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Eco-engineered biofilters for the removal of pesticides from drinking water in Brazil

Laura Orsina Cossu

SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE
DEGREE OF

Doctor of Philosophy (PhD)

James Watt School of Engineering
College of Science and Engineering
University of Glasgow



University
of Glasgow

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Declaration of Originality

I, Laura Orsina Cossu, certify that this thesis presented here for examination for a PhD degree at the University of Glasgow, is my own work, other than where I clearly indicated the contribution of others.

I have read and understood the University of Glasgow PGR Code of Practice, and confirm that no third party has edited this thesis beyond what is permitted according to the PGR Code of Practice.

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I further declare that this thesis has been produced in accordance with the University of Glasgow's Code of Good Practice in Research. I acknowledge that if any issues are raised regarding good research practice, the examination of this thesis may be postponed pending the outcome of any investigation of the issues.

Name: Laura Orsina Cossu

GUID: xxxxxxxxx

Date: 30/06/2025

Chapter 1 – Introduction

Organic micropollutants of anthropogenic origin pose a global threat to drinking water safety. They might enter the environment through point sources, such as industrial waste discharge or accidental spillages, or nonpoint sources, for example agricultural runoff^{1,2}. These compounds are of various origin and include pharmaceuticals, personal care products (PPCPs), endocrine disrupting chemicals, and industrial compounds such as per- and polyfluorinated alkyl substances (PFAS)^{3,4}. Once leaked into the environment, organic micropollutants can be found in surface- and groundwater and ultimately enter drinking water reservoirs^{1,3,5}. Among anthropogenic organic micropollutants, pesticides are of particular concern, as they are widely found in the environment and have been identified as one of the biggest contributors to surface water pollution⁶. Insufficient treatment of drinking water allows pesticides to enter the distribution network and jeopardise the quality and safety of the drinking water that reaches the end consumer^{5,7–10}. The contamination of drinking water with organic micropollutants, specifically pesticides, is a global problem, but it is more prevalent in countries where intensive agriculture is practised, and which are not equipped with advanced drinking water treatment plants (DWTP)¹¹. In a conventional DWTP, the water is treated in a series of steps. These might include sedimentation, chemical coagulation and flocculation, rapid and slow sand filtration, activated carbon, aeration, ozonation or advanced oxidation to obtain high quality drinking water^{5,12–14}. Removal of pesticides, however, is often incomplete in these systems^{5,8,15}, which is a cause for concern, as the negative effects of pesticides in drinking water on human health have not yet been comprehensively researched^{8–10}. While advanced physical-chemical treatment technologies achieve improved pesticide removal, they are accompanied by increased financial and environmental costs, making them unsuitable for implementation in low- and middle-income countries¹¹.

Engineered biofiltration potentially offers a solution to these problems^{5,11}. DWTP slow and rapid gravity filters consist of sand or granular activated carbon (GAC)^{5,13}. These filters may naturally harbour highly diverse microbial biofilms, if not prevented by disinfection, turning them into so called biofilters^{13,16–18}. Some biofilters have shown enhanced pesticide removal, due to the ability of microorganisms to degrade

pesticides and other micropollutants entering the DWTP¹⁹, using them as carbon or energy sources²⁰. Being able to utilise this biodegradation potential to enhance the removal of pesticides and other micropollutants within DWTP biofilters is of great interest. In contrary to advanced physical-chemical treatment technologies, advanced biological micropollutant removal fulfils all criteria of a sustainable technology. Engineered biofiltration is energy- and cost-efficient, does not require the addition of harmful chemicals and offers the possibility of using locally sourced materials^{5,11}, making it suitable for the application in low- and middle-income countries¹¹.

There are two approaches that could be used to promote the degradation of pesticides in biofilters. Firstly, through bioaugmentation, a technique that relies on incorporating microorganisms known to be able to degrade a certain pollutant into a system, in the case of a DWTP this would be the biofilter²¹⁻²⁴ and secondly, biostimulation, in which case the environmental factors are manipulated in such a way that they promote the establishment of desirable microorganisms²⁵⁻³¹.

One of the biggest challenges faced with the bioaugmentation approach is the loss of added degrader strains from the system over time^{22,23,32}. In the past decade, research has been addressing this challenge, but with limited success as of today¹¹. When it comes to biostimulation, deepening the fundamental understanding of the impact of environmental and DWTP operational conditions on biofilter microbial community function and micropollutant removal is crucial to fully utilise the biodegradation potential.

Engineered biofiltration would find its ideal application in Brazil, an agricultural powerhouse, as this approach is both financially and ecologically sustainable and offers improvements to the performance of existing DWTPs without the need to construct new and costly infrastructure. For this reason, Brazil is also at the centre of this thesis, which could be realised through the cooperation of the University of Glasgow with the Federal Universities of Ouro Preto and Minas Gerais in Brazil.

Although engineered biofiltration offers great potential for drinking water treatment, there is currently a lack of established quantitative methods for the investigation and optimisation of biological activities within this system. This thesis contributes to the

understanding of microbial communities from DWTP biofilters and their potential to degrade pesticides. The overarching aim of this thesis is to lay a robust foundation for the reliable study of the pesticide degradation potential of drinking water biofilter by further developing the necessary methods and adapting them for use in this context. In this thesis I decided to focus on the biodegradation of two selected pesticides, namely atrazine and MCPA, and the identification of the functional genes required for this in environmental samples from DWTPs in Scotland and Brazil. Atrazine and MCPA were selected as they are both herbicides, but differ in their persistence in the environment, with MCPA being more easily degradable than atrazine. MCPA is applied worldwide, whereas atrazine has been banned in the EU for over 20 years but is still used frequently in Brazil.

To reach the aim of this thesis, the following objectives were addressed:

1. To highlight the challenge posed on drinking water safety by widespread use of pesticides in combination with incomplete removal of these contaminants during drinking water treatment, illustrated by the example of Brazil
2. To analyse and assess alternative and improved drinking water treatment methods researched in the past decade, evaluating them for their suitability to be applied as sustainable technology in low- and middle-income countries.
3. To establish a protocol for the extraction and quantification of intact microbial cells from biofilter samples, based on an existing protocol that quantifies total microbial cells from biofilter samples.
4. To assess how the storage of biofilter samples impacts the quantification of total and intact cell counts, to minimise the introduction of biases. This is of special importance for samples that are collected from remote areas or that must be shipped overseas prior to analysis.
5. To identify functional genes essential for the biodegradation of atrazine and MCPA and to select suitable primer sets to target those genes to quantify their presence in DWTP biofilters.

6. To amplify and sequence target functional genes relevant for atrazine and MCPA biodegradation identified in biofilter samples collected from Scottish and Brazilian DWTPs.

