# EffluentFit4Food

Towards safe, sustainable irrigation using effluent from wastewater treatment plants (WWTPs)

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# Summary

As climate change intensifies, the Netherlands faces growing pressure on water resources, particularly in balancing agricultural demands with limited water availability. Agriculture and horticulture require 100 to 150 million cubic meters of water annually, reaching up to 256 million cubic meters in dry years, mainly for irrigation. Currently, groundwater supplies 40 to 50 million cubic meters of this need. Other sources include drinking water and surface water [1].

In this context, effluent from wastewater treatment plants (WWTPs) presents an opportunity to mitigate water shortages and address salinisation issues. With approximately 2 billion cubic meters of treated wastewater generated annually [1], this resource could significantly supplement water supplies in agriculture [2].

However, concerns about food safety arise when considering the reuse of effluent, specifically regarding the possible transmission of pathogens, heavy metals, and organic micropollutants (OMPs) from effluent to crops. While effluent reuse is common in drought-prone areas like southern and central Europe, limited studies have explored the potential health risks of OMPs, particularly their effects on crop health and uptake by crops.

Ensuring the safe, sustainable use of WWTP effluent also requires ongoing monitoring for harmful contaminants. Traditional monitoring approaches often rely on labour-intensive, costly, and time-consuming single-point measurements, underscoring the need for faster monitoring technologies for specific components.

This project addressed these concerns by investigating multiple aspects related to effluent reuse. Divided into two parts, Part A focused on:
- reviewing regulations for agricultural effluent reuse;
- evaluating water treatment technologies for removing OMPs;
- examining the effects of raw and treated WWTP effluent on crop health and OMP uptake.

Part B concentrated on developing rapid monitoring techniques for continuously detecting harmful effluent components.

The project's activities and findings are outlined below.

**Part A.**

***Water reuse: EU Regulation and accompanying Guidance Document (Chapter 2)***
The legislative framework for reusing treated municipal wastewater (WWTP effluent) as irrigation water in horticulture focuses on ensuring food safety and environmental protection. The scope of this analysis included legislation related to water reuse, general water quality, hygiene, and specific food products such as onions, pears, and potatoes.

Since June 26, 2023, European Regulation 2020/741 on minimum requirements for water reuse has been in force. These requirements primarily address microbiological risks, setting limits for *E. coli* (<10 number/100 mL) and *Legionella* (<1000 CFU/L when aerosol formation is possible, e.g., during rain or spray irrigation). Additional parameters include basic chemical indicators such as BOD, TSS, and turbidity. The regulation does not cover OMPs, such as pharmaceuticals. However, changes can be expected in the future.

For using reclaimed water for food crop irrigation, a permit is required. This permit must be based on a risk assessment conducted by the permit applicant. This assessment should confirm the nature of potential hazards, evaluate dose-response relationships, assess exposure levels, and characterize the associated risks.

***Selection of treatment technologies for organic micropollutant removal (Chapter 3)***
Treatment technologies had to be chosen for further research in this project. There are various technologies available for the removal of OMP from wastewater. These include advanced oxidation technologies (such as ozonation), membrane technology, adsorption, and nature-based solutions like enzymatic catalysis. The consortium partners utilise technologies such as ozonation + biofiltration by PureBlue and enzymatic catalysis by Pharem.

After conducting a comparative analysis of various technologies based on their costs, removal efficiencies for OMPs, and carbon footprints, we decided to proceed with the ozonation + biofiltration technology, *MicroForce*$^{++}$, developed by PureBlue, and the enzymatic technology, *Zymatic*, from Pharem. The proposed sequence of ozonation plus biofiltration followed by enzymatic catalysis is expected to degrade OMPs effectively without the need for additional technologies. Other options were not selected because they either only remove OMPs without degrading them, or they have higher costs and carbon footprints.

***Evaluation of treatment technologies (Chapter 4)***
A pilot study assessed the removal of OMPs from WWTP effluent. The pilot installation was located at the WWTP Walcheren in Zeeland, the Netherlands. First, both stand-alone ozonation and a combined ozonation-UV treatment were used. The proposed MicroForce$^{++}$ system was introduced later in the project since it was not ready for use at the start of the project.

The ozonation+UV treatment effectively removed OMPs. Out of the 84 OMPs initially detected in raw effluent, 56 were no longer found after treatment. A total of 40 OMPs remained at detectable levels, with some of these being undetected in the raw effluent, likely due to variations in sample timing. Overall, the treatment achieved substantial removal rates for most OMPs, typically between 60-90% and reaching up to 96% for some. However, certain substances - including lamotrigine, metformin, adenosine, fluconazole, and gabapentin - showed removal rates below 60%.

The pilot also revealed that ozonation and UV treatment produced bromate as a by-product, with concentrations ranging from 21 to 51 µg/L. Bromate formation results from the ozonation of bromide, a compound more commonly found in coastal areas like Walcheren.

Untreated and treated WWTP effluent batches were collected to irrigate potatoes, onions and pears for the subsequent part of this project.

Later in the project, the MicroForce$^{++}$ technology was integrated into the pilot. MicroForce$^{++}$, which combines ozonation with biofiltration, proved to be a more sustainable and cost-effective improvement over stand-alone ozonation. It lowered bromate levels to below 0.2 µg/L, reduced $CO_2$ emissions by 50%, and cut treatment costs by 40% (to 0.10 €/m³, based on 2018 prices). These improvements were primarily due to MicroForce$^{++}$ requiring a lower ozone dose (0.43 g $O_3$/g DOC compared to 0.9 g $O_3$/g DOC for stand-alone ozonation), thereby reducing energy consumption and by-product formation.

Due to time constraints, WWTP effluent treated with MicroForce$^{++}$ was not used as irrigation water for crop cultivation.

Unfortunately, no results were obtained for OMP degradation using Pharem's Zymetic technology.

***Effluent exposure study on potato, onion and pear cultivation (Chapter 5)***
Different crops - potatoes, onions, and pears - were cultivated using various irrigation water qualities. This chapter evaluated the impact of irrigation water quality on plant vitality and crop yield.

The types of irrigation water used were raw WWTP effluent, treated effluent with ozone, treated effluent with ozone+UV, and tap water (as a reference). The raw and treated effluents - collected from the previously described pilot - were transported from WWTP Walcheren to the cultivation fields in Randwijk.

A total of 16 beds were cultivated for each crop type, with four beds designated for each irrigation method. Coarse sand served as the soil medium, promoting the rapid movement of irrigation water to the crop roots

while exhibiting minimal chemical activity in breaking down OMPs. This setup represents a worst-case scenario for OMP uptake by crops.

Initially, potato plants demonstrated the highest vitality when irrigated with tap water, while those given treated effluent (using ozone and UV) showed lower vitality. However, by the time of harvest, there were no significant differences in the vitality of potato plants across the various water quality treatments. In contrast, onion plants started with lower vitality when irrigated with tap water, but by harvest time, there were also no notable differences in vitality among the different water quality treatments.

During the harvest, the yield of potatoes varied based on irrigation water quality. The highest yield was achieved with tap water(2.4 kg per pot), followed by treated effluent with ozone and raw effluent (1.8 kg per pot). The lowest yield was recorded with treated effluent that combined ozone and UV treatment (1.2 kg per pot). The reason for these differences is not known.

There was a statistically significant effect of irrigation water quality on onion weight. The heaviest onions were grown with pure effluent (79 grams), followed by the two treated effluents (62 grams and 71 grams). In comparison, onions irrigated with tap water averaged 60 grams. While there were minor differences in the number of onions per pot and the weight of onions per pot across the various irrigation water qualities, these differences were not statistically significant.

No significant effects were observed regarding the yield of pears when comparing different qualities of irrigation water.

***Uptake of organic micropollutants in crops (Chapter 6)***

The crops (potatoes, onions, and pears) grown using various qualities of irrigation water were analyzed for their uptake of organic micro pollutants (OMPs). The crops were freeze-dried, and an extraction process was applied to isolate the potential OMPs. Non-target library screening analyses were performed to detect these substances. The results are presented not as absolute concentrations of OMPs in the crops, but rather as OMP concentrations equivalent to a known indicator. This approach allows for a comparison of OMP uptake across different crop types and irrigation water qualities, despite the absence of absolute concentration values. The findings provide insights into the types and number of unique OMPs absorbed by each crop type, as well as the impact of irrigation water quality on this uptake. For selected OMPs, it was possible to calculate their absolute concentrations in the crops.

The following conclusions were drawn:

- The quality of irrigation water has an impact on the uptake of organic micro pollutants. When using pure effluent, the highest frequencies and concentrations of OMPs are observed. In contrast, when treated effluent (ozonated water or ozonated water combined with UV treatment) is used, the concentration of OMPs is either lower or undetectable;
- Potatoes absorb the most unique types of OMPs, followed by onions. In comparison, pears absorb significantly fewer types of OMPs;
- For several OMPs in potatoes, the absolute concentrations were determined. When raw effluent was used for irrigation, the concentrations ranged from 0.2 to 2.3 ng/g of potato. This range is comparable to findings from other studies where vegetables and fruits were exposed to WWTP effluent. In contrast, when treated effluent was used, the OMPs were either no longer detectable or had concentrations ranging from 0.1 to 0.3 ng/g of potato;
- Pharmaceuticals and their metabolites are the most commonly found OMPs, althoug a drug and an industrial chemical have been occasionally detected;
- A significant number of OMPs were detected that had not been found in the effluent or treated effluent. For many of these OMPs, the quality of irrigation water did not affect their uptake; the levels detected were consistent across all types of irrigation water used, including tap water. These OMPs include pharmaceuticals, pesticides, drugs, industrial chemicals, and hormones. It is likely that these OMPs did not come from the irrigation water itself but were instead introduced through contamination from the spraying of polluted surface water near the fields where the crops were grown.

***Toxicity aspects of organic micropollutants (Chapter 7)***
For an initial assessment of public health risks associated with consuming crops irrigated with wastewater treatment plant (WWTP) effluent, toxicity data of the detected components were reviewed. Initially, Acceptable Daily Intake (ADI) values were considered; however, these values were available for only a limited number of components. Therefore, $LD_{50}$ values were used as an alternative to provide a preliminary indication of toxicity.

The $LD_{50}$ (the dose required to kill 50% of a test population, e.g. rats) for many components found in crops ranges from 100 to several thousand mg/kg body weight, although a few components have a lower $LD_{50}$ (<100 mg/kg). For comparison, caffeine, a compound commonly consumed in coffee, has an LD50 of 127-192 mg/kg. In contrast, the absolute concentrations of components detected in potatoes were substantially lower, ranging from 0.2 to 2.5 ng/g (or µg/kg) potato.

To illustrate, if a person weighing 50 kg consumes 500 grams of potatoes in one day, with a component concentration of 2.5 ng/g potato, the person would ingest a total of 1250 ng of this component. This corresponds to an intake of 25 ng per kilogram of body weight.

***Conclusions, gaps and considerations (Chapter 8)***
The previous chapters of Part A presented the results from various work packages of the EffluentFit4Food project. The consortium discussed these results on April 25, 2024, to determine which conclusions can already be drawn regarding the use of (treated) WWTP effluent as irrigation water for crop cultivation, identify any remaining concerns, and outline the knowledge gaps that need to be addressed before conclusions can be made.

The major research conclusions are the following:
- Some of the substances from the effluent of the WWTP Walcheren are present in the crops irrigated with this water (pears, onions and potatoes);
- Post-treatment of the effluent (with ozone + UV) reduces the concentrations of organic OMPs in the effluent and the crops;
- Specific treatment with ozone (in low concentrations) and biological degradation reduces the concentrations of pharmaceutical residues less than with high ozone doses, but still by almost 70%. The bromate concentration decreases from 21-51 µg/L till levels below 0.2 µg/L; a factor of 250, $CO_2$ emissions reduce by 50% and treatment costs by 40%;
- The concentrations of 11 selected OMPs found in potatoes irrigated with raw effluent remain well below the known LD50 values;
- Uncertainties remain regarding the safe reuse of effluent for irrigation, particularly concerning the combined and long-term effects of detected OMPs and other undetected substances;
- Removal of *E. coli* bacteria from reclaimed water intended for irrigation appears to be necessary from a legislation and regulation point of view;
- Currently, there are no specific legislations and regulations concerning OMPs in reclaimed water used for irrigation, though the presence of OMPs should be mentioned in risk analysis.

The question remains: how much post-treatment is truly necessary? While removing *E. coli* bacteria may suffice in some cases, there are still several gaps and considerations that must be addressed to form a complete picture before making definitive conclusions. These are as follows:
- Identification of substances: Not all OMPs present in the effluent or crops may have been identified, and certain groups of substances, such as PFAS and heavy metals, were not included in this project's analysis;
- Measurement accuracy: Non-target library screening provides relative concentrations rather than absolute ones, affecting the precision of the results;
- Risks to public health: Limited data exist on the cumulative and long-term effects of the micropollutants found in crops, especially regarding interactions between detected and undetected contaminants;
- Representativeness of effluent: The composition of effluent varies significantly between WWTPs, influenced by factors like proximity to industrial facilities or hospitals;
- Soil and crop variation: The results obtained using coarse sand may differ for other soil types, and uptake of contaminants may vary in different crop types, like leafy greens, which tend to absorb higher concentrations;

- Accumulation over time: Long-term use of treated effluent for irrigation may lead to buildup of micropollutants in the soil, impacting future crop safety;
- Regulatory framework: Current regulations primarily focus on *E. coli* levels and do not address OMPs. However, OMPs may be added to regulatory standards in the future and will be included in upcoming risk assessments;
- Responsibility and risk assessment: Clear responsibility for effluent reuse permissions and risk assessment standards remains to be established, as demonstrated by ongoing pilot projects.

**Part B**

***Development of (dis)continuous monitoring of contaminants in waste water (Chapter 9)***

Technology was developed to create a microfluidic device for (dis)continuously detecting target substances, combining innovative design strategies, biochemical experimentation and machine learning techniques. These developments support the feasibility of a microfluidic platform integrated with machine learning for sensitive contaminant detection and lay the groundwork for future testing and development.

- Several microfluidic cell prototypes were explored, each with different characteristics. This includes a cost-effective 3D-printed cell for flexible design iterations, an advanced PDMS cell with fewer leaks and adjustable features, and a simple, low-cost version using double-sided adhesive tape;
- Functionalized beads were developed against a model analyte, and evaluated across different conditions. Initial tests employing supervised machine learning showed a relationship between the ratio of clustered beads to single beads and analyte presence, indicating promise for targeted detection;
- Custom electronics systems were designed to automate device operations, covering system setup, sample handling, controllable focussing, measurement control, image and data acquisition, and remote operation capabilities;
- A data processing pipeline was set up to analyze microscope images, incorporating image pre-processing and investigating both unsupervised and supervised learning methods. Supervised learning using synthetic data showed potential for precise bead detection and cluster identification, suggesting enhanced capabilities for robust microfluidic analysis.

# Collaborating partners











# 1 Introduction

## 1.1 Background

As climate change progresses, with more frequent and severe droughts expected, the delicate balance between water availability and agricultural needs is increasingly strained. In the Netherlands, agriculture and horticulture consume between 100 and 150 million cubic meters of water annually, with peaks reaching 256 million cubic meters during dry years like 2003. Groundwater provides 40 to 50 million cubic meters annually [1]. Other sources include drinking water and surface water. Much of the water used by agriculture and horticulture is used for irrigating crops during dry periods.

Due to the changing climate, resulting in longer periods of drought and higher temperatures, in combination with a higher demand for fresh water due to economic growth, the Dutch National Institute for Public Health and the Environment (RIVM) expects shortages of drinking water availability by 2030 [3]. The RIVM mentions several alternative sources of fresh water that can be used in the future to prevent drinking water shortages and the reuse of the effluent of municipal wastewater treatment plants (WWTP) as irrigation water in agriculture is one of the possible solutions [2]. With around 2 billion cubic meters of purified wastewater generated annually in the Netherlands, predominantly from communal sewage treatment plants [1], there's a significant resource available. This effluent could be used not only in areas affected by drought but also in regions lacking sufficient fresh surface water due to increased salinity.

The primary concern with reusing WWTP effluent for food production is water quality. This raises issues related to food safety, especially regarding the potential transmission of pathogens, heavy metals, and organic micropollutants (OMPs) from the effluent to crops. While effluent reuse is common in drought-prone regions like southern and central Europe, there are limited studies on its potential health risks. In particular, the effects of OMP exposure on crop health and the uptake of OMPs by crops are not well understood.

As of June 26, 2023, the European guideline (2020/741) regarding minimum requirements for water reuse has become legislation. It requires that parties intending to reuse effluent from WWTPs for irrigation must conduct an additional risk assessment related to components that may impact public health.

Safe and sustainable use of effluent also requires constant monitoring for harmful contaminants. Traditional monitoring often relies on laborious, expensive, and time-consuming measurements based on a single time point. Therefore, new, fast monitoring technologies are needed for specific components.

This project provides valuable insights to address these challenges, e.g. as input for risk analyses. This information includes:

- Assessment of technologies capable of removing contaminants;
- Research into the uptake of contaminants by selected crops when irrigated with water of varying qualities (concentrations of contaminants);
- Providing an overview of the regulations that must be complied with to reuse effluent in agriculture;
- Development of rapid monitoring of contaminants.

Ultimately, the knowledge gained from this project will contribute to sustainable water management practices, ensuring both water availability for agriculture and the safety of food production in the face of changing climates.

## 1.2 Activities

This section outlines the research activities conducted in this project and where they can be found in the report, serving as a guide for readers. The chapters in this report each cover separate (sub)studies within this project.

This report is divided into two parts. Part A focuses on legislation related to the reuse of effluent for agricultural applications, technologies for removing OMPs from water, and the transfer of OMPs to crops under different effluent water qualities. Part B addresses the development of new rapid monitoring technologies for detecting contaminants in water.

**Part A**

*Analysis of legislation and regulations (WP 1)*
An inventory of relevant legislation on the use of effluent for irrigation was conducted to assess the extent to which WWTP effluent can be utilised. This includes Dutch and European environmental protection regulations and food safety regulations.

*Selection of model substances for research (WP 2)*
Organic micropollutants (OMPs), such as pesticides and pharmaceutical residues, were identified as potential priority or critical substances. The research in the subsequent stages of the project will focus on these compounds. Other components, such as bromate, heavy metals, and PFAS, were only identified as potential risk factors later in the project and were therefore not included in this study. Additionally, the focus was placed on OMPs rather than microorganisms, as significantly less is known about OMPs.

*Selection and evaluation of purification techniques (WP 5)*
A state-of-the-art overview of technologies for removing OMPs from water streams was made. Additionally, the technologies developed by project partners are explained. Each technology is briefly described and compared based on OMP removal efficiency, costs, and carbon footprint. Based on this comparison, technologies were selected for further work in this project to achieve the complete removal of OMPs from WWTP effluents (**Chapter 3**).

*Research with pilot facility (WP 7)*
Building on the findings from WP 5, a pilot study was conducted at the wastewater treatment plant Walcheren (Ritthem, province of Zeeland, The Netherlands) to explore the removal of OMPs from wastewater effluent. The study employed PureBlue Water's MicroForce$^{++}$ technology, which integrates ozonation with biofiltration. Initially, standalone ozonation and ozonation followed by UV were used, as the MicroForce$^{++}$ system was not yet operational and purified effluent was needed for subsequent experiments. **Chapter 4** presents data on OMP concentrations in both raw and treated effluent, removal efficiencies, and includes an economic analysis.

*Crop research into transfer and transport of components (WP 6)*
Selected crops (potatoes, onions, and pears) were cultivated using different water irrigation qualities. These qualities included raw WWTP effluent, treated effluent with ozonation, treated effluent with ozonation and UV, and tap water as a reference. The effect of irrigation water quality on plant vitality, crop yield, and crop quality was assessed (**Chapter 5**).

Next, the potatoes, onions and pears that were grown using the various irrigation water qualities were analyzed on the presence of OMPs (**Chapter 6**). The analysis uses non-target library screening, which identifies components in a sample without specifically targeting substances. This method matches detected peaks in the sample's spectrum to a database of known components. Although it provides insights into the substances present, it primarily allows for qualitative assessment of concentrations. Despite unknown absolute concentrations, the results are presented in concentrations equivalent to a known indicator, enabling a comparison of OMP uptake per crop and irrigation water type. For selected OMPs, the absolute concentration in crops can be calculated. The results provide information on the types and amounts of OMPs absorbed by each crop type, and the influence of irrigation water quality on the uptake.

The detected OMPs in effluent, treated effluent, and crops are briefly assessed for toxicity based on LD50 values. This evaluation identifies which OMPs pose the greatest immediate risk to health (**Chapter 7**).

A brief discussion on the applicability of the results from Part A is presented in **Chapter 8**. This chapter examines, among other things, the conclusions that can be drawn regarding the safe use of (treated) effluent for agricultural irrigation and identifies what information is still lacking to draw certain conclusions.

**Part B**

*Development and demonstration of in-line (bio)monitoring techniques (WP 3 and 4)*
Technology is being developed towards creating an automated, in-line device that can (dis)continuously monitor effluent. This provides insights into the content of wastewater and allows timely intervention if the effluent is unsafe for use in irrigation. This device employs a microfluidic cell containing contaminant-specific micron-sized beads that cluster in the presence of the target contaminant in the effluent. An optical read-out system images bead interactions, and software algorithms analyze these images to detect and quantify target molecules. The execution of this work package includes the development of a suitable reaction chamber, contaminant-specific functionalised beads, custom electronics for automated operations, and a data processing pipeline for signal quantification (**Chapter 9**).

## 1.3 Project information

Project number: LWV20.21
Project title: EffluentFit4Food
Project period: June 1, 2021 – December 31, 2024

# Part A[1]

[1] Sections of Part A have been adapted for B. Letterie *et al.* - Organic micropollutants in potatoes, onions, and pears: Effects of irrigation with reclaimed wastewater and ozone post-treatment (in preparation)

# 2 Water reuse: EU Regulation and accompanying Guidance Document

## 2.1 Introduction

This chapter examines the legislative framework governing the reuse of municipal wastewater treatment plant (WWTP) effluent as irrigation water in horticulture.

The primary concern with reusing WWTP effluent for food production is water quality. This raises issues related to food safety, especially regarding the potential transmission of pathogens, heavy metals, and organic micropollutants (OMPs) from the effluent to crops. While effluent reuse is common in drought-prone regions like southern and central Europe, there are limited studies on its potential health risks. In particular, the effects of OMP exposure on crop health and the uptake of OMPs by crops are not well understood.

Since June 26, 2023, the European guideline (2020/741) regarding minimum requirements for water reuse has been in force. It requires parties intending to reuse effluent from WWTPs for irrigation to conduct an additional risk assessment related to components that may impact public health.

In this chapter, an inventory of relevant legislation on using effluent for irrigation was conducted to assess how WWTP effluent can be utilised. This includes Dutch and European environmental protection regulations and food safety regulations. Special attention is given to the role of OMPs.

## 2.2 Methods

Legislation is subject to continuous amending and/or changing. Therefore, it is important to consult the latest version of each legislation, which is called the consolidated version. Consolidated versions of legislation were consulted online, using the web portal *Eur-Lex*[2], which is managed by the Publications Office of the European Union.

The search focused on food legislation, specifically on the food products in the project's scope: onion, pear, and potato. Furthermore, legislation related to the reuse of water, water in general, and hygiene was in scope. During the project's duration, developments and changes in legislation and policy were monitored on a regular basis.

## 2.3 Results

### 2.3.1 Minimum requirements for water reuse (Reg EU 2020/741)

Regulation EU 2020/741, which is applicable from 26 June 2023, stresses the need to apply alternatives for food crop irrigation. Minimal requirements posed upon reclaimed water are provided in Table 2-1.

[2] https://eur-lex.europa.eu/homepage.html

***Table 2-1 Reclaimed water quality requirements for agricultural irrigation (from Reg. (EU) 2020/741, Annex I, table 2).***

| Reclaimed water quality class | Indicative technology target | Quality requirements | | | | |
|---|---|---|---|---|---|---|
| | | E. coli (cfu/100 mL) | BOD (mg/L) | TSS (mg/L) | Turbidity (NTU) | Other |
| A | Secondary treatment, filtration, and disinfection | ≤ 10 | ≤ 10 | ≤ 10 | ≤ 5 | *Legionella spp.*: < 1,000 cfu/L where there is a risk of aerosolization<br>Intestinal nematodes (helminth eggs): ≤ 1 egg/L for irrigation of pastures or forage |
| B | Secondary treatment, and disinfection | ≤ 100 | In accordance with Directive 91/271/EEC (Annex I, Table 1) | In accordance with Directive 91/271/EEC (Annex I, Table 1) | - | |
| C | Secondary treatment, and disinfection | ≤ 1,000 | | | - | |
| D | Secondary treatment, and disinfection | ≤ 10,000 | | | - | |

Minimum requirements are laid down for the reuse of water for agricultural irrigation. The number of these minimum requirements is, however, limited. With regards to food safety, the requirements are set on the number of *E. coli* (<10 number/100 mL) and *Legionella* (<1,000 colony forming units/L when there is a risk of aerosol formation, e.g. with rain or spray irrigation).

Regulation EU 2020/741 refers to many other legislative texts. Table 2-2 presents an overview of these other regulations, varying from legislation in the areas of water, food, feed, hygiene, microbiological criteria, contaminants, and pesticides. An overview of all related legislation is provided in Annex 1.

***Table 2-2 Requirements and obligations, as a minimum, to be taken into account in the risk assessment (from Reg. (EU) 2020/741, Annex II).***

| | Type and number | Latest consolidated version | Ref |
|---|---|---|---|
| Reduce and prevent water pollution from nitrates | Directive 91/676/EEC | *2008.12.11* | [4] |
| Quality of water intended for human consumption | >12-01-2023: Regulation 2020/2184/EU | *unchanged* | [5] |
| Environmental objectives | Directive 2000/60/EC (WFD) | *2014.11.20* | [6] |
| Prevent groundwater pollution | Directive 2006/118/EC | *2014.07.11* | [7] |
| Environmental quality standards for priority substances and certain other pollutants | Directive 2008/105/EC | *2013.09.13* | [8] |
| Environmental quality standards for pollutants of national concern, namely river basin specific pollutants | Directive 2000/60/EC (WFD) | *2014.11.20* | [6] |
| Bathing water quality standards | Directive 2006/7/EC | *2014.01.01* | [9] |
| Protection of the environment, and in particular of the soil, when sewage sludge is used in agriculture | Directive 86/278/EEC | *2022.01.01* | [10] |
| Hygiene of foodstuffs | Regulation (EC) No 852/2004 | *2021.03.24* | [11] |
| Microbiological risks in fresh fruits and vegetables at primary production through good hygiene | Guidance doc 2017XC0523(03) | *unchanged* | [12] |
| Feed hygiene | Regulation (EC) No 183/2005 | *2022.01.28* | [13] |
| Comply with the relevant microbiological criteria | Regulation (EC) No 2073/2005 | *2020.03.08* | [12] |
| Maximum levels for certain contaminants in foodstuffs | >25-05-2023: Regulation 2023/915/EU | *2023.08.10* | [14] |
| Maximum residue levels of pesticides in or on food and feed | Regulation (EC) No 396/2005 | *2024.02.26* | [15] |
| Animal health | Regulation (EC) No 1069/2009<br>Regulation (EU) No 142/2011 | *2019.12.14*<br>*2022.04.17* | [16]<br>[17] |

Furthermore, a permit for water reuse for food crop irrigation should be granted based on a risk assessment executed by the permit requester. This risk assessment should include the following steps concerning water reuse for food crop irrigation: confirmation of the nature of the hazards, including, where relevant, the dose-response relationship; assessment of the potential dose range or exposure; characterisation of the risks. These aspects are consistent with those outlined in Table 2-2.

Additional requirements should be set when this risk assessment is carried out. These include additional requirements for the following classes of food hazards: heavy metals, pesticides, disinfection by-products, pharmaceuticals, and other substances of emerging concern, including micropollutants, microplastics, and antimicrobial resistance.

**Guidelines to support the application of Reg EU 2020/741 (2022/C298/01)**

Following the publication of the minimum requirements for water reuse (Regulation EU 2020/741), Guideline 2022/C298/01 was issued to support its application. This Guideline provides detailed information on limit values for reclaimed water quality, drawing on overviews from other legislative texts. It includes examples of common hazards and hazardous events, potential exposure routes, and receptors that may be present in systems for water reuse in agricultural irrigation.

For instance, the Guideline considers quality standards for bathing water, bacteria commonly found in raw wastewater, and specific chemicals in wastewater. However, it does not address the micropollutants analysed in the current project. Additional insights related to these aspects can be found in Annex 2.

### 2.3.2 Reusing wastewater in agriculture: technical specifications for risk management plans

In March 2024, a Delegated Regulation (EU) 2024/1765 supplementing Regulation (EU) 2020/741 regarding technical specifications for the key elements of risk management was adopted. This Delegated Regulation provides technical specifications for the key elements of risk management set out in Annex II of Regulation 2020/741 on minimum requirements for water reuse.

Furthermore, the specifications in the Annex of the Delegated Reg. will help those involved in water reuse projects (including competent authorities, water treatment plant operators, storage and distribution operators, and end users) in preparing robust risk management plans to safely reuse treated wastewater in agriculture.

### 2.3.3 Ongoing developments in the regulatory landscape

The European Commission has proposed an update to the Urban Wastewater Treatment Directive[3]. New pollutants, such as microplastics or micropollutants, have emerged, and outdated limit values need to be updated as a result of technical progress.

Furthermore, there is a proposal for a Directive amending the Water Framework Directive, the Groundwater Directive and the Environmental Quality Standards Directive[4] to review Annex X of the Water Framework Directive, concerning the list of priority substances. The proposal addresses a wide range of pollutants, e.g. pesticides, pharmaceuticals, per- and polyfluoroalkyl substances (PFAS). The idea is that by avoiding water pollution, the proposal will benefit the potential for water reuse.

## 2.4 Conclusions

Current water quality requirements for agricultural irrigation distinguish four quality classes based on the type of crop to be irrigated. These requirements focus primarily on microorganisms (e.g., *E. coli* and *Legionella*) alongside several chemical parameters such as BOD, TSS, and turbidity. However, they do not address micropollutants.

The current regulations may be expanded. RIVM is working on this and recently published a report with recommendations [18]. These recommendations primarily focus on PFAS, not pharmaceutical residues or

[3] https://environment.ec.europa.eu/publications/proposal-revised-urban-wastewater-treatment-directive_en
[4] https://environment.ec.europa.eu/publications/proposal-amending-water-directives_en

other micropollutants. However, a second report (part 2) is expected to be published this year. It is unclear if and when the regulations for water reuse regarding micropollutants will be adjusted.

At present, there are no regulations for micropollutants in water reuse, but changes can be expected in the future. However, these changes are still unclear, and how and when they will be incorporated into legislation is not known.

A permit must be granted based on a risk assessment conducted by the applicant to use reclaimed water for food crop irrigation.

Table 2-3 provides an overview of of available norms or reference values regarding reclaimed water and food.

***Table 2-3 Overview of available norms or reference values.***

| Norms available | Reclaimed water | Food |
|---|---|---|
| Microbiological | Yes (limited) | Yes (more extended) |
| Contaminants | Nitrates, metals, PAHs | In specific foods: mycotoxins, specific alkaloids, metals, nitrates (in lettuce), halogenated persistent organic pollutants |
| Pesticides | No | Yes |
| Drugs | Not found | Not found |
| PFAS (contaminant) | Yes (total) | Not found |

# 3 Selection of treatment technologies for organic micropollutant removal

## 3.1 Introduction

In this chapter, the technologies selected for removing organic micropollutants (OMPs) from the effluent of wastewater treatment plants (WWTPs) within this project are outlined.

This chapter begins by providing an overview of the water technology sector's perspective on the presence of OMPs in WWTPs, with a primary focus on the Netherlands. Following this, various available technologies for OMP removal are briefly outlined.

The chapter then proceeds to detail the technologies offered by consortium partners PureBlue and Pharem. Through a comparative analysis of these technologies, the project will determine which ones to use for experimental purposes. Key performance criteria for selection include OMP removal efficiency, costs, carbon footprint and flexibility.

## 3.2 State-of-the-art review

### 3.2.1 Current initiatives in organic micropollutant management at WWTPs

With increasing concerns about the presence of organic micropollutants in the environment, the water technology sector is actively investing in advanced water treatment technologies aimed at eliminating these substances. Enhanced monitoring and heightened awareness of the associated risks are driving these efforts [19].

The Dutch Waterboards are contributing through the '*Innovation Program Removal of Micropollutants at Wastewater Treatment Plants*' (IPMV), a five-year initiative running from 2019 to 2023, as part of the national *Ketenaanpak Medicijnresten* program initiated by the Dutch Ministry of Infrastructure and Water Management. The IPMV focuses on exploring and advancing novel approaches to address OMPs, with a goal of improving the Technological Readiness Level (TRL) of these techniques. This includes addressing uncertainties related to removal efficiency, costs, and carbon footprint [19].

Key objectives of the IPMV include achieving a minimum removal efficiency of 80%[5] for seven out of eleven selected guide substances (Dutch: gidsstoffen) and ensuring that successful technologies remain operational for at least ten years. To fully integrate these technologies into Dutch WWTPs, additional research is required to address remaining questions and resolve challenges related to plant-specific conditions, OMP concentrations, bromide presence, and budget constraints [20].

### 3.2.2 Available techniques for organic micropollutant removal at WWTPs

Conventional secondary processes in WWTPs are often unable to remove (most) OMPs found in wastewater [21]. Therefore, additional post-treatment installations are necessary to address these remaining OMPs [22]. Researchers have proposed various post-treatment technologies to target OMPs, categorised as:

- Advanced oxidative processes (AOPs);
- Nature-based solutions;
- (Advanced) adsorption technologies;
- Membrane filtration technologies.

[5] This used to be 70%, but was increased to 80% in 2020 due to new EU directives.

Optimal and efficient removal of the complete spectrum of different OMPs often requires the implementation of multiple techniques in series [23].

The subsequent paragraphs will elaborate on each technology category.

**Advanced oxidative processes (AOPs)**

Advanced oxidative processes (AOPs) are chemical processes that utilise in-situ generation of strong oxidants for the removal of organic compounds. Removal takes place through the oxidation of these compounds, leading to their *degradation* [24]. While several types of AOPs exist, the primary technologies for WWTPs are ozonation, hydrogen peroxide, ultraviolet radiation (UV), or combinations of these technologies.

*Ozonation*
Ozonation is an advanced oxidative process in which ozone gas ($O_3$) is introduced into a substance, such as wastewater, for purification and disinfection. Ozone, a reactive gas with low solubility in water, is typically generated on-site and reacts with contaminants and pollutants in the water, breaking them down. A schematic overview of the process is shown in Figure 3-1.

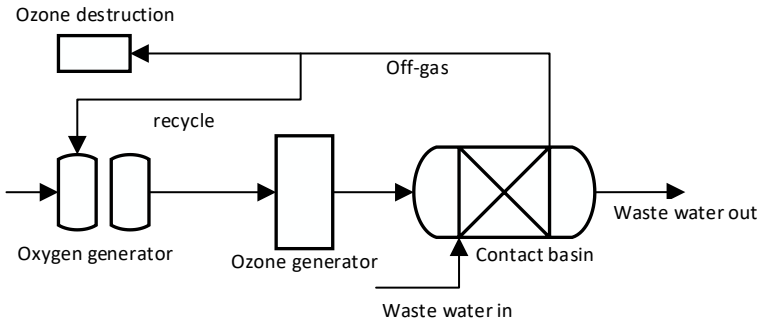



***Figure 3-1 Schematic overview of an ozonation installation where ozone is generated on-site.***

Ozonation is a proven technology already implemented in full-scale installations at WWTPs in Germany and Switzerland as an additional cleaning step, aimed at removing OMPs [25]. In The Netherlands, the first ozonation installation was put into use at WWTP Houten in 2021 [20].

Removal rates of OMPs vary, depending on the type, ranging from very low to almost 100%. Many ozonation processes combine ozone with other techniques, such as sand filters, biofilters [26, 27], ultrasound [28], or even other AOP technologies like hydrogen peroxide and UV [20]. This combination typically removes OMPs by over 80% [27]. An additional benefit is that ozonation also disinfects the water.

However, a disadvantage of ozonation is the formation of (unknown) byproducts, which may be harmful. For example, ozonation of bromide can lead to the formation of bromate. Fortunately, there are already many mitigation strategies to prevent the unwanted formation of bromate. Furthermore, ozone itself is a toxic compound. Another big disadvantage of stand-alone ozonation is the relatively high energy consumption required to generate and dissolve the ozone.

*Hydrogen peroxide treatment and UV radiation*
While hydrogen peroxide ($H_2O_2$) treatment and ultraviolet (UV) radiation are standard for water disinfection, their application for OMP removal is still limited in practice. However, a pilot-scale test of a hydrogen peroxide – UV combination has been conducted and is now commercially available [29].

Pilot studies conducted with the hydrogen peroxide – UV combination at WWTPs showed an average OMP removal efficiency of 41%, which is relatively low compared to other competitive technologies [30, 31]. It is worth noting that removal efficiencies could potentially increase to 80% by improving UV transmission and adjusting parameters within the conventional WWTP process; however, this comes with additional costs that are not economically feasible compared to ozonation [30].

## Nature-based solutions

*Constructed wetlands (CWs)*
Constructed wetlands (CWs) are artificial wetlands used to treat wastewater. Similar to natural wetlands, CWs replicate various conditions such as water intake, but they are under controlled settings [32]. The removal of organic matter in CWs occurs through several processes, including photodegradation and biodegradation by microorganisms. Several tests have been conducted to investigate the removal of OMPs. Most of these tests were performed on a pilot scale. The results indicated that CWs could remove OMPs by 21-98%, depending on factors such as the type of OMP, feed water quality, CW design, and the time of year [25, 33, 34].

The main advantages of CWs include their utilisation of green technology (no chemicals required, low carbon footprint) and low maintenance costs. While it is believed that the costs of CWs are lower than those of conventional water treatment technologies, the exact cost of removing OMPs from a cubic meter of water remains unknown. However, a notable disadvantage of CWs is that they require more space compared to competing technologies [35].

*Enzymatic catalysis*
Enzymes are proteins that function as biological catalysts, accelerating chemical reactions under mild conditions such as low temperature and low pH. Their versatile applications span various industries, including the production of pharmaceuticals, food, polymers, and chemicals. The technology used to utilise enzymes to accelerate chemical reactions is known as enzymatic catalysis [36].

For industrial purposes, enzymes are employed either in their free form or immobilised by attaching them to a carrier. Immobilisation methods include adsorption, physical entrapment, or covalent bonding. Since enzymes act as catalysts and are not consumed during chemical reactions, constant replacement is unnecessary [37]. However, periodic replacement of enzymes is necessary since they can be inactivated by denaturants and inhibitors [38].

Enzymes can also play a role in wastewater treatment, where they can remove OMPs by transforming them into (harmless) products. This means that enzymatic catalysis in this scenario is also a degradation technology. The efficiency of this process depends on factors such as the type of OMP, the specific enzyme used, and process conditions, including residence time, temperature, pH, and the design of the carrier material [39].

The type of chemical reaction accelerated depends on the type of enzyme. Enzymes can be highly specific, breaking down or converting only a single substance, or non-specific, acting on multiple components. However, since wastewater consists of various OMPs, a mixture of different enzymes is essential for targeting all OMPs [40].

While enzymatic catalysis is a well-established field of research, using enzymes for OMP removal in wastewater treatment is a relatively new technique. But, although most tests are conducted on a lab scale, some enzyme technologies are commercially available [41].

The primary advantage of using enzymes lies in their alignment with green technology principles: no additional chemicals are required, resulting in a low $CO_2$ footprint. However, notable disadvantages include operational costs (mainly the replacement of enzymes) and the potential generation of toxic byproducts.

## (Advanced) adsorption technologies

Adsorption is a surface phenomenon in which molecules attach to the surface of a solid (adsorbent) through various processes. This technology thus is a *capture* technology; it removes contaminants without degrading them. Several adsorbents exist for removing OMPs from aqueous streams, with activated carbon being the most commonly used.

*Activated carbon*

Activated carbon consists of a porous structure with internal surface areas ranging between 500 and 2000 $m^2/g$ [42]. This porous structure with a high surface area provides activated carbon with a significant adsorption capacity, making it widely used in many industrial processes. A schematic overview of the porous structure of an activated carbon particle is shown in Figure 3-2.

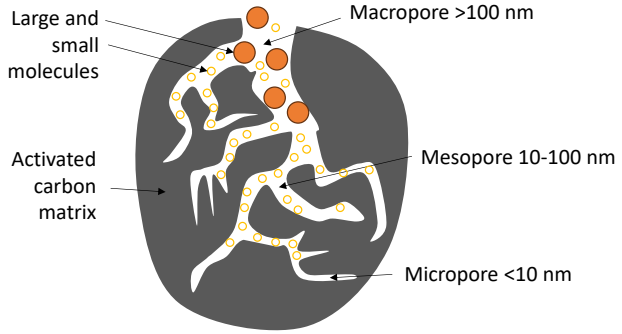



***Figure 3-2 Schematic overview of a porous activated carbon particle (adapted from** [43]**).***

Activated carbon is commercially available in granular (GAC) and powdered (PAC) forms [44]. Both forms consist of the same material but differ in particle size. Granular activated carbon has particles ranging from 0.2 mm to 5 mm [45], while powdered activated carbon (PAC) consists of much smaller particles ranging from 1 to 50 µm [46].

*Powdered activated carbon (PAC)*

Powdered activated carbon is generally employed in two processes for OMP removal in WWTPs:

- Additional Treatment: OMP removal in a special tank after the existing conventional treatment;
- PAC in Activated Sludge (PACAS): OMP removal during the existing secondary treatment by dosing powdered activated carbon into the sludge tanks.

In the first PAC process, the effluent from the conventional WWTP undergoes treatment in a separate facility consisting of a contact tank, settling tank, and filtration unit. PAC is introduced into the contact tank, where it adsorbs OMPs and other remaining organic components. The treated effluent then passes into a settling tank, where it is combined with polyelectrolytes to coagulate the PAC particles. After this, the effluent, now free of contaminants, undergoes subsequent filtration to remove the PAC particles. The sludge or concentrate stream from the PAC installation is partially recycled, while excess sludge is withdrawn for incineration to prevent PAC saturation. [43, 44]. A schematic overview of this process is shown in Figure 3-3.

