dichlorvos C<sub>4</sub>H<sub>7</sub>Cl<sub>2</sub>O<sub>4</sub>P</p> <p>Insecticide log K<sub>ow</sub> = 1.43</p>	N/A	220.97	
<p>Phorate C<sub>7</sub>H<sub>17</sub>O<sub>2</sub>PS<sub>3</sub></p> <p>Insecticide log K<sub>ow</sub> = 3.56</p>	N/A	260.38	

### 3.2. SOLID PHASE EXTRACTION - SPE

To remove unwanted compounds and minimize their effects, the samples undergo an appropriate **solid-phase extraction** (SPE) with, reducing the background matrix interfering with the measurement of the trace organic compounds. Therefore, the stored samples are prefiltered at room temperature (100  $\mu\text{m}$  glass filter, MN GF-5), pH adjusted with either  $\text{H}_2\text{SO}_4$  or  $\text{NaOH}$  ( $\text{pH} = 7,5 \pm 0.05$ ) and 10  $\mu\text{l}$  of an **internal standard** is added.

The internal standard (IS) is composed of three marked deuterated references that represent the chosen OMPs over the whole range of retention times. These IS were added at a known concentration of 0.1  $\mu\text{g/L}$  and accordingly, OMPs' loss from samples through the SPE can be calculated. The following ISs are selected:

- **(S)-Naproxen-d3**
  - **MW:** 233.28 g/mol
  - $\text{C}_{14}\text{H}_{11}\text{D}_3\text{O}_3$
  - 1.0 mg in 1 ml methanol solution
- **Atenolol-d7**
  - **MW:** 273.38 g/mol
  - $\text{C}_{14}\text{H}_{15}\text{D}_7\text{N}_2\text{O}_3$
  - 1.0 mg in 1 ml methanol solution
- **Carbamazepine-d8 (Major)**
  - **MW:** 244.32 g/mol
  - $\text{C}_{15}\text{H}_4\text{D}_8\text{N}_2\text{O}$
  - 1.0 mg in 1 ml methanol solution

After adding the standard, the samples are well mixed prior connection to the SPE-System, seen in Figure 2. Before connecting the samples to the system, the cartridges for extraction require conditioning:

The cartridges (Oasis HLB 500 mg/6 mL) are preconditioned with 6 mL acetonitrile followed by 6 mL nanopore water. All the solvents are HPLC-grade. The cartridge conditioning is conducted without any pressure set up.





*Figure 2: Solid-phase extraction set up with attached cartridges during sample*

The samples are extracted by a suction pipe onto the cartridges installed on the SPE-manifold. The attached vacuum pump is set to reach a flow of about of 3–5 mL/min, which relates to about 1-2 drops per second. After complete extraction the sample bottles are rinsed with 20ml Nanopure and the cartridges are gently dried with nitrogen for 30 min at around 1.5 bar. The samples were then eluted with 8ml of LCMS grade acetonitrile followed by drying under nitrogen gas at 35 °C. Dried and eluted samples were reconstituted with corresponding mobile phase for spiked and non-spiked samples separately. Then samples were then stored in amber coloured 2ml analytical glass vials for LCMS analysis.