An outline of the thesis is given below:

Chapter 2 – Literature Review

Chapter 2 consists of a literature review, which frames the research context of this thesis. It informs about slow and rapid gravity sand filtration for drinking water treatment in general and provides an insight into recent research on microbial communities associated with DWTP biofiltration.

Chapter 3 – Review on Pesticide Contamination and Drinking Water Treatment in Brazil: The Need for Improved Treatment Methods

The research presented in Chapter 3 is a systematic review, that has been published in ACS ES&T Water. The article is titled “Review on Pesticide Contamination and Drinking Water Treatment in Brazil: The Need for Improved Treatment Methods” and can be accessed here: <https://doi.org/10.1021/acsestwater.4c00063>. The article consists of two main sections. The first focuses on the occurrence of pesticide in Brazilian drinking water, whereas the second presents current and novel treatment technologies for the removal of pesticides from drinking water. The article suggests engineered biofiltration as suitable and sustainable drinking water technology for low- and middle-income countries with intense agriculture, such as Brazil.

Chapter 4 – Optimisation of methods for the quantification of microbial communities in biofilters.

Chapter 4 presents method optimisations performed for DNA extractions (Chapter 4.1) and for the flow cytometric quantification and characterisation of microbial communities (Chapter 4.2) from DWTP biofilters. Chapter 4.2 addresses a range of questions concerning the quantification of intact and total cells from DWTP biofilter samples by flow cytometry (FCM). It further addresses the potential bias introduced by storage conditions on cell quantification and FCM fingerprinting signals. This chapter

puts forward and optimised extraction protocol for detaching and analysing both total and intact cells from DWTP biofilter samples.

Chapter 5.1 – Evaluation of primer sets for the quantification of atrazine and MCPA biodegradation genes in DWTP biofilter samples

In Chapter 5.1, relevant functional genes for the biodegradation of atrazine and MCPA were identified. Primer sets targeting these functional genes were collected in a systematic literature search and their suitability for qPCR assays of DNA extracts from DWTP biofilter samples was assessed, both in silico and in the laboratory.

Chapter 5.2 – Analysis of *trzN* and *tfdAa* functional genes amplified from environmental samples

Chapter 5.2 describes the analysis of two functional genes (*trzN* and *tfdAa*) that have been amplified from DNA extracts of DWTP biofilter samples collected in Scotland and Brazil. This chapter focuses on the analysis of targeted functional genes after sequencing. Amplicon Sequence Variants (ASVs) were created using the DADA2 pipeline and subsequently submitted to BLAST searches.

Chapter 6 – Conclusions and Recommendations

Finally, Chapter 6 summarises the main findings of this thesis and highlights the recommendations for and potential of future research.

Chapter 2 – Literature review

Chapter 2 introduces the overall topic of drinking water treatment, focusing on filtration, and outlines the overarching framework within which the experimental chapters are set. Each of the experimental chapters contains its own detailed introduction to its respective main topic.

2.1 Drinking Water Treatment

A typical DWTP consists of a treatment train of consecutive water treatment steps, designed to improve drinking water quality⁵. For a conventional DWTP this often involves aeration, coagulation, flocculation, and sedimentation, rapid and slow sand filtration, and chlorination. More advanced DWTP may also perform advanced oxidation and disinfection and GAC filtration^{5,11,33}.

In the following sections, a brief introduction to conventional drinking water treatment methods is given. The main focus of this thesis is on biofiltration, which will be introduced in detail in section 2.2.

2.1.1 Aeration

Aeration serves several purposes during drinking water treatment. Aeration: 1. increases the amount of dissolved oxygen content in the water; 2. causes gases dissolved in the water, such as hydrogen sulphide, to be released, which can otherwise have a negative effect on the taste and odour of the drinking water; 3. rises the pH and reduces the corrosiveness of the water by decreasing the carbon dioxide content; 4. oxidises iron and manganese causing precipitation; and 5. removes volatile organic compounds. The two main processes of water aeration are to either let water fall through air, or to inject air into the water^{5,14}.

2.1.2 Coagulation, flocculation, and sedimentation

Aeration is typically followed by coagulation, flocculation and sedimentation. This step of the drinking water treatment train is designed to remove substances of natural and anthropogenic origin, such as turbidity, colour, and organic material, like microbes, or biological matter^{5,12}. Hydrophobic micropollutants that have a high K_{ow} (>6) value and low water solubility can successfully be removed from drinking water at this stage too⁵. During the coagulation step, a coagulant, usually aluminium or iron salt, is added to the

water, which forms positively charged aluminium or iron hydroxide complexes, which adsorb to negatively charged particles present in the influent water. In the next step, flocculation, the water is continuously but gently mixed and larger particle flocs are built from the destabilised particles through hydrogen bonding or Van der Waal forces. Finally, flocs are left to settle during sedimentation, which is the first step of removing them from the water phase¹².

2.1.3 Filtration

During the filtration step, residual particles are removed from the influent water and dissolved compounds are transformed while the water is passing through a porous filter bed. Two main types of filters are distinguished; Rapid gravity filters (RGFs) and slow sand filters^{5,13,33}. In addition, the filter medium is naturally colonised by a microbial community, that attaches to the sand or GAC and feeds on nutrients present in the influent water⁵, turning the filter into a biologically active filter, referred to as biofilter in this thesis.

Rapid Gravity Filtration: Rapid gravity filtration generally serves as solid-liquid separation process in drinking water, following coagulation and flocculation. The main mechanism of removal is through physical forces, but physicochemical processes take place too. Sand is the most common filter media and grain sizes range from 0.4 to 1.5 mm, whereby the composition of the sand varies (heterogeneous or homogeneous) depending on water quality and upstream water treatment processes. Alternative filter media are anthracite, granular activated carbon (GAC), garnet, pumice, expanded clay particles or glass¹³. Operational parameters such as filter configuration, filtration rate, flow control, and backwashing regime varies from treatment plant to treatment plant, based on local conditions, which are elaborated in detail in *Twort's Water Supply*¹³. A detailed description of these parameters would go beyond the scope of this thesis.