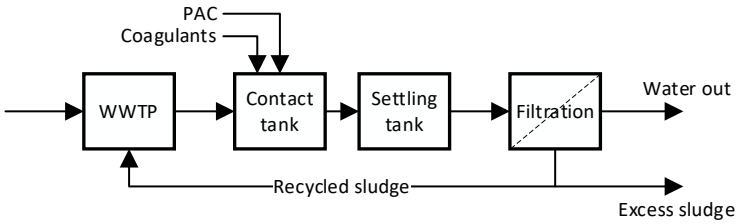



***Figure 3-3 Schematic overview of the PAC process as an additional WWTP treatment.***

In the PACAS process, PAC particles are directly dosed into the sludge tanks of the existing WWTP. During the wastewater's residence time in the WWTP, OMPs adsorb into the PAC particles. The PAC particles leave the WWTP with the sludge, which is partially recycled to the sludge tank and partially withdrawn for incineration. Since PAC-particles are partially still present in the effluent, an additional separation step is required to remove the PAC-particles. This is for example done by membrane filtration [43, 44, 47]. An schematic overview of the process is shown in Figure 3-4.

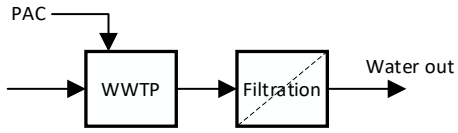



***Figure 3-4 Schematic overview of the PAC in activated sludge (PACAS) process.***

PAC is a proven technology used in over 20 WWTPs in Germany and Switzerland for OMP removal from wastewater. However, research is ongoing to optimise processes and combine activated carbon with other technologies [20].

*Granular activated carbon (GAC)*
Granular activated carbon is often employed as a single filtration step in fixed bed filters. At WWTP plants, a fixed bed filter containing GAC particles follows the existing conventional water treatment process. This fixed bed filter is usually a cylindrical vessel. The effluent flows downward through the bed of GAC particles, with OMPs adsorbing into the GAC particles until saturation [25]. Once saturated, the GAC is replaced and incinerated, typically after 7000-15000 bed volumes, corresponding to an operation time of 4 to 12 months [44]. A schematic overview of the process is shown in Figure 3-5.

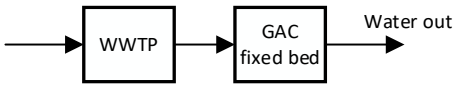



***Figure 3-5 Schematic overview of the GAC process as an additional WWTP treatment.***

Unlike powdered activated carbon, no additional separation unit is required for saturated activated carbon, as the GAC particles remain in the fixed bed filter. The GAC system is, therefore, simpler and easier to operate. GAC particles can also remove macropollutants, which may decrease OMP removal efficiency due to competition for adsorption sites.[44].

GAC is currently used in pilot studies at WWTPs, with a OMP removal between 80% and 85%. Challenges include improving the functioning and lifespan of GAC filters [20].

*Alternative adsorbents*
In addition to activated carbon, alternative adsorbents such as zeolites [48] and cyclodextrins [49] exist for OMP removal from wastewater. These alternatives can offer a lower carbon footprint, as activated carbon originates from fossil fuels. However, their efficacy is not yet proven, and they have undergone only lab- and pilot-scale testing. Alternative sources for manufacturing activated carbon could also reduce the carbon footprint of activated carbon.

### Membrane filtration technologies

Membrane filtration processes utilise pressure to force water molecules through a semi-permeable membrane, effectively retaining dissolved solids, particles, and other undesirable substances. This results in purified water on one side of the membrane (permeate) and concentrated pollutants on the other side (concentrate or retentate). Membrane filtration is thus a technology that *separates* OMPs; it does not degrade them. The filtration mechanism depends on many parameters and the type of membrane, but mainly on the sizes of the components and the dimensions of the pores in the membrane; the membrane retains components larger than its pore sizes [50].

A schematic overview of the filtration technologies and the corresponding targeted components for removal are shown in Figure 3-6. Two membrane filtration technologies capable of removing OMPs are Nanofiltration and Reverse Osmosis.

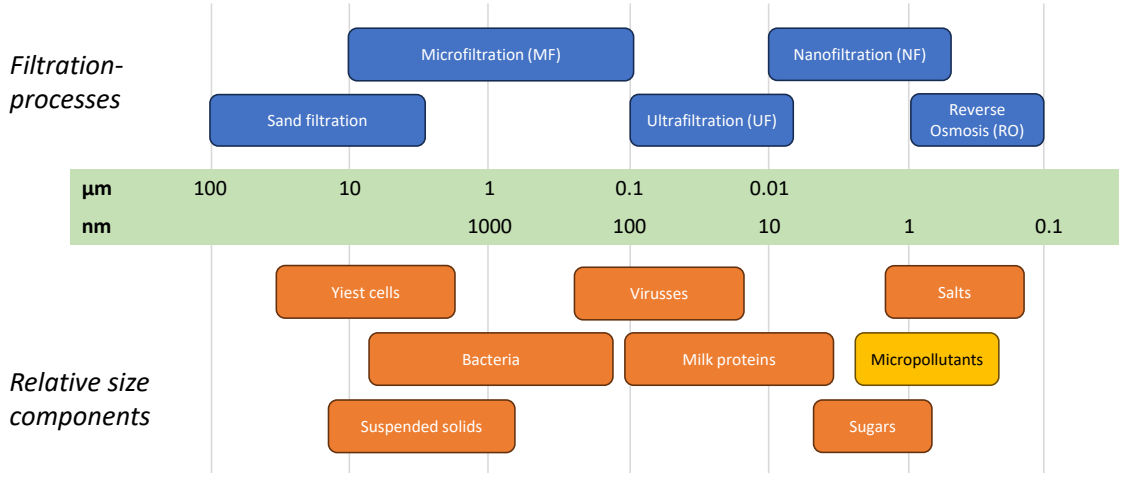



***Figure 3-6 Schematic overview of filtration technologies illustrating the relative sizes of components (including organic micropollutants) targeted for removal by each technique.***

*Nanofiltration (NF)*
Nanofiltration selectively retains components in solutions at the nanometer scale, roughly corresponding to molecules with a molecular weight between 200 and 1000 Daltons, the size range of most OMPs. As such, nanofiltration presents a promising technique for removing OMPs from WWTP effluents. Furthermore, nanofiltration effectively removes viruses and bacteria, providing an additional benefit. It also removes ions, particularly divalent ions, and various other components [50].

Conventional nanofiltration processes typically utilize spiral-wound modules featuring flat-sheet nanofiltration membranes wound around a tube. However, this configuration poses challenges for wastewater treatment as the membranes are prone to clogging or require costly pre-treatment. A recent advancement overcoming these limitations involves a new type of nanofiltration membrane designed in a capillary/hollow-fibre geometry (capNF) [51]

Most research on OMP removal by nanofiltration is conducted at the lab scale. Tests demonstrate that conventional dense nanofiltration membranes retain OMPs between 30% and 90%, depending on the type of OMP. Pilot studies employing capNF membranes at WWTPs have shown similar OMP retention [26].

Challenges include the disposal of the concentrate stream containing the retained OMPs and determining how nanofiltration techniques can be implemented cost-effectively. The OMPs in the concentrate stream could be degraded or captured with other technologies, before recycling the concentrate stream back to the conventional WWTP [26].

*Reverse Osmosis (RO)*
Reverse Osmosis (RO) selectively retains components in solutions with sizes even smaller than those retained by NF, including monovalent ions [50]. RO finds applications in industrial processes and in producing potable (i.e., drinking) water. It is highly effective in removing OMPs from WWTP effluents, as demonstrated by various demonstration plants [52]. An additional benefit is the elimination of viruses and bacteria [50].

Similar to NF, RO processes typically employ spiral-wound modules with flat-sheet RO membranes wound around a tube. However, this configuration presents challenges for wastewater treatment due to membrane clogging or the need for costly pre-treatment [50].

The primary drawback of RO is its high operational costs. Since the pores of RO membranes are smaller than those of NF membranes, RO requires higher pressures to pump water through the membranes. This is because RO membranes can retain monovalent ions, resulting in higher osmotic pressure between the feed and permeate sides than NF processes. To operate, the operating pressure must exceed the difference in osmotic pressure between the feed and permeate sides. Another reason for the high operational costs is the

susceptibility of RO membranes to fouling. Therefore, a pre-treatment step is necessary before RO membranes can efficiently purify WWTP effluent. [50].

### 3.2.3 Technologies of partners

The TKI EffluentFit4Food project consortium comprises two technology partners: PureBlue (The Netherlands) and Pharem (Sweden). This paragraph provides an overview of their respective technologies for removing OMPs.

*MicroForce++ (PureBlue)*
PureBlue is a Dutch company specialising in sustainable, innovative water treatment technologies. Technologies that PureBlue has in its portfolio include ozonation, biofiltration and hydrogen peroxide in combination with UV. Products of PureBlue consist, in general, of a combination of several technologies [53].

MicroForce$^{++}$ is a PureBlue product designed to remove harmful substances from wastewater, such as OMPs. It consists of two ozonation/bioreactor combinations connected in series. Each ozonation/bioreactor combination uses a specific ozone dose. This ozone dose breaks down non-biodegradable molecules into smaller, biodegradable molecules, which are degraded in the successive bioreactor.

A feasibility study conducted on MicroForce$^{++}$ demonstrated its strong performance across various criteria, including OMP removal efficiency, carbon footprint, and costs. The study encompassed both lab-scale tests and pilots at WWTPs [27]. The removal efficiencies of indicator substances are generally higher than 80%, varying depending on the type of OMP. For at least 7 of the 11 indicator substances (Dutch: gidsstoffen), the removal efficiency ranges from 85% to 95% [9]. Furthermore, MicroForce$^{++}$ is highly scalable and adaptable to the capacity of any WWTP. Its compact design with short residence times makes it a space-efficient solution.

Current studies on MicroForce$^{++}$ are promising, and therefore, PureBlue aims to make MicroForce$^{++}$ a standardised water treatment.

*Zymatic (Pharem)*
Pharem Biotech is a Swedish company specialising in water treatment and biotechnology. Pharem uses new biotechnology and enzymatic catalysis developments to remove organic pollutants in municipal treatment plants and industrial processes [54].

One of Pharem's innovative technologies is Zymatic, which utilizes enzymes on a carrier material to break down harmful components in WWTP effluent. Unlike many conventional wastewater treatment technologies, enzyme technologies are not limited to a single treatment mechanism but can have multiple reaction mechanisms to target different OMPs simultaneously. By selecting specific enzymes, a custom-made water treatment process can be created to suit wastewater streams with specific OMPs [41].

Zymatic employs immobilization techniques to bind the enzyme mix to a carrier material. This carrier material with enzymes is utilized as a downstream purification step in a WWTP through a column arrangement similar to a sand filter, facilitating easy scalability [40]. A feasibility study at a WWTPs in Sweden showed removal efficiencies ranging between 13% and 91%, depending on the type of OMP, which is similar to competing technologies [40].

Enzyme-based water treatment technologies currently have a lower Technical Readiness Level compared to other more mature technologies. However, initial tests indicate significant potential for this emerging technology.

### 3.2.4 Overview technologies

Table 3-1 provides an overview of water treatment technologies capable of removing OMPs from WWTP effluent. Technologies included are Conventional Activated Sludge (CAS), capillary nanofiltration (CapNF), spiral-wound nanofiltration (NF SW), Reverse Osmosis (RO), Powdered Activated Carbon (PAC), Powdered

Activated Carbon in Activated Sludge (PACAS), Granular Activated Carbon (GAC), Ozone, $H_2O_2$+UV, constructed wetlands, and the technologies MicroForce$^{++}$ and Zymetic.

Please note that the characteristics presented in the table are subject to variation based on process parameters, types of OMPs, and calculation methods used. For instance, some studies may monitor a broad range of OMPs, while others may focus on a fixed set of components. Similarly, different assumptions regarding costs, such as electricity, capital investment, maintenance, and operating costs, may vary among studies.

***Table 3-1 Overview of removal efficiencies, costs and carbon footprint of technologies for removing organic micropollutants (OMPs).***

| Technology | Removal efficiency OMPs, average* | Removal efficiency OMPs, range* | Costs (€/m$^3$ water) | Carbon footprint (g$CO_2$/m$^3$ water) | Reference |
|---|---|---|---|---|---|
| CAS | 23%[a] | 0–55%[a] | | | [26] |
| NF (CapNF) | 80%[b] | 47-97%[b] | 0.43-0.53 | 200-299 | [26] |
| NF (SW) | 82%[b] | 71-92%[b] | 0.20-0.40 | | [26, 55] |
| RO | 97%[b] | 75-100%[b] | 0.45 | | [26, 56] |
| PAC (+cloth) | 55-92% | | 0.15 | 113 | [57] |
| PACAS | 60–80%[a] | 35–100%[a] | 0.03-0.08 | 116 | [47] |
| GAC | 80-85% | 44-98% | 0.26 | 325 | [20, 58] |
| Ozone | 86% | 25-98% | 0.12 | 150 | [30] |
| $H_2O_2$+UV | 41% | 3-92% | 0.29 | 600 | [30] |
| Constructed wetlands | 52-61%[b] | 8-98%[b] | | | [33, 59] |
| MicroForce$^{++}$ | 85-95% | | 0.06-0.07 | 59 | [27] |
| Zymetic | 56-84% | 13-99% | N/A | N/A | [40] |

* Removal efficiencies, as determined by pilot studies, are typically calculated by comparing the effluent concentrations of the WWTP with those of the pilot system. If specified otherwise, the removal efficiencies are determined using one of the following methods: a) Comparison of influent to the WWTP with the effluent from the pilot system. b) Laboratory studies using artificial water streams.

In general, the table illustrates that all additional technologies can enhance the removal efficiency of OMPs compared to conventional activated sludge (CAS) WWTP. However, it's important to note that each technology exhibits a range of removal efficiencies, which are influenced by the type of OMP and process conditions. Achieving complete removal of all OMPs using a single technology does not appear to be possible.

Regarding costs, there are variations among the different technologies. The cost of treating a cubic meter of water with an additional technology ranges from €0.06 to €0.53. Membrane filtration methods (NF, RO) tend to have slightly higher costs compared to other technologies. However, it is worth mentioning that these membrane filtration technologies offer added value by also removing viruses, bacteria, ions, and other components. On the other hand, the lowest costs are associated with MicroForce$^{++}$ technology (Ozonation+biofiltration) and PACAS. MicroForce$^{++}$ technology is therefore a sustainable alternative for stand-alone ozonation; MicroForce$^{++}$ consumes ca. 50% less ozone due to the biological filtration.

In terms of carbon footprint, most technologies exhibit values falling between 100-300 g $CO_2$/m$^3$ water. The use of peroxide in combination with UV treatment results in a higher carbon footprint of 600 $CO_2$/m$^3$ water. Conversely, MicroForce$^{++}$ technology demonstrates the lowest carbon footprint among the considered technologies.

## 3.3 Research plan of water treatment technologies

The technologies outlined in Table 3-1 demonstrate significant potential for removing OMPs from wastewater, including the technologies developed by the partners in this project, namely MicroForce$^{++}$ by PureBlue and Zymatic by Pharem. Consequently, the technologies available within this consortium offer a solid foundation for developing strategies to address OMP removal from WWTP effluents. Given that no single technology can

entirely eliminate the full spectrum of OMPs, the proposed approach is to utilize MicroForce$^{++}$ (ozonation+biofiltration) as the first technology for OMP removal, followed by the implementation of Zymatic to target the remaining OMPs.

In this project, other technologies are not further considered, as *separation* technologies (NF, RO) and *capture* technologies (PAC, PACAS, GAC) do not degrade OMPs. Consequently, they still necessitate additional *degradation* technologies. The proposed sequence of ozonation followed by biofiltration and enzymatic catalysis should sufficiently degrade OMPs without the requirement for additional technologies. $H_2O_2$+UV is not considered, as ozonation, the competitive AOP technology, demonstrates superior performance in terms of OMP removal, but also costs and carbon footprint.

We first focus on the proven ozone technology. MicroForce$^{++}$ is later introduced in the project as an upgrade to ozonation to make the process cheaper and more sustainable, while also reducing bromate formation, among other benefits. This approach allows for comparable removal efficiencies to stand-alone ozonation.

Since Zymatic can be tailored to target specific components based on enzyme selection, the plan is to deploy Zymatic to address the OMPs that are less effectively removed by ozonation and the MicroForce$^{++}$. By combining the capabilities of MicroForce$^{++}$ and Zymatic, the goal is to achieve comprehensive OMP removal.

At present, the Technical Readiness Level (TRL) differs for the two technologies. While MicroForce$^{++}$ is already available at pilot-scale for deployment at WWTPs, Zymatic will initially rely on insights gained from MicroForce$^{++}$ trials to select enzymes for addressing the remaining OMPs. Consequently, MicroForce$^{++}$ will be implemented as a pilot installation at WWTP Walcheren, while Zymatic will focus at a selection of OMPs with reduced concentrations following treatment by MicroForce$^{++}$.

# 4 Evaluation of treatment technologies for organic micropollutant removal

## 4.1 Introduction

This chapter discusses the results of pilot setups used to treat effluent from the WWTP Walcheren in Ritthem, Netherlands, aimed at removing organic micropollutants (OMPs). The objective was to investigate the extent to which OMPs can be removed from WWTP effluent. As mentioned in the previous chapter, the technologies chosen for further investigation were ozonation (MicroForce$^{++}$) and enzymatic catalysis.

In the first phase of the project, a pilot study was conducted using standard ozonation, followed by ozonation combined with UV. Ozonation is one of the most promising technologies for removing OMPs from wastewater. However, a key concern with chemical treatments for OMPs removal is the formation of by-products that could increase the ecotoxicity of treated water. The most concerning by-product is bromate, which has been classified as potentially carcinogenic to humans [60]. Given that the WWTP Walcheren is located in a coastal area, higher concentrations of bromide, which can be transformed into bromate, were observed. To minimize bromate formation, the ozone dose must be kept as low as possible, while still ensuring sufficient removal of OMPs. Samples of both the raw effluent and effluent treated with ozone and ozone-UV were collected and later used in Work Package 6 to irrigate crops (Chapter 5), after which OMP uptake by the crops was analyzed (Chapter 6).

In addition to by-product formation, chemical oxidation processes are known to be energy-intensive. This has shifted the focus towards environmentally friendly, innovative solutions, such as hybrid systems that combine physical, chemical, and biological processes. These systems aim to improve the limited removal of pharmaceutical residues and other OMPs in conventional WWTPs, expand the range of OMPs removed, and reduce the costs associated with physicochemical treatments. One such hybrid technology is MicroForce$^{++}$, developed by PureBlue Water, which combines ozonation with biological filtration. This technology was found to be a promising, sustainable, cost-effective, and scalable solution for OMP removal. However, at the time this project began, MicroForce$^{++}$ was not yet operational, and a pilot study with this technology was performed in a later stage of the project. Due to time constraints and pilot availability, no effluent from MicroForce$^{++}$ was used for crop irrigation in this research.

An analytical method was selected to identify unknown components in both untreated and treated WWTP effluent, as the specific OMPs present in WWTP effluent were not known, making it difficult to determine which components needed to be quantified.

Unfortunately, no results were obtained for enzymatic catalysis by Pharem in this study.

## 4.2 Methods and materials

In this paragraph, the methodology used to treat and analyse OMPs in the WWTP effluent is described.

### 4.2.1 Ozone pilot and UV treatment

The technology used to treat the WWTP effluent with stand-alone ozonation and UV treatment was provided by PureBlue Water. The pilot installation has a hydraulic capacity of 1-7 $m^3$/h and a dosing range between 7 and 60 g $O_3$/h. The pilot installation is shown in Figure 4-1.

***Figure 4-1 Pilot installation of ozone plus UV treatment on-site at WWTP Walcheren (left) and in workshop PureBlue Water (right).***

To produce irrigation water for the crops, an ozone dose of 0.9 g $O_3$/g DOC was applied. This relatively high dose was chosen to amplify the difference in OMP concentrations between untreated and treated effluent. Based on previous research and PureBlue's experience, this dose is expected to achieve significant and sufficient OMP removal to meet STOWA's targets for indicator substances.

The pilot installation was also equipped with a UV reactor to investigate the effect of disinfection on the quality of the treated effluent. This was mainly introduced to lower the microbiological pressure of the water, since the applied UV dose (ca. 100 mJ/cm$^2$) is not sufficient to accelerate the removal of OMPs. However, given the long-term storage of the treated effluent before application and use for crop irrigation, the extra disinfecting step may have missed its effect as there is a high risk of bacterial recolonization.

A schematic process flow diagram of the ozone pilot is shown in Figure 4-2.

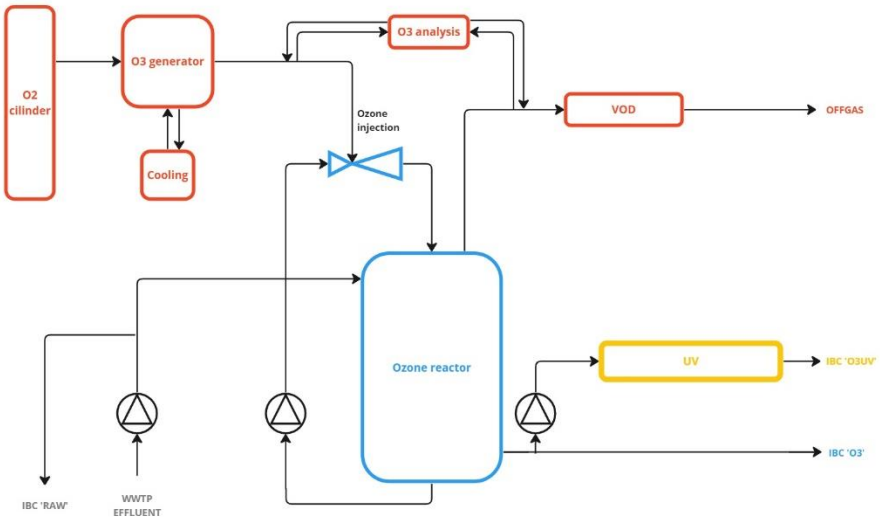



***Figure 4-2 Process flow diagram of the mobile stand-alone ozone pilot + UV.***

### 4.2.2 Pilot location

The pilot was located at the WWTP Walcheren in Ritthem, Zeeland, The Netherlands (Figure 4-3). This facility serves a population equivalent of around 180,000 and is operated by Waterschap Scheldestromen.


***Figure 4-3 Location of the pilot in the province of Zeeland, Netherlands (left) and aerial picture of WWTP Walcheren (right).***

### 4.2.3 Scope of the pilot tests

The following water qualities were produced and used to irrigate the crops:

- Effluent of the WWTP Walcheren (OMPs measured);
- Effluent of the WWTP Walcheren treated with ozone (no OMPs measured);
- Effluent of the WWTP Walcheren treated with ozone and UV (OMPs measured).

The different water qualities that were produced were stored in IBCs, covered to protect the water against sunlight and temperature variations, and transported to Randwijk where the water was used to irrigate the crops. Figure 4-4 gives an impression of the IBCs with cover. With each batch the crops have been irrigated for a duration of two weeks, between April and August 2022.

For both the WWTP effluent and the effluent treated with ozone plus UV the OMPs were analysed using the library screening. Following from the necessity to provide water on a bi-weekly basis to irrigate the crops also the analysis of the OMPs was done bi-weekly from April to August 2022.


***Figure 4-4 IBC with the treated and untreated effluent covered for protection against light and heat.***

The dates when the samples were taken from the raw effluent and from the effluent treated with ozone plus UV are shown in Table 4-1. It can be seen that more analyses were done on the treated effluent than on the raw effluent, and not all dates correspond. To analyse the results of the measurements of the relative concentrations of OMPs, the average values of the different samples of the raw effluent were compared to the average values of the treated effluent and a removal efficiency for each component was calculated. In addition, for the samples taken on the 4$^{th}$ of May and on the 9$^{th}$ of August the concentrations before and after treatment were compared and removal efficiencies were calculated. These results were used to verify the reliability of the average concentrations.

***Table 4-1 Dates of the analysis of raw effluent and treated effluent with ozone plus UV.***

| Date analysis raw effluent (dd-mm-yyyy) | Date analysis treated effluent (dd-mm-yyyy) |
|---|---|
| 09-03-2022 | 04-05-2022 |
| 30-03-2022 | 05-06-2022 |
| 14-04-2022 | 29-06-2022 |
| 04-05-2022 | 12-07-2022 |
| 09-08-2022 | 27-07-2022 |
| | 09-08-2022 |
| | 24-08-2022 |

Next to a visual comparison and calculated standard deviations, T-tests were done to compare average values for each component before and after treatment and a conclusion was drawn regarding the significance of the differences between the means.

Bromide was analysed on the raw effluent of the WWTP and bromate was analysed on the effluent treated with ozone plus UV at a target ozone dose of 0.9 g $O_3$/g DOC. During the ozonation, no specific mitigation strategies for bromate formation were applied in this project, since the effect of potential bromate accumulation in crops was not identified as a potential risk at the start of the project. The results of the several analyses on the formation of bromate are shown in paragraph 4.3.

### 4.2.4 Analysis of broad spectrum micropollutants with UPLC-QTOF library screening

For the analysis of the a broad spectrum of OMPs a UPLC-QTOF library screening was developed by the independent analytical laboratory Aqualab Zuid. The library consists of over 2000 components and a Schymanski level of 2-4 can be achieved [61]. The list of components, or suspects, consists of mainly herbicides, pesticides and pharmaceuticals of which the exact mass and retention time are known. Because of the know retention time a Schymanski level of 2, which represents a probable structure by library spectrum match, can often be achieved [62]. The reported concentrations are relative, meaning the exact concentrations are unknown and the concentrations between the substances cannot be compared. However, the concentrations of one component can be compared between different analyses making it possible to compare concentrations of one component before and after treatment and to calculate removal efficiencies.

### 4.2.5 Analysis of target indicator substances

In addition to the comparison between individual components and the removal efficiencies, the guideline described by Stichting Toegepast Onderzoek Waterbeheer (STOWA) which mentions 11 indicator substances to evaluate technologies to remove OMPs was used to determine the required ozone dose, and to evaluate the performance of the MicroForce$^{++}$. The 11 indicator substances are shown in Table 4-2.

***Table 4-2 List of the 11 guiding substances for evaluating the performance of different technologies** [63].*

| Component |
|---|
| 4- en/of 5-Methyl-1H-benzotriazole |
| Carbamazepine |
| Diclofenac |
| Gabapentin |
| Hydrochlorothiazide |
| Irbesartan |
| Metoprolol |
| Sotalol |
| Trimethoprim |
| Venlafaxine |
| 1,2,3-benzotriazole |

Furthermore, STOWA states that a technology can be considered to work efficiently if more than 70% on average of (at least) 7 out of the 11 guiding substances can be removed by the WWTP, meaning conventional WWTP plus possible quaternary treatment during dry weather conditions [63]. However, STOWA considers a more ambitious threshold of 80% removal. The target of 80% target was used as guideline throughout this pilot study to ensure sufficient OMP removal.

## 4.3 Results and discussion

In this paragraph, the results of the ozone pilot and UV treatment are shown.

### 4.3.1 Organic micropollutants (OMPs)

The results of all the measurements of the library screening are shown in Annex 3. In the raw effluent 84 components were detected and of these 84 components 56 were not found in the effluent treated with ozone plus UV, while in the treated effluent 40 components were detected. This means that 12 components were found in the treated effluent while they were not detected in the raw effluent. A possible reason for this could be that the samples of the raw effluent and of the treated effluent were taken on different days, and averages were used to analyse the results. For the components that were not detected in the treated effluent, it cannot be concluded that these components were removed for 100% because the components might still be present although below the detection limit. Also the library screening differs from target analysis in that sense that if a component is not found, it cannot be concluded with full confidence that the component is not present, but the library screening was simply not able to detect the component. In addition, for the components that seem to spike in the analysis it is not fully certain that they are actually present, as two or more other components may give a similar spike and are incorrectly identified. However, since a Schymanski level of 2 can often be achieved by the library screening, the detected component are probably present in the effluent.

Figure 4-5, Figure 4-6 and Figure 4-7 show the average relative concentrations of the components detected in both the raw effluent and in the effluent treated with ozone plus UV. The error bars represent the standard deviations. If no error bar is shown it means that only in one sample the component was detected. The components are shown in different ranges (below 0.3 µg/L, between 0.3 and 1 µg/L and above 1 µg/L) to enhance the readability of the graphs. Although the relative concentrations can be used to compare the concentrations before and after treatment, and to calculated removal efficiencies, they cannot be used to compare the concentrations of different substances. For example, it cannot be concluded that 1H-benzotriazole is present in a higher concentration in the raw effluent than Fluconazole.

For Adenosine, Caffeine, Cotinine, Fluconazole, Histamine and Theobromine no standard deviations are shown for both the raw effluent and the treated effluent. There is low confidence that the differences before and after treatment can be compared and can be used to calculate average removal efficiencies. Only for the 4$^{th}$ of May and the 9$^{th}$ of August analyses were done of the raw effluent before treatments and after treatment so the removal efficiencies found on these days are considered more reliable.

By visually analysing the graphs, for Melamine, DEET, Adenine, Metformin, 1H-benzotriazole and Ritalic acid it is difficult to say if the average relative concentration before and after treatment show a significant difference. For this reason T-test were performed ($\alpha = 0.05$, equal and unequal variances varying per component based on the outcome of F-tests ($\alpha = 0.05$)). For DEET, Adenine and Ritalinic acid it cannot be concluded that the differences between the means are significant. For this reason, calculated removal efficiencies for these components are considered unreliable.

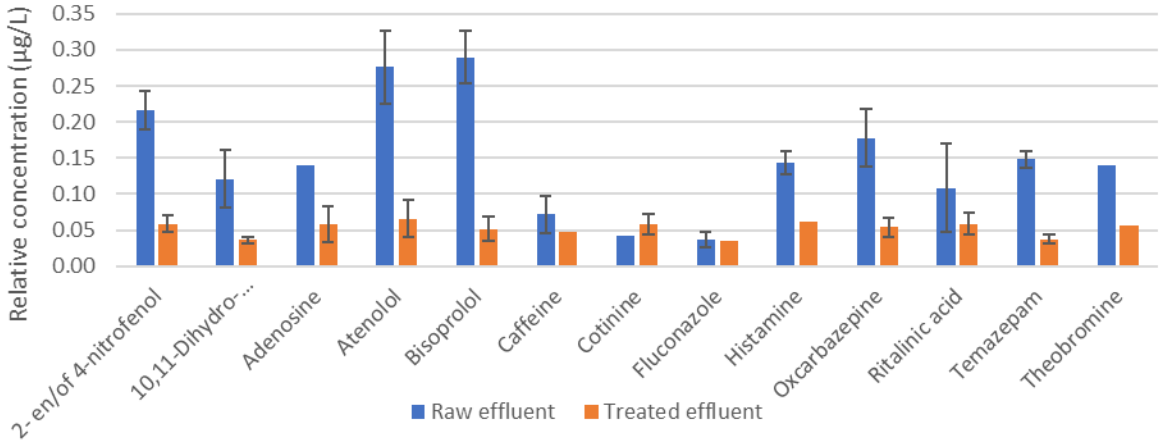



***Figure 4-5*** ***Relative concentrations of the organic micropollutants before and after treatment with ozone plus UV in the range below 0.3 µg/L. The error bars represent standard deviations.***

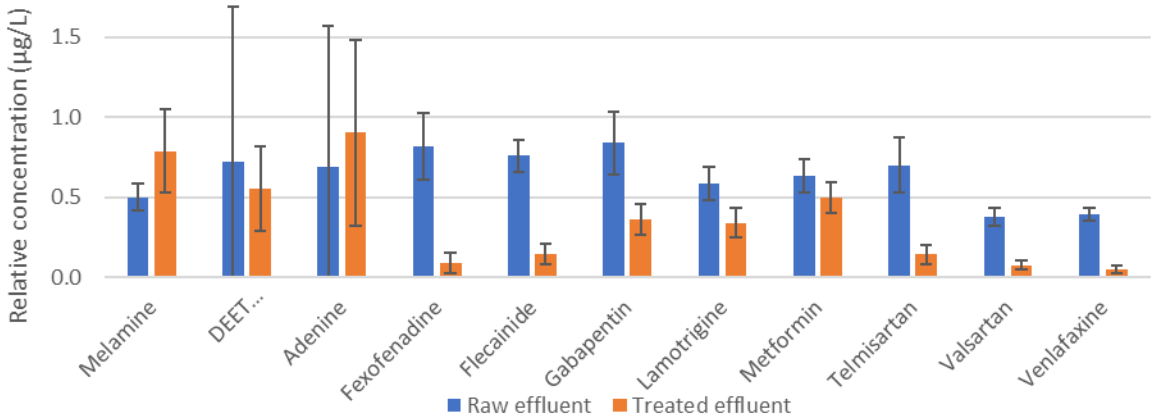



***Figure 4-6*** ***Relative concentrations of the organic micropollutants before and after treatment with ozone plus UV in the range between 0.3 and 1 µg/L. The error bars represent standard deviations.***

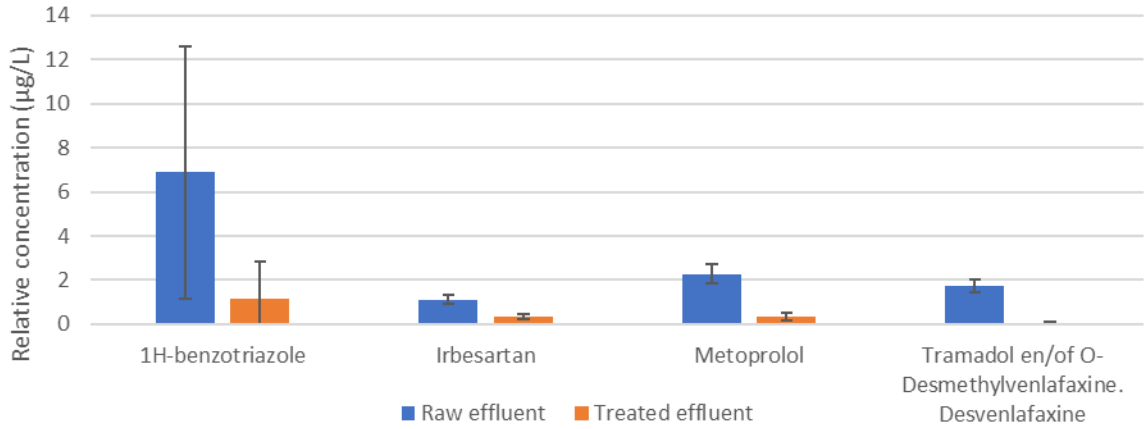



***Figure 4-7*** ***Relative concentrations of the organic micropollutants before and after treatment with ozone plus UV in the range higher than 1 µg/L. The error bars represent standard deviations.***

For the components found in the raw effluent and in the treated effluent, the removal efficiencies were calculated for treatment with ozone plus UV. The results are shown in Table 4-3. The average removal efficiencies based on just one result are considered unreliable, and highlighted in bold. All the removal efficiencies of 100% were removed since the relative concentration might have been below detection limit giving the wrong impression about a full removal of the component. For Adenine all the removal efficiencies

are negative numbers and are therefore considered to be unreliable and suggests that adenine is difficult to measure with the library screening resulting in large standard deviations as can be seen in Figure 4-6. The same conclusion can be drawn for Melamine. For Cotinine the average value of the raw effluent is based on only one measurement and therefore considered unreliable.

***Table 4-3 Removal efficiencies of treatment with ozone plus UV, unreliable average removal efficiencies highlighted in bold (based on only one measurement of insignificant difference between the means).***

| Component | Removal efficiency (%) | | |
|---|---|---|---|
| | Average | 4-5-2023 | 9-8-2022 |
| 1H-benzotriazole | 83% | 71% | 85% |
| 2- en/of 4-nitrofenol | 73% | | 58% |
| Melamine | **-58%** | 9% | **-5%** |
| DEET (Diethyltoluamide) | **24%** | 66% | 65% |
| 10,11-Dihydro-10,11-dihydroxy carbamazepine | 70% | 100% | 78% |
| Adenine | **-31%** | **-507%** | **-76%** |
| Adenosine | **58%** | 51% | |
| Atenolol | 76% | | 82% |
| Bisoprolol | 82% | | 84% |
| Caffeine | **35%** | | |
| Cotinine | **-39%** | | |
| Fexofenadine | 89% | 95% | 93% |
| Flecainide | 81% | 92% | 82% |
| Fluconazole | **7%** | | 22% |
| Gabapentin | 57% | 62% | 52% |
| Histamine | **57%** | 62% | |
| Irbesartan | 69% | 76% | 66% |
| Lamotrigine | 42% | 57% | 43% |
| Metformin | 21% | 29% | 25% |
| Metoprolol | 85% | 93% | 85% |
| Oxcarbazepine | 70% | 77% | 78% |
| Ritalinic acid | **46%** | 67% | 65% |
| Telmisartan | 79% | 89% | 81% |
| Temazepam | 75% | 75% | 75% |
| Theobromine | 60% | | |
| Tramadol en/of O-Desmethylvenlafaxine. Desvenlafaxine | 96% | | 98% |
| Valsartan | 80% | | 78% |
| Venlafaxine | 88% | | 92% |

The majority of the OMPs are removed efficiently with the ozonation and UV treatment, only lamotrigine, metformin, adenosine, fluconazole and gabapentin have removal efficiencies below 60%. For all the OMPs with removal efficiencies shown in bold, it can only with a low confidence level be concluded that the removal is low.

### 4.3.2 Bromate

Bromide in the raw effluent and bromate in the water treated with ozone were analysed on different sampling days. The results are given in Figure 4-8. Bromate concentrations fluctuate between 20 and 50 µg/L. These results fall within expectations given the fact that very high ozone doses were applied (0.7 g $O_3$/g DOC for first test and 0.9 g $O_3$/g DOC for all other tests) and no mitigation measures were taking in the stand-alone ozonation pilot. Furthermore, a venturi side stream injection of ozone was used, which is known for its high tendency to generate bromate. Next to that, bromide concentrations in the WWTP are found to be very high at WWTP Walcheren due to its coastal geographical location.

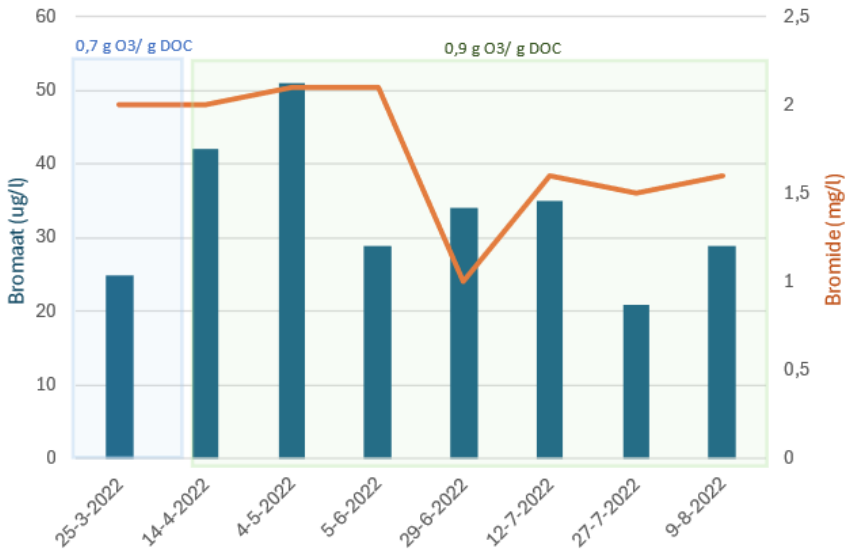



***Figure 4-8 Results bromide and bromate during sampling days. First test day at ozone dose 0.7 g $O_3$/g DOC, all other test days 0.9 g $O_3$/g DOC, with compact ozone pilot using venturi injection and using no bromate mitigation strategies.***

## 4.4 MicroForce$^{++}$ as a sustainable and cost-effective alternative for ozonation

As mentioned before, stand-alone ozonation is a well-established technology that can efficiently remove medicinal residues from WWTP effluent. In order to lower the carbon footprint and financial impact of the post-treatment, a more sustainable hybrid ozone technology was tested, called MicroForce$^{++}$. More information about the MicroForce$^{++}$ technology, and the tests done on the effluent on WWTP Walcheren can be found in the report STOWA 2023-49 Rapport Pilotonderzoek MicroForce$^{++}$ [64].

Furthermore, this research revealed that MicroForce$^{++}$ excels in terms of $CO_2$ footprint and Total Costs of Ownership (TCO). The $CO_2$ footprint is 66 g $CO_2/m^3$, representing a reduction of approximately 50% compared to the reference technology (130 g $CO_2/m^3$ for Ozone + sand filtration). The primary savings in $CO_2$ footprint result from the lower ozone dosage (0.43 g $O_3$/g DOC compared to 0.9 g $O_3$/g DOC for stand-alone ozonation [64]) required for component removal, allowing for lower energy and resource consumption. Additionally, the sludge production or the use of backwash water by the biofilm reactors makes a negligible contribution to the overall $CO_2$ footprint.

The estimated cost of MicroForce$^{++}$ is approximately 0.10 €/$m^3$ (price level of 2018), which is a reduction of about 40% compared to the reference technology using ozone in combination with a sand filter. This cost reduction is primarily attributed to the lower ozone demand and, consequently, reduced energy consumption.

Finally, this study demonstrated that the modular concept of MicroForce$^{++}$ allows for high adaptability at wastewater treatment hotspots in the Netherlands. MicroForce$^{++}$ aims to be a standardised water treatment technology that can be easily installed and comes in various available formats to provide a solution regardless of the size of the WWTP. The compact design is made possible by the short residence time in the biological system and intelligent ozone dosing.

## 4.5 Conclusions

It can be concluded that post-treatment with ozone and UV significantly improves the effluent quality in terms of OMPs. This means the treated effluent used for irrigation had significantly less OMPs. The effect of this on crop accumulation, toxicity and crop yield will be investigated in WP6 (Chapters 5 and 6).