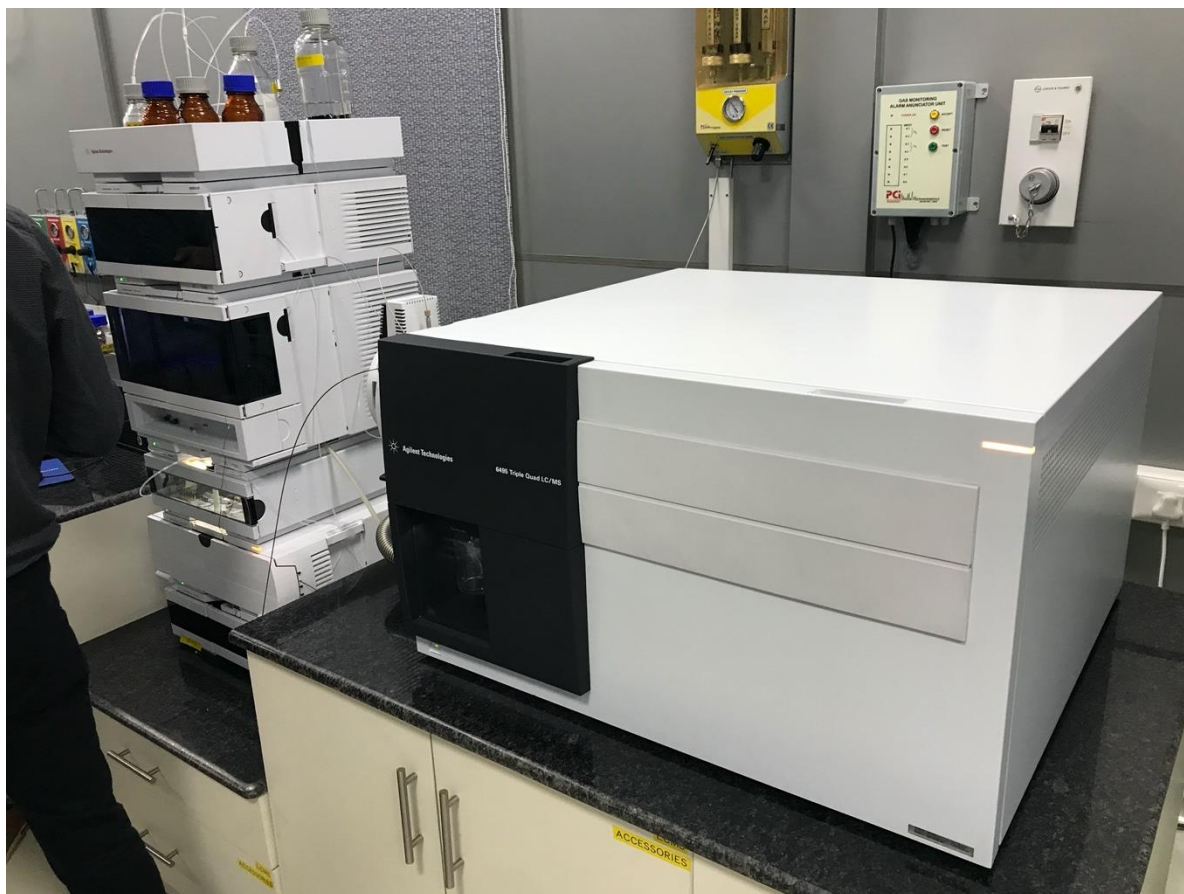
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### 3.3. HPLC WITH MASS SPECTROMETRY (LC-MS)

Based on a literature review of methods used for MP detection in wastewater, a new method following the proceedings of an existing procedure from literature was developed (Vanderford and Snyder 2006; Li et al. 2019). For the measurements of the samples, a liquid chromatography (LC) (Agilent HPLC with a 6495 Triple Quad LCMS) is used (Figure 3).



*Figure 3: Liquid chromatography-mass spectrometry unit (Agilent HPLC with a 6495 Triple Quad LCMS) with Eluates at the IIT Delhi LOTUS HR laboratory.*

The LC is responsible for the separation of the different substances within the samples, while the MS offers identification and quantification of the occurring substances. These instruments are very sensitive; a pre-treatment of the samples had to be conducted, as the matrices of the wastewater samples are highly loaded with organic constituents.



Chromatography was performed using Agilent's 1260 infinity system. Four chromatographic methods were developed to maximise sensitivity and achieve good chromatography for the targeted analytes exhibiting a broad range of chemistries. The compounds were selected to methods as per table 6.

**Method 1.** For protonated compounds, separation was done using ACN and Buffer (5mM Ammonium acetate with 0.02% Acetic acid). Initially, the buffer was kept at 95% for 3 minutes then reduced to 5% over the next 17 minutes. Then these conditions were kept constant for the next 5 minutes and then returned to initial conditions in the till overall run reached 30 minutes.

**Method 2.** For de-protonated compounds, separation was done using ACN and Buffer (0.005% Acetic acid). Initially, the buffer was kept at 98% and reduced to 2% in the next 9 minutes. Then these conditions were kept constant for the next 13 minutes and then returned to initial conditions in the next 2 minutes and kept constant till the overall run reached 22 minutes.

**Method 3.** For antibiotic compounds, separation was done using 0.1% Formic acid MeOH and Buffer (0.1 % Formic acid). Initially, the buffer was kept at 98% for 2 minutes and reduced to 85% in the next 2 minutes followed by a reduction of 5% over the next 2 minutes. It was then again reduced to 75 over the next 2 minutes followed by a reduction of 5% over the next 0.5 minutes and another 5% over the next 4.5 minutes which was reduced to zero over the next 5 minutes. These conditions were kept constant over the next 8 minutes and then returned to original conditions in the next 0.1 minutes which was kept constant till the overall run reached 30 minutes.