Slow Sand Filtration: Slow sand filtration is named after its comparatively low flow rate, which is about one twentieth or less of the flow rate of rapid gravity filtration. Slow sand filtration is a historically proven and effective method to produce high quality drinking water, by removing pathogens and other unwanted compounds, which reduces the load of chlorination needed before distribution of drinking water. During slow sand filtration, a combination of physical and microbiological purification takes

place, which extends below the surface of the filter bed. Slow sand filtration is a single-stage filter system that follows a relatively simple concept. It can be installed as part of a drinking water treatment train, or on its own in rural areas. A slow sand filter consists of three zones, the surface coating (Schmutzdecke), the autotrophic zone, and the heterotrophic zone¹³. Influent water is slowly and continuously introduced to the filter bed, to create a head of water that rises approximately 1.0 to 2.0 meters above the filter bed^{34,35}. The relatively slow, gravity-driven flow rate of the influent water passing through the filter bed ensures a retention time long enough for biological and physical interactions to occur³⁶. The main advantages of slow sand filtration are the reduction of physical, chemical and microbiological load of raw water, without the need for intense disinfection, producing effluent water free of harmful microbes³⁶.

2.1.4 Disinfection and oxidation

In many DWTP the treatment is finalised with a disinfection and oxidation process to ensure biological quality of the finished drinking water and to remove colour and odour issues. Chemical oxidants applied in this process might be hydrogen peroxide, free chlorine, chloramines or ozone. Chemical oxidants remove microbial load from the drinking water and may also react with natural organic matter or micropollutants⁵. This can lead to the production of disinfection by-products that potentially have equal or even greater toxicity than their parent compounds^{5,12,37,38}.

2.2 Biofilter microbial communities

This section provides an insight into recent research findings, focussing on DWTP biofilters and their associated microbial communities. It is intended to provide an introduction to the topic of biofilters, but is not a comprehensive representation of the current state of research.

2.2.1 Factors shaping microbial communities within DWTP biofilters

Relevant factors determining the assembly of microbial communities on DWTP filter media were studied in order to improve the understanding of how these forces affect the drinking water quality produced by biofiltration^{39,40}. Vignola et al. 2018³⁹ found in an laboratory-scale experiment, that rapid gravity filter communities were assembled by selection, rather than stochastic processes, as only 28% of the operational taxonomic

units (OTUs) of the biofilter communities were shared with the influent water. The authors further found, that GAC biofilters harboured a more diverse community in comparison with sand biofilters³⁹. Oh et al 2018⁴⁰ analysed microbial communities from different biofilter media of full scale DWTP. Metagenome sequencing was performed for microbial communities from a rapid gravity filter, GAC filter, slow sand filter and the Schmutzdecke of the slow sand filter. Similar to Vignola et al. 2018³⁹, Oh et al. 2018⁴⁰ found that microbial communities differ significantly from those in the influent water. The study further revealed filter-specific colonization pattern of bacterial groups, with GAC differing from the three sand filters. The authors suggest that GAC potentially selects fast-growers⁴⁰.

Not only the material of the filter medium shapes the microbial communities of biofilters. Carpenter et al. 2017⁴¹ found, that three distinct operational conditions applied to three groups of biofilter columns for over a year, resulted in significant differences in microbial community assembly. Microbial biomass at the surface of the filter column was lower, when higher superficial velocities were applied, but higher at a deeper level of the biofilter. Higher superficial velocities also resulted in higher overall biomass of the biofilter⁴¹.

In a study of two full-scale slow sand filters, Haig et al. 2015⁴², found that microbial community composition of the biofilter is also shaped by the filter depth and age, as well as environmental conditions, such as the month in which the filter was sampled⁴².

2.2.2 Microbial community compositions and activities within DWTP biofilters

Microbial processes within biofilters are still poorly understood. Two recent studies addressed this knowledge gap by investigating the vertical distribution of microbial communities within slow sand filters⁴³ and a bench scale biofilter set up⁴⁴. Attiani et al. 2025⁴³ found, that while prokaryotic communities were evenly distributed horizontally at each depth of the studied slow sand filters, community composition differed significantly vertically. They found that the Schmutzdecke contained higher biomass and diversity, in comparison to deeper filter layers⁴³. Similar results were found by Shi et al. 2024⁴⁴ who observed, that the microbial community collected from the bottom of a 30 cm laboratory-scale GAC biofilter, showed lower diversity. However, this community exhibited a higher degree of interconnectedness among the community members,

compared to the microbial community taken from the top of the GAC biofilter⁴⁴. Both studies further state, that the spatially distinct microbial communities lead to degradation of specific compounds at different filter depths^{43,44}. Shi et al. 2024 observed further, that even though the bottom microbial community only made up 20% of the total cell abundance of the biofilter, it utilised up to 60% more dissolved organic carbon in comparison to the top community, including high-molecular-weight humic substances, which have not been utilised by the top community at all⁴⁴. A similar result was found by Vignola et al. 2023⁴⁵, who run a 90 cm laboratory-scale GAC biofilter for 12 weeks, investigating the removal of dissolved organic matter by microbial communities from the top, middle and bottom of the biofilter. The study revealed, that the bottom community utilised the more complex fractions of the dissolved organic matter⁴⁵.

2.2.3 Functions of DWTP biofilters

Biofilters influence the quality of drinking water in two ways: 1. they seed the effluent water and therefore influence the microbial composition within the drinking water¹⁶, and 2. they contribute to the removal of contaminants from influent water⁴¹.

Pinto et al. 2012¹⁶ found that bacterial taxa from biofilter seeded the filter effluent and were able to persist, even though the treated water was disinfected before being fed into the distribution system. Biofilter microbial communities therefore directly impact the microbial composition and quality of drinking water that reaches the end consumer^{16,39,46,47}. GAC showed better control of microbial growth in direct comparison with sand, showing higher bacterial decay rates, harbouring less bacterial cells and performing better pathogen control³⁹.

Biofilter microbial communities are further responsible for the biodegradation of dissolved nutrients and contaminants in the influent water, improving the drinking water quality^{40,41,44,45,47,48}. Ma et al. 2020⁴⁷ argue, that biofilter microbial communities have a stronger impact on the effluent water quality by bio transforming influent water nutrients and pollutants, rather than by altering the microbial community composition. The removal of micropollutants from drinking water through biofiltration is discussed in depth in chapter 3 of this thesis.

Chapter 3 – Review on pesticide contamination and drinking water treatment in Brazil: the need for improved treatment methods

Laura O Cossu^a, Sérgio Francisco De Aquino^b, César Rossas Mota Filho^c, Cindy J Smith^a and Marta Vignola^{a,*}

^a Infrastructure and Environment, James Watt School of Engineering, Advanced Research Centre (ARC), University of Glasgow, Glasgow, G11 6EW, United Kingdom

^b Chemistry Department, Institute of Exact and Biological Sciences, Federal University of Ouro Preto, Ouro Preto MG, 35402-163, Brazil.

^c Department of Sanitary and Environmental Engineering, Federal University of Minas Gerais, Belo Horizonte MG, 31270-901, Brazil

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Abstract

Pesticide pollution of surface water is a global threat to drinking water safety. The need for improved drinking water treatment methods is discussed using Brazil as a case study. Brazil's agriculture is intensive, and pesticide consumption is high, while current drinking water treatment methods are inadequate for effectively removing pesticides.