84 components were detected in the raw effluent. Of these 84 components, 56 were not detected in the effluent treated with ozone plus UV, while 40 components were detected in the treated effluent.

The library screening gives a good indication of the removal efficiencies achieved for the OMPs found in both the raw effluent and the effluent treated with ozone plus UV. Most OMPs are efficiently removed with ozonation and UV treatment; only lamotrigine, metformin, adenosine, fluconazole, and gabapentin have removal efficiencies below 60%.

During the experiments with the pilot, bromate formation occurred, with measured concentrations between 21 and 51 µg/L. These results fall within expectations given the fact that very high ozone doses were applied and no mitigation measures were taken in the stand-alone ozonation pilot (i.e. venturi ozone injection).

MicroForce$^{++}$ technology can be considered a sustainable and cost-effective alternative for stand-alone ozonation. It results in a 50% lower $CO_2$ footprint, a 40% cost reduction (0.10 €/$m^3$, price level of 2018), and bromate levels below 0.2 µg/L.

# 5 Effluent exposure study on potato, onion and pear cultivation

## 5.1 Introduction

In Work Package 6 (WP6) of this project, we investigated the extent to which organic micropollutants (OMPs) from effluent originating from WWTP Walcheren enter the crop products of potatoes, onions, and pears through drip irrigation, as well as the effect of effluent treatments with ozone and/or UV on reducing this uptake.

This chapter details the cultivation experiments, where the crops were irrigated with various water quality types, including effluent, treated effluents (as described in Chapter 4), and tap water. We assessed plant vitality and crop yields to determine the impact of irrigation water quality. The harvested crops were analyzed for OMP uptake in a subsequent part of the project (Chapter 6).

## 5.2 Methods and materials

### 5.2.1 Description of effluents

In an exposure test, three types of effluents, and tap water were tested for the uptake of contaminants and pathogens in the crop products of pear, potato, and onion. The different irrigation waters were:

1. Raw effluent WWTP Walcheren;
2. (reserve; tap water WUR-location Randwijk);
3. Effluent WWTP Walcheren additionally treated with ozone;
4. Effluent WWTP Walcheren additionally treated with ozone and UV;
5. Tap water WUR-location Randwijk.

Details of the different effluent streams and the impact of the treatment technologies on organic micropollutant removal can be found in Chapter 4. Note that irrigation water type 2 was initially planned to be effluent treated with the MicroForce$^{++}$ technology; however, as the pilot was not operational at the start of the cultivation season, this option was omitted.

The effluent and treated effluents were transported from WWTP Walcheren to Randwijk. On April 12, May 12, June 24, and July 13, 2022, one or more IBC tanks with effluents from Zeeland were received. Per treatment, one IBC tank was placed on the test field for drip irrigation. The IBC tanks were placed in insulating covers (Figure 5-1 and Figure 5-2). The remaining IBC tanks were stored in a storage shed until they were also needed.

***Figure 5-1 The effluents in IBC tanks ready for transport at the Ritthem location.***

***Figure 5-2 IBC tanks with different types of irrigation water (left) and the cabinet for the control unit for the irrigation.***

### 5.2.2 Experimental setup

The experimental layout is shown in Figure 5-3. The experiment was originally set up for five treatments but was ultimately conducted with four treatments. It was first divided into three sections for the three crops (pear, onion, and potato). The field experiment was further structured for each crop as a block trial, featuring four blocks per irrigation type. The different irrigation water types were randomly assigned along the blocks.

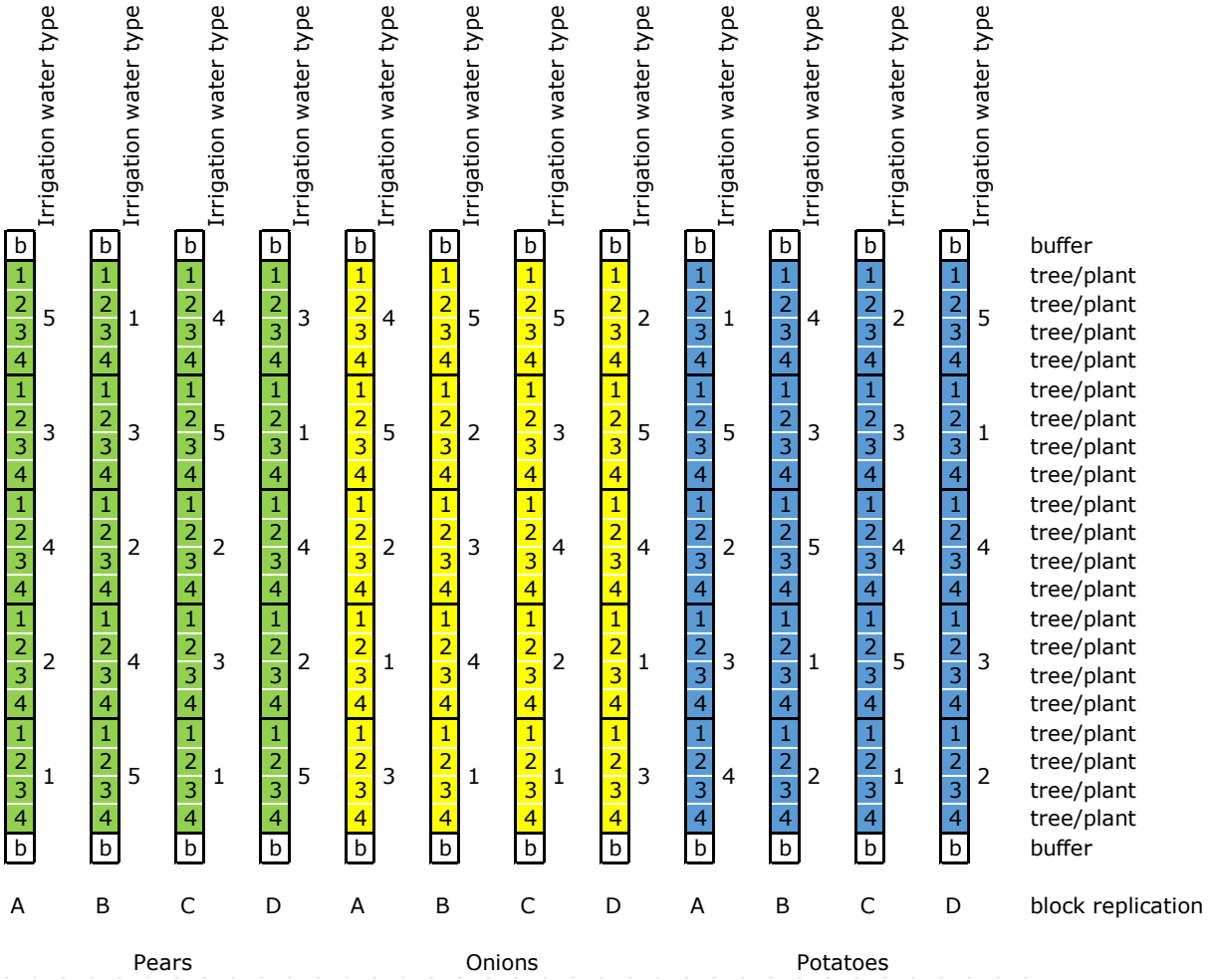



***Figure 5-3*** ***The experimental layout of the cultivation field consists of cells, each containing a pot. The numbers inside the cells (1, 2, 3, 4) indicate the repetitions within a block. The letters A, B, C, or D represent the different block repetitions. The number next to a block (1, 2, 3, 4, 5) denotes the type of irrigation water used for that block.***

### 5.2.3 Substrates

The substrate for the experiment consisted of coarse sand. Compared to clay soil as a substrate, this substrate has less influence on the composition of the effluent in the substrate: the effluent reaches the roots faster, and coarse sand is likely less chemically active than clay soil. The pH of the substrate affects the effluent. Therefore, a high pH was chosen, which is also commonly found in the soils of Zeeland.

For the pear crop, trees were planted in pots a year earlier, in 2021, to have more fruit on the trees in the experimental year (Figure 5-4). The pots and substrate were identical to those in which the onions and potatoes were planted a year later (Figure 5-5). The onions and potatoes were planted on April 19. Five onions and one potato were planted per pot.

Because the pear trees' flowering and fruit set were disappointing, many trees were replaced with five-year-old trees from a trench cultivation system. These older trees were grown in a different substrate (DenOuden Substrado Trench Soil; for an analysis, see Annex 4). They were transplanted into larger pots, bringing along much of the DenOuden substrate. Additionally, the sandy substrate from the experiment was used to fill these pots. The pear trees from the trench cultivation system were transplanted into the pots on April 5 and 6. Only these older, well-bearing pear trees were used for observations and sampling.

***Figure 5-4 The pear crop in 2021, before the year with the effluent treatments.***

***Figure 5-5 Experimental overview on May 16, 2022.***

### 5.2.4 Fertilisation, irrigation and crop protection

Potato and onion were fertilised with Osmocote-Pro-High-K granules, which were mixed into the substrate before planting. The dosage and composition are shown in Table 5-1.

***Table 5-1 Fertilisation used for the cultivation of potato and onion.***

| Crop | # plants per pot | Pot volume (L) | Fertiliser type | Fertilizer amount (g/pot) | N/plant (g) | $P_2O_5$/plant (g) | $K_2O$/plant (g) |
|---|---|---|---|---|---|---|---|
| Potato | 1 | 15 | Osmocote Pro High K | 52.5 | 5.8 | 5.3 | 10.0 |
| Onion | 5 | 15 | Osmocote Pro High K | 15 | 1.7/5 plants | 1.5/5 plants | 2.9/ 5 plants |

The fertilisation of the older pear trees is shown in Table 5-2. The fertiliser granules were applied to the substrate on May 5.

***Table 5-2 Fertilisation used for the pear trees.***

| Fertiliser type | Fertilizer amount (g/pot) | N/plant (g) | $P_2O_5$/plant (g) | $K_2O$/plant (g) |
|---|---|---|---|---|
| Horti cote Topdress (21+5+10) | 86 | 18 | 4.3 | 8.6 |

Irrigation with the effluents and tap water was performed using an automated dosing system. The irrigation was done with five drippers per pot (see Figure 5-6). The water supply was adjusted to ensure there was always drain water coming out of the bottom of the pots. Occasionally, this was not the case for a few days.

***Figure 5-6 Five drippers per pot for irrigation.***

### 5.2.5 Unintended exposure

Unfortunately, the crops were unintentionally exposed to surface water by spray drift. The pear trees from the experiment were sprayed against night frost on April 3 and 27 with Lingewater for several hours at night, along with all other pear orchards in the experimental garden. The pots with potatoes and onions, planted on April 19, were not directly sprayed. However, because the air was very humid due to the spraying (containing tiny water droplets), spray water ended up on these pots on April 27 as well. It is also possible that some nearby pots with onions were sprayed when the pear trees from the experiment were sprayed.

On July 14 and 18 and August 11 to 14, the experimental gardens at Randwijk were sprayed daily for several hours with water from the Linge river during the day to prevent heat damage. The pear, potato, and onion plants from the experiment were not sprayed during this time. However, because the air was again humid from sprays, spray water likely ended up on all the crops in the experiment.

In the experimental year 2022, no sprays were applied to the three crops with crop protection products, foliar fertilizers, thinning agents, growth regulators, or other hormones. However, such treatments were

applied in the vicinity of the experiment, so it is possible that traces of these substances ended up on the three crops.

On July 21, it was reported from Zeeland that legionella contamination had been detected in the effluent. No alarming concentrations were found after sampling the effluent in Randwijk on July 25. At that time, the water temperature in the outdoor IBC storage containers was 24-25 °C.

### 5.2.6 Measurements and statistics

On June 21 and August 15, vitality scores were given per block or pot, with meanings ranging from 1 (almost dead) to 10 (very vital).The crops were harvested on August 22 and 23. For pears, fruits damaged by birds in the weeks leading up to harvest were removed.

The total weight of pears, potatoes, or onions per pot was measured during harvest. The number of products per pot was also counted for pears and onions, allowing the product weight to be calculated. For potatoes, only the weight per pot was measured because there was sometimes regrowth of tubers with very low weights. For pears, rotten fruits were not counted. In this trial, pear rot on the tree was mainly due to bird damage. A bird net was installed in the weeks leading up to harvest, and damaged pears were regularly removed.

Statistical calculations were performed using the Genstat program (22nd edition) via analysis of variance for each crop. Significance at p=0.05 is indicated by "S"; "NS" means "not significant at p=0.05". In cases of significance, letters were used to indicate which treatment effects differed significantly from each other: if all letters between two treatments were different, there was significance.

## 5.3 Results and discussion

### 5.3.1 Vitality

The crops had a good start to the growing season. However, on June 21, a decline in vitality was observed in some potato plots (Figure 5-7). This was recorded by assigning a vitality score to each plot. The same was done for the onions. For the general condition of the crops on June 21, see Figure 5-8. Additionally, on August 15, shortly before the harvest, another vitality score was assigned to all crops (for the condition of the crops on August 18, see Figure 5-9).

***Figure 5-7 Observed decline in vitality in some potato plants on June 21.***

***Figure 5-8 Condition of the pear (left), onion (middle) and potato (right) crops on June 21, 2022.***

***Figure 5-9 Condition of the crops on August 18, 2022.***

The results of the vitality assessments are shown in Table 5-3 and Table 5-4.

***Table 5-3 Vitality assessment on June 21, 2022. Significance is indicated by 'S' (significant) and 'NS' (not significant). For significant results, treatments are labeled with letters (A, B, C) to indicate differences: treatments sharing the same letter do not differ significantly, while treatments with different letters do.***

| Water type | Potato | Onion | Pear |
|---|---|---|---|
| 1. Effluent | 4.8 – B | 6.5 – C | |
| 2. Tap water (reserve) | 6.5 – C | 5.0 – AB | |
| 3. Effluent+Ozone | 5.0 – B | 6.0 – BC | |
| 4. Effluent+Ozone+UV | 3.3 – A | 6.0 – BC | |
| 5. Tap water | 7.0 - C | 4.0 – A | |
| Significance | S | S | |

***Table 5-4 Vitality assessment on August 15, 2022. Significance is indicated by 'S' (significant) and 'NS' (not significant).***

| Water type | Potato | Onion | Pear |
|---|---|---|---|
| 1. Effluent | 6.3 | 9.0 | 9.0 |
| 2. Tap water (reserve) | 6.7 | 8.3 | 9.0 |
| 3. Effluent+Ozone | 4.8 | 9.0 | 9.0 |
| 4. Effluent+Ozone+UV | 5.7 | 9.0 | 9.0 |
| 5. Tap water | 5.3 | 9.0 | 8.8 |
| Significance | NS | NS | NS |

On June 21, it was found that treatment 4 (effluent treated with Ozone and UV) for potatoes showed a significantly lower vitality compared to the other treatments. Tap water (treatments 2 and 5) had significantly higher vitality than the effluent treatments. For onions, it was the opposite: the raw effluent (treatment 1) showed higher vitality than the tap water treatments.

On August 15, no significant differences in vitality were observed in the potatoes. The vitality did recover since June 21 for the treatments that showed a lower vitality then. In general, the vitality of the plants were limited across all treatments. On the other hand, the decline in vitality did not continue, and the plants remained partially green and productive until the harvest.

The differences observed in onions on June 21 were also no longer apparent on August 15. The scores for onions on August 15 were notably higher compared to June 21. These scores reflect more of a relative estimate of crop volume than vitality; at this stage of growth, the above-ground parts of the plants were already dying off and were no longer vital.

For pears, no differences in vitality were observed on August 15.

### 5.3.2 Harvest

The results of the harvest are presented in Table 5-5. For all crops, the product yield was on the low side.

***Table 5-5 Results of harvest of potatoes, onions and pears. Results include the yield (amount of products/pot and kg/pot), average weight of individual crop (g/product) and amount of rotten crops/plot. Significance is indicated by 'S' (significant) and 'NS' (not significant). For significant results, treatments are labeled with letters (A, B, C) to indicate differences: treatments sharing the same letter do not differ significantly, while treatments with different letters do.***

| | Amount product/pot | kg/pot | g/product | Rotten product/pot |
|---|---|---|---|---|
| **Potato** | | | | |
| 1. Effluent | | 1.79 *B* | | |
| 3. Effluent+Ozone | | 1.84 *B* | | |
| 4. Effluent+Ozone+UV | | 1.22 *A* | | |
| 5. Tap water | | 2.36 *C* | | |
| *Significance* | | *S* | | |
| **Onion** | | | | |
| 1. Effluent | 14.5 | 1.14 | 79 *B* | 3.3 |
| 3. Effluent+Ozone | 16.5 | 1.02 | 62 *A* | 2.3 |
| 4. Effluent+Ozone+UV | 14.8 | 1.05 | 71 *A* | 3.0 |
| 5. Tap water | 15.5 | 0.94 | 60 *A* | 1.3 |
| *Significance* | *NS* | *NS* | *S* | *NS* |
| **Pear** | | | | |
| 1. Effluent | 83 | 9.4 | 113 | |
| 3. Effluent+Ozone | 69 | 8.1 | 117 | |
| 4. Effluent+Ozone+UV | 81 | 8.8 | 108 | |
| 5. Tap water | 71 | 8.6 | 121 | |
| *Significance* | *NS* | *NS* | *NS* | |

For potatoes, a significant effect of irrigation water type on the harvest yield was demonstrated: tap water resulted in higher production (kg potato/pot) than all other treatments. Effluent treated with ozone + UV led to a decrease in production. In the potato plots, occasional plants with rotten potatoes were observed. The observed differences align reasonably well with the vitality differences noted on June 21. Any potential application of effluent for drip irrigation in open fields should be further investigated.

For the other crops, no effect of effluents on production (kg or amount of crop/pot) was observed. However, for onions, a positive effect on onion weight was observed: with raw effluent, the weight of an onion was higher than with tap water or treated effluent (thus, the actual production was likely higher, although not statistically significant). The observed differences align reasonably well with the vitality differences noted on June 21.

Since pears are a perennial crop, where a tree is already present at the start of the experiment, it stands to reason that any negative effects of effluents would be less apparent compared to annual crops.

The following photos in Figure 5-10 provide an impression of the harvest and sampling of the crop products.

***Figure 5-10 Impression of the harvest of crop products.***

## 5.4 Conclusions

For the effect of irrigation water quality on growing crops, the following is concluded regarding plant vitality and crop yields.

Vitality of plants:
- Potatoes: Best vitality with tap water initially, but no significant differences among water types at harvest.
- Onions: Lower initial vitality with tap water; no significant differences among water types at harvest.
- Pears: no scores were recorded, as the pear trees (perennial plant) had already grown before the experiments on pear growth started."

Yield crops:
- Potatoes: Highest yield with tap water, followed by treated effluent with ozone and raw effluent. Lowest yield with treated effluent with ozone + UV. No clear general trend linking water quality to yield.
- Onions: Heaviest onions with raw effluent, followed by treated effluents and lightest with tap water. Significant effect on weight but not on overall production.
- Pears: No significant effects of water quality on yield.

There is no clear, uniform pattern in the effect of irrigation water type on vitality and yield across all crops. The variability observed suggests that further experiments are needed to obtain a more statistically robust understanding of the impact of water quality on crop performance.

# 6 Uptake of organic micropollutants in potatoes, onions and pears

## 6.1 Introduction

In Work Package 6 (WP6) of this project, we investigated the extent to which organic micropollutants (OMPs) from effluent originating from WWTP Walcheren enter the crop products of potatoes, onions, and pears through drip irrigation, as well as the effect of effluent treatments with ozone and ozone + UV on reducing this uptake.

This chapter presents the results of the accumulation of organic micro-pollutants in crops—specifically potatoes, onions, and pears—cultivated using various irrigation water qualities. The crops were irrigated with water of four different qualities: untreated municipal wastewater treatment plant (WWTP) effluent, effluent treated with ozone, effluent treated with both ozone and UV, and tap water serving as a control.

Details regarding the cultivation process can be found in Chapter 5 and details regarding effluent treatment can be found in Chapter 4.

The chapter begins with a description of the experimental setup followed by an exploration of the results related to OMP presence in crops. This section is divided into two parts:

1. The initial segment focuses on OMPs found both in the WWTP Walcheren effluent and crops, enabling clear insights into the impact of water quality on OMP accumulation in crops. OMP analysis utilised a library screening approach, focusing solely on equivalent concentrations to elucidate the effect of water quality. Furthermore, for a selected set of OMPs, precise concentrations in potatoes were determined and compared with literature values, providing valuable data for risk assessments.
2. Notably, apart from OMPs detected in the WWTP Walcheren effluent, additional OMPs were found in the crops, suggesting alternative sources. The second part of the chapter explores OMPs present in crops that are not native to the effluent, along with a brief discussion on potential sources leading to their presence in crops.

Through this detailed analysis, this chapter aims to provide insights into the accumulation of OMPs in crops under varying irrigation water qualities, offering valuable information for scientific discourse and practical risk management strategies.

## 6.2 Methods and materials

### 6.2.1 Sample preparation

Sample preparation was performed by WFBR. The QuEChERS (abbreviation for Quick Easy Cheap Effective Rugged Safe) method [65] was used to analyse potential OMPs from crop samples, and unused soil and fertiliser granules used for the cultivation of the crops. Initially developed to analyse veterinary drugs in animal tissues, the QuEChERS method is now used to detect pesticides in plant material [66].The QuEChERS method is also intensively used to detect pharmaceuticals and other OMPs [67, 68].

*Crop samples*
All crop samples were freeze-dried (including peels) prior to preparation of the extract. 10 mL of Milli-Q water and 10 mL of acetonitrile were added to 2 grams of freeze-dried sample and mixed with a vortex for 1 minute. Subsequently, 4 grams of $MgSO_4$ and 1 gram of NaCl were added to the solution and mixed again with the vortex for 1 minute. Internal standards, 200 µL of Linuron (1 mg/L in acetonitrile) and 200 µL of Gabapentin (1 mg/L in methanol), were added and mixed for 1 minute. The extract was centrifuged with a

benchtop centrifuge at 4000 rpm for 10 minutes using a swing-out rotor. Two layers were formed: an organic (acetonitrile) layer and a water layer. The solvent layer was then transferred to a clean vial.

For removal of interfering components, 150 mg of $MgSO_4$ and 25 mg of PSA (Primary and Secondary Amine) were added to the solvent extract and mixed. The extract was centrifuged to settle down the solid particles. Finally, the extract was transferred to an HPLC vial and stored at 4°C until analysis at Aqualab Zuid B.V., Werkendam, The Netherlands.

For each irrigation type per crop, 4 samples were prepared.

*Soil samples*
The QuEChERS strategy was employed for soil extraction, similar to the method used for crops. The only distinction was the use of 5 grams of soil for extraction. The extraction process was conducted in triplicate. Before extraction, the soil's dry matter content was determined by drying the soil at 50°C until no further weight loss was observed. The average moisture percentage was found to be 3.5%.

*Fertiliser granules samples*
The fertiliser granules were pretreated with a grinder (Krups silent mixer, 10 pulses of 1 second each) to obtain approximately 50 grams of finely homogeneous powder. The QuEChERS strategy was employed for the extraction of the fertilizer granules, similar to the method used for crops. This process was conducted in triplicate.

### 6.2.2 Analysis

*Non-target library-screening analyses*
Aqualab Zuid B.V. conducted non-target library-screening analyses on the extraction samples. These analyses focus not on concentrations of specific substances but on the presence of peaks in an analysis spectrum. Such a spectrum of peaks provides an overview of the number of substances present and the substance's relative concentration (peak height). Substances are identified by comparing data of the peaks (e.g. molecular weight and retention time) to a database with such substance information (library). The amount of identified substances depends on the extensiveness of the library. The library of Aqualab Zuid contains around 2000 substances. UPLC-QTOF-MS (Ultra High Performance Liquid Chromatography – Quadrupole Time-Of-Flight - Mass Spectrometry) is used to obtain the spectrum of peaks. The detection limit (LOD) is 0.01 µg/L indicator.

The analysis is qualitative. Obtained concentrations are expressed relative to the peak area of a reference substance (atrazine d5 for the positive ionisation run or bentazone d6 for the negative ionisation run). It is only allowed to compare these concentrations if they are of the same chemical component and the same type of crop. Thus, the relative effect of water quality on OMP uptake per crop can be investigated.

*Absolute concentration in crops*
Absolute concentrations of OMPs in extracts (in µg/L) were determined for selected OMPs. These OMPs were selected after the non-target library-screening analyses, and had to comply with the following criteria:
- Present in effluent WWTP Walcheren;
- Present in crops that were irrigated with (treated) effluent;
- Calibration possible at AquaLab Zuid B.V.

Calibration-factors for selected OMPs were determined by AquaLab Zuid B.V. It turned out that OMPs meeting the criteria stated above were detected only in potatoes. The exact concentrations of OMPs in potatoes (in ng/g potato) were determined using the exact concentrations in the extracts, the amount of freeze-dried crop weighed for extraction, the quantity of extraction solvent, and the moisture loss of the crop due to freeze-drying.

A list with selected OMPs and their corresponding Limit Of Detection is presented in Annex 6.

## 6.3 Crop uptake of organic micropollutants found in WWTP effluent

This section presents the findings regarding the uptake of OMPs detected in the effluent of WWTP Walcheren by various crops, using different water qualities for irrigation. The section is divided by crop type (potatoes, onions, and pears).

### 6.3.1 Potatoes

Figure 6-1 shows the equivalent concentrations of OMPs in potatoes, categorised by irrigation water type. It should be noted once again that equivalent concentrations do not represent the actual concentrations found in crops, but rather concentrations equivalent to a specific indicator. Therefore, equivalent concentrations of different OMPs cannot be directly compared with each other. However, the concentrations can be compared per OMP for different irrigation water types.

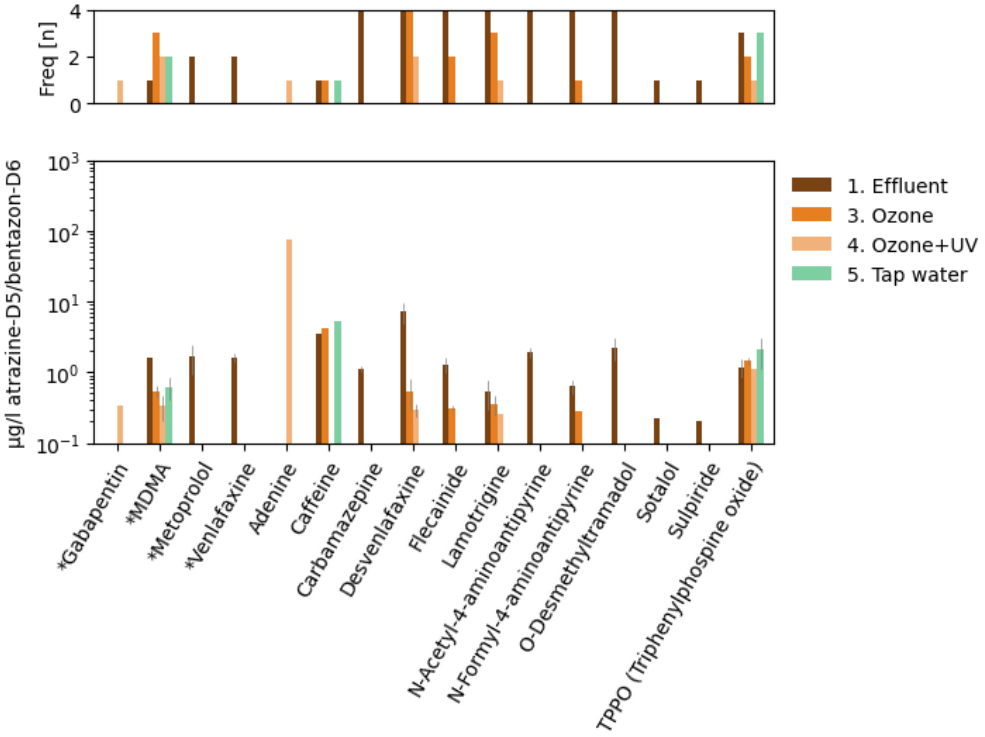



***Figure 6-1*** ***Bottom: Average equivalent concentration of organic micropollutants in potatoes per irrigation type. Bars represent standard deviation, and components with an asterisk (*) indicate indicative identification. Top: Detection frequencies (maximum 4 samples per irrigation water type per crop type).***

In potatoes, 16 OMPs were detected that were also found in the effluent of WWTP Walcheren. Four of these OMPs were identified indicatively.

Most OMPs show the highest equivalent concentrations when effluent is directly used for irrigation. The use of treated effluent (ozone or ozone+UV) or tap water for irrigation results in lower equivalent concentrations or the absence of detection for certain OMPs.

Metropolol (indicative), venlafaxine (indicative), carbamazepine, N-acetyl-4-aminoantipyrine, o-desmethyltramadol, sotalol and sulpiride are not detected anymore in crops irrigated with effluent treated with ozone and ozone+UV. However, due to detection limits of the analysis method, a complete absence of OMPs cannot be guaranteed. Other components also show a reduction when the irrigation water is treated. The OMPs reductions can be attributed to the (partial) removal of OMPs in treated effluent, leading to much lower concentrations than those in untreated effluent.

It is also assumed that tap water does not contain any OMP or contains them at negligible concentrations, and therefore crops irrigated with tap water should not uptake OMPs. Some OMPs appear in crops when they are irrigated with tap water. However, the source of these OMPs is not the tap water, as will be discussed further in this chapter.

Previous studies (Chapter 4, 0) have demonstrated that in effluent treated with ozone + UV, the concentration of OMPs are reduced or are not detected anymore. This holds for, among others: carbamazepine, flecainide, N-acetyl-4-aminoantipyrine, N-formyl-4-aminoantipyrine, sotalol, sulpiride, desvenlafaxine, and venlafaxine. These compounds show reduced uptake in crops when irrigated with treated effluent. Consequently, employing additional treatment technologies in WWTPs such as ozonation significantly diminishes the absorption of OMPs by crops, thereby mitigating potential health risks associated with the use of WWTP effluent for irrigation purposes.

The detection frequency indicates how often an OMP is found in a crop when the OMP is present in the irrigation water. The maximum detection frequency is 4, as 4 samples per crop have been taken for each irrigation water type. The substances carbamazepine, desvenlafaxine, flecainide, lamotrigine, n-acetyl-4-aminoantipyrine, n-formyl-4-aminoantipyrine, and o-desmethyltramadol have all been detected 4 times when irrigated with effluent. These components are therefore quickly absorbed by potatoes, and thus, the effect of irrigation water quality can be more confidently determined from these components. All these components show a decrease in uptake by potatoes when the effluent is treated (ozone and ozone+UV) or are no longer detected. It is also observed that when a component is found in treated effluent, the detection frequency is often lower than 4, indicating less frequent detection. However, it is possible that the component is still present but at concentrations below the detection limit.

Examining the list of indicator components (Dutch: gidsstoffen) defined by STOWA [69], we observe the presence of carbamazepine, metoprolol (indicative), venlafaxine (indicative), sotalol, and gabapentin (indicative) in potatoes. Additionally, desvenlafaxine, a metabolite of venlafaxine, is detected. All of these components are medicines. Except for gabapentin (present in only one sample for potatoes irrigated with ozone+UV treated effluent), all components show the influence of irrigation water on crop uptake, as described previously. It is worth noting that all STOWA indicator components are included in the OMP library.

Some OMPs deviate from the previously described trend. Caffeine and TPPO (Triphenylphosphine oxide) show equivalent concentrations of the same order of magnitude for all irrigation types, suggesting no significant effect of the type of irrigation water on OMP uptake in potatoes. Caffeine is a stimulant that is found in drinks such as coffee; TPPO is an industrial chemical used for several purposes, including as a catalysis and a coordinating solvent used to activate crystallisation [70]. This anomaly is unexpected, considering the trends observed for other OMPs. Since the concentration of caffeine and TPPO is lower in treated effluent (0), it should result in lower uptake in potatoes. Furthermore, caffeine and TPPO should not be present in tap water, and hence, potatoes irrigated with tap water should not contain these OMPs. Therefore, it appears that the source of caffeine and TPPO is something other than the irrigation water.

MDMA (indicative) also shows distinct behaviour. While the equivalent concentration is highest for effluent irrigation, it remains noticeable for other irrigation types, including tap water, where MDMA should not be present. Also, it was previously shown that ozonation removes 100% of MDMA from effluent. Additionally, the detection frequency for effluent (1x) is lower than that for other irrigation types. This trend suggests that the source of MDMA is also likely something other than the irrigation water.

### 6.3.2 Onions

In onions, five different OMPs were detected, which were also found in the effluent of WWTP Walcheren (Figure 6-2). Among these, three OMPs were indicatively identified. None of these OMPs aligns with STOWA indicators.

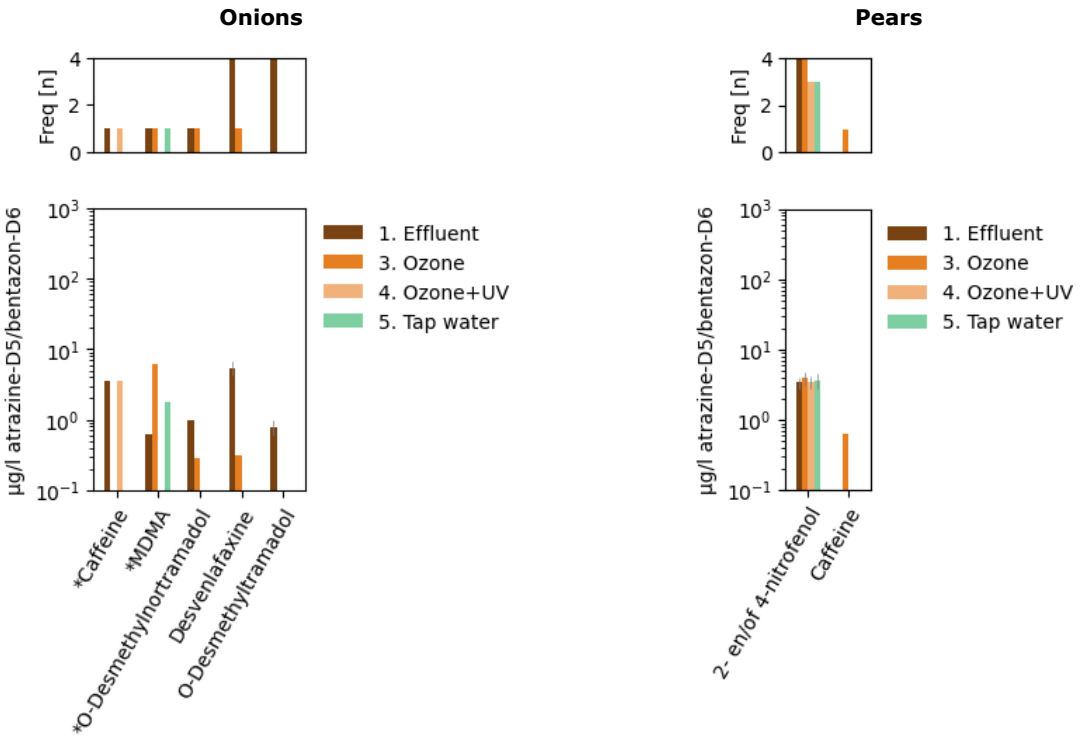



***Figure 6-2 Bottom: Average equivalent concentration of organic micropollutants in onions and pears per irrigation type. Bars represent standard deviation, and components with an asterisk (*) indicate indicative identification. Top: Detection frequencies (maximum 4 samples per irrigation water type per crop type).***

Desvenlafaxine, O-desmethyltramadol and O-desmethylnortramadol (indicative) show a similar trend as seen with OMP uptake in potatoes: crops irrigated with effluent show a high concentration of OMPs compared with crops irrigated with treated effluent or tap water, where the OMP concentration is significantly lower or non-existing. Thus, also here the effect of water quality is evident.

MDMA (indicative) and caffeine (indicative) were also detected in potatoes, and show the same deviating behaviour here with the onions. The OMPs are only detected in one of the four samples per irrigation type, and do not show a clear effect of irrigation type on uptake by crops. MDMA is even again found in samples irrigated with tap water, which should not contain this OMP. Also here, it is likely that the source of these two OMPs is something other than the irrigation water.

### 6.3.3 Pears

In pears, only two different OMPs were detected, which were also found in the effluent of WWTP Walcheren (Figure 6-2). They are not on the list of indicators of STOWA [69].

Caffeine was detected in only one sample, obtained from crops irrigated with ozone-treated effluent.
2 and/or 4-nitrofenol are industrial components with diverse applications, such as their use in the synthesis of dyes and pesticides [70]. The equivalent concentrations of this component are similar for all irrigation types, which suggests no effect of irrigation water on OMP uptake. Considering the expected lower concentrations for treated water (previously shown that ozonation has a removal rate of 73% for 2 and/or 4-nitrofenol in effluent, see Chapter 4) and the absence of this component in tap water, it is likely that the source of this component is something other than the irrigation water.

Given that no other components were detected in pears, drawing conclusions about the impact of irrigation water on the uptake of OMPs in pears is not possible.

### 6.3.4 Types of OMPs

Figure 6-3 shows the number and type of unique components detected per crop type and irrigation method. Categories per irrigation method include reliable and indicative (*) identification. The types of components are classified as: organic compounds, industrial substances, drugs, medicines, and metabolites.

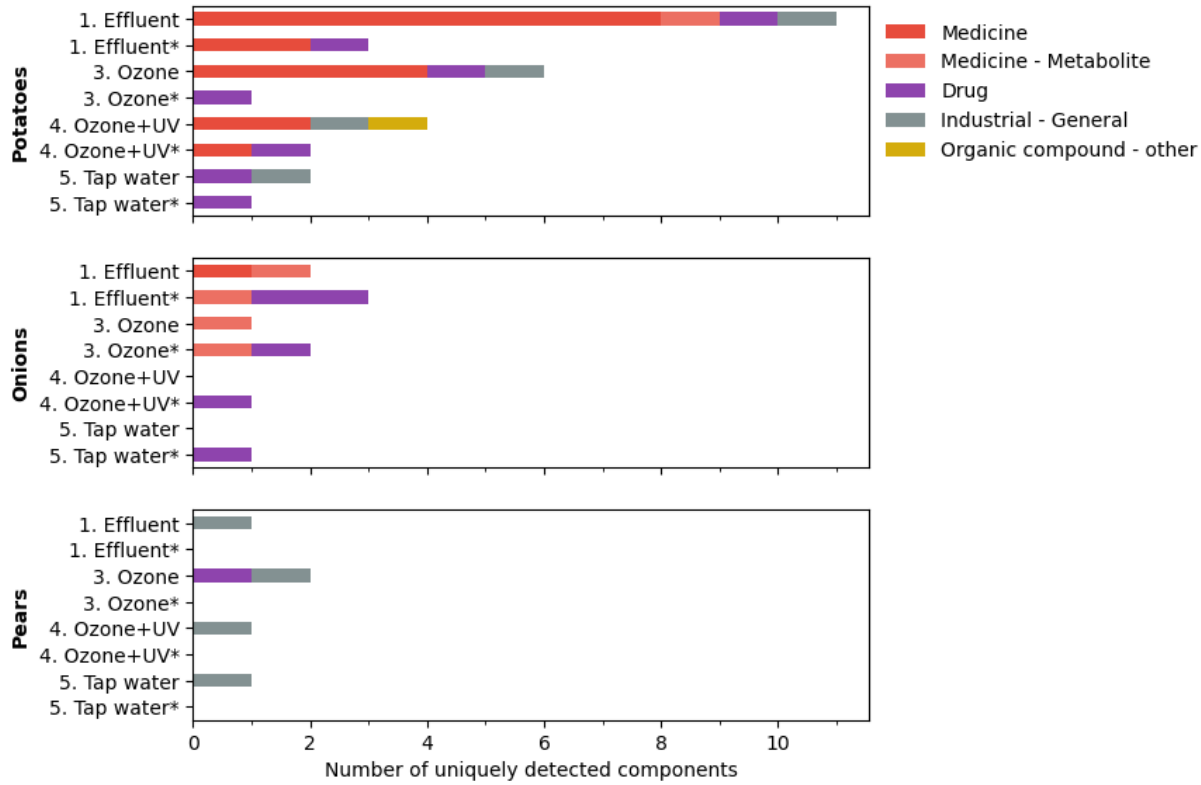



***Figure 6-3 Number of uniquely detected components per crop type and irrigation method. Irrigation types are further categorised into reliable identification and indicative identification (*). The count of uniquely detected compounds per category is classified by the type of organic micropollutant: organic compound-other, industrial – general, drug, medicine – metabolite, and medicine.***

The analysis of uniquely detected components across various crops and irrigation types revealed distinct patterns. Potatoes show the highest number of uniquely detected components, irrespective of the irrigation type of water employed. After potatoes, onions have the most uniquely detected components. Least amount of uniquely detected components were detected in pears.

This means that OMPs uptake highly depends on crop type, since all crops were exposed to the same amount an variety of OMPs. Other studies where crops were grown using reclaimed water for irrigation also noticed an effect of crop type on OMP uptake [71]. The uptake of OMPs per crop type depends on plant physiology, as well as the processes and functions within plants that govern the uptake and translocation of OMPs. It is worth noticing that the type of soil also affects the uptake of OMPs by crops [72]. In our case, the pear trees (perennial plant) had a different soil than potatoes and onions (annual plants). This could have affected the difference in amount of uniquely detected components as well.

There is a clear effect on irrigation water and number of uniquely detected components. This is especially clear when looking at potatoes, where most of the components were detected. Most components were detected in crops irrigated with effluent. This makes sense, since this type of water should have the highest concentrations of OMPs. Treated effluent contains lower amounts of components. Of the treated effluent types, there is not much difference between ozone and ozone + UV. This can be explained by the intention of using UV to disinfect, rather than breaking down OMPs. In crops irrigated with tap water, still some OMPs were detected. It is likely that the source of these OMPs is something other than the irrigation water.

Figure 6-3 also shows what type of OMPs were detected in the crops. Most of the detected OMPs are medicines or their metabolites. This is particularly evident when considering the potatoes and, to a lesser extent, the onions.

An overview of the detection of different OMPs, divided into categories, along with their respective usage[6], is shown in Table 6-1.