**Method 4.** For pesticides compounds, separation was done using MeOH and Buffer (10mM ammonium formate). Initially, the buffer was kept at 100% for 0.2 minutes and reduced to 45% in the next 2 minutes followed by a reduction of 20% over the next 3.5 minutes. It was then again reduced to 15% over the next 2 minutes followed by a reduction to 0% over the next 1 minute which was kept constant till the next 3.5 minutes. The condition was returned to the original conditions in 0.1 minutes which were kept constant till the run reached 15 minutes which was kept constant for the next 5 minutes.

Positive and negative mode methods used reversed-phase Poroshell C18 column (2.1mm x 100mm, 2.7 $\mu$ m particle size; Agilent) maintained at 40°C. Antibiotic methods used reversed-phase column Poroshell C18 column (3.0 mm x 150mm, 2.7 $\mu$ m particle size; Agilent) maintained at 40°C. The mobile phase flow rate was 0.3mL min<sup>-1</sup> and the injection volume was 40 $\mu$ L for positive mode and 50  $\mu$ L for negative mode. The mobile phase flow rate was 0.5 mL min<sup>-1</sup> and the injection volume was 20  $\mu$ L for the antibiotic method and 0.4 mL min<sup>-1</sup> flow rate and 10  $\mu$ L as injection volume was used for pesticides method. All four methods were coupled with 6495QQQ with a fixed fragmenting voltage of 380 where the gas temp was kept at 250°C with a flow rate of 16mL min<sup>-1</sup> for positive mode, 200°C with a flow rate of 12 mL min<sup>-1</sup> for negative mode, 200°C with a flow rate of 11mL min<sup>-1</sup> for antibiotic method and 180°C with a flow rate of 20mL min<sup>-1</sup> for pesticides method. 95% pure nitrogen gas was used as nebulising gas and 99.9% pure nitrogen gas was used as collision gas for all the methods.

The retention time and MRMs detected for the compounds were as per table 6.



Table 6: Retention time and MRMs detected for the selected compounds

	RT (min)	Molar Mass	Precursor ion	MRM (quantifier)
1. ESI (+) mode				
<b>Metformin</b>	1.28	129.1	130.1	60
<b>Atenolol</b>	1.29	266.2	267.2	145.1
<b>Trimethoprim</b>	1.35	290.2	291.2	123.2
<b>Erythromycin</b>	14.79	733.5	734.5	83.1
<b>Carbamazepine</b>	15.05	236.1	237.1	194.1
2. ESI (-) mode				
<b>Sulfamethoxazole</b>	7.8	253	252	156
<b>Methyl Paraben</b>	8.9	152	151	135.9
<b>Naproxen</b>	10.7	230	229	210.8
<b>Diclofenac</b>	11.6	295	294	250
<b>Triclosan</b>	12.5	287.9	286.9	226.1
3. Antibiotic method				
<b>Ciprofloxacin</b>	11.5	331	332	314
4. Pesticide method				
<b>Dichlorvos</b>	2.85	220.9	221.9	109.1
<b>Phorate sulfoxide</b>	3.66	276.4	277.4	143
<b>Atrazine</b>	3.91	215.7	216.7	174
<b>Butachlor</b>	8.04	311.8	312.8	238

#### Method Performance

Linearity was established across four methods ranging from 7.8 ppt to 500 ppt for positive and negative methods whereas 0.78 ppb to 50 ppb for the antibiotic and pesticide method. The calibration curves ranged from 9 to 8 points on the calibration scale for positive, negative, and antibiotic, pesticides methods respectively. Instrument detection limits and Instrument quantitation limits were calculated according to the lowest concentration which gave a signal to noise ratio of  $\geq 3$  and  $\geq 10$  respectively.

The application of the method developed at IIT D lab to environmental samples is ongoing after series of interruption due to Covid-19 between March 2020 and the end of the year 2021. It is further planned to confirm the results with experienced analytical partners in Europe such as TU Delft, University Cyprus, and other reference labs.