Available data of surface water contamination in Brazil shows widespread occurrence of pesticides in natural waters, thereby putting pressure on the water treatment system and threatening the quality and safety of drinking water. Pesticide concentrations in drinking water frequently exceeded maximum permissible concentrations if EU regulations (0.1 µg/L) were applied, highlighting the need for improvements in drinking water treatment. (Advanced) drinking water treatment for the removal of pesticides has been researched intensely over the past decade. However, challenges such as high cost and energy intensity, as well as the production of hazardous by-products must be assessed critically.

Safely managed drinking water is crucial to the sustainable development of low- and middle-income countries and can only be achieved through appropriate technology. Engineered biofiltration has been put forward as a sustainable alternative to conventional and advanced drinking water treatment. This systematic review highlights the promising potential of engineered biofiltration and associated challenges.

Keywords

Drinking water, Pesticide, Advanced treatment, Biofiltration, Low- and middle-income country, Sustainable technology

Synopsis

Pesticide pollution of surface water poses a significant threat to drinking water safety due to insufficient drinking water treatment. This review proposes engineered biofiltration as an appropriate technology for low- and middle-income countries, emphasizing both its potential and associated challenges.

Introduction

Organic micropollutants are a group of anthropogenic chemical compounds. They can enter the environment via point sources, like industrial waste discharges or accidental

spills, or non-point sources, such as agricultural run-off^{1,2}; and include pharmaceuticals, personal care products (PPCPs), endocrine disrupting chemicals, and industrial compounds like per- and polyfluorinated alkyl substances (PFAS)^{3,4}. Due to adverse effects on ecosystems, the fate of micropollutants in the environment is of concern as they are potentially biologically active in trace concentrations^{2,49,50}. Among organic micropollutants, pesticides are classified as of particular importance and priority¹. Pesticides are pure or mixed substances designed to prevent, mitigate, repel, or destroy pests and can be classified in herbicides, insecticides, and fungicides⁵¹. Pesticides are widely found in the environment; agricultural run-off of pesticides has previously been described as one of the biggest contributors to surface water pollution⁶. Pesticides have also been detected in water bodies used as drinking water sources^{1,3,52}, which is an issue as conventional drinking water treatment plants (DWTPs), often fail to successfully remove pesticides from raw water, impacting drinking water quality^{53,54}. The effects of such compounds on human health are still under debate and their presence in drinking water sources calls for caution⁵⁴⁻⁵⁶.

Scope of this review

Pesticides in drinking water is a growing global issue but exacerbated in countries such as Brazil. In Brazil, the extreme use of pesticides - applying high pressure on the environment, drinking water sources and treatment - coexists with conventional treatment technologies that are often ineffective at removing such micropollutants. The growing pressure in Brazil makes it an ideal case study on the impact of emerging contaminants on drinking water quality and the associated challenges for water treatment. The review examines regulation and control management, and considers potential sustainable solutions to address this challenge, not just in Brazil, but globally. After providing the general context and introducing the legal framework of the Brazilian drinking water directive, the review is composed of two main sections titled 3. *Pesticide occurrence in treated drinking water in Brazil* and 4. *Drinking Water Treatment*.

In the first section, pesticide contamination of Brazilian drinking water is assessed. With the help of a systematic literature search, relevant and potentially harmful pesticide concentrations in drinking water were identified. Further, an overview of the data availability and research priorities of the past 20 years was obtained. A recently

published review⁵⁷ addresses the general chemical contamination extent of Brazilian drinking water, whereas this review focuses exclusively on pesticides. Data on pesticide contamination is placed in context of local legal regulations, and these are discussed from a global perspective. This section highlights the challenges associated with intense use of pesticides and their incomplete removal during drinking water treatment.

In the second part of the review, conventional drinking water treatment methods and their shortcomings with respect to pesticide treatment are briefly discussed (4.1. State of the art). In 2013, a comprehensive review was published on existing technologies for pesticide removal⁵². Since then, interest in this field has grown. This review explores the main research drivers and examines the alternative and enhanced treatment methods that have been proposed over the past decade (4.2. Advanced and novel treatment methods). Treatment methods are grouped into physical-chemical treatment and biological treatment. A second systematic literature search was conducted to gather relevant publications.

Finally, the two sections are connected in the discussion, elaborating on potential alternatives for sustainable but effective treatment of drinking water in low- and middle-income countries with intense agriculture, such as Brazil.

Case Study: Brazil

Brazil is one of the greatest agricultural powerhouses worldwide⁵⁸. In 2020, Brazil consumed 5.94 kg of pesticides per hectare of cultivated land, which amounts to 377,176 tonnes of pesticides, ranking the country as second-largest consumer of pesticides globally⁵⁹. From 2009 to 2018 the top three sold pesticides were glyphosate, 2,4-D, and atrazine⁶⁰. The most important agricultural regions in Brazil are the South, Southwest and Midwest, accounting for roughly 80% of Brazil's pesticide consumption⁶¹. Brazil is a middle-income country with a relatively high level of potabilization. According to the WHO/UNICEF joint monitoring program >99% of the population had access to at least basic water supplies and 86% had access to safely managed drinking water in 2020⁶². Most drinking water systems in Brazil rely on conventional treatment methods, which usually perform insufficient pesticide removal. The potential application of sustainable technologies to improve pesticide removal during drinking water treatment is therefore of interest, for Brazil and other low- and middle-income countries.

Pesticides in drinking water – legal framework

Drinking water legislations exist to protect consumers from health hazards caused by (pesticide) contamination in drinking water. Often drinking water regulations impose maximum concentrations of pesticides in drinking water which must not be exceeded. However, an international comparison of threshold limits for 25 pesticides showed great variance in regulations⁶³. Three main clusters were identified, namely countries implementing EU, WHO or USEPA guidelines. For most of the pesticides analysed, the most conservative regulations stem from countries implementing EU recommendations⁶³.

In Brazil, the National Council for the Environment (Conselho Nacional do Meio Ambiente, CONAMA) adopted resolution No. 357/2005, which protects water resources for a multitude of uses⁵⁸. The Brazilian Drinking Water Guidelines provide legal quality standards, including maximum allowable concentration for a range of substances⁶⁴. However, many emerging contaminants, including various pesticides, are not regulated⁶⁵; ordinance no 2914/2011 set maximum permissible concentrations in drinking water for only 28 of all nationally approved pesticides, based on Quantitative Chemical Risk Assessment (QCRA). From 2019 to 2020 the ordinance has been revised and was replaced by Ordinance no 888/2021, which set standards for 54 pesticides or their by-products. Regulated compounds were also chosen based on QCRA which took into consideration both pesticide toxicity and the probability of their occurrence in drinking water. The latter was estimated by considering pesticide physical-chemical properties (environmental fate), sales data, and existing monitoring information^{66,67}. Water quality monitoring falls within the remit of the environmental state agencies⁵⁸.