***Table 6-1 Overview[6] of all components detected in potatoes, onions, and pears, categorised into medicines, metabolites, drugs, industrial, and organic components. An 'X' in a column denotes whether the components have been detected in the corresponding crop. The asterisk '*' denotes substances that have been indicatively identified. An asterisk within parentheses '(*)' denotes substances that have been indicatively identified in some, but not all crops.***

| Component | Use | Potatoes | Onions | Pears |
|---|---|---|---|---|
| **Medicine** | | | | |
| Carbamazepine | Anticonsulvant | X | | |
| Lamotrigine | Anticonsulvant | X | | |
| *Gabapentin | Anticonsulvant | X | | |
| Flecainide | Antiarrhythmic | X | | |
| Sotalol | Antiarrhythmic | X | | |
| *Metoprolol | Beta-blocker | X | | |
| Desvenlafaxine | Antidepressant | X | X | |
| *Venlafaxine | Antidepressant | X | | |
| Sulpiride | Antipsychotic | X | | |
| **Medicine metabolites** | | | | |
| N-Acetyl-4-aminoantipyrine | Metamizole (painkiller) | X | | |
| N-Formyl-4-aminoantipyrine | Metamizole (painkiller) | X | | |
| O-Desmethyltramadol | Tramadol (painkiller) | X | X | |
| *O-Desmethylnortramadol | Tramadol (painkiller) | | X | |
| **Drug** | | | | |
| (*)Caffeine | Stimulant | X | X | X |
| *MDMA | Psychoactive substance | X | X | |
| **Industrial** | | | | |
| TPPO (Triphenylphospine oxide) | Reagent | X | | |
| 2- and/or 4-nitrophenol | Chemical intermediate | | | X |
| **Organic compound** | | | | |
| Adenine | RNA, DNA building block | X | | |

Among the pharmaceuticals detected were carbamazepine and gabapentin, both anticonvulsants used to suppress epileptic seizures. Venlafaxine and desvenlafaxine, antidepressants, as well as metoprolol, a beta-blocker commonly prescribed for heart conditions, were also detected. Additionally, flecainide and sotalol, both antiarrhythmics used to treat heart rhythm disorders, were found. The anti-epileptic lamotrigine and the antipsychotic sulpiride were also among the detected medications.

In addition to these pharmaceuticals, several metabolites were found, including o-desmethyltramadol and o-desmethylnortramadol, both breakdown products of tramadol, a narcotic painkiller. N-formyl-4-aminoantipyrine, a metabolite of various painkillers, was also detected. Another metabolite, n-acetyl-4-aminoantipyrine, was linked to metamizole, another type of painkiller.

A total of two drugs were identified: MDMA, a psychoactive substance with similar effects to ecstasy, and caffeine, a well-known stimulant present in beverages such as coffee and tea.

[6] Type of organic micropollutants is present in the database of AquaLab Zuid B.V. Additionally, the uses of organic micropollutants were found in databases of ChemicalBook [70] and PubChem [104]

One industrial component was detected: TPPO. TPPO is utilised for various industrial purposes, including as a catalyst and a coordinating solvent in crystallisation processes.

Lastly, the organic compound adenine was found. Adenine is a fundamental component of RNA and DNA produced by mammals.

### 6.3.5 Absolute concentrations in potatoes

Absolute concentrations in potatoes were determined for selected OMPs. The criteria for the selection of these OMPs were: detected in effluent; detected in crops; calibration possible at AquaLab Zuid B.V. Figure 6-4 shows the absolute concentrations for 11 OMPs in potatoes, that were cultivated using different water qualities. None of the selected OMPs were detected in onions and pears.

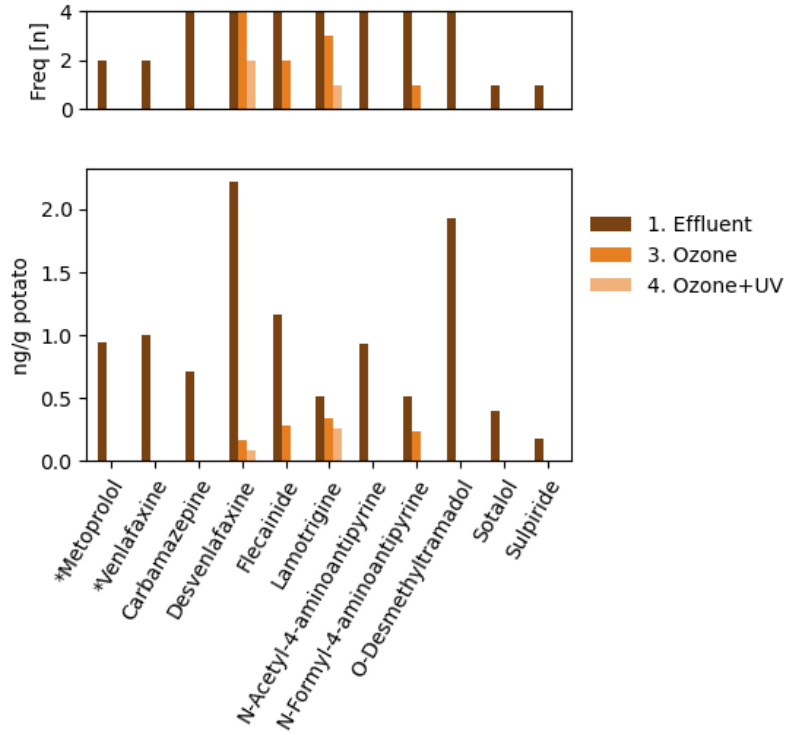



***Figure 6-4 Absolute concentrations of organic micropollutants in potatoes cultivated using different water qualities. Components with an asterisk (*) indicate indicative identification.. Detection frequencies are shown in the top graph ($n_{max}$=4).***

Since absolute concentrations are correlated with equivalent concentrations (Figure 6-1), similar patterns emerge here. The highest concentrations of OMPs are found when pure effluent is used for irrigation. When treated effluent (ozone and ozone+UV) is used, either the concentrations of the OMPs are lower or the OMPs are not detected at all. The effect of water quality on OMPs uptake by potatoes thus is clear; treating effluent significantly reduces the uptake of OMPs.

In terms of concentrations, all OMPs show concentrations within the range of 0.2 – 2.3 ng/g potato. However, it's important to acknowledge that the exact concentrations may vary, as some uncertainty arises from matrix effects during analysis. Therefore, the results presented in Figure 6-4 should emphasise the magnitude of the concentrations rather than their exact values.

*Comparative analysis of current findings with other studies*
The order of magnitude of OMP concentrations in potatoes is similar to found concentrations in other studies that cultivated crops using WWTP effluent as irrigation water. Ben Mordechay *et al.* [71] found concentrations between 0.8-3 ng/g for carbamazepine, gabapentin, lomatrigine and nicotine in potatoes in a case study in Israel. In other types of crops, higher concentrations were found, with OMP concentrations up to 20 ng/g crop in tangerines, avocados, tomatoes and bananas, concentrations up to 100 ng/g crop in

carrots and oranges, and even concentrations up to 900 ng/g crop in leafy greens. The differences in uptake concentrations between different types of crops were attributed to wastewater source, soil properties and plant physiology. The authors suggest to upgrade WWTPs for removing OMPs, in order to reduce human health risks when crops are irrigated with reclaimed water.

Christou *et al.* [73] performed a 3 year study using WWTP effluent for the cultivation of tomatoes , focusing on three selected OMPs: diclofenac (DCF), sulfamethoxazole (SMX) and trimethoprim (TMP). The highest concentrations found during the study were 11.63 ng/g (DCF), 5.26 ng/g (SMX) and 3.4 ng/g (TMP). This falls in the same order of magnitude of the OMP concentrations found in this study, even though crop type and possible other conditions are different. It was found that concentrations increased with each year for some OMPs, most probably due to accumulation of OMPs in the soil. A brief risk analysis concluded that consuming tomatoes cultivated with WWTP effluent possesses a *de minimus* risk (a risk that is so small or insignificant that it is deemed negligible and not worth considering in practical terms) to human health for the three OMPs.

García-Valverde *et al.* [74] also studied the effect of the use of reclaimed water from WWTPs on OMP accumulation in soil and crops. They found concentrations of carbamazepine, lidocaine and caffeine in the range of 0.1-1.7 ng/g crop in cucumbers, peppers, tomatoes and courgettes, which are in the same range of the concentrations of OMPs found in potatoes in this study. Based on average daily intake of crops and acceptable daily intake (ADI) values of the found OMPs, the researchers concluded that the appearance of OMPs in this concentration range possesses no threat to human health.

Manasfi *et al.* [75] studied the uptake of OMPs by leafy greens that were irrigated with reclaimed water from WWTPs. Several OMPs were detected in the leaves, with highest concentration of 29 ng/g (citalopram), 660 ng/g (carbamazepine), 7 ng/g (metoprolol), 18 ng/g (hydrochlorothiazide), 129 ng/g (clarithromycine), 11 ng/g (climbazole), 12 ng/g (benzotriazole), 132 ng/g (acesulfame) and 632 ng/g (sucralose). The order of magnitude of concentrations is for some OMPs several times higher than the concentrations found in this study, but this can be explained by the type of crop. Ben Mordechay *et al.* [71] observed similar higher uptakes of OMPs by leafy greens. Even though, the authors concluded that these concentrations possess a *de minimis* human health risk, based on ADI values [76].

A similar study where leafy greens were cultivated using reclaimed water was performed by Ponce-Robles *et al*. [77]. This study focused on four commonly found OMPs: carbamazepine, diclofenac, naproxen and ketoprofen. These OMPs were detected in the crops, in the range of 0-45 ng/g crop. Also in this study, a risk assessment by the researchers concluded that there is no health risk for humans.

Prosser and Sibley [78] conducted a risk assessment on the use of reclaimed water for crop irrigation on human health, based on literature values. They compared found concentrations of OMPs in crops with ADI values, and concluded that the majority of individual OMPs in the edible tissue of crops due to wastewater irrigation has a *de minimis risk* to human health, but that the effect of mixtures of OMPs could potentially be a hazard.

## 6.4 Crop uptake of organic micropollutants not found in WWTP effluent

In this section, we briefly discuss the uptake of organic micropollutants not detected in the effluent of WWTP Walcheren by potatoes, onions, and pears. As most of these OMPs likely originate from a different source than the irrigation water, these results cannot be used to assess the impact of irrigation water on OMP uptake. The potential sources of these OMPs are briefly discussed.

### 6.4.1 Potatoes

In potatoes, 44 OMPs were detected that were not found in the effluent of WWTP Walcheren (Figure 6-5). Of these, 26 OMPs were indicatively identified.

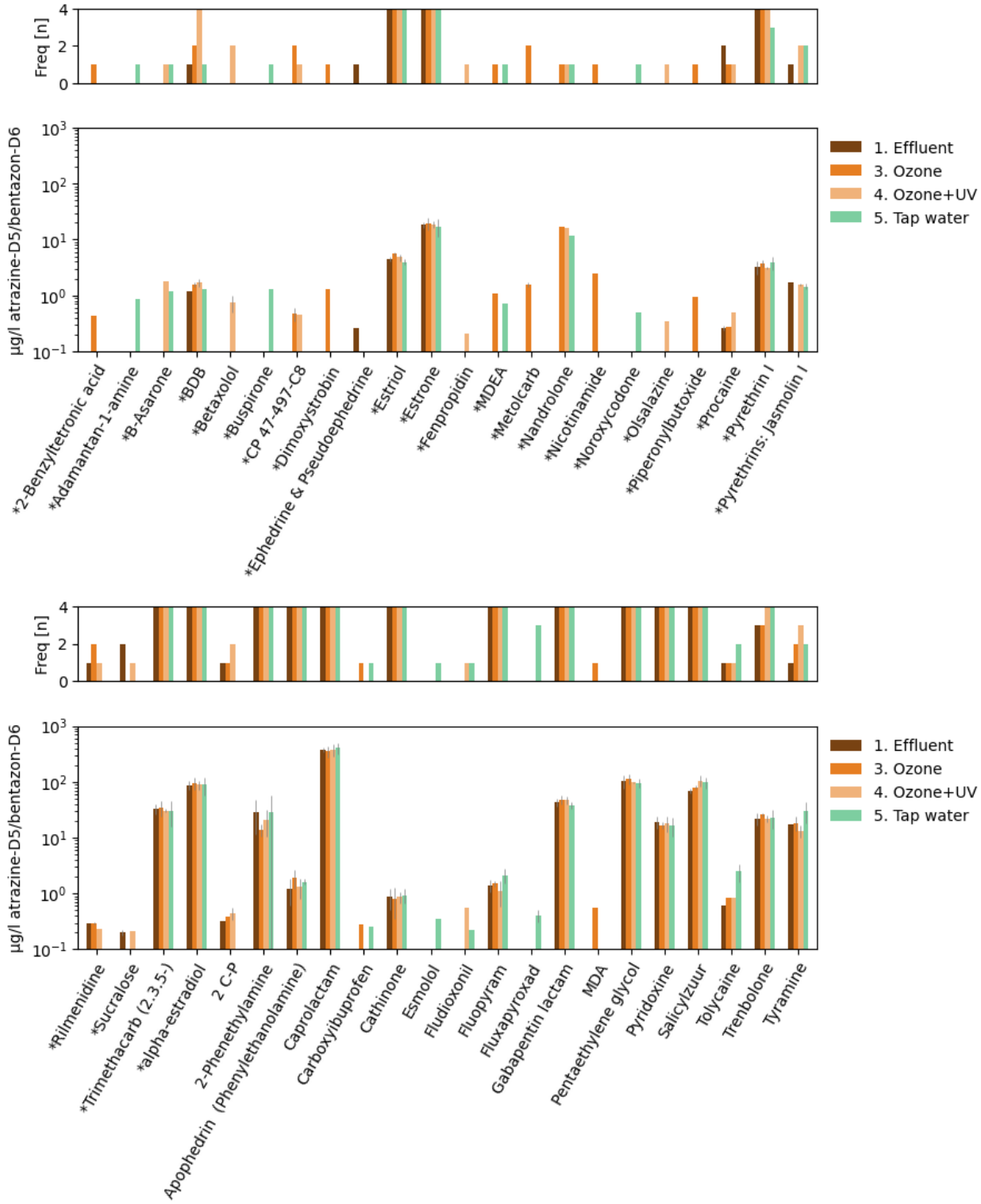



***Figure 6-5*** ***Bottom: Average equivalent concentration of organic micropollutants in potatoes per irrigation type, that were not detected in the effluent of WWTP Walcheren. Bars represent standard deviation, and components with an asterisk (*) indicate indicative identification. Top: Detection frequencies (maximum 4 samples per irrigation water type per crop type).***

Only one component, ephedrine (indicative), shows the pattern where the highest concentration is found when pure effluent is used for irrigation, and lower concentration or no detection when other irrigation qualities are used. However, this component is only one time detected.

In general, four other patterns can be observed when examining concentrations per irrigation type:

1. Concentrations are similar for all four irrigation types, including tap water, and detection frequencies are high for all irrigation types (3 or 4). This pattern is observed for 18 OMPs. Since no effect of irrigation type on OMP uptake is observed, even when tap water is used where no OMPs should be present, the source of these OMPs are not the irrigation water.
2. Components appear only when ozone or ozone+UV treated effluent was used, and have low detection frequencies (1 or 2). This pattern is observed for 10 OMPs.
3. Components appear when tap water was used and, in some cases, when another irrigation type was used, with low detection frequencies (1 or 2). This pattern is observed for 10 OMPs. Since no OMPs are expected to be present in tap water, the source of these components is something other than the irrigation water.
4. Components appear when raw effluent and one or more other irrigation methods were used, with similar concentrations. The detection frequencies are low (1-2). This pattern is observed for five components. Since an effect of irrigation water is expected, and no OMP uptake is expected when tap water is used, the source of these components is something other than the irrigation water.

For the components that only appear when using ozone and/or ozone+UV (pattern 2), it is possible that these components are transformation products of chemical reactions where ozone breaks down OMPs. It is known that ozonation of OMPs can lead to a variety of transformation products [79].

For the components that follow the other patterns, the source is something different then the irrigation water. Therefore, those results cannot be used to draw conclusions about the effect of irrigation type on OMP uptake by crops. However, it is interesting to investigate some potential sources of specific OMPs, as will be discussed in the following paragraph.

### 6.4.2 Onions

In onions, 44 OMPs were detected that were not found in the effluent of WWTP Walcheren (Figure 6-6). Of these, 26 OMPs were tentatively identified.

In general, the same patterns can be observed as with potatoes. There are several OMPs with high detection rates that are present in all irrigation methods used. Additionally, some OMPs only appear when using ozone and/or ozone+UV treated effluent as irrigation water. A couple of components only appear when pure effluent is used: 4-MEC, 5/6 APB, a metabolite of carbamazepine, DMAA (all indicative), deacetyldiltiazem, and fenpropidin. However, the detection frequency of all these components is low (1).

As described in the previous paragraph, the source of most components is most likely not the irrigation water, and for some components, we may be dealing with transformation products of ozonation. Therefore, the results cannot be used to draw conclusions about the effect of irrigation type on OMP uptake by crops.

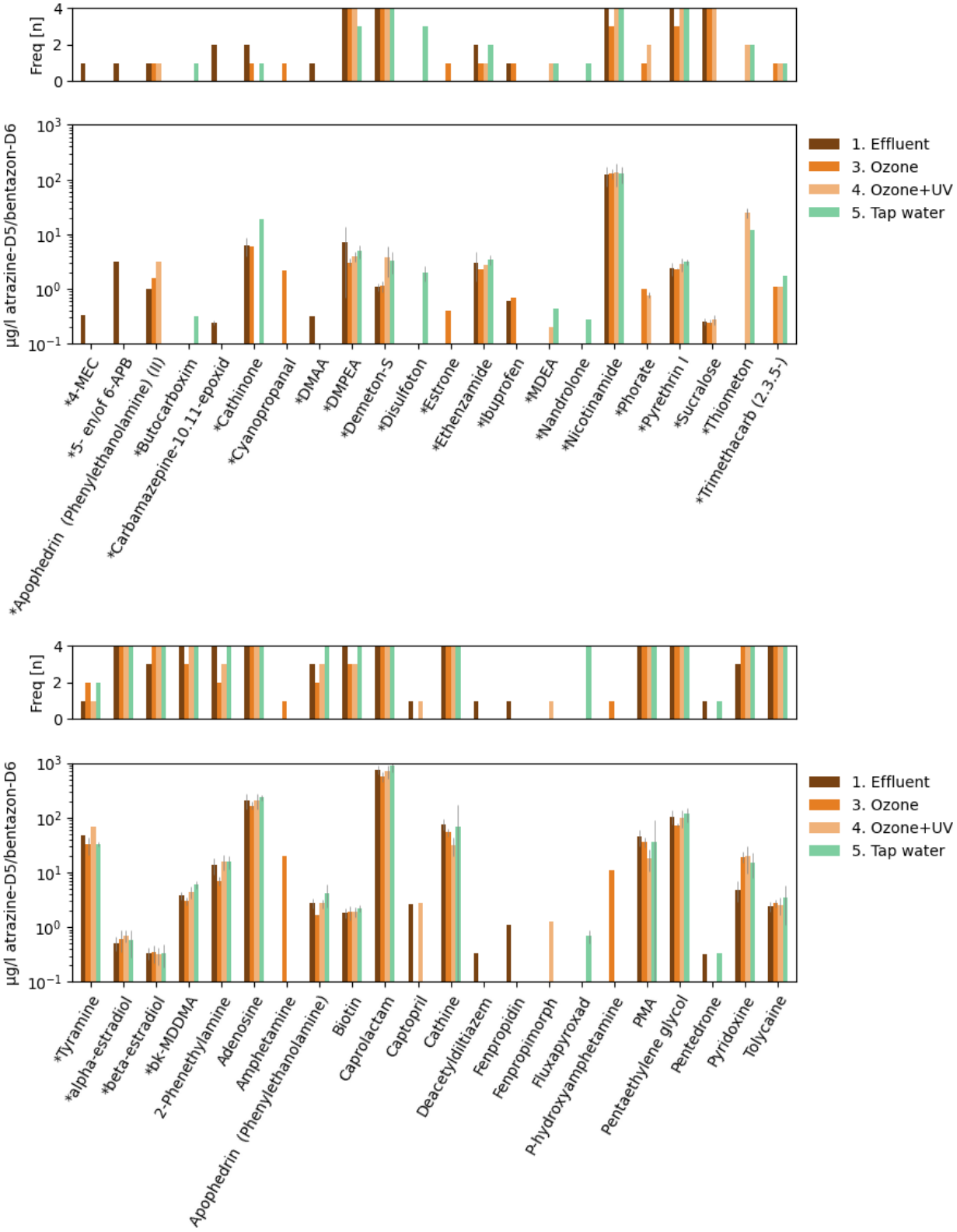



***Figure 6-6*** ***Bottom: Average equivalent concentration of organic micropollutants in onions per irrigation type, that were not detected in the effluent of WWTP Walcheren. Bars represent standard deviation, and components with an asterisk (*) indicate indicative identification. Top: Detection frequencies (maximum 4 samples per irrigation water type per crop type).***

### 6.4.3 Pears

In pears, 20 OMPs were detected that were not found in the effluent of WWTP Walcheren (Figure 6-7). Of these, 11 OMPs were indicatively identified. These numbers are lower than those of potatoes and onions.

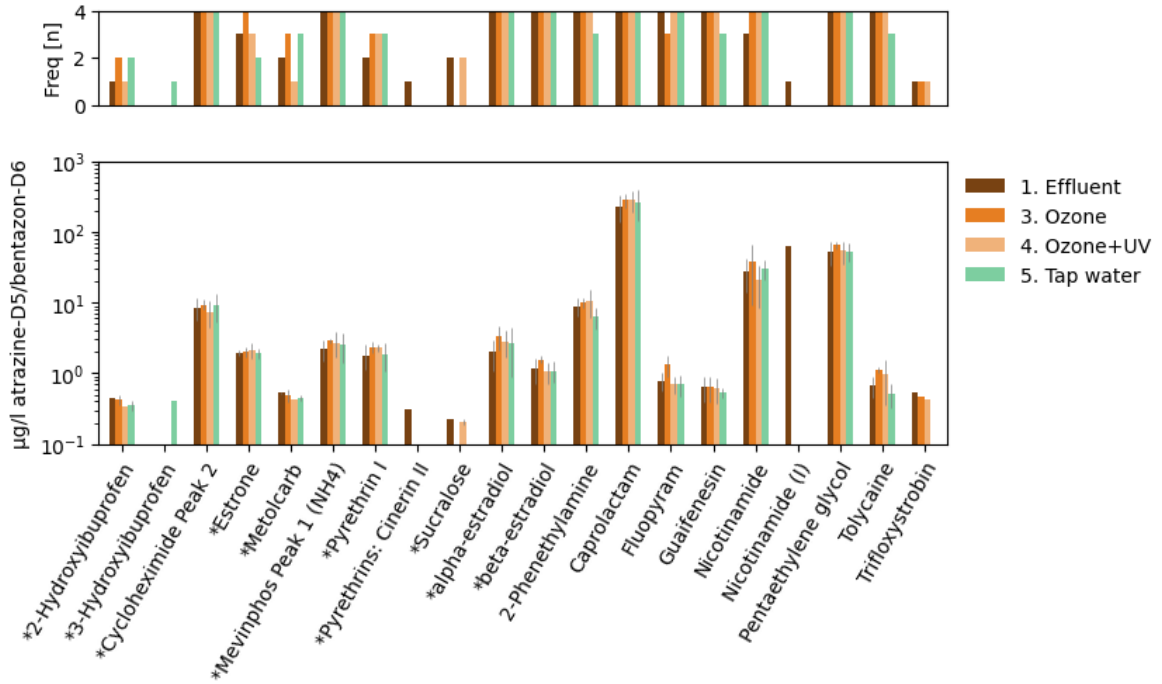



***Figure 6-7*** ***Bottom: Average equivalent concentration of organic micropollutants in pears per irrigation type, that were not detected in the effluent of WWTP Walcheren. Bars represent standard deviation, and components with an asterisk (*) indicate indicative identification. Top: Detection frequencies (maximum 4 samples per irrigation water type per crop type).***

Fifteen OMPs show the general trend of appearing in every irrigation type, including tap water, suggesting that the source of these OMPs is something other than the irrigation water. There are also a couple of components in pears that only appear when effluent is used as an irrigation type. However, since the detection frequency is low (1), it is not possible to draw conclusions about the effect of irrigation type on OMP uptake by crops in the case of pears as well.

### 6.4.4 Types of OMPs

Figure 6-8 shows the number and type of unique components detected per crop type and irrigation method. Categories per irrigation method include reliable and indicative (*) identification. The types of components are classified as: organic compound (hormone, vitamin, other), food additive, industrial, pesticide (fungicide, insecticide), drug, medicine, metabolites and cosmetics.

When focussing on a single crop and compare the irrigation methods, similar types and numbers of OMPs are commonly found. This can be explained by the observation from previous analyses, where it was found that many OMPs were present across all irrigation methods, including tap water.

When we compare the types of OMPs per crop, we see that the types and numbers of OMPs for potatoes and onions are very similar. In the case of pears, we observe a noticeably lower number of detected components. Also, no drugs were found in pears. This could be attributed to pears being a different type of crop compared to potatoes and onions, resulting in different water uptake patterns. Additionally, pear trees were not cultivated from the beginning specifically for this project and have experienced varying soil conditions.

Compared to the results of OMPs detected in crops, as well in the WWTP effluent, we notice the addition of new categories: cosmetics, pesticides, food additives, hormones, and vitamins. An overview of the different detected OMPs and their corresponding uses is shown in Annex 5.

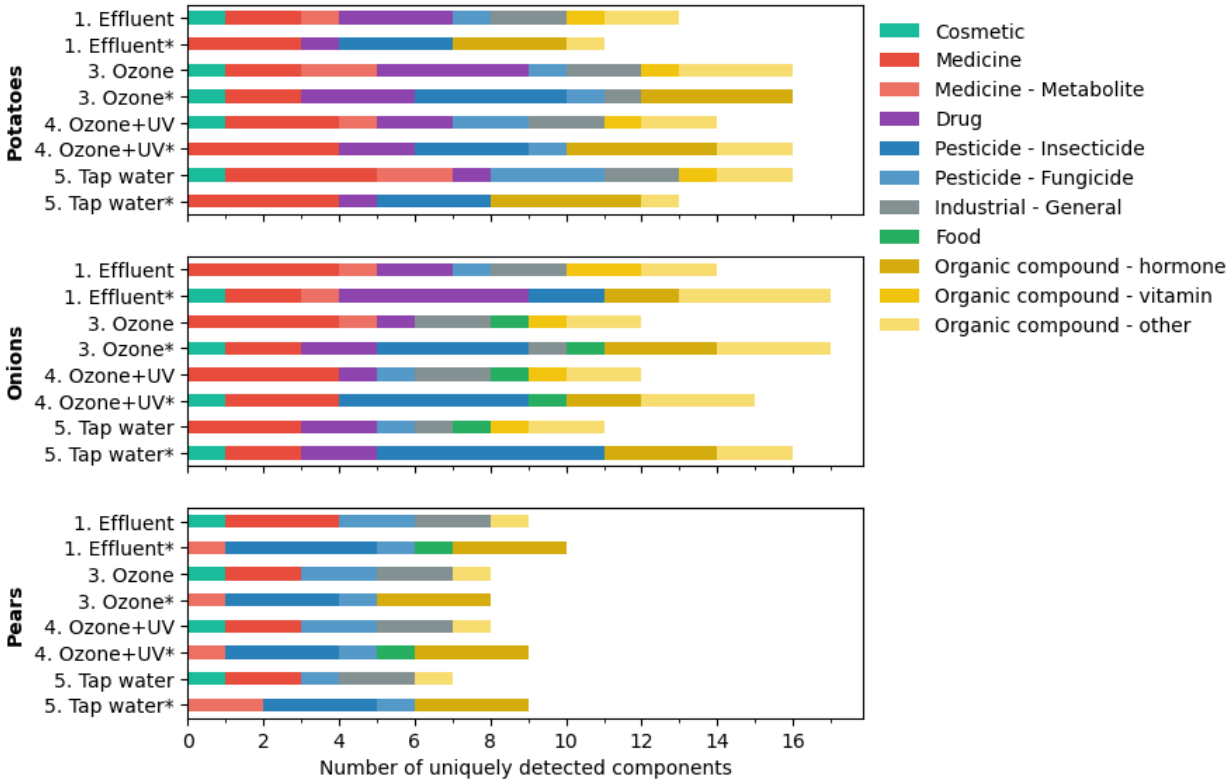



***Figure 6-8*** ***Number of uniquely detected components per crop type and irrigation method. Irrigation types are further categorised into reliable identification and indicative identification (*). The count of uniquely detected compounds per category is classified by the type of OMP: organic compound (hormone, vitamin, other), food additive, industrial, pesticide (fungicide, insecticide), drug, medicine – metabolite, medicine and cosmetics.***

### 6.4.5 Possible sources of OMPs

In the following, several potential sources other than the used irrigation water are discussed. For some potential sources, analysis was performed to check whether this source indeed contains OMPs.

- **Fertiliser granulates**: All crops used the same type of fertiliser granulates, and could thus be a possible source for contaminants. A non-target library-screening analysis of the fertiliser granulates revealed the presence of several components: 2- and/or 4-nitrofenol, 2,3- and/or 2,4- en of 2,6-dinitrofenol, 4-aminophenol, caffeine, 3-keto-carbofuran, estrone, isocyclemone E, pentaethylene glycol, piperonyl methyl keton, pyrethrin I and salicylic acid. However, only three of these components were also detected in crops and not in the effluent: estrone, pentaethylene glycol and pyrethrin I. Thus, the fertiliser granulates are not the main other source of OMPs.
- **Soil**: All crops used the same type of soil, noting that the pear trees were previously cultivated in a different type of soil. A non-target library-screening analysis of the soil revealed the presence of pentaethylene glycol. This component was also detected in all crops and not in the effluent. Since only one OMPs was detected in the soil, we can conclude that the soil is not the main other source of OMPs.
- **Air**: The crops were cultivated in an agricultural area where the use of pesticides is not unlikely. Therefore, it is possible that some of these pesticides have drifted onto our crops. However, this would only explain the presence of pesticides and not all OMPs.
- **Sprinklers**: In a late stage of the project, it was discovered that during the cultivation of the crops, crops in adjacent fields were sprayed several times to protect against frost (in April 2022) and heat (in July and August) (see Chapter 5). Although our crops were not directly sprayed, the high humidity caused the spray water to also reach our crops. In total, spraying occurred for 9 days over multiple hours. Surface water from a nearby river (the Linde), extracted near a WWTP in Zetten, was used for irrigation. This surface water, potentially containing OMPs from WWTP effluent and pesticides from the surroundings, thus represents a probable source of the pharmaceutical residues found in the crops.

## 6.5 Conclusions

Various OMPs have been identified in potatoes, onions, and pears. While some of these OMPs are also present in the effluent of WWTP Walcheren, others are not. The results concerning the components found in the effluent yield the following conclusions.

*Clear impact of water quality on OMPs uptake*
- When using raw effluent, crops absorb multiple OMPs;
- The detection of these OMPs is minimal or occurs at significantly lower concentrations when the effluent is treated with ozone and ozone+UV;
- When raw effluent is used for irrigation, the OMPs in potatoes occur in absolute concentrations ranging from 0.2 to 2.3 ng/g potato. This range of concentrations is comparable to other studies where vegetables and fruits were exposed to WWTP effluent.

*Contaminant distribution across crops*
- Potatoes show the highest detection of contaminants, followed by onions;
- Pears show significantly fewer detected contaminants.

*Unexpected OMPs presence:*
- Some OMPs are found in crops that were not detected in the effluent of WWTP Walcheren. Many of these components are found in equal concentrations across all irrigation methods and likely originate from a source other than irrigation water. Some substances have also been found in fertiliser granulates and the used soil, but most have not. A likely source for these substances is surface water, which was used to spray crops in adjacent fields to protect against frost (in the spring) and heat (in the summer). This surface water may have potentially contained OMPs from the effluent of a nearby WWTP and pesticides from the agricultural surroundings;
- Some OMPs were occasionally detected when ozone is applied for irrigation, suggesting they could be degradation products;
- In future studies similar to this one, it is essential to ensure that measures are taken to prevent cross-contamination of OMPs.

Although a clear understanding has been gained regarding the presence of various types of OMPs in crops irrigated with different qualities of water, it is not possible to conclude whether WWTP effluent or treated WWTP effluent is safe for use as irrigation water. While some studies suggest that the risk of accumulated OMPs in crops to human health is minimal, further research is necessary to investigate the (long-term) risks of consuming crops containing OMPs. The results of this chapter can serve as input for such an analysis.

# 7 Toxicity aspects of organic micropollutants

## 7.1 Introduction

This chapter briefly assesses the detected organic micropollutants (OMPs) in effluent, treated effluent, and crops for toxicity aspects based on their $LD_{50}$ values. This evaluation identifies which OMPs pose the greatest immediate risk to health.

We build on the results described in Chapter 6, which reported the relative concentrations of OMPs from effluent originating from WWTP Walcheren entering the crop products (potatoes, onions, and pears). The crops were irrigated with water of four different qualities: untreated municipal wastewater treatment plant (WWTP) effluent, the effluent treated with ozone, the effluent treated with both ozone and UV, and tap water, which served as a control. Details regarding the effluent treatment and the cultivation process can be found in Chapter 4 and Chapter 5, respectively.

The results of the accumulation of OMPs in crops, with a focus on potatoes, onions, and pears, cultivated using various irrigation water qualities were used to determine the toxicological potency of those recovered OMPs.

## 7.2 Methods

Two different routes were investigated to determine the toxicological potency of the recovered OMPs. We started looking for intake reference values, such as acceptable daily intakes. These values are common for nutrients such as vitamins and minerals but less broadly available for contaminants.

### 7.2.1 Hazard characterisation

Reference points and reference values are used for hazard characterisation. The Open FoodTox database from the European Food Safety Authority was used as a source to retrieve those data. EFSA OpenFoodTox [80] is a chemical hazards database which contains information about toxicity, reference points (NOAEL, BMD, $LD_{50}$, etc.) and reference values (ADI, TDI, etc.). Information about substances is collected from EFSA outputs, including information on food ingredients, pesticides, feed contaminants, and food contact materials. Definitions and descriptions for the reference values ADI, TDI, and NOAEL are presented here [80]:

- *Acceptable Daily Intake (ADI)*
  This is an estimate of the amount of a substance in food or drinking water that can be consumed daily over a lifetime without presenting an appreciable risk to health. It is usually expressed as milligrams of the substance per kilogram of body weight per day and applies to chemical substances such as food additives, pesticide residues and veterinary drugs;
- *Tolerable Daily Intake (TDI)*
  This is an estimate of the amount of a substance in food or drinking water that is not added deliberately (e.g., contaminants) and can be consumed over a lifetime without presenting an appreciable risk to health;
- *No Observed Adverse Effect Level (NOAEL)*
  This represents the greatest concentration or amount of a substance at which no detectable adverse effects occur in an exposed population.

Only limited information was available for OMPs detected in WWTP effluent, and no information was available for OMPs detected in crops. However, for many OMPs, another measure for toxicity is available, that is the $LD_{50}$ value.

### 7.2.2 Measure for toxicity: $LD_{50}$ rat-oral

To compare OMPs for toxicity aspects, $LD_{50}$ values were collected. $LD_{50}$ is the median lethal dose, a toxicological unit that measures the lethal dose of a given substance. The value of $LD_{50}$ for a substance is the dose required to kill 50% of the members of a tested population after a specified test duration. $LD_{50}$ values are often used as a general indicator of the acute toxicity of a compound. A lower $LD_{50}$ value corresponds with increased toxicity. The $LD_{50}$ value depends on numerous variables, such as the route of administration, which can be oral, subcutaneous, intravenous, or otherwise. Furthermore, the $LD_{50}$ value varies per type of species: rat, mouse, rabbit, and so on.

PubChem [81] was used to retrieve $LD_{50}$ values for OMPs. It is a large collection of freely accessible chemical information, including safety and toxicity information. If no information was available for a specific OMP, the Google Search engine was used to identify other websites with $LD_{50}$ values for specific OMPs.

PubChem was checked for all library compounds presented in Annex 7. For many OMPs, it appeared that an $LD_{50}$ value from rats, administered via the oral route ($IC_{50}$ rat-oral), was available, and less often also an $LD_{50}$ value from mice, administered via the oral route ($IC_{50}$ mouse-oral). For many other OMPs, no data were available. It was decided to continue this exercise with the $LD_{50}$ rat-oral.

To get an idea of how poisonous a compound is in comparison with other substances, Figure 7-1 is presented. It is a logarithmic scale, covering a very broad spectrum. Botulinum toxin is one of the most toxic compounds, with an $LD_{50}$ value of 1 ng/kg. Conversely, water is not very toxic, with an $LD_{50}$ value of over 90 g/kg. Ibuprofen, one of the many OMPs detected in the effluent, has an $LD_{50}$ value of 636 mg/kg.

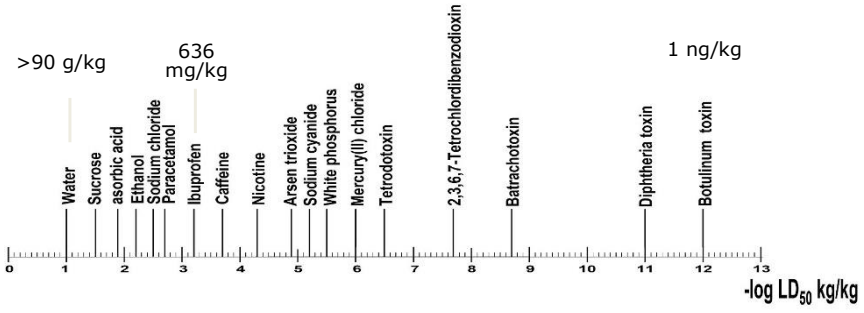



***Figure 7-1 The Poison scale[7].***

## 7.3 Results

This section describes the safety parameters investigated to determine the toxicological potency of the recovered OMPs.

### 7.3.1 Hazard characterisation

The preferred way to compare substances found in crops originating from WWTP effluent or in the effluent itself is to compare intake reference values based on human studies. However, only limited information was available for OMPs detected in WWTP effluent (see Table 7-1), and no information for OMPs detected in crops. Diuron has the lowest ADI, which makes it, for humans, the least favourable substance on this list.

[7] Addapted from *https://en.m.wikipedia.org/wiki/File:Poison-Scale-long.jpg*

***Table 7-1 Overview of OMP detected in WWTP effluent but not in crops. Data taken from EFSA's OpenFoodTox.***

| OMP | Type of OMP | Reference value (human) | Value (mg/kg bw#/day) | Refers to source |
|---|---|---|---|---|
| Melamine | industrial | TDI | 0.5 | http://dx.doi.org/10.2903/j.efsa.2007.1047 |
| Saccharine | industrial | ADI | 3.8 | http://dx.doi.org/10.2903/j.efsa.2017.4722; http://dx.doi.org/10.2903/j.efsa.2014.3787 |
| Bupirimate | pesticide | ADI | 0.05 | http://dx.doi.org/10.2903/j.efsa.2010.1786 |
| Chloridazone | pesticide | ADI | 0.1 | http://dx.doi.org/10.2903/j.efsa.2007.108r |
| Chlorpropham | pesticide | ADI | 0.05 | http://dx.doi.org/10.2903/j.efsa.2017.4903 |
| Cyprodinil | pesticide | ADI | 0.03 | http://dx.doi.org/10.2903/j.efsa.2006.51r |
| Diuron | pesticide | ADI | 0.007 | http://dx.doi.org/10.2903/j.efsa.2005.25r |
| Ethofumesate | pesticide | ADI | 1 | http://dx.doi.org/10.2903/j.efsa.2016.4374 |
| Flutolanil | pesticide | ADI | 0.09 | http://dx.doi.org/10.2903/j.efsa.2008.126r |
| Fluxapyroxad | pesticide | ADI | 0.02 | http://dx.doi.org/10.2903/j.efsa.2012.2522 |
| Metamitron | pesticide | ADI | 0.03 | http://dx.doi.org/10.2903/j.efsa.2008.185r |
| Metobromuron | pesticide | ADI | 0.008 | http://dx.doi.org/10.2903/j.efsa.2014.3541 |
| Thiabendazole | pesticide | ADI | 0.1 | http://dx.doi.org/10.2903/j.efsa.2014.3880 |

#bw: body weight

For TTPO, only one reference value based on rat studies was available (see Table 7-2), showing the No Observed Adverse Effect Level. However, based on only one value, it is impossible to compare substances.

***Table 7-2 OMP detected in WWTP effluent and in potatoes . Data taken from EFSA's OpenFoodTox.***

| OMP | Type of OMP | Reference value (human) | Value (mg/kg bw#/day) | Refers to source |
|---|---|---|---|---|
| TPPO (Triphenylphospine oxide) | industrial | NOAEL | 2 | http://dx.doi.org/10.2903/j.efsa.2012.2737 |

### 7.3.2 $LD_{50}$ rat-oral values

**Guide substances**

The list of guide substances and their respective $LD_{50}$ values (rat, oral) are presented in Table 7-3. This list contains the same substances as the list of guide substances in Chapter 3, proposed by the Ministry of I&W [82], but expanded with eight other substances added during this project. Notably, these $LD_{50}$ values should not be seen as absolute values but rather as a comparison of the order of magnitude of their toxicological effect. Different colours were used to show that effect. Red values indicate the most toxic compounds in this comparison (between 10 and 100 mg/kg), purple values indicate lesser toxic compounds in this comparison (between 100 and 1,000 mg/kg), while blue values indicate even less toxic compounds (between 1,000 and 10,000 mg/kg).

Table 7-3 also presents which guide substances used for WWTP effluent are present in which crop. In potatoes, four different OMPs that are also guide substances were detected, namely carbamazepine, metoprolol, and indicatively, gabapentin and venlafaxine. From these OMPs, venlafaxine is the least favourable substance, indicated by the lowest $LD_{50}$ value.