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## CHAPTER 4 HEAVY METAL MEASUREMENT

Heavy metal measurement is conducted by a range of methods:

- High-resolution inductively coupled plasma mass spectrometry (Agilent Technologies 7900 ICP-MS) available at the IIT Delhi laboratory (Figure 4).
- Microwave plasma - atomic emission spectrometry (Agilent MP-AES 4200), available at the IIT Kanpur laboratory (Figure 5).

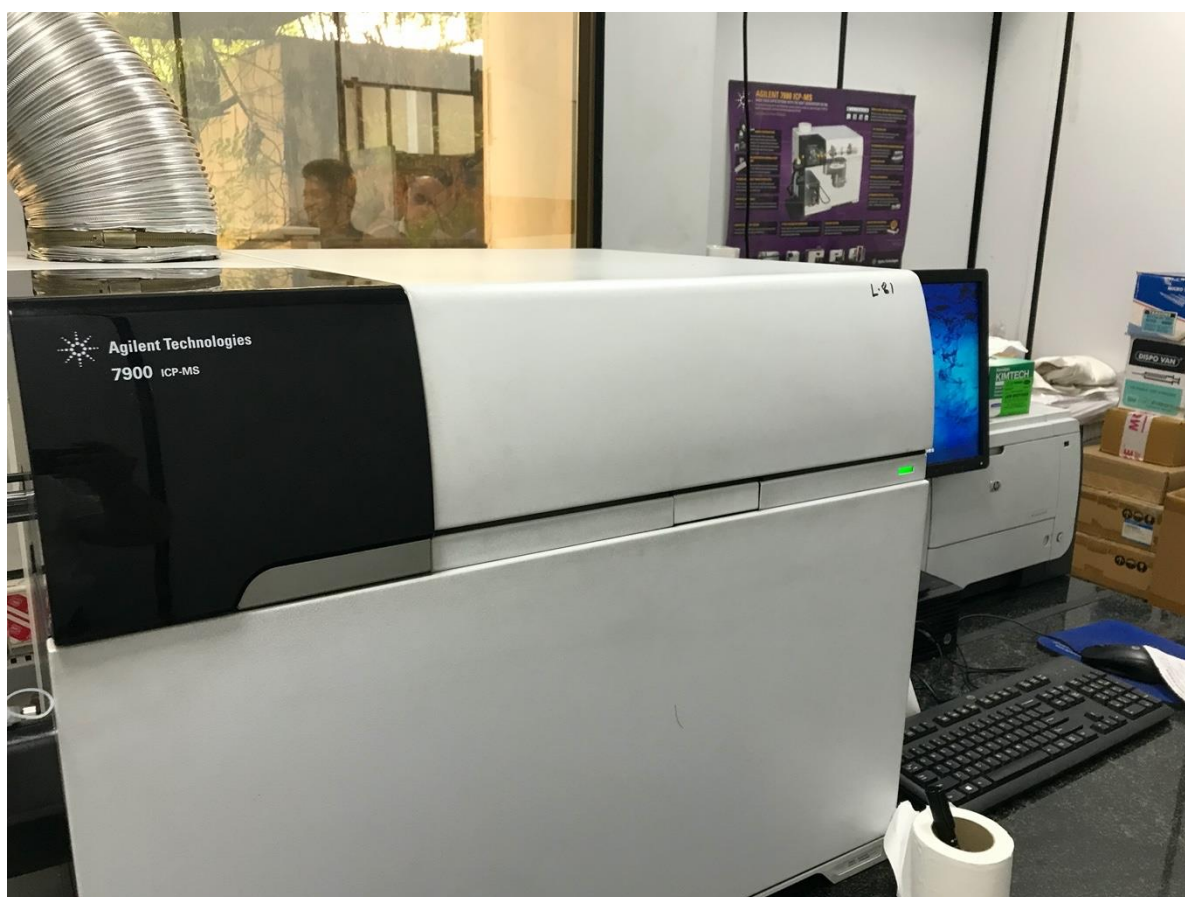


Figure 4: Agilent Technologies 7900 ICP-MS available at the IIT Delhi laboratory.





*Figure 5: Microwave plasma - atomic emission spectrometry (Agilent MP-AES 4200) at the IIT*

Sample preparation and analytical methods follows the established protocols at the respective labs as published earlier.

Details of the adapted methods will be reported in the updated version of 5.2 when running the samples from the project with tailored procedures after re-opening the labs post COVID-19.



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