In global comparison, more restrictive legislations are established elsewhere; EU regulations serve as an example, where the presence of pesticides in drinking water was generally limited by the Drinking Water Directive (Council Directive 98/83/EC) in November 1998. The maximum permissible concentration is 0.1 µg/L for individual pesticides and 0.5 µg/L for the sum of all pesticides present at any given time. Each member state must run a monitoring program, and the quality of drinking water is reviewed by the European Commission every three years⁶⁸.

The drinking water legislations of Brazil and the EU differ fundamentally in comprehensiveness and threshold concentrations set for pesticides. A global comparison showed that the EU has the most restrictive pesticide limit value - following the precautionary principle, which is well below the human health risk model uncertainty range⁶³. The Brazilian regulations on the other hand are based on a QCRA, setting individual threshold values for selected pesticides and other contaminants^{66,67}. Table 1 provides an overview of drinking water frameworks for maximum permissible pesticide concentrations from Brazil, the European Union, US Environmental Protection Agency, and World Health Organisation.

Table 1 Overview of drinking water frameworks for maximum permissible pesticide concentrations from Brazil, the European Union, US Environmental Protection Agency, and World Health Organisation.

Origin	Drinking water regulation	Description
Brazil	Ordinance no 888/2021 ⁶⁶	Maximum permissible concentrations set for 54 pesticides or their by-products individually, based on the QCRA approach.
EU	Drinking Water Directive (Council Directive 98/83/EC) ⁶⁸	Maximum permissible concentrations set for all pesticides: 0.1 µg/L - individual pesticides, 0.5 µg/L - sum of all pesticides, based on the precautionary principle.
US EPA	National Primary Drinking Water Regulations ⁶⁹	Maximum permissible concentrations for 22 pesticides in drinking water. Two sets of limits are established: 1. Maximum Contaminant Level Goals (MCLG), which are non-enforceable public health goals, and 2. Maximum Contaminant Levels (MCL) that must not be exceeded in drinking water and consider both human health and the best available treatment technology and economic costs.

WHO	Guidelines for drinking-water quality: fourth edition incorporating the first and second addenda (ISBN 978-92-4-004506-4) ⁷⁰	Maximum permissible concentrations set for 31 pesticides or their by-products individually, ranging from 0.03 to 200 µg/L.
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Method: Systematic Literature Review

A systematic literature search based on the PRISMA (Preferred Reporting Items for Systematic reviews and Meta-Analyses)⁷¹ workflow was performed to gather the literature presented in this review. Two searches were conducted; the first to gain an understanding of the pesticide occurrence in Brazilian drinking water, and the second to find advanced or novel DWT methods proposed and investigated in the last decade. The process is described in detail in the supporting information.

Results and Discussion

Pesticide occurrence in treated drinking water in Brazil

The literature search identified seven recently published studies (I – VII) that examined treated drinking water for residues of pesticides and other EC, predominantly in the regions of Southern Brazil. Three more studies (VIII – X) sampled various drinking water sources, in the course of analytical method development. A brief description of the studies included in this review (I – X) is given below.

- I) Brucker et al. 2021⁷² tested drinking water samples taken in a rural area of Rio Grande do Sul State, for 57 pesticides while evaluating alternative bioassays. Water samples were taken in two different periods: before and after agricultural application of pesticides.
- II) In a 10-year survey starting in 2006, Montagner et al. 2019⁷³ examined the occurrence of 58 emerging contaminants including 17 pesticides in raw and treated sewage, surface, ground, and drinking water samples from Sao Paulo State.
- III) In a 4-year study from 2011 to 2014 conducted by Caldas et al. 2019⁶⁵ the occurrence of 51 pesticides and PPCPs in surface and drinking water in the area of Rio Grande do Sul State was evaluated.

- IV) Between 2014 and 2015 De Souza et al. 2019⁷⁴ sampled the intake and effluent water of a DWTP located in the City of Londrina, Parana State. 14 pesticides were analysed.
- V) Almeida et al. 2019⁶⁴ monitored nine different water sources in the city of Arapongas, Parana State, including six freshwater sites and one drinking water tap. 12 pesticides, whose presence was previously detected by an extended screening, were quantified in four different time points over a year-long monitoring campaign (2015).
- VI) In a nation-wide survey Machado et al. 2016⁶¹ analysed 100 drinking water and seven source water samples from 22 Brazilian state capitals for the presence of EC, including one pesticide: atrazine. Samples were taken during two sampling periods in 2011 and 2012.
- VII) Rosim Monteiro et al. 2014⁷⁵ assessed the DWT performance of the Piracicaba (Sao Paulo state) treatment plant, by evaluating the concentration of 5 herbicides in source, filtered and treated drinking water.
- VIII) Dovidauskas et al. 2020⁷⁶ focused on the development of a cost-effective and fast method for the quantification of glyphosate and AMPA, a metabolite of glyphosate. More than 1000 tap water samples from 989 municipalities in the state of Sao Paulo were tested.
- IX) Do Carmo et al. 2020⁷⁷ developed a method for the simultaneous analysis of 23 pesticides regulated by the Brazilian Ordinance No 2914/2011. Drinking water samples from Santa Catarina State were tested.
- X) During analytical method development for the simultaneous detection of multiple compounds, Souza Caldas et al. 2013⁷⁸ examined various drinking water samples from Rio Grande do Sul state.