***Table 7-3 Overview of the guide substances and their respective $LD_{50}$ value.***

| Micropollutant | Type of OMP | $LD_{50}$ (rat, oral) (mg/kg) | $LD_{50}$ (mouse, oral) (mg/kg) | Present in crop |
|---|---|---|---|---|
| 1H-benzotriazole | industrial | 560 | 615 | |
| 4-Methyl-1H-benzotriazole | industrial | no data | no data | |
| 5-Methyl-1H-benzotriazole | industrial | 1600 | no data | |
| Amisulpride | drug | no data | 1024 | |
| Azithromycin | drug | >2000 | >3000 | |
| Carbamazepine | drug | 1975 | 529 | potatoes |
| Citalopram | drug | no data | no data | |
| Diclofenac | drug | 62.5 | 170 | |
| Gabapentin | drug | >8000 | 8053 | potatoes* |
| Hydrochlorothiazide | drug | 2750 | 1175 | |
| Irbesartan | drug | no data | no data | |
| Metoprolol | drug | 3090-4670 | 1050 | potatoes* |
| Propranolol | drug | 660 | 289 | |
| Sotalol | drug | no data | no data | |
| Sulfamethoxazole | drug | 6200 | 2300 | |
| Trimethoprim | drug | >5300 | 2764 | |
| Venlafaxine | drug | 350-700 | no data | potatoes* |

* Component indicatively identified

Table 7-4 shows an overview of the other OMPs detected in both crops and WWTP effluent, with their respective $LD_{50}$ value.

***Table 7-4 Other OMPs (than guide substances) detected both in crop and in WWTP effluent***

| Micropollutant | $LD_{50}$ (rat, oral) (mg/kg) | Present in crop |
|---|---|---|
| 2- and/or 4-nitrophenol | 334, and 202 respectively | pears |
| Adenine | 227 | potatoes |
| Caffeine | 192 | potatoes, onions*, pears |
| Desvenlafaxine | 350(f)-700(m)# | potatoes, onions |
| Flecainide | 50-498 | potatoes |
| Lamotrigine | 245 | potatoes |
| Sulpiride | 9800 | potatoes |

# female (f) or male (m) rat

* Component indicatively identified

Table 7-4 also shows that in some cases, a range was found for the $LD_{50}$ value. For the comparisons made in the two next sections, the following decisions were made:

- Only components with an $LD_{50}$ value are included (there are a lot of OMPs for which no data were found);
- $LD_{50}$ values such as ">5000" have been changed into 5000;
- An average is included when multiple $LD_{50}$ values were found;
- Components are again subdivided into "detected in effluent" and "not detected in effluent";
- A logarithmic scale is used, as the $LD_{50}$ values have different orders of magnitude;
- Components with an asterisk (*) are indicatively identified by AquaLab Zuid.

### $LD_{50}$ of OMPs found in crops and in WWTP effluent

This section presents the findings regarding the $LD_{50}$ values of OMPs detected in the effluent of WWTP Walcheren, as well as in various crops, using different water qualities for irrigation. The section is divided by crop type (potatoes, onions, and pears).

Figure 7-2 shows the $LD_{50}$ values and relative concentrations of organic micropollutants found in both potatoes and WWTP effluent, categorised by irrigation water type. It comprises additional information to Figure 6-1. However, not for all components mentioned in Figure 6-1 *$LD_{50}$ rat-oral* values were available.

Therefore, only the 10 OMPs for which $LD_{50}$ values were available are presented, with those values plotted on a logarithmic scale above the graph of relative concentrations in potatoes.

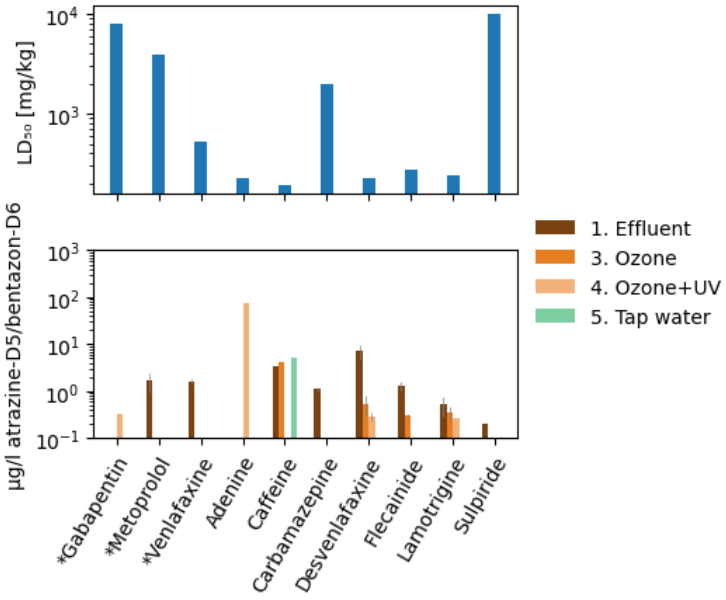



***Figure 7-2*** ***Components in potatoes, also detected in WWTP effluent. Bottom: Average equivalent concentration of organic micropollutants in potatoes per irrigation type. Bars represent standard deviation, and components with an asterisk (*) are indicatively identified. Top: $LD_{50}$ value (oral, rat). A lower $LD_{50}$ value corresponds with increased toxicity.***

The oral rat $LD_{50}$ values provide insight into the order of magnitude of the toxicity of the detected OMPs, and the detected concentrations themselves are equivalent concentration values. Therefore, one should bear in mind that the outcomes are not absolute.

For some OMPs found in potatoes, the absolute concentrations in potatoes were determined. *$LD_{50}$ rat-oral* values were available for four of these components, which are shown in Table 7-5.

***Table 7-5*** ***$LD_{50}$ values of organic micropollutants found in potatoes as well as in WWTP effluent.***

| Micropollutant | Type of micropollutant | $LD_{50}$ (rat, oral) (mg/kg) |
|---|---|---|
| Metoprolol* | Drug | 3090-4670 |
| Venlafaxine* | Drug | 350-700 |
| Carbamazepine | Drug | 1975 |
| Sotalol | Drug | No data |

* Component indicatively identified

The concentrations of the detected OMPs are ranging from 0.2 – 2.5 ng/g (or µg/kg). For comparison, if a person weighing 50 kg consumes 500 g of potatoes containing a component at 2.5 µg/kg, this person would ingest 1250 ng of this component. This corresponds to an intake of 25 ng per kilogram of body weight, significantly lower than the toxic levels ($LD_{50}$) reported for four OMPs, which are in the range of milligrams per kilogram of body weight.

Figure 7-3 shows the $LD_{50}$ values of OMPs found in onions and pears, and in WWTP effluent, categorised by irrigation water type. It comprises additional information to Figure 6-2 for the components for which *$LD_{50}$ rat-oral* data were available. All components have $LD_{50}$ values around 200 mg/kg, which are in the category of less toxic compared to all $LD_{50}$ values found in this project.

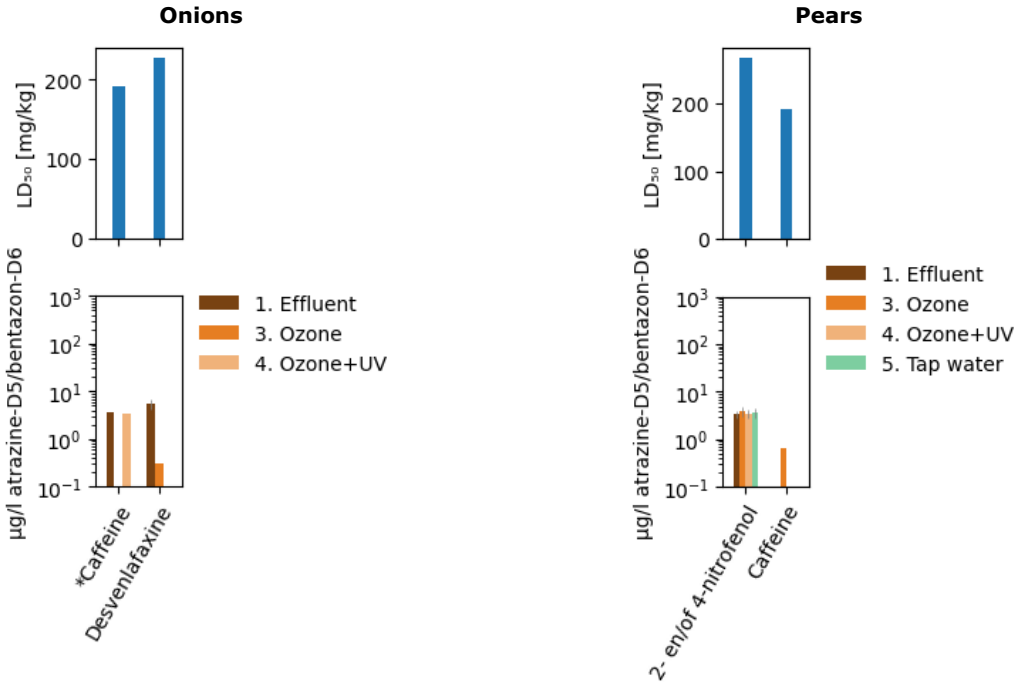



***Figure 7-3*** ***Components in onions and pears, also detected in WWTP effluent. Bottom: Average equivalent concentration of organic micropollutants in onions per irrigation type. Bars represent standard deviation, and components with an asterisk (*) are indicatively identified. Top: $LD_{50}$ value (oral, rat). A lower $LD_{50}$ value corresponds with increased toxicity.***

### $LD_{50}$ of OMPs found in crops but not found in WWTP effluent

There is also a group of micropollutants that was not found in the effluent from WWTP Walcheren, but was detected in the crops. For this group, the source of these micropollutants is not the irrigation water (see Chapter 5). Where available, *$LD_{50}$ rat-oral* values for these OMPs not originating from the WWTP effluent are provided in the figures below (Figure 7-4, Figure 7-5 and Figure 7-6).

$LD_{50}$ values of the components again vary. The lowest $LD_{50}$ values (most toxic) are for the indicative identified substances buspirone (medicine, 136 mg/kg), phorate (insecticide, 1 mg/kg) and cycloheximide (fungicide, 2-133 mg/kg).

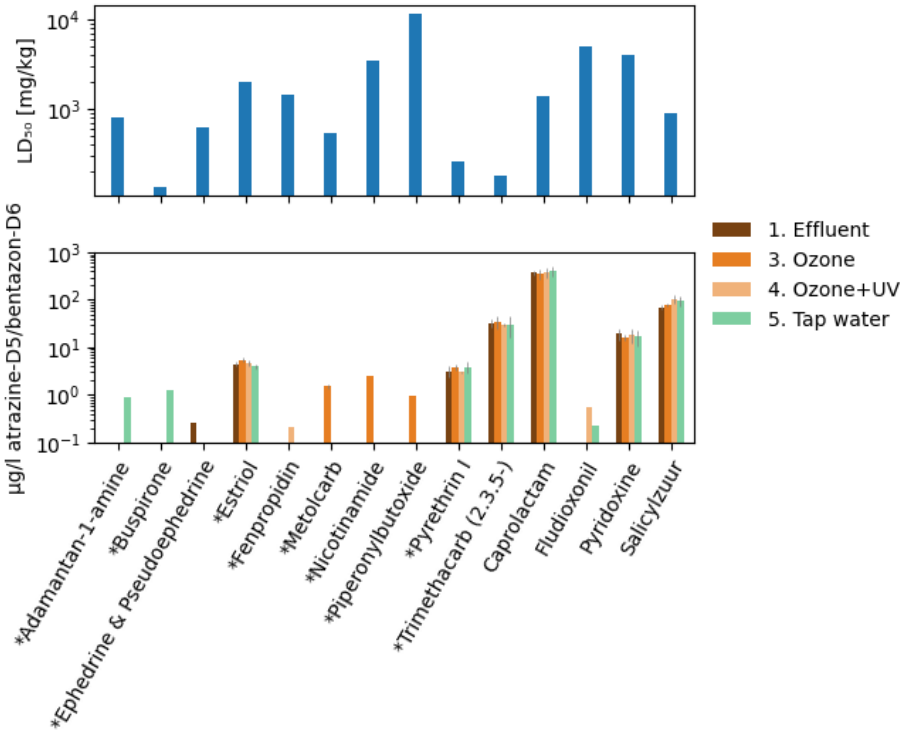



***Figure 7-4*** ***Components in potatoes, but not detected in WWTP effluent. Bottom: Average equivalent concentration of organic micropollutants in potatoes per irrigation type. Bars represent standard deviation, and components with an asterisk (*) are indicatively identified. Top: $LD_{50}$ value (oral, rat). A lower $LD_{50}$ value corresponds with increased toxicity.***

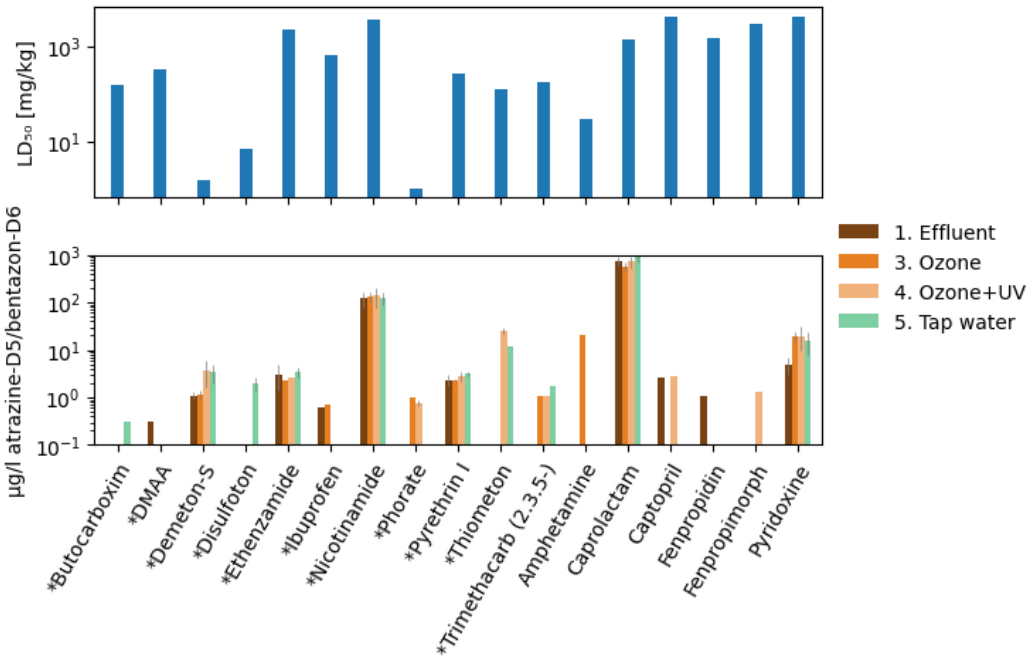



***Figure 7-5*** ***Components in onions, but not detected in WWTP effluent. Bottom: Average equivalent concentration of organic micropollutants in onions per irrigation type. Bars represent standard deviation, and components with an asterisk (*) are indicatively identified. Top: $LD_{50}$ value (oral, rat).***

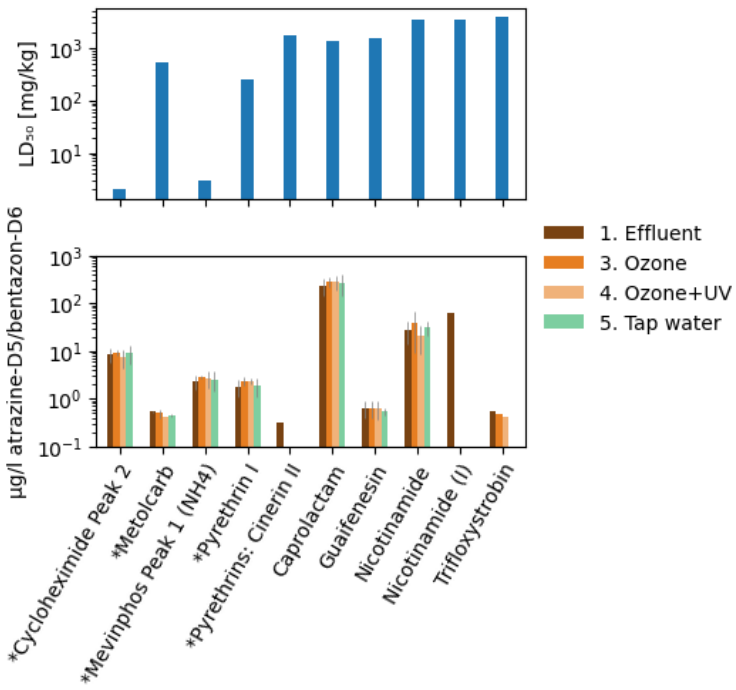



***Figure 7-6*** ***Components in pears, but not detected in WWTP effluent. Bottom: Average equivalent concentration of organic micropollutants in pears per irrigation type. Bars represent standard deviation, and components with an asterisk (*) are indicatively identified. Top: $LD_{50}$ value (oral, rat).***

## 7.4 Conclusions

In this chapter rat oral $LD_{50}$ values were used to provide an indication for toxicity of organic micropollutants. Preferably, we would have used reference values regarding daily or weekly human intake to provide more insight into the long-term effects caused by the consumption of crops irrigated with treated effluent. However, the latter reference values were hardly available.

Because of their availability, rat oral $LD_{50}$ values were chosen to quickly assess the toxicity of components detected in effluent or crops.

For the OMPs analyzed quantitatively in potatoes, the concentrations ranged from 0.2 to 2.5 µg/kg potato. For comparison, if a person weighing 50 kg consumes 500 g of potatoes containing a component at 2.5 µg/kg, this person would ingest 1250 ng of this component. This corresponds to an intake of 25 ng per kilogram of body weight, significantly lower than the toxic levels ($LD_{50}$) reported for four OMPs, which are in the range of milligrams per kilogram of body weight.

The oral rat $LD_{50}$ values provide insight into the order of magnitude of the toxicity of the detected OMPs. The detected concentrations themselves in crops and effluent are equivalent concentration values. Therefore, one should bear in mind that the outcomes are not absolute.

Furthermore, the risk of combined and long-term intake of micropollutants is still unknown and has not been taken into account in the present study.

# 8 Conclusions, gaps and considerations

## 8.1 Introduction

The previous chapters of Part A presented the results from various work packages of the EffluentFit4Food project. These results were discussed by the consortium on April 25, 2024, to determine which conclusions can already be drawn regarding the use of (treated) WWTP effluent as irrigation water for crop cultivation, identifying any remaining concerns, and outlining the knowledge gaps that need to be addressed before conclusions can be made. The findings from this discussion are incorporated into this chapter, which also serves as a summary of the chapters from Part A.

## 8.2 Incentive

Due to increasing drought and salinisation of groundwater and surface water, effluent from WWTPs is seen as a possible additional freshwater source. It is an attractive source because it provides a fairly constant water flow in the summer. Furthermore, techniques are available to remove residual contaminants. This additional purification is also attractive because of changing regulations for the discharge of domestic wastewater.

## 8.3 Project overview

The EffluentFit4Food project investigated whether organic micropollutants (OMPs) from wastewater effluent can be detected in crops (potatoes, onions, and pears) irrigated with this effluent. It also examined whether these contaminants can be efficiently removed from the effluent using a combination of ozone ($O_3$) and UV treatment. Since $O_3$ has the drawback of forming the carcinogenic compound bromate, the effectiveness of a combined treatment using low concentrations of $O_3$ and biological filtration was also evaluated, partially based on findings in literature. This approach focused on the breakdown of OMP residues, particularly pharmaceuticals. A preliminary food safety analysis was conducted, comparing LD50 values of OMPs found in potatoes, onions, and pears, along with an assessment of the absolute concentrations of some OMPs in these crops.

## 8.4 Summary research results

- Some of the substances from the effluent of WWTP Walcheren are present in the crops irrigated with this water (pears, onions and potatoes);
- Post-treatment of the effluent (with ozone and UV) reduces the concentrations of organic OMPs in the effluent and the crops;
- Specific treatment with ozone (at low concentrations) combined with biological degradation reduces the concentrations of pharmaceutical residues less effectively than high ozone doses. However, OMPs still achieve a reduction of almost 70%, and for 7 out of 11 indicator substances, the target of 80% is met. The bromate concentration decreases from 21–51 µg/L to levels below 0.2 µg/L, a factor of 250. Additionally, $CO_2$ emissions are reduced by 50%, and treatment costs by 40%.
- The concentrations of 11 selected OMPs found in potatoes irrigated with raw effluent remain well below the known LD50 values;
- Uncertainties remain regarding the safe reuse of effluent for irrigation, particularly concerning the combined and long-term effects of detected OMPs and other undetected substances;
- Removal of *E. coli* bacteria from reclaimed water intended for irrigation appears to be necessary from a legislation and regulation point of view;

- Currently, there are no specific legislations and regulations concerning OMPs in reclaimed water used for irrigation, though the presence of OMPs should be mentioned in risk analysis. However, some individual OMPs are mentioned in RIVM guidelines and could potentially become part of future regulations.

The question is, how much post-treatment is necessary? It may be sufficient to remove *E. coli* bacteria. This would significantly stimulate the use of effluent for agricultural use.

The advisory group has the following comments on the research carried out:

**Not all substances have been identified**

The water and crops within the project have been tested for over 2000 components/substances using a so-called library screening. As a result, the number of substances examined is large. Nevertheless, some substances or groups of substances (may) have not been included in the research, of which PFAS is the best-known example.

**Relative and absolute concentrations**

The library screening referred to is less accurate than a target substance analysis and gives equivalent concentrations (relative to an indicator) and not absolute concentrations. Therefore, the research results only indicate whether the vast majority of substances are present and the mutual differences between the different irrigation sources.

In addition, absolute concentrations in potatoes have been calculated for eleven OMPs residues using a conversion factor, namely:
- 4- and/or 5-Methyl-1H-benzotriazole
- Carbamazepine
- Diclofenac
- Gabapentin
- Hydrochlorothiazide
- Irbesartan
- Metoprolol
- Sotalol
- Trimethoprim
- Desvenlafaxine
- 1,2,3-benzotriazole

**Risks to public health**

The absolute levels in the crops are in the order of 0.2-2.3 ng/g and are individually well below the lethal dose (LD50 values) per substance. Other studies that used raw WWTP effluent for crop irrigation detected OMPs at similar concentrations and concluded that the risk to public health was *de minimis* [73, 76–78, 83]. However, the picture regarding the actual risks to public health is incomplete, because little is known about the risks of additional intake of:
- the detected individual substances other than the 11 substances mentioned above;
- the detected substances together;
- the effect of intake of the detected substances in the short and long term (cumulation).

**Approach to assessing risks of individual substances**

To provide a rough indication of risk, a comparison was made between the measured values of Desvenlafaxine in potatoes and the LD50 value for caffeine, as LD50 values are not available for all substances.

The highest concentration of Desvenlafaxine found in potatoes was 2.5 ng/g. If a person weighing 50 kg consumes 500 grams of potatoes in one day, this person would ingest 1250 ng, or 25 ng per kilogram of body weight. Compared to the lowest LD50 value found for caffeine (100 mg/kg), this intake represents only 1/4,000,000 of the LD50 for this substance.

While this calculation provides some context, it is important to recognise the limitations of this approach, especially given the uncertainties surrounding the combined and long-term effects of OMPs. Insight into these risks is important before moving forward with the actual application of effluent for irrigation. Here, guidelines from the National Institute for Public Health and the Environment (Dutch: Rijksinstituut voor Volksgezondheid en Milieu, RIVM) are considered.

### Other crops

Three regionally important crops were assessed in this study: potatoes, onions, and pears. The uptake of OMPs from the (post-treated) effluent appeared to be higher in bulb/tuber crops, such as potatoes and onions, compared to pears. However, specific data on the absolute content of these substances was only obtained for potatoes. Additionally, it remains unclear to what extent these results can be generalised to other crop types. For example, green leafy vegetables, such as lettuce and spinach, are known to absorb a wider range of components, often in higher concentrations [71].

### Other types of soil

In this project, crop cultivation was carried out using coarse sand as the soil medium. This type of soil is characterized by rapid water (and contaminant) transport to the crop roots, with no significant soil activity in degrading components. Other soil types, such as clay, are likely to affect transport and degradation processes differently, potentially influencing the uptake of OMPs by crops through the roots.

### Representativeness of used effluent

For the research conducted within the EF4F project, effluent from WWTP Walcheren was used. It is known that effluent composition can vary significantly between different WWTPs, depending on the domestic-to-industrial wastewater ratio. The presence of nursing homes or hospitals can also influence this composition.

The WWTP Walcheren effluent contains bromide, which reacts with ozone ($O_3$) to form the carcinogenic compound bromate. Consequently, the presence of bromide limits the possibilities for using $O_3$ in treatment processes. Bromide is often found at higher concentrations in WWTP effluents from coastal regions [84], such as the WWTP Walcheren. Bromate formation during ozonation can be avoided by using alternative technologies to remove OMPs or by applying mitigation strategies to limit bromate formation. Mitigation strategies include innovative ozone injection methods, adding low concentrations of hydrogen peroxide, or combining technologies (e.g., $O_3$ + biological filtration) to reduce ozone dose. Alternative technologies have drawbacks, such as higher costs or a larger ecological footprint.

A final note on the representativeness of the effluent used in this study concerns the frequency of its use for irrigation. In this research, a worst-case scenario was applied, where irrigation was done exclusively with (treated) effluent. In real-world situations, (treated) effluent would likely only be used during periods of drought, with other water sources used at other times. As a result, the exposure of crops to OMPs in this study is likely higher than it would be under normal circumstances.

### The Time Factor

In the trials, irrigation occurred shortly after the post-treatment of the effluent. However, if there is a larger time gap in practice—such as when the water needs to be buffered in basins—there is a significant chance that *E. coli* concentrations will increase again.

Another time-related factor concerns the accumulation of OMPs in the soil over time. It is unknown how the concentration of OMPs in the soil would evolve if irrigated with contaminated effluent for several consecutive years, or how this might affect the uptake of OMPs by crops.

### Regulatory gaps and future considerations

Current regulations mention *E. coli* as a quality standard for irrigating crops with effluent, but not OMPs. While this is the case for now, future regulations may become stricter and include requirements for (certain)

OMPs. When defining the scope of this project, there was no clear legal framework in the Netherlands on this matter

**Responsibilities**

The responsibilities surrounding the reuse of effluent for irrigating crops remain unclear. While it is evident that provinces serve as the licensing authorities, a comprehensive assessment framework is still lacking. This issue is currently being addressed in a pilot project for reusing effluent near Westenschouwen. In this project, the three involved authorities will collaboratively develop a risk assessment to serve as the basis for the permit.

# Part B

# 9 Development of (dis)continuous monitoring of contaminants in waste water

## 9.1 Introduction

Effectively monitoring effluent quality is crucial for safe and sustainable water reuse practices. However, traditional monitoring methods can be time-consuming, labour-intensive, expensive and too much concentrated on a single measurement point as representative of a longer period. Within WP3 and WP4, Wageningen University & Research (WUR) and HAN University of Applied Sciences (HAN UAS) have focused on developing a novel in-line (bio)monitoring system that contains (1) no living organisms, (2) is robust and highly automated, and (3) is cheap to purchase and direct operating costs. By piggybacking on the wave of open-source tools available today as well as the tremendous possibilities of 3D printing, we are looking to build an innovative instrument that can be easily configured to new experiments or environments. But most importantly, the envisioned system can be rebuilt anywhere in the world where people have access to rapid prototyping systems, thus bringing tools for improving public health and safety to places that currently cannot afford expensive laboratories or devices.

The system will function by continuously passing a small portion of the effluent through a microfluidic cell. This cell will integrate a biochemical assay and an electronic sensor. Data processing algorithms then interpret the sensor signals, providing real-time information about the specific component(s) being monitored in the effluent. By focusing on automation and readily available materials, WP4 strives to develop a practical and cost-effective solution for continuous effluent quality assessment, paving the way for improved water management and resource recovery. To achieve these goals, two promising technical approaches have been identified and explored.

*Approach 1: Microfluidic cell with functionalised microparticles*
This approach utilises functionalised micron-sized beads to detect specific components in the effluent. Beads are equipped with ligands such as antibodies, enzymes, or nucleotide probes, allowing for specific binding to target molecules. The system (dis)continuously flows a small portion of the effluent through the microfluidic cell, where the beads interact with the target molecules. An optical read-out system is used to image bead interactions by electronically positioning the lens. Image analysis and software algorithms are used to detect and quantify the presence of bound beads. This approach offers several advantages, including:

- High specificity due to the use of tailored ligands;
- Real-time monitoring capabilities;
- Potential for low-cost implementation using open-source and consumer-grade materials, and commercially available capture probes.

*Approach 2: Multi-spectral optical detection*
This alternative approach employs a multi-spectral optical detector to detect fluorescent probes specifically targeting certain microorganisms. Although this approach is well-known and described on the state of the art, the inventive step in this WP is the application of open-source and consumer-grade electronics. The main advantages of this approach include:

- Potential for simultaneous detection of multiple microorganisms;
- Use of commercially available probes, making the system cost-effective;
- Rapid and accurate detection of microorganisms.

## 9.2 System overview

The system developed in WP4, regardless of the specific detection approach used (functionalised beads or multi-spectral), relies on several core components:

- Microfluidic cell: A miniaturised reaction chamber, where the necessary biochemical reagents are mixed with a small sample of effluent;
- Biochemical reagents: Specific to the chosen detection approach, these reagents could be functionalised beads or fluorescent probes designed to interact with target components;
- Optical detectors: Sensors that analyse the interaction between the effluent and the reagents within the microfluidic cell. The type of detector will vary based on the chosen approach (e.g., image sensor for bead detection, spectral sensor for fluorescent probes);
- Temperature control unit: This unit regulates the temperature within the microfluidic cell, potentially enhancing the efficiency and specificity of the biochemical reactions;
- Sampling system: A mechanism responsible for extracting a small, controlled sample from the main effluent stream and delivering it to the microfluidic cell within the cartridge;
- Cartridge system: This replaceable unit houses the microfluidic cell, reagents, and potentially other components like filters. The cartridge design will allow for easy replacement while minimising reagent waste and downtime;
- Control and data processing unit: An integrated system that manages various aspects of the device, including:
  - Optical detector control: Optimises parameters like lens position illumination;
  - Temperature control electronics: Regulates the temperature control unit based on predefined settings;
  - Sensor read-out and data processing: Analyses the raw data collected by the optical detectors and translates it into meaningful results;
  - Data output: Publishes the processed data through various channels, such as an internet connection, for real-time monitoring and analysis.

The system overview diagram in Figure 9-1 depicts the key components of the continuous (bio)monitoring system.

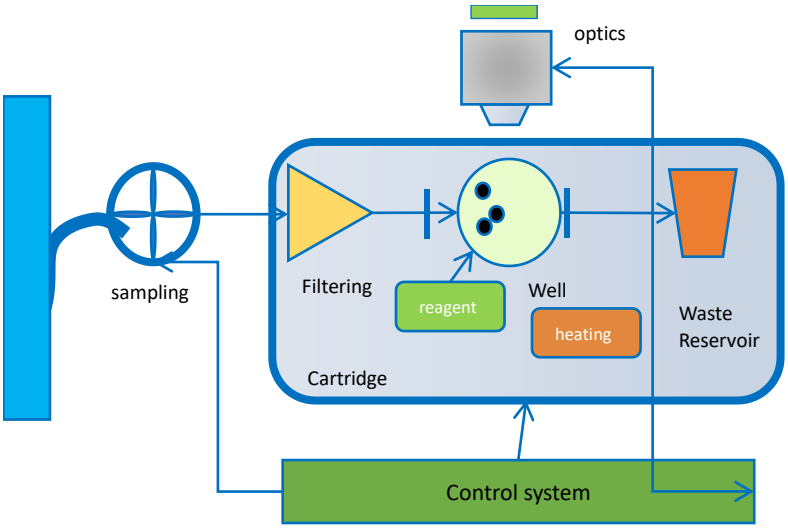



***Figure 9-1 Schematic overview diagram of a (dis)continuous monitoring system.***

## 9.3 System operation - Sample and measurement cycle

The system operates in a (dis)continuous and automated cycle, ensuring real-time monitoring of the effluent quality. Here's a breakdown of the key steps in each cycle:

1. Chamber flush: The system begins by flushing the microfluidic cell within the cartridge with a cleaning buffer. This step removes any residual sample or reagents from the previous cycle, ensuring minimal carryover and maintaining the integrity of the measurement;

2. Reagent introduction: Precisely controlled valves then introduce a small, controlled volume of fresh biochemical reagents into the microfluidic cell. The specific type of reagent will depend on the chosen detection approach (e.g., functionalised nanoparticles or fluorescent probes);
3. Sample injection: Following reagent introduction, a tiny sample of effluent is drawn from the mainstream and injected into the microfluidic cell. The controllable valves ensure precise sample volume, minimising waste and maximising measurement accuracy;
4. Measurement and analysis: Once the sample and reagents are mixed within the microfluidic cell, the optical detectors take measurements. The data acquisition and processing unit analyses this data using pre-programmed algorithms, translating the raw signal into meaningful information about the target component(s) in the effluent.

This cyclical process repeats continuously, providing a constant stream of real-time data on the effluent quality. The system's automation ensures consistent performance and minimizes the need for manual intervention.

A schematic diagram of the monitoring system is shown in Figure 9-2.

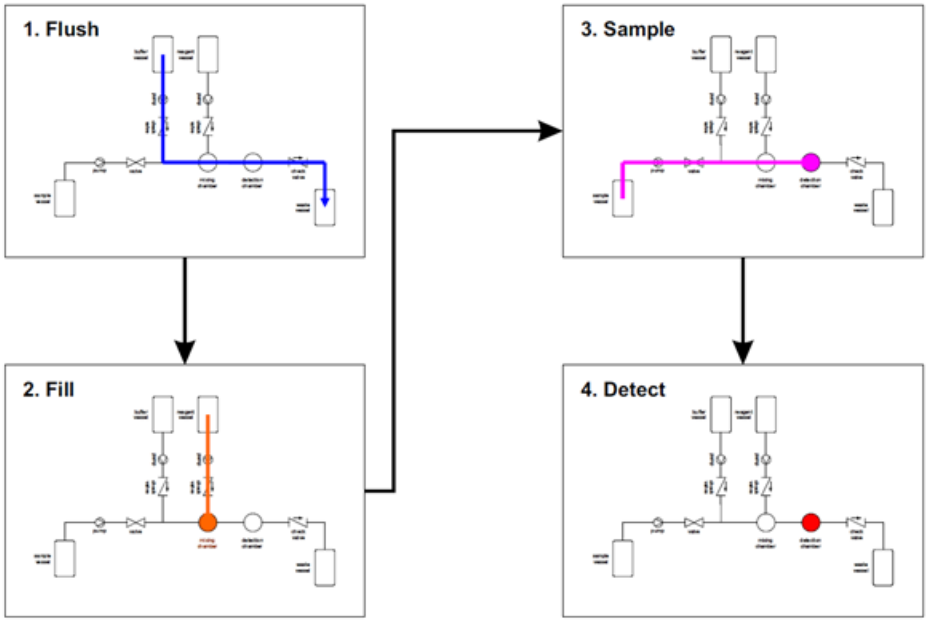



***Figure 9-2 Behavioural diagram of a (quasi)-continuous monitoring system.***

## 9.4 Sample and measurement control

The sample and measurement control system represents a crucial aspect of the (dis)continuous (bio)monitoring system. Through several iterations, a basic proof-of-concept of the sample and measurement control system was realized. The sample system consists of the following components:

- Sample delivery: By selecting a suitable peristaltic pump with minimal pulsation and implementing precise control mechanisms, we successfully integrated a peristaltic pump for accurate sample delivery. This approach offers advantages like contamination-free operation;
- Reagent injection: By utilizing a stepper motor controlled syringe pump system, accurate injection of pre-defined volumes of reagent liquid is enabled. The readily available nature of syringe pumps, commonly used in medical settings, simplifies system integration and maintenance. Here, we use an open-source syringe pump design and replicated an improved version using rapid prototyping;
- Reagent management and refilling: As the reagent liquid is a consumable, a user-friendly refilling mechanism is essential. While replacing the entire syringe initially seemed like an option, the complexity and potential for user error associated with this approach were considered. A more elegant solution involves a separate reagent reservoir from which the syringe pump automatically refills itself by reversing the motor direction. This approach avoids introducing unnecessary T-connectors into the tubing system,

which could compromise system integrity or lead to sample contamination. To prevent backflow and potential sample contamination during the refilling process, the system incorporates small one-way valves. These valves, integrated into a compact block, ensure fluid flow only in the intended direction (from the reservoir to the syringe and then to the microfluidic device).

Note that early designs envisioned a self-contained cartridge housing the microfluidic cell, reagents, and waste reservoir. However, through progressive insight gained from testing and evaluation, we determined that separating the reagent compartment and waste reservoir from the cartridge offers several advantages. This approach allows for:

- Simplified cartridge replacement: The microfluidic cell, which experiences wear and tear over time, can be replaced more easily without discarding the remaining reagents or generating unnecessary waste;
- Enhanced reagent management: Separate reagent compartments enable easier refilling and potentially extend the cartridge lifespan;
- Improved system efficiency: A dedicated waste reservoir simplifies waste collection and disposal, optimizing system operation.

Photos of the set-up are shown in Figure 9-3, Figure 9-4 and Figure 9-5.

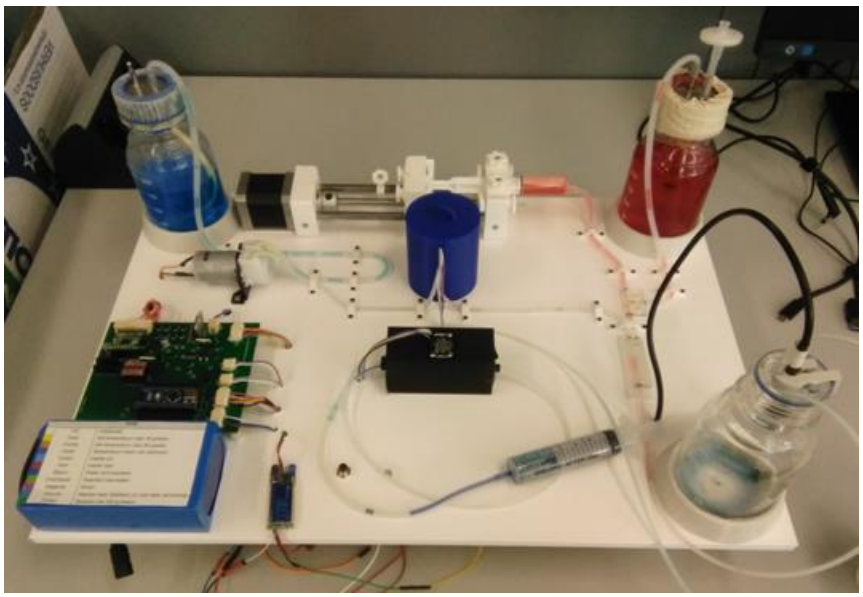


***Figure 9-3 An experimental set-up showing the effluent stream (blue fluid), a reagent reservoir (red fluid), a measurement unit (black box), control electronics, and waste disposal jar.***

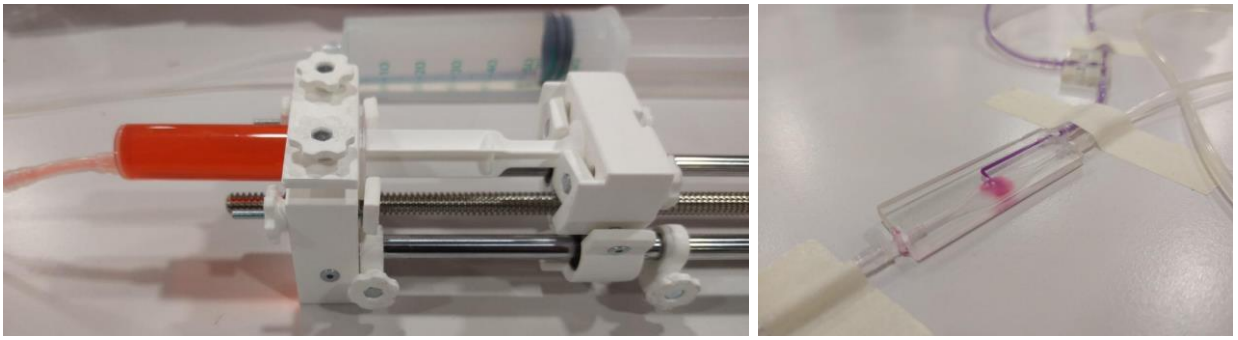


***Figure 9-4 Close up picture of the syringe pump (left) and microfluidic cell (right). In the right figure, diffusion of a reagent substitute (purple fluid) can be observed.***

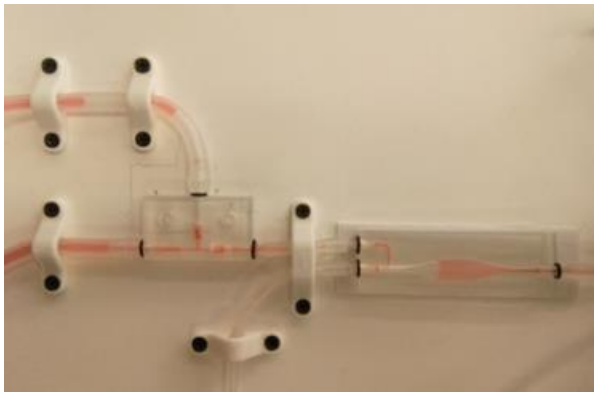


***Figure 9-5 Close up picture of the back-flow protection (left) and microfluidic cell (right).***

## 9.5 Resin printed microfluidic cell

In WP4 we have investigated a miniaturized reaction chamber, where the necessary biochemical reagents are mixed with a small sample of effluent. Microfluidic devices offer exciting possibilities for miniaturized and efficient (bio)chemical analysis. However, commercially available options are often expensive due to low production volumes, limiting accessibility for research labs with budgetary constraints. We sought to overcome this barrier by exploring the potential of 3D printing for microfluidic cell development. Within the realm of 3D printing, resin printers offer a compelling approach for microfluidic device fabrication. These transparent plastic chips can be designed with intricate internal channels and chambers, allowing for precise manipulation of fluids within the device. This technology presents several key advantages:

- Cost-effectiveness: Compared to commercially available options, resin-printed microfluidic chips are significantly more affordable, particularly during the prototyping phase;
- Rapid iteration: The ease and speed of 3D printing enable rapid design changes and iterations. This allows for swift optimization of the microfluidic cell's internal geometry to achieve the desired functionalities;
- Accessibility: The availability of resin printers within HAN and WFBR facilitates in-house microfluidic cell development, fostering greater control over the design process and minimizing dependence on external vendors.