Table 2: List of pesticides tested in drinking water samples of Brazil. Bold: pesticides regulated by the Brazilian Ordinance No 888/2021, LOD: Limit of detection, LOQ: Limit of quantification

Pesticide	Min conc. (ng/L)	Max conc. (ng/L)	Freq. (%)	LOD (ng/L)	LOQ (ng/L)	State	Ref.
α-Endosulfan	< LOD	-	0	6	20	Santa Catarina	⁷⁷
β-Endosulfan	< LOD	-	0	6	20	Santa Catarina	⁷⁷

Alachlor	< LOD	-	0	606 ^b	2000 ^b	Santa Catarina	77
Aldrin	< LOD	-	0	6	20	Santa Catarina	77
Ametrine	<	-	0			Sao Paulo	73
	<	-	0	24	74	Parana	74
	10	10	NA	3	10	Parana	64
	< LOQ	-	0	NA	8	Sao Paulo	75
Ampa (metabolite of glyphosate)	< LOD	-	0	8'000'0 00 ^b	25'000 0'000 ^b	Sao Paulo	76
Atrazine	< LOQ	-	0	6	20	Rio Grande do Sul	72
	1	687 ^b	73			Sao Paulo	73
	101 ^b	241 ^b	16	30	90	Parana	74
	5	37	40	NA	4	Rio Grande do Sul	65
	20	30	NA	3	10	Parana	64
	< LOD	24	75			Nation wide	61
	7240 ^b	7240 ^b	NA	NA	8	Sao Paulo	75
	92.3	92.3	10		4	Rio Grande do Sul	78
	< LOD	-	0	60	200 ^b	Santa Catarina	77
Azoxystrobin	1	2	10			Sao Paulo	73
	15	38	58	4	13	Parana	74
	100	192 ^b	6	NA	40	Rio Grande do Sul	65
	< LOD	< LOQ	NA	10	100	Parana	64
Bentazone	30	30	2	NA	8	Rio Grande do Sul	65
	< LOQ	-	0		8	Rio Grande do Sul	78
Bromacil	0.7	0.7	3			Sao Paulo	73
Carbendazim	2	15	9			Sao Paulo	73
	86	86	4	5	16	Parana	74

	< LOD	-	0	NA	8	Rio Grande do Sul	65
	< LOD	10	NA	3	10	Parana	64
	< LOQ	-	0		8	Rio Grande do Sul	78
Carbofuran	< LOD	-	0	6	20	Parana	74
	20	50	10	NA	8	Rio Grande do Sul	65
	8.9	23.8	30		8	Rio Grande do Sul	78
	< LOD	-	0	212 ^b	700 ^b	Santa Catarina	77
Carbofuran-3-hydroxide	< LOQ	-	0	6	20	Rio Grande do Sul	72
Chlordane	< LOD	-	0	6	20	Santa Catarina	77
Chlorpyrifos	< LOD	-	0	1	4	Parana	74
	< LOD	-	0	909 ^b	3000 ^b	Santa Catarina	77
Clomazone	162 ^b	229 ^b	100	6	20	Rio Grande do Sul	72
	0.6	158 ^b	37			Sao Paulo	73
	< LOD	-	0	33	99	Parana	74
	40	164 ^b	19	NA	40	Rio Grande do Sul	65
	40	123.9 ^b	40		40	Rio Grande do Sul	78
Cyproconazole	10	14	6	NA	8	Rio Grande do Sul	65
Dieldrin	< LOD	-	0	6	20	Santa Catarina	77
Difenoconazole	2	2	3			Sao Paulo	73
	< LOD	-	0	NA	8	Rio Grande do Sul	65
Diuron	7.1	8.2	4	8	24	Parana	74
	40	490 ^b	4	NA	40	Rio Grande do Sul	65
	160 ^b	170 ^b	NA	3	10	Parana	64

	95.8	95.8	10		40	Rio Grande do Sul	⁷⁸
Endrin	< LOD	-	0	6	20	Santa Catarina	⁷⁷
Endosulfan sulfate	< LOD	-	0	6	20	Santa Catarina	⁷⁷
Epoxiconazole	14	14	3			Sao Paulo	⁷³
	70	70	4	NA	40	Rio Grande do Sul	⁶⁵
	46.7	83	20		40	Rio Grande do Sul	⁷⁸
Fipronil	1	13	35			Sao Paulo	⁷³
	< LOD	-	0	NA	0.8	Rio Grande do Sul	⁶⁵
Fluquinconazole	6	18	6			Sao Paulo	⁷³
Glyphosate	< LOD	> LOQ	3	5'000'0 00 ^b	15'000' 000 ^b	Sao Paulo	⁷⁶
Hexazinone	< LOQ	-	0	7	23	Parana	⁷⁴
	< LOD	-	0	3	10	Parana	⁶⁴
Imazapic	12	15	10	NA	8	Rio Grande do Sul	⁶⁵
Imazethapyr	12	37	15	NA	8	Rio Grande do Sul	⁶⁵
	< LOD	< LOQ	NA	10	50	Parana	⁶⁴
Imidacloprid	3	63	61			Sao Paulo	⁷³
	26	47	8	6	18	Parana	⁷⁴
	< LOD	10	NA	3	10	Parana	⁶⁴
Imizaquin	10	10	NA	3	10	Parana	⁶⁴
Iprodion	< LOD	-	0	NA	40	Rio Grande do Sul	⁶⁵
Irgarol	4	4	2	NA	4	Rio Grande do Sul	⁶⁵
	7.2	7.2	10		4	Rio Grande do Sul	⁷⁸
Lindane	< LOD	-	0	6	20	Santa Catarina	⁷⁷

Malathion	5	70	23			Sao Paulo	73
	< LOD	-	0	25	76	Parana	74
Metconazole	< LOQ	-	0	6	20	Rio Grande do Sul	72
Metolachlor	< LOD	-	0	303 ^b	1000 ^b	Santa Catarina	77
Molinate	< LOD	-	0	181 ^b	600 ^b	Santa Catarina	77
Parathion methyl	< LOD	-	0	272 ^b	900 ^b	Santa Catarina	77
Pendimethalin	< LOD	-	0	606 ^b	2000 ^b	Santa Catarina	77
Permethrin	< LOD	-	0	606 ^b	2000 ^b	Santa Catarina	77
Picloran	< LOQ			NA	80	Sao Paulo	75
Picoxystrobin	< LOD	-	0			Sao Paulo	73
Pirimiphos-methyl	< LOQ	-	0		8	Rio Grande do Sul	78
Profenofos	< LOD	-	0	1818 ^b	6000 ^b	Santa Catarina	77
Propanil	10	10	2	NA	8	Rio Grande do Sul	65
Propiconazole	< LOD	-	0	NA	4	Rio Grande do Sul	65
	10	10	NA	3	10	Parana	64
Pyraclostrobin	< LOD	-	0			Sao Paulo	73
Pyrazosulfuron-ethyl	< LOD	-	0	NA	4	Rio Grande do Sul	65
Pyrimethanil	< LOQ	-	0	6	20	Rio Grande do Sul	72
Quinclorac	124 ^b	248 ^b	6	NA	80	Rio Grande do Sul	65
Simazine	4	40	32			Sao Paulo	73
	< LOQ	-	0	8	23	Parana	74
	< LOD	-	0	NA	4	Rio Grande do Sul	65
	1360 ^b	1360 ^b	NA	NA	80	Sao Paulo	75
Tebuconazole	85	85	12.5	6	20	Rio Grande do Sul	72
	2	42	23			Sao Paulo	73

	< LOQ	-	0	9	27	Parana	⁷⁴
	40	460 ^b	46	NA	40	Rio Grande do Sul	⁶⁵
	60	60	NA	3	10	Parana	⁶⁴
	53	76.7	20		40	Rio Grande do Sul	⁷⁸
	< LOD	-	0	5454 ^b	18000 ^b	Santa Catarina	⁷⁷
Tebuthiuron	< LOQ	-	0	6	19	Parana	⁷⁴
	< LOD	-	NA	3	10	Parana	⁶⁴
	< LOQ			NA	8	Sao Paulo	⁷⁵
Terbufos	< LOD	-	0	36	120 ^b	Santa Catarina	⁷⁷
Trifloxystrobin	< LOD	-	0			Sao Paulo	⁷³
	303 ^b	303 ^b	4	NA	8	Rio Grande do Sul	⁶⁵
Trifluralin	< LOD	-	0	606 ^b	2000 ^b	Santa Catarina	⁷⁷
4,4'-DDD (metabolite DDT)	< LOD	-	0	6	20	Santa Catarina	⁷⁷
4,4'-DDE (metabolite DDT)	< LOD	-	0	6	20	Santa Catarina	⁷⁷

^b= exceeding EU legislation (100 ng/L)

Table 2 provides a summary of pesticide concentrations in drinking water from the studies introduced above. It consists of 112 entries, composed of 10 studies that investigated a total of 56 pesticides and 3 metabolites in drinking water samples. None of the concentrations regulated in the Brazilian ordinance were exceeded. However, considering the more stringent EU limits atrazine, azoxystrobin, clomazone, diuron, quinclorac, simazine, tebuconazole and trifloxystrobin exceeded the permitted values in one or several samples. After excluding non-detected samples with a LOD higher than the EU limits, 99 entries remain. Of those, 14% exceed the EU limit of 0.1 µg/L for one or multiple samples, as highlighted in table 2. Irrespective of the concentrations identified, atrazine, carbendazim, diuron, fipronil, malathion, imidacloprid and simazine were highlighted as of particular concern, due to their toxicity and frequency of occurrence^{65,73}. Except imidacloprid all of these have been included or maintained in the Brazilian Ordinance 888/2021⁶⁶.

Data on the contamination of surface and drinking water by pesticides in Brazil was identified as sparse^{58,79}. As a comparison, the literature search on pesticide contamination of drinking water in Brazil returned 10 times less results than the same search for Europe, indicating unequal data availability.

In recent years, three published review papers have discussed pesticide contamination in freshwater⁵⁸ and the occurrence of emerging contaminants in various water matrixes including fresh, waste, and drinking water^{80,81} respectively.

Albuquerque et al. 2016⁵⁸ reviewed 17 papers published before December 2015; from a total of 43 investigated pesticides, 34 were detected at least once above the LOQ of the analytical method applied. They further concluded that data on the occurrence of pesticides in Brazilian freshwater is insufficient, as, at the time, only 11% of the nationwide approved pesticides have been investigated, and studies were limited to only 5 of the 27 Brazilian states. Starling et al. 2018⁸⁰ and Marson et al. 2022⁸¹ pointed out, that published studies on water contaminants largely focused on Brazilian states in the south and south-east of the country, such as São Paulo, Rio de Janeiro and Rio Grande do Sul. Marson et al. 2022⁸¹ reviewed 74 studies published between 2011 and 2021, analysing emerging contaminants and persistent organic pollutants (POPs) in various Brazilian water matrixes, including drinking water. A total of 157 contaminants were detected in samples from various water sources, of which 58 were pesticides. A spatial analysis showed that pesticide contamination was often reported in the states of Mato Grosso, Rio Grande do Sul and Parana, corresponding with intense agricultural activities in these regions. Atrazine was one of the most frequently identified pesticides, detected in 18.4% of the studies⁸¹.

The National Drinking Water Quality Surveillance Program (Vigiagua) is a monitoring program coordinated by the Brazilian Ministry of Health (MH). The water quality norm (ordinance 888/2021, former 2914/2011) introduced by the MH requires the monitoring of specified contaminants including 54 pesticides or their by-products in drinking water⁶⁶. Municipalities are responsible for collecting the data and storing it in the Sisagua (Information System of Water Quality Monitoring) database⁷⁹.

Information on pesticide monitoring available on the Sisagua database has been analysed in two meta studies by Barbosa et al. 2015⁷⁹ and Panis et al. 2022⁵⁵. Barbosa et

al. 2015⁷⁹ analysed drinking water quality data available on Sisagua for the years 2007 to 2010. This study showed that the 27 pesticides subjected to the monitoring program accounted for less than 10% of the active ingredients authorised for use in Brazil. The number of pesticides monitored has since been increased to 54 under ordinance 888/2021⁶⁶. Barbosa et al. 2015⁷⁹ further observed an incompleteness of the data set; of the expected 980,000 entries, only 169,000 existed. This represents non-compliance with the minimum analysis of pesticides required by the authorities. Most data entries were provided by municipalities in the centre-west, southeast and south of Brazil. Very little information was given by municipalities in the north and north-east. Of the existing entries, Barbosa et al. 2015⁷⁹ classified 10 to 30% as not valid, mostly due to incomplete information or inconsistencies in the data set. Pesticide concentrations above the drinking water standards were observed for 0.1 to 0.4% of the recorded results. Barbosa et al. 2015⁷⁹ concluded that a final assessment of public exposure to pesticides in drinking water would require a more complete and comprehensive data set.

Panis et al. 2022⁵⁵ evaluated the extent of pesticide contamination of drinking water in the state of Parana based on the 2018 Sisagua report, which included drinking water quality data from 2014 to 2017. 127 out of 399 communities showed contamination with at least one of 11 selected pesticides that are proven, probable or possible carcinogens. Further analysis of those 127 municipalities showed extensive contamination of drinking water with pesticides currently approved for agricultural use but also some that were banned decades ago. Pesticide limits for single compounds as set by the EU were exceeded in most municipalities and the limit for the sum of pesticides was exceeded in all municipalities⁵⁵. Based on the monitored pesticide contamination and the benchmark cancer risk, they estimated expected cancer cases. For glyphosate and diuron, a strong positive correlation between the estimated cancer risk and the reported breast cancer cases in the corresponding period was found⁵⁵.

Panis, Zanetti et al. 2022⁵⁶ stress further, that pesticides in Brazilian drinking water are a case for concern, as 13% of all water samples analysed between 2018 and 2021 showed contamination, of which 25% were taken directly from household taps, as published in the Sisagua report of 2022. Further, pesticide concentrations in drinking water of 50 Brazilian municipalities exceeded maximum limits set by the Brazilian MH. Panis, Zanetti

et al. 2022⁵⁶ point out the lack of straightforward and low-cost methods for the removal of pesticides from food and water and emphasise the need for preventive measures to reduce intensive spraying of pesticides⁵⁶.