The various iterations depicted here showcase the development towards a final, optimized microfluidic cell design (Figure 9-6). Through rapid prototyping using a resin printer, we were able to explore different design concepts, identify potential challenges, and ultimately refine the cell to meet the specific requirements of the continuous (bio)monitoring system.

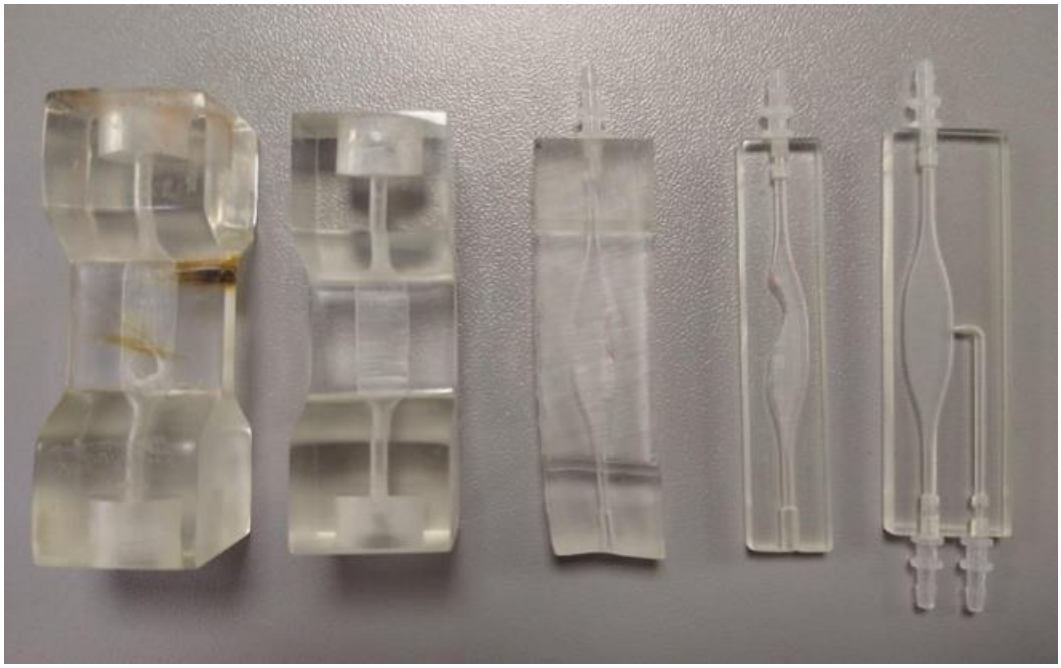


***Figure 9-6 Various iterations of a resin printed microfluidic cell, the device on the right shows the final version.***

## 9.6 Tape microfluidic cell

In addition to the 3D-printed microfluidic cell, in WP4 we investigated a simple and cost-effective approach using double-sided adhesive tape. This method utilizes ultra-thin double-sided tape, with patterns cut using common rapid prototyping techniques, to create microfluidic channels with heights ranging from tens to hundreds of micrometres. A key advantage of this approach lies in the ease of surface modification. Since the layer stack is self-made, we can readily apply hydrophobic coatings to the top or bottom walls for optimized fluid flow characteristics.

The device itself leverages readily available materials like glass or plastic sheets for the top and bottom walls. This basic "sandwich" structure can be further expanded by adding additional layers, such as an interface layer designed for robust fluid port connections. Laser cutting a thin piece of plexiglass or using a cutting plotter with a thin piece of double backed adhesive, are both very quick and easy methods of producing simple microfluidic devices, but they have the disadvantage of being two-dimensional only. They don’t allow for any complex geometry and require special connectors to be able to hook up small tubes for the in- and outlets of the device.

For the optical window, polycarbonate (PC) emerged as the preferred material. PC offers excellent light transmission in the visible and near-infrared range, and is easily machined, making it well-suited for the application. We explored different double sided tapes, resulting in further reduction in channel height from 100 micron down to 10 micron, favouring a lower overall profile for the microfluidic system.

A 3-layer polycarbonate microfluidic cartridge was successfully produced using double-sided adhesive tape and silicone rubber grommets for detachable tubing connections (see Figure 9-7). This exploration demonstrates the potential of double-sided adhesive tape as a simple and affordable technique for microfluidic prototyping.

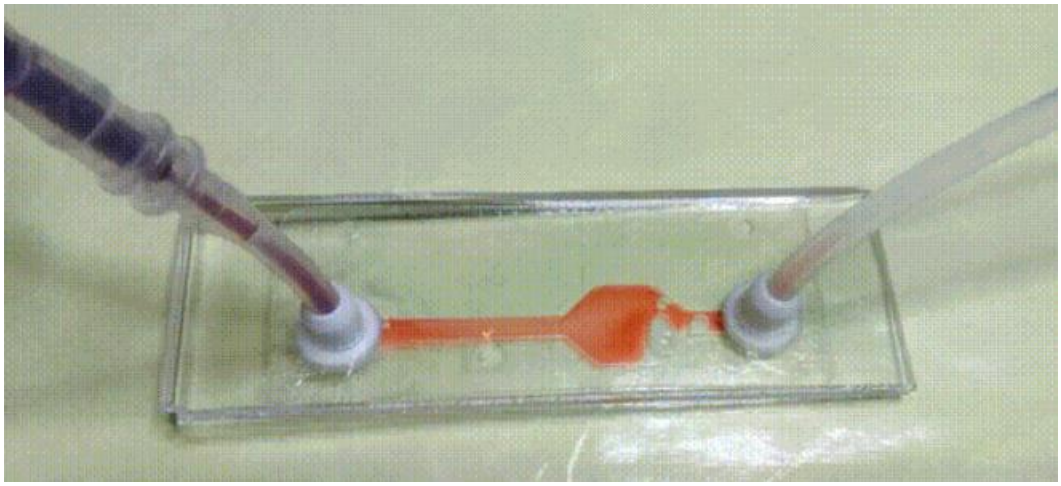


***Figure 9-7 A tape microfluidic cell with silicon tubing attached. Flow of a sample substitute (red fluid) can be observed.***

## 9.7 PDMS device

In addition to the previous types of devices, our exploration extended to Polydimethylsiloxane (PDMS) microfluidics. PDMS is a colourless silicone-based polymer, widely used for making microfluidics. The advantages of working with PDMS include that it offers great freedom of shape and that it is possible to bind PDMS to glass or itself without the need for tape, which can cause leaks.

PDMS is a two part resin that consists of the base resin and a curing agent. After the base and the curing agent are mixed the PDMS becomes a silicon. Because of this it is possible to pour the PDMS in a unique designed mold, and make unique microfluidics. The steps are schematically shown in Figure 9-8.

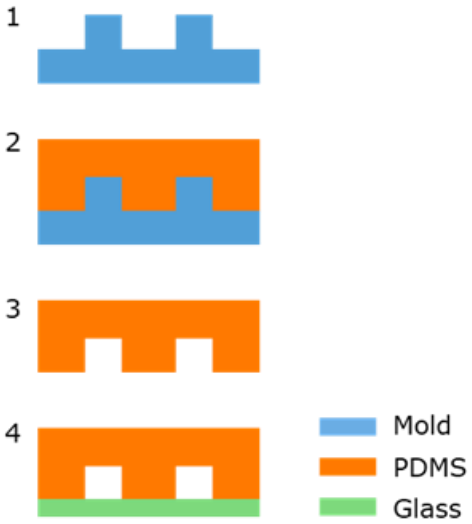



***Figure 9-8 Global steps how to make a PDMS microfluidic.***

### 9.7.1 Device creation

Before it is possible to make the device, a mould has to be made. This mould is designed and drawn using SolidWorks, a modelling software. Once the mould design is finalized, the mould is produced. This was done using an SLA 3D printer (Formlabs Form 2), along with the Formlabs clear v4 resin. This printer was chosen because of the capability of printing with a layer height of 25 microns. After the mould is printed, washed and cured, the mould is ready for use.

The next step involves pouring the PDMS into the mould and allowing it to cure. This way, you can easily make a fully custom microfluidic. To seal the channels, a microscope cover glass is covalently bonded to the PDMS, which ensures a dense system. A cover glass was chosen because the focusing distance of the microscope has to be taken into account.

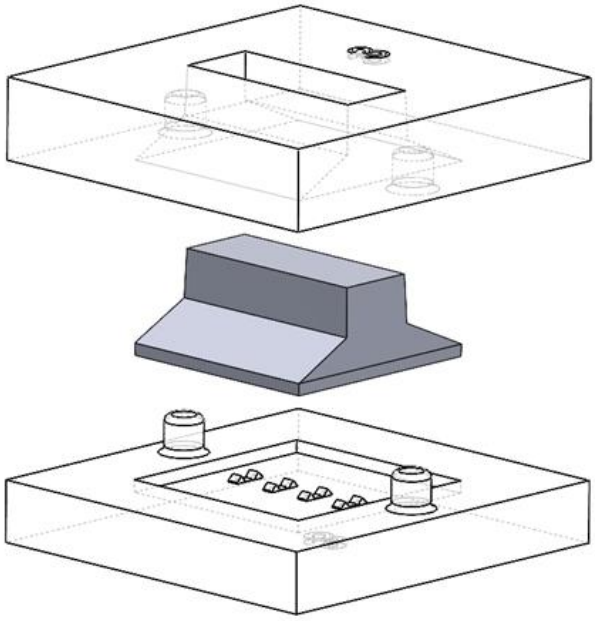


***Figure 9-9 Digital model of the PDMS mold.***

### 9.7.2 Results PDMS device

Several products of the model depicted in Figure 9-9 were made for testing. The tests showed that PDMS does indeed give a lot of freedom of shape, allowing many shape variations to be made. The model above incorporates four chambers, each with a different height (25, 50, 75 and 100 µm). Test results have shown that we get the best image results when the chamber is as low as possible. With our capabilities, 25 µm is the lowest possible to produce, as this is the smallest layer height the SLA 3D printer can print.

Because the PDMS can be covalently coupled to the microscope slide, the chamber was much more resistant to leaks. Also, the silicone properties of the PDMS ensured that the inlets to the chamber were sealed and also remained leak-tight.

In the next stage for device development, more features will have to be added to make the chamber work. This is where the PDMS device is most suitable. This is because it is also possible to link PDMS covalently to PDMS. This ensures that filters and other features can be easily applied.

## 9.8 Functionalized beads

The detection of specific targets within the microfluidic cell relies on the specific clustering of functionalized polystyrene beads in the presence of a target analyte. The technology employs two sets of beads, each coated with a target-specific ligand. These ligands allow the beads to bind the target molecule from opposite sides with high specificity, creating a so called "sandwich" (Figure 9-10). Unlike typical scenarios where beads bind to a surface, these beads move freely and exhibit a tendency to cluster together in the presence of the analyte. Therefore, clustering is hypothesised to be dependent on analyte concentration. The clustering can be visualized by the optical detector and quantified using imaging software, providing the analyte concentration in the effluent.

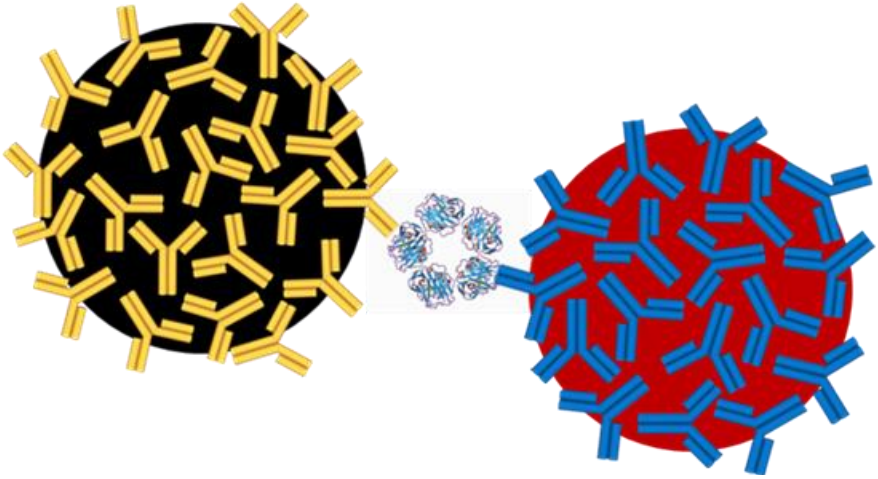


***Figure 9-10 Two functionalized polystyrene beads binding C-reactive protein, forming a sandwich (CRP not drawn to scale).***

The specificity of the system relies on the ligands attached to the beads. Equipping the beads with different ligands allows the system to detect other analytes. For the development of this technology, the detection of C-reactive protein (CRP) was selected as a model. As an inflammatory marker, CRP is not directly relevant for measurement in effluent, yet there are certain considerations which make it a convenient target for test development. Firstly, CRP as a model has been extensively utilized in our laboratory, and antibodies with a range of binding affinities to CRP were previously assessed, and are readily accessible. Moreover, being a pentameric protein, CRP presents multiple copies of the same epitope, thereby enhancing the probability of antibody attachment and enhancing the signal readout. These considerations make CRP a suitable and convenient initial model for the development of the technology.

### 9.8.1 Production of beads

The two sets of bead suspensions were prepared by physically adsorbing either anti-CRP antibody C6cc or C7 onto polystyrene microspheres of 1 micron in size. These microspheres were obtained from Polysciences (15712-15), while the antibodies were obtained from Hytest (C6cc, C7). The microspheres and antibody solutions were suspended in a low salt borate buffer, followed by incubation overnight, allowing adsorption of the antibodies to the bead's surface. Subsequent washing with a blocking buffer aimed to cover unoccupied binding sites and remove any unbound antibodies. Additionally, the beads underwent quick bursts of sonication to prevent nonspecific clustering and enhance the yield of individual beads. Bead functionality was assessed by their performance in lateral flow and agglutination assays.

As an alternative production technique, beads were synthesized via the covalent coupling of antibodies to the polystyrene beads. However, this method resulted in substantial clustering of the beads, rendering them inseparable by sonication. Given the critical importance of single bead availability for optimal technology performance, the covalent coupling approach was deemed impractical and thus abandoned as a viable production method.

### 9.8.2 Assessment of bead functionality

To make an initial assessment of bead functionality, an agglutination assay was performed. In this assay, the two bead suspensions were incubated together in conical-bottom wells, either in the presence or absence of the analyte of interest, CRP. Beads without the analyte mostly remained suspended, while in the presence of CRP functional beads formed clusters, causing them to sink and aggregate at the bottom of the wells. Additionally, the experiment included an assessment of suitable running buffers, with tests conducted using either PBS or borate buffer, and varying concentrations (10%, 1%, or none) of blocking protein (BSA).

Figure 9-11 illustrates the results of the bead functionality and buffer component assessment. The presence of clear bead agglutination in the CRP-positive samples confirmed the functionality of the beads in reacting to the analyte. Regarding the buffer assessment, differences were observed among the various concentrations

of BSA. A high concentration (10%) of BSA resulted in minimal distinction between positive and negative samples, likely due to BSA hindering bead-analyte binding. Conversely, the absence of blocking protein led to non-specific bead agglutination in PBS but not in borate buffer. Overall, conducting tests with 1% BSA yielded the most discernible results, with borate buffer demonstrating slightly superior outcomes, thus suggesting it as an optimal choice for further experimentation.

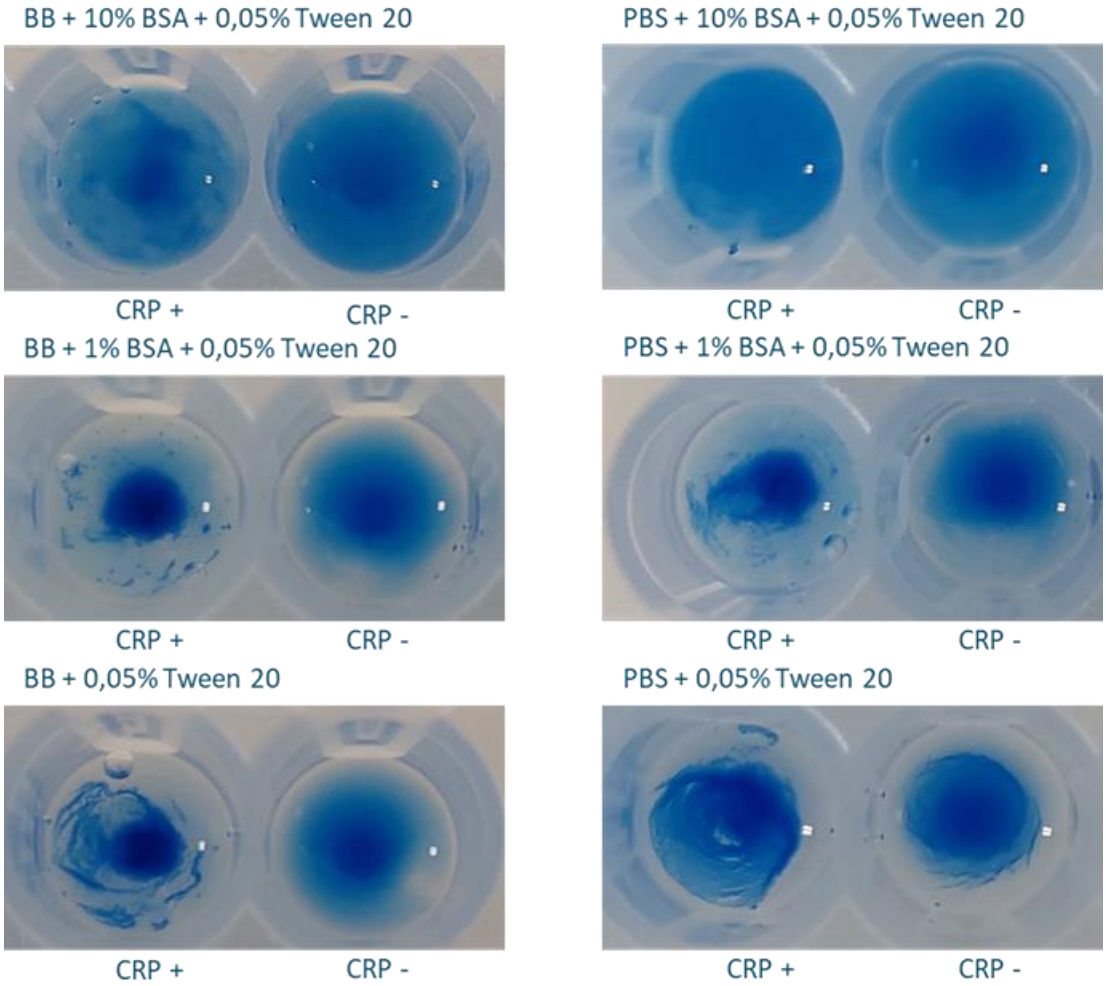



***Figure 9-11 Assessment of bead functionality and buffer components.***

### 9.8.3 Bead performance in a reaction chamber

The next step in the development of this technology was to test the beads in a reaction chamber and observe them under a microscope. The 1 µm beads were clearly visible at 400x magnification. Since the development of the device for this technology was performed in parallel with the development of the beads, these experiments were performed with other reaction chambers, such as Counting Chambers (Bürker). During these experiments, two concentrations of CRP were tested as well as a blank, and a variety of conditions.

As can be seen in Figure 9-12, clustering of the beads increased with the CRP concentration. This was the first indication that clustering is dependent on CRP concentration. This provided a proof of concept. In the initial iteration, the beads were diluted at a ratio of 1:20, and images were captured after 60 minutes. Additionally, it became apparent that the height of the reaction chamber is an important factor for signal interpretation, as many beads will not be in focus in higher chambers. In Figure 9-12, white dots can be observed which are beads that are out of focus. This underlines the pivotal role for device design with regards to chamber dimensions and focusing of the optical sensors.

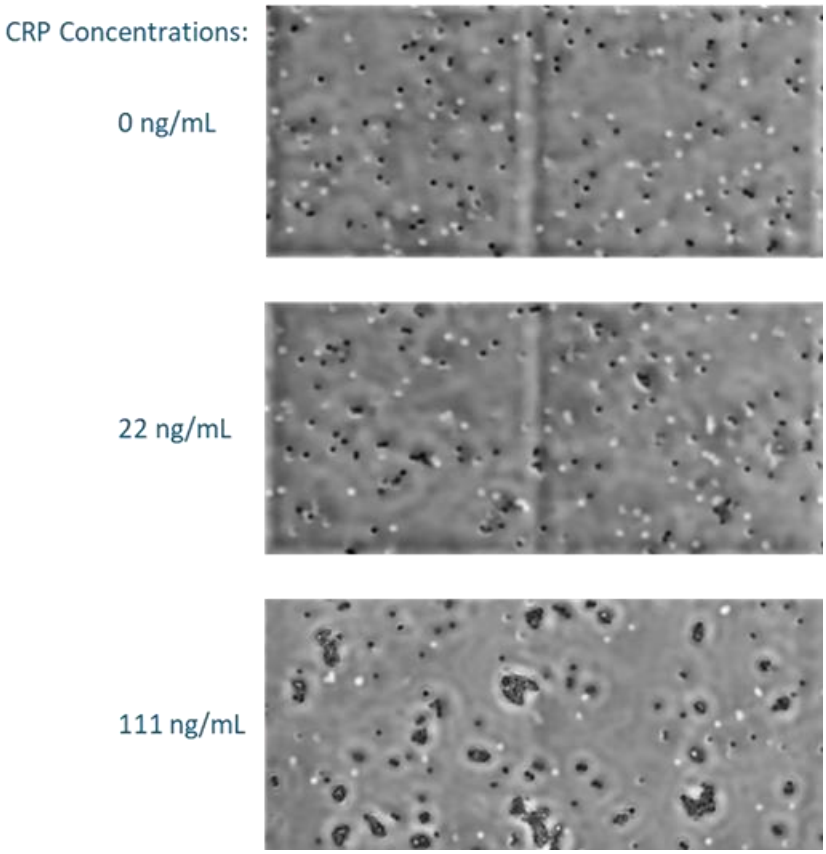



***Figure 9-12 Microscopic images of bead clustering in response to different concentrations of CRP (0, 22, 111 ng/mL). Images were taken after 60 minutes, with a bead dilution of 1:20, using a chamber with a depth of 0.2 mm.***

In subsequent testing, a higher concentration of beads was utilized, allowing a reduction of the incubation time. Additionally, these experiments were conducted in a reaction chamber with a height of 0.1 mm, half of the height used before. Similar to the previous experiment, the beads were subjected to several concentrations of CRP (0, 10, 1,000 and 100,000 ng/mL). As can be seen in Figure 9-13, bead clustering was dependent on CRP concentration, and was already apparent 5 minutes after the addition of the analyte. These images were subjected to signal quantification, as is further discussed in paragraph 9.11. Further optimization will need to be done to shorten this time-span even further, allowing continuous monitoring.

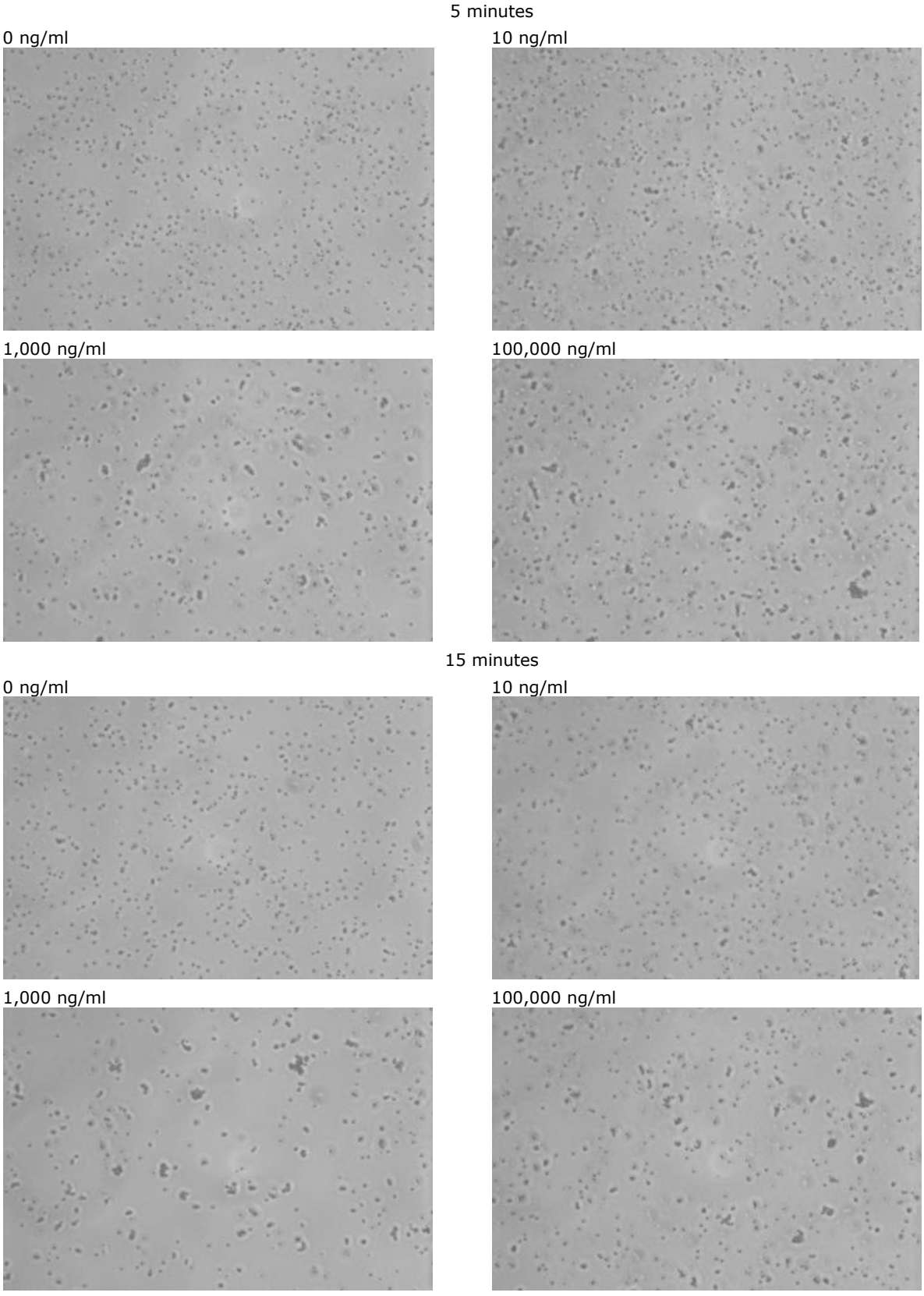



***Figure 9-13 Microscopic image of bead clustering in response to different concentrations of CRP (0, 10, 1000 and 100,000 ng/mL). Images were taken 5 and 15 minutes after the addition of the analyte, with a bead dilution of 1:5, using a chamber with a depth of 0.1 mm.***

## 9.9 Bead imaging and focusing

While high magnification (40x) is essential for visualizing micron-sized beads within the microfluidic channels, it presents a challenge: limited depth of field. This narrow focal plane, typically around 1 micron, results in blurry objects outside of the focus zone, significantly reducing image contrast and quality. To overcome this limitation, a controllable focus system becomes crucial.

### 9.9.1 Initial prototype with repurposed optics

Initially, we explored an inverted bright-field microscope design utilizing a Toshiba PHR 803T optical pick-up unit salvaged from a DVD reader. This approach, previously successful in other open-source projects, featured custom drive electronics for actuating the objective's voice coils, enabling focal adjustments. This autofocus system offered potential for continuous sample monitoring, applicable to cell viability testing or observing dynamics in immunoassays. The project's design philosophy emphasizes affordability and accessibility, leveraging open-source tools and rapid prototyping to enable replicability in resource-constrained settings.

### 9.9.2 Refinement with commercial objective lens

Unfortunately, the repurposed optical pick-up unit's quality fell short of the project's requirements. In WP4 we redesigned the system around a commercially available (COTS) objective lens. To achieve precise focusing of the beads within the microfluidic chamber, a 3D-printed stage equipped with three miniature stepper motors was devised (Figure 9-14). This movable stage was then integrated with a Raspberry Pi camera and an illumination stage to create a new version of the bright-field microscope. The entire system was housed within a custom 3D-printed enclosure designed to interface seamlessly with the microfluidic cell (Figure 9-15). Figure 9-16 shows an example of microbeads that are now focused and imaged by the system.

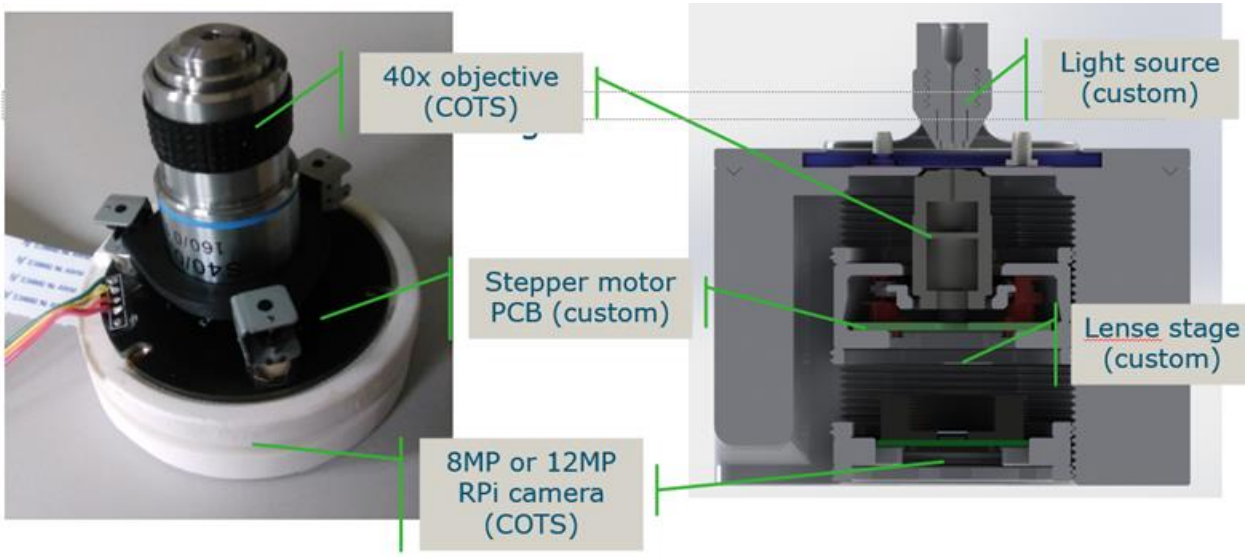



***Figure 9-14 A movable objective stage for bead focusing using a triple stepper motor configuration.***

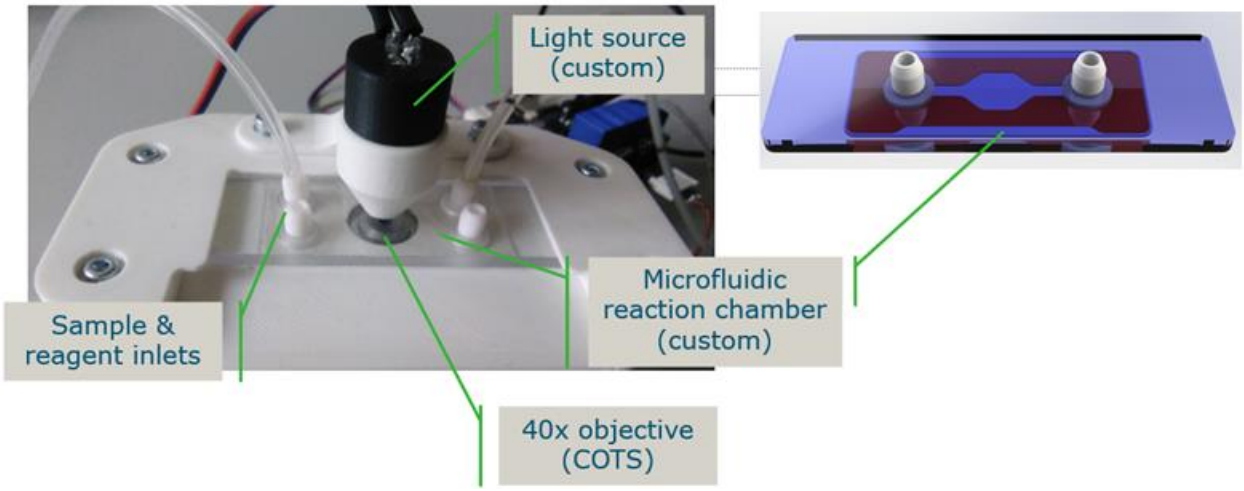



***Figure 9-15 The objective stage is integrated into a housing that also interfaces to the microfluidic cell.***

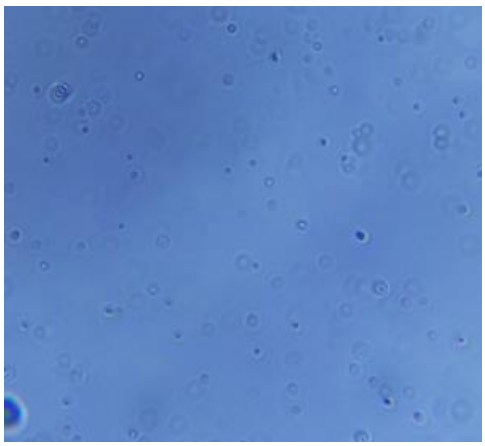


***Figure 9-16 Micron-sized beads are focused and imaged by the system.***

## 9.10 Control electronics

The control electronics play a critical role in automating and coordinating the various system components to achieve the intended operation. The project leverages a Raspberry Pi as the core of the electronics system and develops a custom add-on board, or "hat", to extend its functionalities. This approach offers flexibility for adapting the system to specific requirements.

Here's a breakdown of some key functionalities:

- System preparation: The electronics initiate system preparation tasks, including flushing the microfluidic cell to ensure cleanliness and regulating the temperature to the desired level for optimal chemical reactions;
- Sample and measurement: Once the system is prepared and the fluids are ready, the electronics control the pumps (likely stepper motor-driven) to precisely deliver precise volumes of sample and reagents into the microfluidic chamber for analysis;
- Data acquisition: During the measurement phase, the electronics trigger the capture of optical images or videos using the integrated camera. This captured data serves as the basis for subsequent analysis;
- Data storage and management: The captured data can be stored locally on a storage medium or uploaded to cloud-based storage for remote access and analysis. This ensures data safety and facilitates further processing or sharing.

Additional functionalities:

- Precise control: Stepper motor controllers are integrated within the electronics to ensure precise control over various functionalities, including:
  - Pump operation: Precise control of the pumps guarantees accurate and repeatable sample and reagent volumes;
  - Focus system: Stepper motors controlled by the electronics enable precise adjustments of the focusing stage for sharp bead imaging;
- Temperature regulation: The electronics interface with heating and cooling devices, such as thermoelectric modules (TEMs), to maintain the desired temperature within the microfluidic cell;
- Illumination control: The electronics regulate the illumination stage, optimizing lighting conditions for image capture;
- Remote Connectivity: The Raspberry Pi-based electronics platform facilitates remote system control. This allows users to initiate measurements, monitor progress, and access captured data remotely through a network connection. Additionally, the system can be configured to send results or raw data to designated locations for further analysis.

Software applications designed for real-time particle detection (e.g., micron-sized beads or cells), image annotation, and object counting have been developed using Python3, PyQt5, and OpenCV2, and run directly on the Raspberry Pi. This software is configurable to different target analytes, enabling versatility in the system's application. Future advancements include object tracking functionalities to study particle dynamics and interactions, potentially leveraging machine learning packages like TensorFlow.

The electronics design process prioritizes robustness by incorporating ESD protection circuitry. Additionally, a custom PCB (printed circuit board) was created to ensure reliable and compact electronics integration. Figure 9-17 shows the design and realization of the custom control electronics.

By combining readily available components like the Raspberry Pi with custom-designed elements, the project achieves a cost-effective and powerful electronics system tailored to the specific needs of the continuous (bio)monitoring system. Furthermore, the open-source approach to software development fosters collaboration and knowledge sharing within the scientific community.

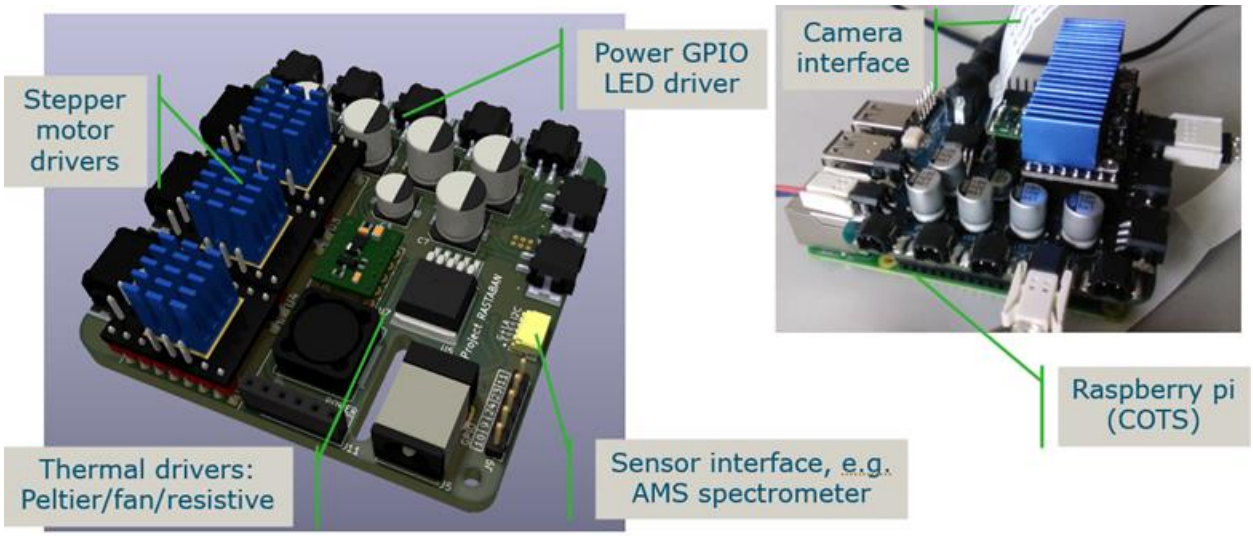



***Figure 9-17 Design (left) and realization (right) of custom control electronics.***

## 9.11 Data processing pipeline

Extracting meaningful information from the captured microscope images and videos is crucial for understanding the behaviour of beads within the microfluidic cell. This section delves into the data processing pipeline designed to analyse these images and ultimately quantify the target substance concentration.

As illustrated above, the functionalized beads form clusters depending on target substrate concentrations (Figure 9-18). In WP4, we explore the hypothesis that the ratio of clustered beads to single beads within the microfluidic chamber correlates directly to the concentration of the target substance present in the sample fluid.

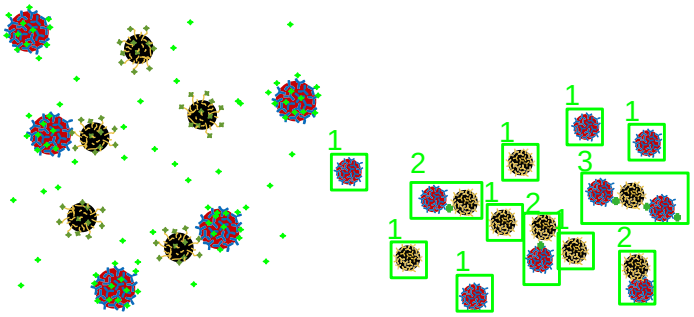
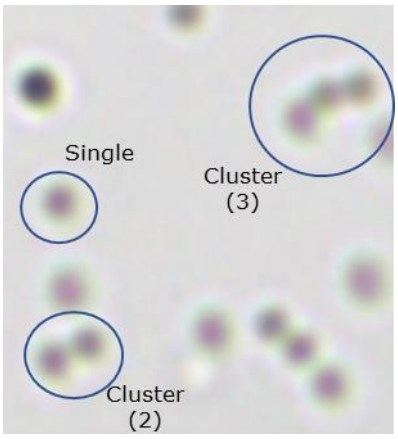


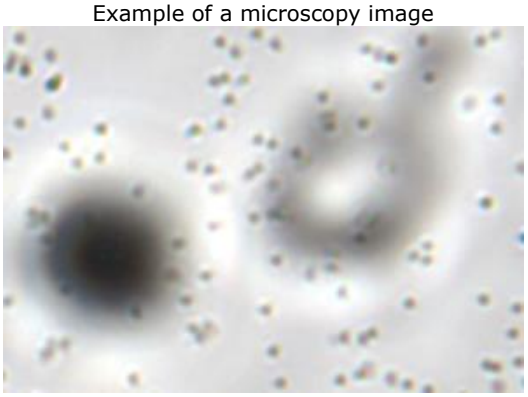
***Figure 9-18 The presence of the analyte is hypothesized to induce increased clustering of beads with differentially attached ligands.***

In practice, processing microscopy images can be challenging due to varying illumination conditions (Figure 9-19), focus planes, the presence of debris, etc. Before applying any machine learning technique, the project employs various image processing methods to prepare the image data for analysis. These fundamental techniques aim to enhance the quality of the images and facilitate accurate detection of features of interest, including noise cleaning, and applying filters to enhance specific features of the beads while suppressing irrelevant background information.

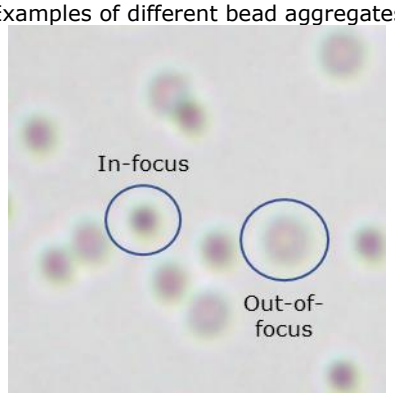
Example of a microscopy image




Examples of different bead aggregates



Examples of debris




Examples of different single beads

***Figure 9-19 Example microscopy images showing single beads, clusters and debris.***

In this WP, we further investigate the potential of machine learning techniques to automate the analysis of these images and videos. Machine learning encompasses three primary categories: supervised, unsupervised, and reinforcement learning. Here, we will apply only two:

- Supervised Learning: This approach requires labelled datasets where each data point is categorized into a specific class, either single bead or cluster.
- Unsupervised Learning: In contrast, unsupervised learning deals with unlabelled datasets where the underlying structure of the data needs to be discovered.

### 9.11.1 Related studies

As part of their studies, several students of HAN UAS have conducted a literature study focusing on the application of machine learning techniques for the analysis of clusters in a biological context. This study provides an overview of previous applications, and provides insight into relevant factors, as well as various approaches within this domain. It can be found in Annex 8.

### 9.11.2 Unsupervised learning

First, we explored unsupervised learning techniques, specifically focusing on popular clustering algorithms commonly used in microbiology, K-means and DBSCAN:

- K-means: This widely used algorithm partitions the image data into a predefined number (k) of clusters based on the mean feature values within each cluster. In our case, K-means could potentially differentiate between single beads and clusters based on features like size or intensity;
- DBSCAN (Density-Based Spatial Clustering of Applications with Noise): This algorithm excels at identifying clusters of arbitrary shapes in noisy data. This was particularly relevant for our project, given the potential presence of unfocused beads.

Next, we investigated a combined approach utilizing both K-means and DBSCAN algorithms. This strategy aimed to leverage the strengths of each method: K-means for its efficiency in partitioning data and DBSCAN for its ability to handle noise and identify clusters of irregular shapes. The different steps of this approach are shown in Figure 9-20. This combined approach demonstrated promising results in distinguishing between single and clustered beads, even for images with some degree of blur.

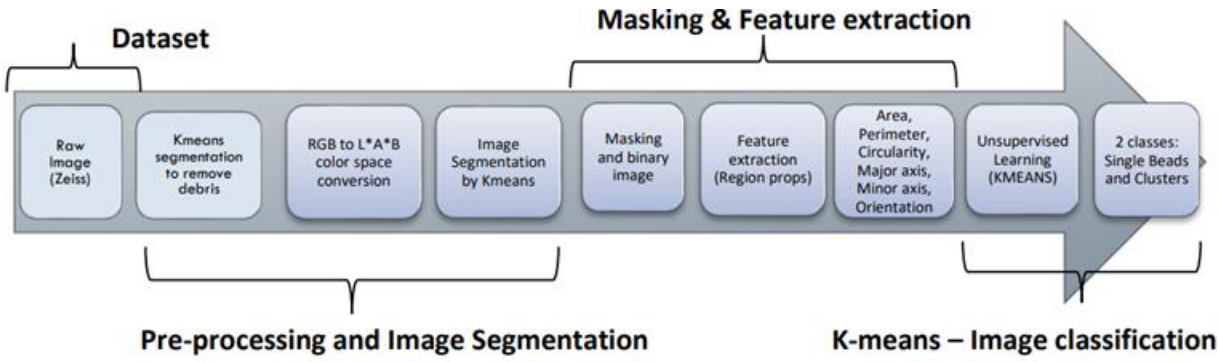



***Figure 9-20 Unsupervised learning pipeline.***

While unsupervised learning offered valuable insights into the data, a key challenge emerged: limited ground truth data for validation. Since the project primarily focused on the presence or absence of the analyte, the exact number of single beads and clusters in each image remained unknown. This lack of information regarding the "correct" cluster distribution made it difficult to definitively assess the unsupervised learning models' performance. Examples of cases specifically investigated to check the unsupervised learning results are shown in Figure 9-21.

Despite the limitations encountered, the exploration of unsupervised learning has been a valuable exercise. The insights gained regarding cluster characteristics and the combined K-means and DBSCAN approach offer a solid foundation for future development. One strategy would be to apply active learning, which allows the model to iteratively query for labels on the most informative data points, potentially enabling efficient learning with minimal human intervention.

This approach unfortunately was not pursued due to lack of time.

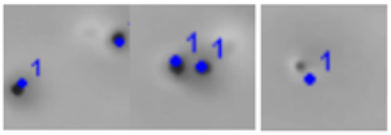
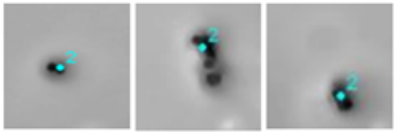
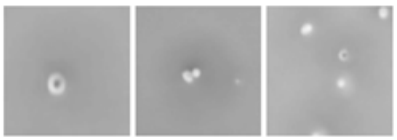
| | |
|---|---|
|  | The images provided are examples of single beads, which have been manually annotated as such. The second image is considered to be two beads that are located close to each other. Additionally, beads that are surrounded by white layers are considered to be of high density based on visual assessment – third image. |
|  | The images provided are considered clusters, as they depict two or more beads that are attached together. |
|  | In the images provided, complete white spots are not annotated as they are not within the scope of the project. Additionally, black spots that are surrounded by white spots and are small or low-density are not taken into consideration during the annotation process. |

***Figure 9-21 Some cases, specifically investigated to check the unsupervised learning results.***

### 9.11.3 Supervised learning with synthetic data

Another approach to deal with unlabelled data, lies in the utilization of synthetic data. In WP4, we looked into supervised learning as a potential approach for accurate bead detection and cluster identification.

Traditional supervised learning relies on real-world image datasets for training models. However, in our case, acquiring a large dataset with accurately labelled single beads and clusters proved challenging. To overcome this limitation, the project explored the generation of synthetic data. This approach leverages the understanding that bead images can be approximated as 2D Gaussian kernels with varying sizes and color intensities depending on their location within the focal plane. By exploiting this insight, the project can synthetically create a vast number of training images featuring single beads and clusters with different characteristics.

Within this synthetic environment, clusters are defined as beads positioned in close proximity, while single beads represent isolated objects. This clear definition of ground truth facilitates the labelling process crucial for supervised learning.

Note that while synthetic data offers significant advantages in overcoming limitations with real-world data availability, it is important to acknowledge potential drawbacks. Synthetic data may not fully capture the complexities and variations present in real-world images. Therefore, future work should involve incorporating real-world data, potentially using techniques like transfer learning or domain adaptation, to refine the model and enhance its generalizability.

*Feature extraction and exploration*
Before feeding the synthetic data into the learning model, a pre-processing step is applied. This step involves the following:

- Binary Blob Extraction: Individual beads are identified and isolated as binary blobs within the images;
- Blob contour analysis: The shape of each blob is characterized by extracting its contour;
- Geometric feature computation: A set of basic geometric features is computed for each blob, including area, perimeter, aspect ratio, extent, solidity, and Hu moments.

Examples of these steps are shown in Figure 9-22.

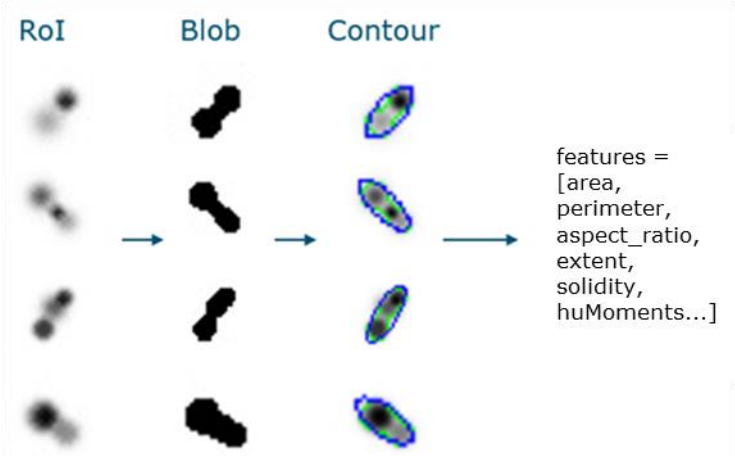



***Figure 9-22 Examples of synthetic bead images in a Region of Interest, from which blobs, contours and features are extracted.***

*Feature extraction and exploration*

By analysing the extracted features (e.g., through scatterplots and correlation heat maps, Figure 9-23), we identified a simple yet effective approach for distinguishing single beads from clusters. This approach relies primarily on the aspect ratio of the blob, a valuable feature as it remains unaffected by image scale or rotation.

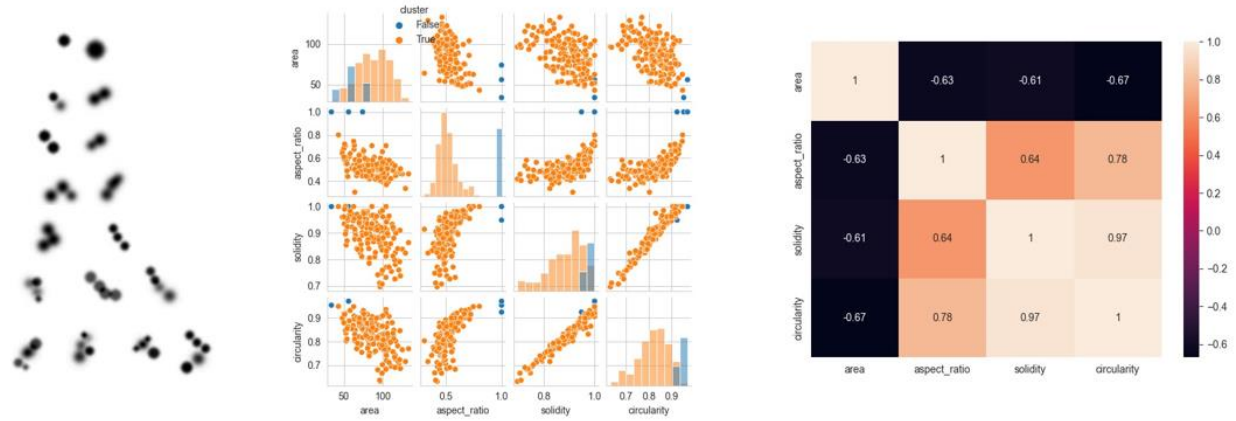



***Figure 9-23 Examples of scatterplots and correlation heatmap of various blob features.***

Once the supervised learning method had been trained on with synthetic data, the method was then applied to hundreds of microscopy images that were obtained from real-life experiments. Example of a real microscope image processing is shown in Figure 9-24.

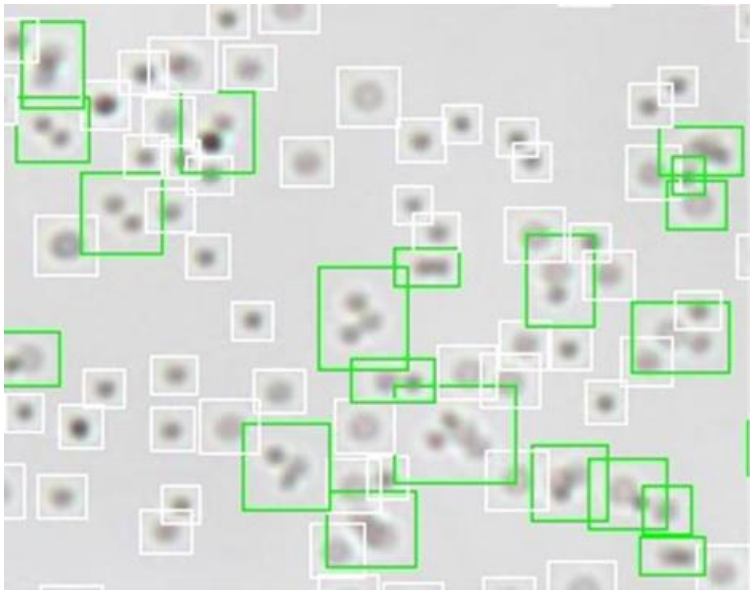


***Figure 9-24 Example of a real microscope image processing, indicating single beads (white box) and clusters (green box).***

Overall, the exploration of supervised learning with synthetic data demonstrates a promising avenue for achieving robust bead detection and cluster identification, paving the way for a highly accurate microfluidic analysis system.

Finally, the method has been applied to a series of experiments, as outlined in paragraph 9.8. Beads were incubated with the target analyte, CRP, and a control fluid. Figure 9-25 depicts the successful application of a supervised machine learning method for detecting CRP. The data presented suggests a correlation between the ratio of clustered beads to single beads and the presence of CRP in the sample fluid.

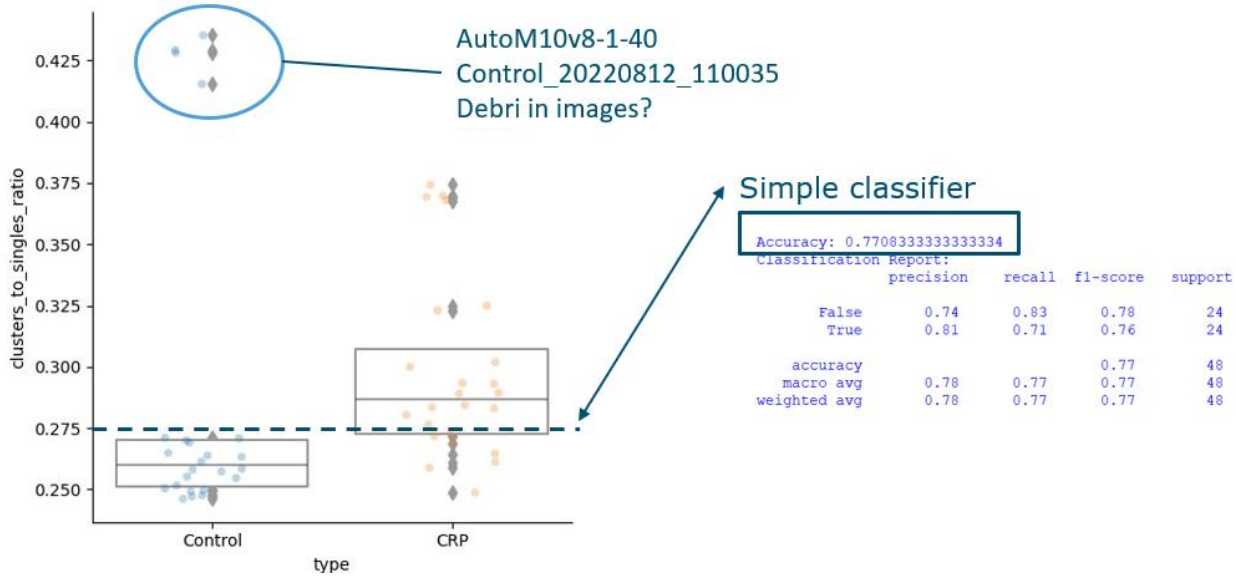



***Figure 9-25 Example of CRP detection using the proposed supervised machine learning method.***

In essence, the presence of CRP appears to influence the aggregation behaviour of the beads, leading to a higher formation of clusters at higher CRP concentrations. These preliminary measurements provide promising evidence for the feasibility of the approach.

The method was further applied to a more intricate dose-response experiment, the images of which can be found in Figure 9-13 in paragraph 9.8.3. The algorithm's output is depicted in Figure 9-26. With the given parameters, the algorithm effectively distinguishes between the various cluster-to-singles ratios in the

images, and thus visualize the difference in CRP-concentrations. The images taken 5 minutes after the addition of CRP show an exponential increase in signal intensity, though more experiments are required to validate this. Interestingly, the curve of the images taken 15 minutes after the addition of CRP exhibits a decline in signal intensity at high CRP concentrations, although there is clear clustering in the images. It is hypothesized that this decline is due to a lower number of clusters, but an increase in cluster size. The algorithm considers only the number of clusters, but does not take their size into account. Therefore, when many small clusters aggregate together into one big cluster, the signal output will change. It is hypothesized that this effect is caused by the differences in speed at which the beads and clusters travel through the chamber. Single beads move at a similar speed and in a similar direction, whereas clusters gain more mass, and move slower. These dynamics make it more likely for single beads to bump into the slow moving clusters which have already formed, rather than forming new clusters with other single beads. Thus, in the current setup, cluster size appears to become a significant parameter at high CRP concentrations.

These experiments provide a proof of concept, however further validation with a larger dataset is likely necessary to confirm the reliability and generalizability of the observed correlation. Additionally, further optimization is required before the technology can be tested in the field.

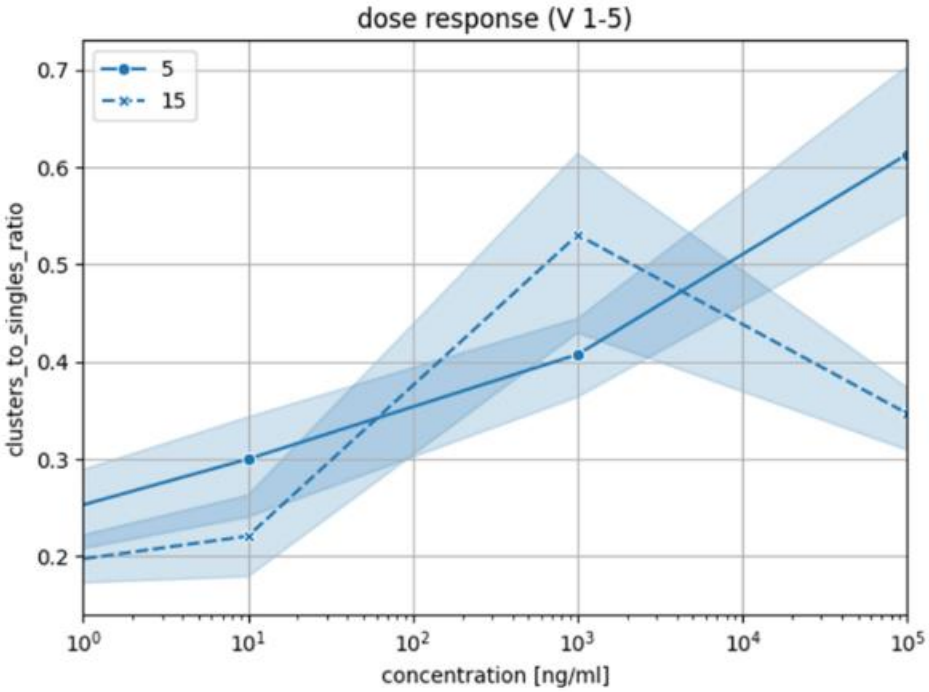



***Figure 9-26 Dose-response curve of CRP, employing the proposed machine learning method. The signal readout was obtained after 5 and 15 minutes after the addition of the target. The area around the line depicts the standard deviation.***

## 9.12 Conclusions

WP4 explored the development of a microfluidic device for (dis)continuously detecting target substances using a combination of innovative design strategies, biochemical experimentation and machine learning techniques.

The initial deliverables for this WP (D4.1) aimed to showcase promising techniques for biomonitoring applications. The work conducted aligns well with this objective by demonstrating the feasibility of a microfluidic platform coupled with machine learning for sensitive and specific analyte detection. This lays the groundwork for future pilot-scale testing and development (D4.3), which has only been partially addressed.

Key achievements:

- 3D-printed microfluidic cell: a cost-effective and rapidly prototyped 3D-printed microfluidic cell was developed, offering flexibility for design iterations;
- PDMS microfluidic cell: an advanced prototyped microfluidic cell was developed, as a sequel to the 3D printed device, offering fewer leaks and more future flexibility to adjust filters and other features;
- Double-sided tape approach: an alternative fabrication method using double-sided adhesive tape was investigated, demonstrating potential for simple and low-cost microfluidic prototyping;
- Production of functionalized beads: functionalized beads using CRP as a model were produced, and their performance was assessed in a variety of settings;
- Controllable focusing system: a controllable focusing system was designed and implemented to address the challenge of limited depth of field in high-magnification microscopy, enabling sharp bead image capture;
- Automated electronics system: a custom electronics system was designed to automate various aspects of the device operation, including system preparation, sample and measurement control, image and data acquisition, and remote control capabilities;
- Data processing pipeline: a data processing pipeline was established to analyse the captured microscope images, employing image pre-processing techniques and exploring unsupervised and supervised learning approaches;
- Supervised learning with synthetic data: supervised learning with synthetically generated data demonstrated promise for accurate bead detection and cluster identification, potentially leading to robust microfluidic analysis;
- Preliminary CRP detection: preliminary measurements utilizing the supervised machine learning method indicated a correlation between the ratio of clustered beads to single beads and the presence of CRP, suggesting the potential for specific analyte detection.

Future directions:

- Supervised learning refinement: further development of the supervised learning model by incorporating real-world data and techniques like transfer learning can enhance its generalizability;
- Multiplexing capabilities: future iterations could explore incorporating multiplexing functionalities within the microfluidic cell to enable simultaneous detection of various target molecules;
- Sensitivity and specificity optimization: additional studies are needed to refine the system's sensitivity and specificity for accurate and reliable analyte detection.

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90. EC: Council Directive 98/83/EC of 3 November 1998 on the quality of water intended for human consumption. Consolidated text 2015.10.27, http://data.europa.eu/eli/dir/1998/83/2015-10-27
91. EC: Commission Implementing Decision (EU) 2022/1307 of 22 July 2022 establishing a watch list of substances for Union-wide monitoring in the field of water policy pursuant to Directive 2008/105/EC of the European Parliament and of the Council (notified under document C(2022) 5098), http://data.europa.eu/eli/dec_impl/2022/1307/oj
92. EC: Proposal for a Directive of the European Parliament and of the Council amending Directive 2000/60/EC establishing a framework for Community action in the field of water policy, Directive 2006/118/EC on the protection of groundwater against pollution and deterioration and Directive 2008/105/EC on environmental quality standards in the field of water policy. COM/2022/540 final, https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A52022PC0540
93. EC: Proposal for a Directive of the European Parliament and of the Council concerning urban wastewater treatment (recast). COM(2022) 541 final, https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A52022PC0541
94. EP, EC: Regulation (EC) No 396/2005 of the European Parliament and of the Council of 23 February 2005 on maximum residue levels of pesticides in or on food and feed of plant and animal origin and amending Council Directive 91/414/EEC. Consolidated text 2024.05.11, http://data.europa.eu/eli/reg/2005/396/2024-05-11
95. EC: Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. Consolidated text 2020.03.08, http://data.europa.eu/eli/reg/2005/2073/2020-03-08
96. EC: Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. Consolidated text 2023.05.25, http://data.europa.eu/eli/reg/2006/1881/2023-05-25

97. EC: Commission Regulation (EU) 2023/915 of 25 April 2023 on maximum levels for certain contaminants in food and repealing Regulation (EC) No 1881/2006. Consolidated text 2024.07.22, http://data.europa.eu/eli/reg/2023/915/2024-07-22
98. EP, E.: Commission Regulation (EU) No 142/2011 of 25 February 2011 implementing Regulation (EC) No 1069/2009 of the European Parliament and of the Council laying down health rules as regards animal by-products and derived products not intended for human consumption and implementing Council Directive 97/78/EC as regards certain samples and items exempt from veterinary checks at the border under that Directive. Consolidated text 2024.07.11, http://data.europa.eu/eli/reg/2011/142/2024-07-11
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# Annex 1 Overview of all legislation related to water reuse for irrigation

***Table A1-1. Overview of all legislation related to water reuse for irrigation, categorised by water reuse, water, food and animal health.***

| **Water reuse** | **Consolidated version** | **Ref** |
|---|---|---|
| Regulation (EU) 2020/741 on minimum requirements for water reuse<br>*Since 26-6-2023* | No<br>http://data.europa.eu/eli/reg/2020/741/oj | [85] |
| Commission Notice Guidelines to support the application of Regulation 2020/741 on minimum requirements for water reuse 2022/C 298/01 | No<br>https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:52022XC0805(01) | [86] |
| Reusing wastewater in agriculture – technical specifications for risk management plans. Draft delegated Regulation: Ares(2024)181349 | No<br>https://ec.europa.eu/info/law/better-regulation/have-your-say/initiatives/13846-Reutilizacion-de-las-aguas-residuales-en-la-agricultura-especificaciones-tecnicas-para-los-planes-de-gestion-del-riesgo_en<br>https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=PI_COM%3AAres%282024%29181349&qid=1713531500739 | [87] |
| Deleg Reg 2024-01261_Reusing wastewater in agriculture<br>*No longer in force* | No<br>https://eur-lex.europa.eu/eli/reg_del/2024/1261/oj | |
| 2024-90303_Corrigendum to Deleg Reg (EU) 2024-1261 | No<br>https://eur-lex.europa.eu/eli/reg_del/2024/1261/corrigendum/2024-05-15/oj | |
| Deleg Reg 2024-01765_Reusing wastewater in agriculture | No<br>https://eur-lex.europa.eu/eli/reg_del/2024/1765/oj | |
| COM/2024/442 final. Report on the exercise of the delegation conferred on... | Nvt<br>https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX:52024DC0442&qid=1731960758720 | |
| Reusing wastewater in agriculture – technical specifications for risk management plans. Delegated Regulation: C(2024)1454 final. 11-3-2024 | No<br>https://ec.europa.eu/info/law/better-regulation/have-your-say/initiatives/13846-Reusing-wastewater-in-agriculture-technical-specifications-for-risk-management-plans_en<br>https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=PI_COM%3AC%282024%291454&qid=1713531500739 | [88] |
| **Water** | **Consolidated version** | **Ref** |
| Directive 2000/60/EC establishing a framework for Community action in the field of water policy.<br>WFD, kaderrichtlijn water | 2014.11.20<br>http://data.europa.eu/eli/dir/2000/60/2014-11-20 | [6] |

| | | |
|---|---|---|
| Directive 91/676/EEC concerning the protection of waters against pollution caused by nitrates from agricultural sources | 2008.12.11<br>http://data.europa.eu/eli/dir/1991/676/2008-12-11 | [4] |
| Directive 91/271/EEC concerning urban waste-water treatment UWWTD | 2014.01.01<br>http://data.europa.eu/eli/dir/1991/271/2014-01-01 | [89] |
| Directive 98/83/EC on the quality of water intended for human consumption (until 2023) | 2015.10.27<br>http://data.europa.eu/eli/dir/1998/83/2015-10-27 | [90] |
| Directive (EU) 2020/2184 on the quality of water intended for human consumption (recast) | No<br>http://data.europa.eu/eli/dir/2020/2184/oj | [5] |
| Directive 2006/118/EC on the protection of groundwater against pollution and deterioration | 2014.07.11<br>http://data.europa.eu/eli/dir/2006/118/2014-07-11 | [7] |
| Directive 2008/105/EC on environmental quality standards in the field of water policy, amending | 2013.09.13<br>http://data.europa.eu/eli/dir/2008/105/2013-09-13 | [8] |
| Directive 2006/7/EC concerning the management of bathing water quality | 2014.01.01<br>http://data.europa.eu/eli/dir/2006/7/2014-01-01 | [9] |
| Directive 86/278/EEC on the protection of the environment, and in particular of the soil, when sewage sludge is used in agriculture | 2022.01.01<br>http://data.europa.eu/eli/dir/1986/278/2022-01-01 | [10] |
| Implementing Decision (EU) 2022/1307 establishing a watch list of substances for Union-wide monitoring in the field of water policy pursuant to | No<br>http://data.europa.eu/eli/dec_impl/2022/1307/oj | [91] |
| COM/2022/540 final: Proposal for a Directive….. on the protection of groundwater against pollution and deterioration and …. on environmental quality standards in the field of water policy | No<br>https://environment.ec.europa.eu/publications/proposal-amending-water-directives_en<br><br>https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A52022PC0540 | [92] |
| COM(2022) 541 final: Proposal for a Directive concerning | No<br>https://environment.ec.europa.eu/publications/proposal-revised-urban-wastewater-treatment-directive_en | [93] |

| | | |
|---|---|---|
| urban wastewater treatment (recast) | https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A52022PC0541 | |
| **Food** | **Consolidated version** | **Ref** |
| Regulation (EC) No 852/2004 on the hygiene of foodstuffs | 2021.03.24<br>http://data.europa.eu/eli/reg/2004/852/2021-03-24 | [11] |
| Regulation (EC) No 396/2005 on maximum residue levels of pesticides in or on food and feed of plant and animal origin | 2024.0511<br>http://data.europa.eu/eli/reg/2005/396/2024-05-11 | [94] |
| Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs | 2020.03.08<br>http://data.europa.eu/eli/reg/2005/2073/2020-03-08 | [95] |
| Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs (until 24/05/2023) | 2023.05.25<br>http://data.europa.eu/eli/reg/2006/1881/2023-05-25 | [96] |
| Regulation (EU) 2023/915 on maximum levels for certain contaminants in food | 2024.07.22<br>http://data.europa.eu/eli/reg/2023/915/2024-07-22 | [97] |
| Commission notice on guidance document on addressing microbiological risks in fresh fruits and vegetables at primary production through good hygiene 2017XC0523(03) | No<br>https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX:52017XC0523(03) | [12] |
| **Animal health** | **Consolidated version** | **Ref** |
| Regulation (EC) No 183/2005 laying down requirements for feed hygiene | 2022.01.28<br>http://data.europa.eu/eli/reg/2005/183/2022-01-28 | [13] |
| Regulation (EC) No 1069/2009 laying down health rules as regards animal by-products and derived products not intended for human consumption and repealing…. | 2019.12.14<br>http://data.europa.eu/eli/reg/2009/1069/2019-12-14 | [16] |
| Regulation (EU) No 142/2011 laying down health rules as regards animal by-products and derived products not intended for human consumption and implementing…. | 2024.11.07<br>http://data.europa.eu/eli/reg/2011/142/2024-07-11 | [98] |

# Annex 2 Examples of common hazards and hazardous events for reuse of water

***Table A2-1. Examples of common hazards and hazardous events, potential exposure routes, and receptors that may be present in systems for water reuse in agricultural irrigation [source: Guideline 2022/C298/01, Annex 2]***

**Quality standards for intestinal enterococci and E. coli set in the Bathing Water Directive (2006/7/EC)**

| | Intestinal enterococci (CFU/100ml) | | *E. coli* (CFU/100ml) | |
|---|---|---|---|---|
| Quality Class | Inland waters | Coastal and transitional waters | Inland waters | Coastal and transitional waters |
| Excellent | 200 (1) | 100 (1) | 500 (1) | 250 (1) |
| Good | 400 (1) | 200 (1) | 1 000 (1) | 500 (1) |
| Sufficient | 330 (2) | 185 (2) | 900 (2) | 500 (2) |

(1) 95th percentile of measured concentrations.
(2) 90th percentile of measured concentrations.
*Source:* Directive 2006/7/EC; selected in JRC (2019) (1)

**List of microbial hazards usually detected in raw waste water and their effect on health and reference pathogens (Table A.1 of ISO 20426:2018) (1)**

| Pathogen | Examples | Disease | Reference pathogen (1) |
|---|---|---|---|
| *Bacteria* | *Shigella* | Shigellosis (bacillary dysentery) | *E. coli* O157:H7<br>*Campylobacter* |
| | *Salmonella* | Salmonellosis, gastroenteritis (diarrhoea, vomiting, fever), reactive arthritis, typhoid fever | |
| | *Vibrio cholera* | Cholera | |
| | Pathogenic *E.coli* | Gastroenteritis and septicaemia, haemolytic uremic syndrome | |
| | *Campylobacter* | Gastroenteritis, reactive arthritis, Guillain-Baré syndrome | |
| *Protozoa* | *Entamoeba* | Amoebiasis (amoebic dysentery) | *Cryptosporidium* |
| | *Giardia* | Giardiasis (gastroenteritis) | |
| | *Cryptosporidium* | Cryptosporidiosis, diarrhoea, fever | |
| Helminths | *Ascaris* | Ascariasis (roundworm infection) | Intestinal nematodes (helminth eggs) |
| | *Ancylostoma* | Ancylostomiasis (hookworm infection) | |
| | *Necator* | Necatoriasis (roundworm infection) | |
| | *Trichuris* | Trichuriasis (whipworm infection) | |
| Viruses | *Enteroviruses* | Gastroenteritis, heart anomalies, meningitis, respiratory illness, nervous disorders, others | *Rotavirus* |
| | *Adenovirus* | Respiratory disease, eye infection, gastroenteritis | |
| | *Rotavirus* | Gastroenteritis | |

(1) Source: Minimum quality requirements for water reuse in agricultural irrigation and aquifer recharge, JRC (2017)

**Examples of some chemical parameters listed in the Drinking Water Directive potentially present in urban waste water**

| Parameter | Value |
|---|---|
| Nitrate ($NO_3$) | 50 mg/L |
| Copper | 2,0 mg/L |
| Uranium | 30 µg/L |
| Chromium | 25 µg/L |
| Nickel | 20 µg/L |
| Arsenic, Tri- and Tetrachloroethene | 10 µg/L |
| Selenium | 20 µg/L |
| Cadmium, Lead | 5 µg/L |
| Antimony | 10 µg/L |
| 1, 2 - dichloroethane | 3 µg/L |
| Mercury, Benzene | 1,0 µg/L |
| Vinyl chloride | 0,50 µg/L |

(') JRC, 2019. Water quality in Europe: effects of the Urban Wastewater Treatment Directive. JRC Science for Policy Report.

| | |
|---|---|
| PFAS Total (totality of per- and polyfluoroalkyl substances) | 0,50 µg/L |
| Sum of PFAS (the sum of per- and polyfluoroalkyl substances considered a concern as regards water intended for human consumption) | 0,10 µg/L |
| Acrylamide, polycyclic aromatic hydrocarbons (PAHs), Epichlorohydrin | 0,10 µg/L |
| Benzo(a)pyrene | 10 ng/L |
| Bisphenol A, | 2,5 µg/L |
| Trihalomethanes Total | 100 µg/L |
| Haloacetic acids (HAAs) | 60 µg/L |

Source: Annex I, Part B of Directive 2020/2184 (Minimum requirements for parametric values used to assess the quality of water intended for human consumption). Selected in JRC (2019) and adapted considering revisions of the new Drinking Water Directive and substances that could be found after disinfection.
Directive 2020/2184 provides a watch list mechanism to address compounds of emerging concern, such as endocrine-disrupting compounds, pharmaceuticals and microplastics. The Commission Implementing Decision of 19 January 2022 establishes, for the watch list of substances and compounds of concern for water intended for human consumption, the following endocrine-disrupting compounds:

— 17-beta-estradiol ≤ 1 ng/L
— nonylphenol: ≤ 300 ng/L

**Example of priority pollutants listed in the Environmental Quality Standard Directive potentially present in urban waste water (')**

| Parameter | Annual average (AA) value (µg/L) | | Maximum allowable concentration (µg/L) | | µg/kg wet weight |
|---|---|---|---|---|---|
| | Inland surface waters (') | Other surface waters | Inland surface waters (') | Other surface waters | Biota |
| Anthracene | 0,1 | 0,1 | 0,1 | 0,1 | - |
| Benzene | 10 | 8 | 50 | 50 | - |
| Brominated diphenyl-lethers (sum of the concentrations of congener numbers 28, 47, 99, 100, 153 and 154) | - | - | 0,14 | 0,14 | 0,0085 |
| Cadmium and its compounds (depending on water hardness classes) | 0,08 to 0,25 | 0,2 | 0,45 to 1,5 | 0,45 to 1,5 | - |
| C10-13 Chloro-alkanes (No indicative parameter is provided for this group of substances. The indicative parameter(s) must be defined through the analytical method) | 0,4 | 0,4 | 1,4 | 1,4 | - |
| 1,2-Dichloroethane | 10 | 10 | not applicable | not applicable | - |

| | | | | | |
|---|---|---|---|---|---|
| Dichloromethane | 20 | 20 | not applicable | not applicable | - |
| Di(2- ethylhexyl)- phthalate (DEHP) | 1,3 | 1,3 | not applicable | not applicable | - |
| Fluoranthene | 0,0063 | 0,0063 | 0,12 | 0,12 | 30 |
| Hexachloro-benzene | - | - | 0,05 | 0,05 | 10 |
| Hexachloro-butadiene | - | - | 0,6 | 0,6 | 55 |
| Lead and its compounds | 1,2 (bioavail-able concen-trations of the substances) | 1,3 | 14 | 14 | - |
| Mercury and its compounds | - | - | 0,07 | 0,07 | 20 |
| Naphthalene | 2 | 2 | 130 | 130 | - |
| Nickel and its compounds | 4 (bioavailable concentra-tions of the substances) | 8,6 | 34 | 34 | - |
| Nonylphenols (4-Nonylphenol) | 0,3 | 0,3 | 2,0 | 2,0 | - |

**Example of priority pollutants listed in the Environmental Quality Standard Directive potentially present in urban waste water (1)**

| Parameter | Annual average (AA) value (µg/L) | | Maximum allowable concentration (µg/L) | | µg/kg wet weight |
|---|---|---|---|---|---|
| Octylphenols ((4-(1,1′,3,3′-tetramethylbutyl)-phenol)) | 0,1 | 0,01 | not applicable | not applicable | - |
| Pentachloro-benzene | 0,007 | 0,0007 | not applicable | not applicable | - |
| PAH Benzo(a)pyrene (3) | $1,7 \times 10^{-4}$ | $1,7 \times 10^{-4}$ | 0,27 | 0,027 | - |
| Tributyltin compounds (Tributyltin-cation) | 0,0002 | 0,0002 | 0,0015 | 0,0015 | - |
| Trichloro-benzenes | 0,4 | 0,4 | not applicable | not applicable | - |
| Trichloro-methane | 2,5 | 2,5 | not applicable | not applicable | - |
| Perfluorooctane sulfonic acid and its derivatives (PFOS) | $6,5 \times 10^{-4}$ | $1,3 \times 10^{-4}$ | 36 | 7,2 | 9,1 |
| Hexabromo-cyclododecanes (HBCDD) | 0,0016 | 0,0008 | 0,5 | 0,05 | 167 |

(1) Selected among the 45 priority substances set by the EQS Directive that includes pesticides, and household and industrial chemicals. *Source:* EQS Directive 2013/39/EU; selected in JRC, 2019.
(2) Inland surface waters encompass rivers and lakes and related artificial or heavily modified water bodies
(3) For the group of priority substances of polyaromatic hydrocarbons (PAH) (No 28), the biota EQS and corresponding AA-EQS in water refer to the concentration of benzo(a)pyrene, on the toxicity of which they are based. Benzo(a)pyrene can be considered as a marker for the other PAHs, hence only benzo(a)pyrene needs to be monitored for comparison with the biota EQS or the corresponding AA-EQS in water.

# Annex 3 Organic micropollutants in raw effluent WWTP Walcheren and treated effluent

***Table A3-1 Relative concentrations of the organic micropollutants in raw effluent WWTP Walcheren and treated effluent (ozone + UV).***

| | Raw effluent (µg/L) | | | | | | | Ozone plus UV (µg/L) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Component | 9-3-2022 | 30-3-2022 | 14-4-2022 | 4-5-2023 | 9-8-2023 | Average: | STDEV: | 4-5-2022 | 5-6-2022 | 29-6-2022 | 12-7-2022 | 27-7-2022 | 9-8-2022 | 24-8-2022 | Average: | STDEV: |
| 1H-benzotriazole | 3,40 | 4,70 | 5,70 | 17,00 | 3,60 | 6,88 | 5,73 | 5,00 | 0,78 | 0,20 | 0,49 | 0,63 | 0,53 | 0,42 | 1,15 | 1,71 |
| 2- en/of 4-nitrofenol | 0,22 | 0,23 | 0,24 | 0,22 | 0,17 | 0,22 | 0,03 | | | 0,05 | 0,05 | 0,05 | 0,07 | 0,07 | 0,06 | 0,01 |
| 4- en/of 5-Methyl-1H-benzotriazole | 1,30 | 1,40 | 1,30 | 1,30 | 1,00 | 1,26 | 0,15 | | | | | | | | | |
| Melamine | 0,44 | 0,44 | 0,45 | 0,53 | 0,63 | 0,50 | 0,08 | 0,48 | 0,65 | 1,20 | 0,58 | 0,97 | 0,66 | 0,97 | 0,79 | 0,26 |
| N-Butylbenzeensulfonamide | | 0,11 | 0,09 | 0,11 | 0,14 | 0,11 | 0,02 | | | | | | | | | |
| Saccharine | | | | | | | | | | | | | | 0,17 | 0,17 | |
| TPPO (Triphenylphospine oxide) | 0,04 | 0,05 | 0,05 | 0,06 | 0,07 | 0,05 | 0,01 | | | | | | | | | |
| Bupirimate | | | | | | | | | | | | | | 0,05 | 0,05 | |
| Chloridazone | | | | | | | | | | | 0,08 | 0,04 | | | 0,06 | 0,03 |
| Chlorpropham | | | | | | | | | | | | | | 0,05 | 0,05 | |
| Climbazole | 0,05 | 0,08 | 0,06 | 0,06 | 0,08 | 0,06 | 0,02 | | | | | | | | | |
| Cyprodinil | | | | | | | | | | | | | | 0,06 | 0,06 | |
| DEET (Diethyltoluamide) | 0,16 | 0,17 | 0,15 | 0,73 | 2,40 | 0,72 | 0,97 | 0,25 | 0,29 | 0,32 | 0,60 | 0,87 | 0,83 | 0,70 | 0,55 | 0,26 |
| Diuron | | | | | 0,06 | 0,06 | | | | | | | | | | |
| Ethofumesate | | | | | | | | | 0,04 | | | | | | 0,04 | |
| Flutolanil | | | | 0,05 | | 0,05 | | | | | | | | | | |
| Fluxapyroxad | | | | | | | | | | | | | | 0,06 | 0,06 | |
| Metamitron | | | | | | | | | 0,19 | | | | | | 0,19 | |
| Metobromuron | | | | | 0,04 | 0,04 | | | | | | | | | | |
| Metolachlor | | | | | | | | | 0,06 | | | | | | 0,06 | |
| Thiabendazole | | | | | 0,04 | 0,04 | | | | | | | | | | |
| 10,11-Dihydro-10,11-dihydroxy carbamazepine | 0,11 | 0,10 | 0,08 | 0,14 | 0,18 | 0,12 | 0,04 | | | | | 0,04 | 0,04 | 0,03 | 0,04 | 0,00 |
| 10-Hydroxycarbamazepine | 0,06 | 0,07 | 0,06 | 0,06 | 0,10 | 0,07 | 0,02 | | | | | | | | | |
| Aciclovir | | | | | 0,04 | 0,04 | | | | | | | | | | |
| Adenine | | 1,70 | | 0,28 | 0,09 | 0,69 | 0,88 | 1,70 | 0,89 | 0,40 | 0,87 | 1,40 | 0,16 | | 0,90 | 0,58 |
| Adenosine | | | | 0,14 | | 0,14 | | 0,07 | 0,09 | 0,03 | 0,04 | 0,06 | | | 0,06 | 0,02 |
| Aliskiren | 0,16 | 0,14 | 0,14 | 0,18 | 0,21 | 0,17 | 0,03 | | | | | | | | | |
| Amisulpiride | 0,03 | 0,03 | 0,04 | 0,04 | 0,04 | 0,04 | 0,01 | | | | | | | | | |
| Amitriptyline | | 0,12 | | | | 0,12 | | | | | | | | | | |
| Arecoline | | | | | | | | | 0,04 | | 0,05 | 0,06 | | | 0,05 | 0,01 |
| Atenolol | 0,20 | 0,27 | 0,27 | 0,30 | 0,34 | 0,28 | 0,05 | | 0,10 | | 0,04 | 0,07 | 0,06 | | 0,07 | 0,03 |
| Atorvastatin | 0,03 | | | | | 0,03 | | | | | | | | | | |
| Azithromycin | 0,06 | 0,07 | 0,06 | 0,04 | 0,04 | 0,05 | 0,01 | | | | | | | | | |
| Bisoprolol | 0,23 | 0,32 | 0,28 | 0,31 | 0,31 | 0,29 | 0,04 | | 0,08 | | 0,04 | 0,05 | 0,05 | 0,04 | 0,05 | 0,02 |
| Caffeine | 0,09 | 0,05 | | | | 0,07 | 0,03 | | | | | 0,05 | | | 0,05 | |
| Carbamazepine | 0,58 | 0,67 | 0,54 | 0,69 | 0,82 | 0,66 | 0,11 | | | | | | | | | |
| Celiprolol | 0,08 | 0,10 | 0,08 | 0,12 | 0,11 | 0,10 | 0,02 | | | | | | | | | |
| Cetirizine | 0,20 | 0,30 | 0,25 | 0,36 | 0,31 | 0,28 | 0,06 | | | | | | | | | |
| Cimetidine | 0,10 | 0,12 | 0,11 | 0,11 | 0,13 | 0,11 | 0,01 | | | | | | | | | |
| Citalopram | 0,14 | 0,19 | 0,15 | 0,13 | 0,15 | 0,15 | 0,02 | | | | | | | | | |
| Clindamycin | | | | | 0,04 | 0,04 | | | | | | | | | | |
| Clopidogrel | 0,08 | 0,10 | 0,09 | 0,10 | 0,10 | 0,09 | 0,01 | | | | | | | | | |
| Cotinine | | 0,04 | | | | 0,04 | | 0,05 | | 0,04 | | 0,07 | 0,08 | 0,06 | 0,06 | 0,01 |
| Diclofenac | 0,03 | 0,03 | 0,04 | 0,04 | 0,04 | 0,04 | 0,00 | | | | | | | | | |
| Diltiazem | 0,12 | 0,13 | 0,10 | | 0,08 | 0,11 | 0,02 | | | | | | | | | |
| Disopyramide | 0,08 | 0,11 | 0,07 | 0,09 | 0,11 | 0,09 | 0,02 | | | | | | | | | |
| Doxylamine | | | 0,03 | | 0,04 | 0,04 | 0,01 | | | | | | | | | |
| EDDP | 0,09 | 0,11 | 0,07 | | 0,05 | 0,08 | 0,02 | | | | | | | | | |
| Ephedrine en/of Pseudoephedrine | 0,03 | 0,04 | | 0,04 | | 0,04 | 0,01 | | | | | | | | | |

| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Fexofenadine | 0,52 | 0,88 | 0,70 | 1,00 | 1,00 | 0,82 | 0,21 | 0,05 | 0,20 | | | 0,08 | 0,07 | 0,05 | 0,09 | 0,06 |
| Flecainide | 0,66 | 0,80 | 0,64 | 0,82 | 0,87 | 0,76 | 0,10 | 0,06 | 0,24 | | 0,10 | 0,16 | 0,16 | 0,15 | 0,15 | 0,06 |
| Fluconazole | | | | 0,03 | 0,05 | 0,04 | 0,01 | | | | | | 0,04 | | 0,04 | |
| Gabapentin | 0,71 | 1,10 | 0,73 | 0,66 | 0,99 | 0,84 | 0,19 | 0,25 | 0,31 | 0,26 | 0,32 | 0,45 | 0,48 | 0,45 | 0,36 | 0,10 |
| Histamine | | 0,14 | 0,13 | 0,16 | | 0,14 | 0,02 | 0,06 | | | | | | | 0,06 | |
| Histidine | | | | 0,06 | | 0,06 | | | | | | | | | | |
| HMMA | | | | 0,04 | | 0,04 | | | | | | | | | | |
| Hydrochlorothiazide | 0,06 | 0,08 | 0,06 | 0,08 | 0,08 | 0,07 | 0,01 | | | | | | | | | |
| Irbesartan | 0,90 | 1,10 | 0,96 | 1,20 | 1,40 | 1,11 | 0,20 | 0,29 | 0,47 | 0,19 | 0,30 | 0,39 | 0,47 | 0,29 | 0,34 | 0,10 |
| Ketamine | 0,04 | 0,04 | | | | 0,04 | 0,00 | | | | | | | | | |
| Labetalol | 0,06 | | 0,06 | | 0,06 | 0,06 | 0,00 | | | | | | | | | |
| Lamotrigine | 0,47 | 0,58 | 0,52 | 0,61 | 0,75 | 0,59 | 0,11 | 0,26 | 0,39 | 0,18 | 0,35 | 0,37 | 0,43 | 0,40 | 0,34 | 0,09 |
| Lidocaine | 0,15 | 0,23 | 0,21 | 0,22 | 0,25 | 0,21 | 0,04 | | | | | | | | | |
| Losartan | 0,53 | 0,69 | 0,59 | 0,60 | 0,74 | 0,63 | 0,08 | | | | | | | | | |
| MDMA | 0,15 | 0,24 | 0,13 | 0,35 | 0,31 | 0,24 | 0,10 | | | | | | | | | |
| Metformin | 0,69 | 0,75 | 0,56 | 0,49 | 0,68 | 0,63 | 0,11 | 0,35 | 0,47 | 0,46 | 0,49 | 0,67 | 0,51 | 0,54 | 0,50 | 0,10 |
| Methocarbamol | | | 0,03 | | 0,06 | 0,05 | 0,02 | | | | | | | | | |
| Metoclopramide | 0,04 | | | | 0,04 | 0,04 | 0,00 | | | | | | | | | |
| Metoprolol | 1,80 | 2,40 | 1,90 | 2,40 | 2,90 | 2,28 | 0,44 | 0,17 | 0,64 | 0,10 | 0,28 | 0,43 | 0,43 | 0,36 | 0,34 | 0,18 |
| Mycophenolic acid | 0,04 | | | | | 0,04 | | | | | | | | | | |
| N-Acetyl-4-aminoantipyrine | 0,33 | 0,52 | 0,72 | 0,76 | 0,88 | 0,64 | 0,22 | | | | | | | | | |
| Naproxen | | 0,03 | | | 0,04 | 0,04 | 0,01 | | | | | | | | | |
| N-Formyl-4-aminoantipyrine | 0,14 | 0,22 | 0,29 | 0,30 | 0,37 | 0,26 | 0,09 | | | | | | | | | |
| Nicotinamide | | | | | | | | 0,22 | 0,25 | 0,27 | 0,27 | 0,57 | 0,49 | | 0,35 | 0,15 |
| Nordiltiazem | | | | | 0,04 | 0,04 | | | | | | | | | | |
| O-Desmethylnortramadol | 0,17 | 0,22 | 0,18 | 0,23 | 0,26 | 0,21 | 0,04 | | | | | | | | | |
| O-Desmethyltramadol | 0,39 | 0,47 | 0,39 | 0,48 | 0,55 | 0,46 | 0,07 | | | | | | | | | |
| Oxazepam | 0,06 | 0,05 | 0,05 | 0,05 | 0,06 | 0,05 | 0,01 | | | | | | | | | |
| Oxcarbazepine | 0,16 | 0,16 | 0,16 | 0,16 | 0,25 | 0,18 | 0,04 | 0,04 | 0,07 | | 0,05 | 0,06 | 0,05 | | 0,05 | 0,01 |
| Pantoprazole | 0,04 | 0,04 | | | | 0,04 | 0,00 | | | | | | | | | |
| Propranolol | | 0,12 | 0,10 | | 0,12 | 0,11 | 0,01 | | | | | | | | | |
| Ritalinic acid | 0,04 | 0,10 | 0,08 | 0,13 | 0,20 | 0,11 | 0,06 | 0,04 | 0,06 | | 0,04 | 0,08 | 0,07 | 0,05 | 0,06 | 0,01 |
| Rivaroxaban | 0,05 | 0,06 | 0,05 | 0,05 | 0,06 | 0,05 | 0,00 | | | | | | | | | |
| Salicylzuur | | | | | | | | | | | | 0,43 | | | 0,43 | |
| Sitagliptin | | 0,04 | | | 0,05 | 0,04 | 0,00 | | | | | | | | | |
| Sotalol | 0,39 | 0,51 | 0,40 | 0,54 | 0,62 | 0,49 | 0,10 | | | | | | | | | |
| Sulfamethoxazole | 0,05 | 0,08 | 0,07 | 0,09 | 0,13 | 0,09 | 0,03 | | | | | | | | | |
| Sulfapyridine | 0,28 | 0,37 | 0,30 | 0,33 | 0,59 | 0,37 | 0,13 | | | | | | | | | |
| Sulpiride | 0,08 | 0,10 | 0,07 | 0,08 | 0,09 | 0,08 | 0,01 | | | | | | | | | |
| Telmisartan | 0,47 | 0,81 | 0,57 | 0,77 | 0,88 | 0,70 | 0,17 | 0,08 | 0,23 | 0,06 | 0,14 | 0,19 | 0,17 | 0,14 | 0,14 | 0,06 |
| Temazepam | 0,15 | 0,15 | 0,13 | 0,15 | 0,16 | 0,15 | 0,01 | 0,04 | 0,05 | | | 0,03 | 0,04 | 0,03 | 0,04 | 0,01 |
| Theobromine | 0,14 | | | | | 0,14 | | | | | | 0,06 | | | 0,06 | |
| Tramadol en/of O-Desmethylvenlafaxine. | | | | | | | | | | | | | | | | |
| Desvenlafaxine | 1,40 | 1,80 | 1,50 | 1,90 | 2,10 | 1,74 | 0,29 | | 0,13 | | | 0,05 | 0,04 | 0,04 | 0,06 | 0,04 |
| Trimethoprim | 0,12 | 0,13 | 0,12 | | | 0,12 | 0,01 | | | | | | | | | |
| Valsartan | 0,31 | 0,34 | 0,36 | 0,43 | 0,44 | 0,38 | 0,06 | | 0,10 | 0,04 | 0,06 | 0,08 | 0,10 | | 0,08 | 0,02 |
| Valsartanic acid | | | | | 0,04 | 0,04 | | | | | | | | | | |
| Venlafaxine | 0,34 | 0,44 | 0,37 | 0,42 | 0,40 | 0,39 | 0,04 | | 0,09 | | | 0,04 | 0,03 | 0,03 | 0,05 | 0,03 |

# Annex 4 Analysis soil of pear trees

***Tabel A4-1 Analysis of soil used to grow pear trees. Analysis performed by Eurofins Agro, Kortingsregeling.***

| Resultaat | | Eenheid | Resultaat | Streeftraject | laag | vrij laag | goed | vrij hoog | hoog |
|---|---|---|---|---|---|---|---|---|---|
| Chemisch | N-totale bodemvoorraad | kg N/ha | 6520 | 4040 - 6410 | | | | | |
| | C/N-ratio | | 15 | 13 - 17 | | | | | |
| | N-leverend vermogen | kg N/ha | 85 | 95 - 145 | | | | | |
| | S-plantbeschikbaar | kg S/ha | 57 | 20 - 30 | | | | | |
| | S-totale bodemvoorraad | kg S/ha | 2830 | 655 - 1115 | | | | | |
| | C/S-ratio | | 34 | 50 - 75 | | | | | |
| | S-leverend vermogen | kg S/ha | 45 | 20 - 30 | | | | | |
| | P-plantbeschikbaar | kg P/ha | 13,1 | 7,2 - 10,0 | | | | | |
| | P-bodemvoorraad | kg P/ha | 1215 | 425 - 545 | | | | | |
| | K-plantbeschikbaar | kg K/ha | 185 | 420 - 530 | | | | | |
| | K-bodemvoorraad | kg K/ha | 720 | 450 - 605 | | | | | |
| | Ca-plantbeschikbaar | kg Ca/ha | 200 | 200 - 470 | | | | | |
| | Ca-bodemvoorraad | kg Ca/ha | 9885 | 8230 - 12350 | | | | | |
| | Mg-plantbeschikbaar | kg Mg/ha | 375 | 220 - 275 | | | | | |
| | Mg-bodemvoorraad | kg Mg/ha | 745 | 340 - 625 | | | | | |
| | Na-plantbeschikbaar | kg Na/ha | 135 | 100 - 140 | | | | | |
| | Na-bodemvoorraad | kg Na/ha | 85 | 65 - 95 | | | | | |
| | Si-plantbeschikbaar | g Si/ha | 82160 | 16720 - 72460 | | | | | |
| | Fe-plantbeschikbaar | g Fe/ha | 7360 | 6970 - 12540 | | | | | |
| | Zn-plantbeschikbaar | g Zn/ha | 360 | 1390 - 2090 | | | | | |
| | Mn-plantbeschikbaar | g Mn/ha | 720 | 2790 - 3620 | | | | | |
| | Cu-plantbeschikbaar | g Cu/ha | 115 | 110 - 180 | | | | | |
| | Co-plantbeschikbaar | g Co/ha | < 5 | 15 - 20 | | | | | |
| | B-plantbeschikbaar | g B/ha | 1390 | 280 - 420 | | | | | |
| | Mo-plantbeschikbaar | g Mo/ha | 40 | 280 - 13930 | | | | | |
| | Se-plantbeschikbaar | g Se/ha | < 5,9 | 9,8 - 13 | | | | | |
| Fysisch | Zuurgraad (pH) | | 7,4 | 5,4 - 5,7 | | | | | |
| | C-organisch | % | 3,5 | | | | | | |
| | Organische stof | % | 6,2 | | | | | | |
| | C/OS-ratio | | 0,56 | 0,45 - 0,55 | | | | | |
| | Koolzure kalk | % | 1,7 | 2,0 - 3,0 | | | | | |
| | Klei (<2 µm) | % | 6 | | | | | | |
| | Silt (2-50 µm) | % | 9 | | | | | | |
| | Zand (>50 µm) | % | 77 | | | | | | |
| | Slib (<16 µm) | % | 9 | | | | | | |
| | Klei-humus (CEC) | mmol+/kg | 207 | > 119 | | | | | |
| | CEC-bezetting | % | 100 | > 95 | | | | | |
| | Ca-bezetting | % | 86 | 80 - 90 | | | | | |
| | Mg-bezetting | % | 11 | 6,0 - 10 | | | | | |
| | K-bezetting | % | 3,2 | 2,0 - 5,0 | | | | | |
| | Na-bezetting | % | 0,6 | 1,0 - 1,5 | | | | | |
| | H-bezetting | % | < 0,1 | < 1,0 | | | | | |
| | Al-bezetting | % | < 0,1 | < 1,0 | | | | | |
| | Verkruimelbaarheid | rapportcijfer | 9,6 | 6,0 - 8,0 | | | | | |
| | Verslemping | rapportcijfer | 7,9 | 6,0 - 8,0 | | | | | |
| | Stuifgevoeligheid | rapportcijfer | 7,0 | 6,0 - 8,0 | | | | | |

| | | Eenheid | Resultaat | Streeftraject | laag | vrij laag | goed | vrij hoog | hoog |
|---|---|---|---|---|---|---|---|---|---|
| Biologisch | Vochthoudend vermogen | mm | 57 | | | | | | |
| | Microbiële biomassa | mg C/kg | 210 | 310 - 930 | | | | | |
| | Microbiële activiteit | mg N/kg | 75 | 60 - 80 | | | | | |
| | Schimmel/bacterie-ratio | | 0,5 | 0,6 - 0,9 | | | | | |

# Annex 5 Overview organic micropollutants in crops, not detected in effluent

***Table A5-1 Overview of all components detected in potatoes, onions, and pears, but not detected in the effluent of WWTP Walcheren, categorised into medicines, metabolites, drugs, industrial, and organic components. An 'X' in a column denotes whether the component has been detected in the corresponding crop.***

| Component | Use | Potatoes | Onions | Pears |
|---|---|---|---|---|
| Cosmetics | | | | |
| Salicylic acid | Skincare (anti-inflammatory) | X | | |
| (*)Nicotinamide | Skincare (anti-inflammatory) | X | X | X |
| Medicines | | | | |
| Apophedrin | Cardiovascular | X | X | |
| *Rilmenidine | Antihypertensive medication | X | | |
| Tolycaine | Anasthetic drug | X | X | X |
| *Procaine | Anasthetic drug | X | | |
| *Olsalazine | Anti-inflammatory | X | | |
| *Ethenzamide | Anti-inflammatory | | X | |
| *Ibuprofen | Anti-inflammatory | | X | |
| *Adamantan-1-amine | Antiviral and antiparkinsonian | X | | |
| *Betaxolol | Beta-blocker | X | | |
| Esmolol | Beta-blocker | X | | |
| *Buspirone | Anti-anxiety | X | | |
| *Ephedrine & Pseudoephedrine | Central nervous system stimulant | X | | |
| Amphetamine | Central nervous system stimulant | | X | |
| Guaifenesin | Expectorant | | | X |
| PMA | Antidepressant | | X | |
| Medicine-metabolite | | | | |
| Gabapentin lactam | Gabapentin - anticonsulvant | X | | |
| *Carbamazepine-10.11-epoxid | Carbamazepine – anticonsulvant | | X | |
| Carboxyibuprofen | Ibuprofen – anti-inflammatory | X | | |
| *2-Hydroxyibuprofen | Ibuprofen – anti-inflammatory | | | X |
| *3-Hydroxyibuprofen | Ibuprofen – anti-inflammatory | | | X |
| *Noroxycodone | Oxycodone - opioid analgesic | X | | |
| Deacetyldiltiazem | Diltiazem, calcium channel blocker | | X | |
| P-hydroxyamphetamine | Amphetamine - nervous system stimulant | | X | |
| Drugs | | | | |
| *BDB | Entactogenic | X | | |
| *4-MEC | Entactogenic | | X | |
| (*)Cathinone | Stimulant | X | X | |
| *bk-MDDMA | Stimulant | | X | |
| *DMAA | Stimulant | | X | |
| Pentedrone | Stimulant | | X | |
| *CP 47-497-C8 | Analgesic effects | X | | |
| 2 C-P | Psychedelic | X | | |
| Trenbolone | Veterinary steroid | X | | |
| MDA | Psychoactive substance | X | | |
| *MDEA | Psychoactive substance | X | X | |
| Cathine | Psychoactive substance | | X | |
| *5- and/or 6-APB | Psychoactive substance | | X | |
| Captopril | ACE inhibitor | | X | |
| Pesticides | | | | |

| Component | Use | Potatoes | Onions | Pears |
|---|---|---|---|---|
| Fluopyram | Fungicide | X | | X |
| *Dimoxystrobin | Fungicide | X | | |
| *Fenpropidin | Fungicide | X | X | |
| Fludioxonil | Fungicide | X | | |
| Fluxapyroxad | Fungicide | X | X | |
| *Cycloheximide | Fungicide | | | X |
| Trifloxystrobin | Fungicide | | | X |
| Fenpropimorph | Fungicide | | X | |
| *Trimethacarb | Insecticide | X | X | |
| *Pyrethrin I | Insecticide | X | X | X |
| *Pyrethrins: Jasmolin I | Insecticide | X | | |
| *Pyrethrins: Cinerin II | Insecticide | | | X |
| *Metolcarb | Insecticide | X | | X |
| *Piperonylbutoxide | Insecticide | X | | |
| *Mevinphos | Insecticide | | | X |
| *Demeton-S | Insecticide | | X | |
| *Thiometon | Insecticide | | X | |
| *Butocarboxim | Insecticide | | X | |
| *Disulfoton | Insecticide | | X | |
| *Phorate | Insecticide | | X | |
| Industrial | | | | |
| Caprolactam | Chemical intermediate | X | X | X |
| *Cyanopropanal | Chemical intermediate | | X | |
| Pentaethylene glycol | Chemical intermediate and solvent | X | X | X |
| *2-Benzyltetronic acid | Use in syntheses | X | | |
| Food | | | | |
| *Sucralose | Antificial sweetener | X | X | X |
| (*)2-Phenethylamine | Found in foods | X | X | X |
| (*)Tyramine | Found in foods and beverages | X | X | |
| *B-Asarone | Found in plants | X | | |
| Organic compounds | | | | |
| *alpha-estradiol | Human hormone | X | X | X |
| *beta-estradiol | Human hormone | | X | X |
| *Estriol | Human hormone | X | | |
| *Estrone | Human hormone | X | X | X |
| *Nandrolone | Anabolic steroid | X | X | |
| Pyridoxine | Vitamin – supplement | X | X | |
| Biotin | Vitamin – supplement | | X | |
| Adenosine | DNA, RNA building block | | X | |
| *DMPEA | | | | |

# Annex 6 Detection limits analysis organic micropollutants

***Table A6-1 Organic micropollutants that are selected for calculating the absolute concentrations in potatoes (ng/g potato), along with their corresponding Limit Of Detection (LOD).***

| Component | Limit of Detection (LOD) [ng/g potato] |
|---|---|
| Carbamazepine | 0.005 |
| Flecainide | 0.01 |
| Lamotrigine | 0.01 |
| Metoprolol | 0.006 |
| N-Acetyl-4-aminoantipyrine | 0.005 |
| N-Formyl-4-aminoantipyrine | 0.008 |
| O-Desmethyltramadol | 0.008 |
| Sotalol | 0.02 |
| Sulpiride | 0.02 |
| Desvenlafaxine | 0.003 |
| Venlafaxine | 0.006 |

# Annex 7 $LD_{50}$ values for all library compounds

***Table A7-1 Overview of $LD_{50}$ values performed for all library compounds. Blue background indicates: compound detected in product but not in effluent***

| Compound name in PubChem | Pubchem compound number | rat, oral, $LD_{50}$ (mg/kg) | mouse, oral, $LD_{50}$ (mg/kg) | rat, oral, $LD_{50}$ (mg/kg) | source |
|---|---|---|---|---|---|
| 10,11-Dihydro-10,11-dihydroxy carbamazepine | 13726064 | no data | no data | | |
| 10-Hydroxycarbamazepine | 114709 | no data | no data | | |
| 1H-benzotriazole | 7220 | 560 | 615 | | |
| 2,5-Dimethoxy-4-propylphenethylamine | 44350080 | no data | no data | | |
| 2-Nitrophenol | 6947 | 334 | 1297 | | |
| 4-Nitrophenol | 980 | 202 | 282 | | |
| 3734-22-3 | 54678489 | no data | no data | | |
| 2-Hydroxyibuprofen | 10443535 | no data | no data | | |
| 2-Phenethylamine | 1001 | no data | no data | | |
| 3-Hydroxyibuprofen | 71312545 | no data | no data | | |
| 4-Methyl-1H-Benzotriazole | 122499 | no data | no data | | |
| 5-Methyl-1H-Benzotriazole | 8705 | 1600 | no data | | |
| 4-Aminoantipyrine | 2151 | 1700 | 800 | | |
| 4-MEC (4-Methylethcathinone) | 52988259 | no data | no data | | |
| 5-APB (5-(2-Aminopropyl)benzofuran)) | 9837232 | no data | no data | | |
| 6-APB (6-(2-Aminopropyl)benzofuran)) | 9794343 | no data | no data | | |
| Acesulfame | 36573 | 7430 | no data | | |
| Acetaminophen (=paracetamol) | 1983 | 1944 | 338 | | |
| Aciclovir | 135398513 | >20000 | >10000 | | |
| Adamantan-1-amine hydrochloride | 64150 | 800 | 700 | | |
| Adenine | 190 | 227 | 783 | | |
| Adenosine | 60961 | no data | >20000 | >20000 | A |
| Aliskiren | 5493444 | no data | no data | 1320 - 6690 | B |
| alpha-estradiol | 68570 | no data | no data | | |
| Alprazolam | 2118 | 1220 | 1020 | | |
| Amisulpride | 2159 | no data | 1024 | 1187 - 2769 | C |
| Amitriptyline | 2160 | 320 | no data | | |
| Amphetamine | 3007 | 30 | 22 | | |
| Apophedrin | 1000 | no data | no data | | |
| Apophedrin | 1000 | no data | no data | | |
| Arecoline | 2230 | 2500 | 550 | | |
| Aspartame | 134601 | >10000 | >10000 | | |
| Atenolol | 2249 | 2000 | 2000-3000 | | |
| Atorvastatin | 60823 | no data | no data | | |
| Azithromycin | 447043 | >2000 | >3000 | | |
| beta-Asarone | 5281758 | no data | no data | | |
| BDB (1-(3,4-Methylenedioxyphenyl)-2-butanamine) | 129870 | no data | no data | | |

| Compound name in PubChem | Pubchem compound number | rat, oral, $LD_{50}$ (mg/kg) | mouse, oral, $LD_{50}$ (mg/kg) | rat, oral, $LD_{50}$ (mg/kg) | source |
|---|---|---|---|---|---|
| Benzotriazole | 7220 | 600 | 615 | | |
| beta-estradiol | 5757 | no data | no data | | |
| Betaxolol | 2369 | no data | no data | | |
| Bezafibrate | 39042 | 1082 | 723 | | |
| Biotin | 171548 | *>1.45 mmol/kg* | >10000 | | |
| Bisoprolol | 2405 | no data | 730 | | |
| bk-MDDMA (Dimethylone) | 9794472 | no data | no data | | |
| Bupirimate | 38884 | 4000 | 4000 | | |
| Buspirone | 2477 | 136 | no data | | |
| Butocarboxim | 5360962 | 153 | no data | | |
| Caffeine | 2519 | 192 | 127 | | |
| Caprolactam | 7768 | 1200-1600 | 2100-2500 | | |
| Captopril | 44093 | 4245 | 2500 | | |
| Carbamazepine | 2554 | 1975 | 529 | | |
| Carbamazepine-10,11-epoxide | 2555 | no data | no data | | |
| Carboxyibuprofen | 10444113 | no data | no data | | |
| Cathine | 441457 | no data | no data | | |
| Cathinone | 62258 | no data | 400 | | |
| Celiprolol | 2663 | no data | no data | | |
| Cetirizine | 2678 | 365 | no data | | |
| Chloridazon | 15546 | 3600 | 2500 | | |
| Chlorpropham | 2728 | 1200 | 3650 | | |
| Cimetidine | 2756 | 5000 | 2550 | | |
| Ciprofloxacin | 2764 | >2000 | 5000 | | |
| Citalopram | 2771 | no data | no data | | |
| Clarithromycin | 84029 | 1270 | 1230 | | |
| Climbazole | 37907 | 400 | no data | | |
| Clinafloxacin | 60063 | no data | no data | | |
| Clindamycin | 446598 | 2619 | no data | | |
| Clofribic acid | 2797 | 897 | 1170 | | |
| Clopidogrel | 60606 | *no data* | *no data* | | |
| Codeine | 5284371 | 427 | 250 | | |
| Cotinine | 854019 | no data | 1604 | | |
| 70434-82-1 | 125835 | no data | no data | | |
| Crotamiton | 688020 | 1500 | 1600 | | |
| Cyanopropanal | 77049 | no data | no data | | |
| Cycloheximide | 6197 | 2 | 133 | | |
| Cyprodinil | 86367 | 2000 | no data | | |
| Deacetyldiltiazem | 91638 | no data | no data | | |
| Diethyltoluamide | 4284 | 1950 | 1170 | | |
| Demeton-S | 24723 | 1,5 | no data | | |
| Diazepam | 3016 | 1200 | 700 | | |
| Diclofenac | 3033 | 62,5 | 170 | | |
| Digoxigenin | 15478 | no data | no data | | |

| Compound name in PubChem | Pubchem compound number | rat, oral, $LD_{50}$ (mg/kg) | mouse, oral, $LD_{50}$ (mg/kg) | rat, oral, $LD_{50}$ (mg/kg) | source |
|---|---|---|---|---|---|
| Digoxin | 2724385 | 28,27 | 17,8 | | |
| 35079-97-1 | 83852 | no data | no data | | |
| Diltiazem | 39186 | no data | 740 | | |
| Dimoxystrobin | 10936292 | no data | no data | | |
| Diphenhydramine | 3100 | 500 | 160 | | |
| Disopyramide | 3114 | 333 | 352 | | |
| Disulfoton | 3118 | 6,8 | 4,8 | | |
| Diuron | 3120 | 1017 | no data | | |
| 2680-03-7 | 17587 | 316 | 460 | | |
| 120-20-7 | 8421 | no data | no data | | |
| Doxazosin | 3157 | no data | no data | | |
| Doxylamine | 3162 | 470 | 250 | | |
| 30223-73-5 | 115159 | no data | no data | | |
| Enrofloxacin | 71188 | 5000 | 4336 | | |
| Ephedrine | 9294 | 600 | 689 | | |
| Pseudoephedrine | 7028 | 660 | 500 | | |
| Ephedrine | 9294 | 600 | 689 | | |
| 36507-30-9 | 2555 | no data | no data | | |
| Erythromycin | 12560 | 4600 | 2580 | | |
| Esmolol | 59768 | no data | no data | | |
| Estriol | 5756 | >2000 | no data | | |
| Estrone | 5870 | no data | no data | | |
| Ethenzamide | 3282 | 2200 | 700 | | |
| Ethofumesate | 33360 | 1130 | >1600 | | |
| Fenpropidin | 91694 | 1447 | >2600 | | |
| Fenpropimorph | 93365 | 3000 | 5980 | | |
| Fexofenadine | 3348 | no data | no data | | |
| Flecainide | 3356 | 50-498 | no data | | |
| Fluconazole | 3365 | 1271 | 1408 | | |
| Fludioxonil | 86398 | >5000 | >5000 | | |
| Flumequine | 3374 | 1753 | 1630 | | |
| Fluopyram | 11158353 | no data | no data | | |
| Fluoxetine | 3386 | 825 | 464 | | |
| Flutolanil | 47898 | 10000 | >10000 | | |
| Fluxapyroxad | 16095400 | no data | no data | | |
| Gabapentin | 3446 | >8000 | 8053 | | |
| Gabapentin lactam | 47457 | no data | no data | | |
| Guaifenesin | 3516 | 1510 | 690 | | |
| Histamine | 774 | no data | 220 | | |
| Histidine | 6274 | >15000 | >15000 | | |
| HMMA (hydrochloride) / 4-Hydroxy-3-methoxy Methamphetamine Hydrochloride | 71748871 | no data | no data | | |
| Hydrochlorothiazide | 3639 | 2750 | 1175 | | |
| Ibuprofen | 3672 | 636 | 740 | | |
| Irbesartan | 3749 | no data | no data | | |

| Compound name in PubChem | Pubchem compound number | rat, oral, $LD_{50}$ (mg/kg) | mouse, oral, $LD_{50}$ (mg/kg) | rat, oral, $LD_{50}$ (mg/kg) | source |
|---|---|---|---|---|---|
| Ketamine | 3821 | no data | no data | | |
| Ketoprofen | 3825 | 62,4 | 360 | | |
| Kojic acid | 3840 | no data | no data | | |
| Labetalol | 3869 | >2000 | 660 | | |
| Lamotrigine | 3878 | 245 | 205 | | |
| Lidocaine | 3676 | 317 | 102 | | |
| Lincomycin | 3000540 | 1000 | 13900 | | |
| Lomefloxacin | 3948 | 3800 | >4000 | | |
| Losartan | 3961 | no data | no data | | |
| MDA (Methylenedioxyamphetamine of: Tenamfetamine) | 1614 | no data | 13,3 | | |
| MDEA (3,4-Methylenedioxy-N-ethylamphetamine) | 105039 | no data | no data | | |
| MDMA (3,4-Methylenedioxymethamphetamine) | 1615 | no data | no data | 180 | D |
| Melamine | 7955 | 3161 | 3296 | | |
| Metamitron | 38854 | 1447 | 1450 | | |
| Metformin | 4091 | 1000 | 1450 | | |
| Methocarbamol | 4107 | 1320 | 812 | | |
| Methylparaben | 7456 | >5600 | >8000 | | |
| Metobromuron | 18290 | 2000 | 2098 | | |
| Metoclopramide | 4168 | 750 | 270 | | |
| Metolachlor | 4169 | 2200 | 1150 | | |
| Metolcarb | 14322 | 498-580 | 109 | | |
| Metoprolol | 4171 | 3090-4670 | 1050 | | |
| Mevinphos | 5355863 | 3 | 7-18 | | |
| Mycophenolic acid | 446541 | 352 | 1000 | | |
| N-Acetyl-4-aminoantipyrine (syn.: 4-Acetamidoantipyrine) | 65743 | no data | no data | | |
| Nandrolone | 9904 | no data | no data | | |
| Naproxen | 156391 | 248 | no data | | |
| N-Butylbenzenesulfonamide | 19241 | 2050 | 2500 | | |
| 1672-58-8 | 72666 | no data | no data | | |
| Nicotinamide | 936 | 3500 | 2500 | | |
| Nicotinamide | 936 | 3500 | 2500 | | |
| Nicotine | 89594 | 188 | 24 | | |
| Nordiltiazem | no | not present | not present | | |
| Norfloxacin | 4539 | >4000 | 4000 | | |
| Noroxycodone | 5489120 | no data | no data | | |
| O-Desmethylnortramadol | no | not present | not present | | |
| O-Desmethyltramadol | 9838803 | no data | no data | | |
| Levofloxacin | 149096 | 1478 | 1803 | | |
| Ofloxacin | 4583 | 3590 | 3266 | | |
| Olsalazine | 22419 | no data | no data | | |
| Ormetoprim | 23418 | no data | no data | | |
| Oxazepam | 4616 | >8000 | 1540 | | |
| Oxcarbazepine | 34312 | no data | no data | | |
| Pantoprazole | 4679 | 747 | no data | | |

| Compound name in PubChem | Pubchem compound number | rat, oral, $LD_{50}$ (mg/kg) | mouse, oral, $LD_{50}$ (mg/kg) | rat, oral, $LD_{50}$ (mg/kg) | source |
|---|---|---|---|---|---|
| Pentaethylene glycol | 62551 | no data | no data | | |
| Pentedrone | 57501499 | no data | no data | | |
| Phorate | 4790 | 1,0 | 2,25 | | |
| P-hydroxyamphetamine | 3651 | no data | no data | | |
| Piperonylbutoxide | 5794 | 11500 | 2600 | | |
| 64-13-1 | 31721 | no data | no data | | |
| Procaine | 4914 | no data | 350 | | |
| Propranolol | 4946 | 660 | 289 | | |
| Pseudoephedrine | 7028 | 660 | 500 | | |
| Pyrethrin I | 5281045 | 260 | no data | | |
| Cinerin II | 5281548 | 1030-2370 | no data | | |
| Jasmolin I | 12304687 | no data | no data | | |
| Pyridoxine | 1054 | 4000 | no data | | |
| Rilmenidine | 68712 | no data | no data | | |
| Ritalinic acid | 86863 | no data | no data | | |
| Rivaroxaban | 9875401 | no data | no data | | |
| Roxithromycin | 6915744 | 830 | 665 | | |
| Saccharin | 5143 | 14200 | 17500 | | |
| salicylic acid | 338 | 891 | 480 | | |
| Sildenafil | 135398744 | no data | no data | | |
| Sitagliptin | 4369359 | >3000 | 4000 | | |
| Sotalol | 5253 | no data | no data | 3450 | E |
| Sucralose | 71485 | no data | no data | | |
| Sulfadiazine | 5215 | no data | 1500 | | |
| Sulfadimethoxine | 5323 | no data | 3200 | | |
| Sulfamerazine | 5325 | no data | 25000 | | |
| Sulfamethazine | 5327 | no data | 50000 | | |
| Sulfamethoxazole | 5329 | 6200 | 2300 | | |
| Sulfanilamide | 5333 | 3900 | 3000 | | |
| Sulfapyridine | 5336 | 15800 | 16600 | | |
| Sulpiride | 5355 | 9800 | 1700 | | |
| Telmisartan | 65999 | no data | no data | | |
| Temazepam | 5391 | 1833 | 1963 | | |
| Theobromine | 5429 | 1265 | 837 | | |
| Thiabendazole | 5430 | 2080 | 1300 | | |
| Thiometon | 12541 | 120-130 | 37 | | |
| Tolycaine | 72137 | no data | no data | | |
| TPPO (Triphenylphospine oxide) | 13097 | no data | 1380 | | |
| Tramadol | 33741 | 228 | 270 | | |
| Desvenlafaxine | 125017 | no data | no data | 350(f)-700(m) | F |
| Trenbolone | 25015 | no data | no data | | |
| Trifloxystrobin | 11664966 | >4000 | no data | | |
| 2,3,5-Trimethacarb | 25550 | 125-232 | >250 | | |
| Trimethoprim | 5578 | >5300 | 2764 | | |

| Compound name in PubChem | Pubchem compound number | rat, oral, $LD_{50}$ (mg/kg) | mouse, oral, $LD_{50}$ (mg/kg) | rat, oral, $LD_{50}$ (mg/kg) | source |
|---|---|---|---|---|---|
| Triphenylphosphine oxide | 13097 | no data | 1380 | | |
| Tylosin | 5280440 | >5000 | 10000 | | |
| Tyramine | 5610 | no data | no data | | |
| Tyramine | 5610 | no data | no data | | |
| Valsartan | 60846 | >2000 | no data | | |
| 164265-78-5 | 19388302 | no data | no data | | |
| Venlafaxine | 5656 | 350-700 | no data | | |
| Virginiamycin M1 | 5459319 | no data | no data | | |
| Warfarin | 54678486 | 1,6 | 3 | | |

Pubchem references: https://pubchem.ncbi.nlm.nih.gov/compound/xxxxx
in which xxxxx is the compound number

References to other webpages than PubChem

A https://echa.europa.eu/nl/registration-dossier/-/registered-dossier/23062/7/3/2

B https://www.hpc-standards.com/msds/688601_1627395352_Aliskiren_MSDS_EN_HPC-Standards.pdf

C https://www.hpc-standards.com/msds/687449_1600937380_Amisulpride_MSDS_EN_HPC-Standards.pdf

D https://www.aatbio.com/resources/toxicity-lethality-median-dose-td50-ld50/methylenedioxymethamphetamine

E https://go.drugbank.com/drugs/DB00489

F https://imgcdn.mckesson.com/CumulusWeb/Click_and_learn/SDS_9HIKMP_DESVENLAFAXINE_ER_TAB_50MG_30_BT.pdf

# Annex 8 Literature study machine-learning for the analysis of clusters in a biological context

Mohapatra *et al.*[99] applied colour-based clustering for WBC (white blood cells) nucleus segmentation of stained blood smear images followed by relevant feature extraction for leukaemia detection. They have applied several unsupervised machine learning techniques such as K-means, K-medoid, and fuzzy c-mean for colour-based segmentation. Their process includes as follows: Blood smear image grabbed by a digital microscope, followed by applying K-means segmentation to get sub-images in which one WBC (white blood cell) is excited per sub-image. After that, they applied selective filtering and unsharp masking as preprocessing, followed by conversion of RGB to L*a*b colour space. In their methodology for feature extraction, different features were examined including shape, texture, and colour features, the latter being considered the most important feature. Satisfactory results were obtained for detecting leukaemia with the proposed features using the SVM classifier. They have mentioned that including more shape features such as elongation and compactness which can be determined from the cell's area and perimeter could result in more robust results.

In the study by Chen *et al.* [100] on detecting leukemia using microscopic blood images, a novel clustering algorithm was proposed. This algorithm incorporates stimulating discriminant measures (SDM) based on within- and between-cluster scatter variances to achieve robust segmentation of the nucleus and cytoplasm in lymphocytes and lymphoblasts. Their overall system achieves superior recognition rates of 96.72% and 96.67% accuracies using bootstrapping and 10-fold cross-validation with Dempster-Shafer and SVM, respectively.

In another study on unsupervised learning, Liu *et al.* [101] presented an efficient algorithm for segmenting microscopic images of Phellodendron. As a first step in the pre-processing phase, they employed a technique called super-pixel segmentation to divide the images into smaller, homogeneous regions. In this process, they found that the Felzenszwalb method was superior to other image segmentation methods such as watershed and Quick Shift. Next, the authors extracted features from the segmented images using a combination of techniques including Sobel's operator for edge detection, the lab colour space for colour information, and Harris corner detection for identifying salient points in the image. Following feature extraction, the authors then applied a spectral clustering method to group the segmented regions based on their similarity. They then labelled the objects and performed post-processing to refine the segmentation results. The proposed algorithm was thoroughly validated, and the results indicated that it achieved a precision of 81% and a recall of 93%, demonstrating its effectiveness in accurately segmenting Phellodendron microscopic images. Despite its performance, this method can be computationally expensive.

Chayadevi and Raju [102] examined the automatic detection of microbes and the extraction of bacterial clusters through statistical and neural network approaches. Bacterial patterns in the images were detected and counted through contour detection and the freeman chain algorithm. Digital images collected from different hospital sources were pre-processed using binarization and thresholding to eliminate blur, irregular and noisy images. From pre-processing, 81 features of the bacteria were extracted but some potential features such as circularity, eccentricity, tortuosity, major and minor axis, and perimeter of the bacteria were selected for segmentation. After image segmentation, the freeman chain contour algorithm was used to count and recognize the bacteria. The algorithm scans the segmented image one by one from left to right and top to bottom and extracts the pixel of the bacteria into an array using the 8-neighbor connectivity indicating 8 directions. Clustering relates to unsupervised learning, where the data has no class labels.

Norouzia *et al.* [103] used K-means and SOM means, two of the most used clustering algorithms in medical applications. These neural network methods use a set of input vectors and generate an output vector of clusters using the inter and intra-cluster distances that map each input vector to a cluster. Experimental results for both clustering methods were discussed. The results of the 320 bacterial images were evaluated

with the manual counting taken by doctors. The results showed that the proposed methods were more accurate than human counting.


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