In summary, scientific studies published in the past decade and the nationwide monitoring program Sisagua, showed the widespread occurrence of pesticides, exerting intensive pressure on the quality and safety of Brazilian drinking water. If more stringent drinking water regulations (i.e., EU guidelines) were to be applied, Brazilian water utilities would have to face the burden to retrofit their current DWTPs with advanced treatment, resulting in increased energy and financial bills. Therefore, the development of sustainable and effective treatment technologies for pesticides removal is a timely and pressing challenge. The effectiveness of current traditional water treatment technologies in removing pesticides and the recent developments of new technologies is analysed and discussed in the further sections of this review.

Drinking Water Treatment

State of the art

This section provides a summary of conventional drinking water treatment and their limitations. A more extended overview of common drinking water treatment technologies, including micropollutant removal performance is given by Benner et al. 2013⁵². A list of technical terms and their descriptions can be found in the supporting information.

In conventional DWTPs a range of different treatment steps are carried out to provide safe drinking water to the end consumer. Treatment potentially consists of sedimentation, chemical coagulation and flocculation, rapid and slow sand filtration, activated carbon, aeration, ozonation or advanced oxidation²⁰. Water clarification (coagulation, flocculation, settling and sand filtration) and disinfection (chlorination) are the main steps of conventional treatment in low- and middle-income countries. Conventional drinking water treatment steps make use of physio-chemical processes to reduce turbidity, eliminate pathogens and control taste and odour issues. These treatment steps provide subsidiary benefits in reducing the load of micropollutants from the source water, but removal is often erratic and incomplete^{15,52,54}.

Physical-chemical treatment

Coagulation-Flocculation

Coagulation-flocculation is applied as an initial stage of the treatment process and removes organic matter, soluble metals, and suspended materials. The addition of a chemical coagulant to the raw water helps forming larger flocs, which can be removed in settlement tanks²⁰. Pesticide removal through coagulation-flocculation is insufficient, due to a lack of adsorption of many pesticides to colloidal or particulate material. The adsorption capacity and therefore removal efficiency of pesticides depends on their physio-chemical properties; more hydrophilic compounds show lower adsorption rates. Organic micropollutants not attached to agglomerated particles are unlikely to be removed during the coagulation-flocculation process^{2,20,54}.

Filtration (adsorptive media / GAC)

Water filtration using sand is a conventional treatment process where fine particles and soluble organic matter are removed from the raw water while passing through a porous bed of filter medium²⁰. Granular activated carbon (GAC) filter may be installed as advanced, tertiary treatment process, downstream of rapid gravity filters^{20,52}. While most micropollutants are effectively removed from water by GAC filters, the adsorption capacity of GAC is limited for some highly polar pharmaceuticals and pesticides. To prevent breakthroughs of those substances, frequent GAC reactivation may be needed, which enhances operation and maintenance costs of water treatment^{20,52}. As an example, breakthrough of most pesticides and disinfection by-products (DBPs) may occur within 6 to 12 months of operation (empty bed contact time (EBCT) of 10 to 30 minutes)²⁰. GAC filtration is considered a very promising technology for micropollutant removal, but its application is limited by high costs, which is of particular concern in low- and middle-income countries^{52,82}. Further, the adsorption process does not destroy the contaminants; the pollution still exists and has solely been transferred onto another medium⁸³.

Oxidation (Chlorination, Ozonation and AOPs)

Oxidants conventionally implemented in drinking water treatment such as chlorine and ozone are capable of successfully breaking down certain organic pesticides, but may

lead to the formation of by-products and, in the case of ozone, requires on-site generation²⁰. Advanced Oxidation Processes (AOPs) use highly reactive free radicals, holding unpaired valence electrons to oxidize a broad spectrum of micropollutants. In water treatment, hydroxyl free radicals ($\cdot\text{OH}$) are often the radicals of choice. Compared to other oxidants (ozone 2.07 V, chlorine 1.36 V), free hydroxyl radicals generate a more powerful oxidation potential (2.80 V). Usually, persistent organic substances, including those resistant to ozone (like some pesticides), can be oxidized by AOPs. AOPs increased the removal rate of atrazine from 15 - 40% (via ozonation) to 70 – 85%²⁰. AOPs require pre-treatment for highest efficiency and post-treatment to remove readily biodegradable compounds, oxidation by-products and potential excess oxidants (e.g. hydrogen peroxide) or activators/catalysts (e.g. TiO_2 , iron species) from the treated water²⁰. The production of potentially toxic and persistent (by-)products is of great concern in oxidative treatment. Additionally, oxidation and AOPs are cost-intensive due to the continuous application of chemicals and high energy demands^{52,84}.

Biological treatment

Sand and GAC filter were found to naturally harbour highly diverse and complex microbial communities and favour the establishment of biofilms^{16–18}. The filter no longer serves as adsorptive medium alone but takes over an additional role supporting a biomass substrate composed of microbes that feed on the readily available carbon present in the system²⁰. Biofilters are referred to as biologically active filters (BAF) or biologically active carbon filter (BAC). Abundance of microorganisms in drinking water treatment systems was detected to be as high as 10^5 cells/mL⁸⁵. Lab and pilot-scale studies have shown that bacterial communities associated with sand filters are potentially able to degrade pesticides and other micropollutants. Although biofilters are not specifically designed to remove pesticides, similar to coagulation-flocculation, an ancillary effect of enhanced pesticide removal has been observed in these systems. However, autochthonous bacterial communities of biologically active filters are often not able to degrade micropollutants sufficiently^{19,86}. This may be because the dominant species do not have the required metabolic properties⁵². Because of incomplete biodegradation, traces of the contaminants or their degradation products can still be detected in treated water^{19,86,87}.

Advanced and novel treatment methods

As highlighted in previous sections, pesticides were found in Brazilian drinking water in concentrations exceeding the limits imposed by EU legislation. The removal of pesticides in conventional drinking water treatment plants is often erratic and incomplete, therefore advanced treatment is required. However, these advanced treatment methods come with limitations and shortcomings of their own. Figure 1 presents a schematic overview of conventional vs. advanced drinking water treatment methods and their benefits and shortcomings are highlighted. Research of the past decade addressing the potentials and challenges of novel and improved DWT technologies for pesticide removal has been analysed and findings are presented in this section. Key drivers for research were identified and used to organise the literature. Each study has been focussing on one or more core aspects of improving DWT technology, as discussed below. The aim of this section is to highlight current research interests and different approaches to improve DWT, not to provide an exhaustive list of studies.

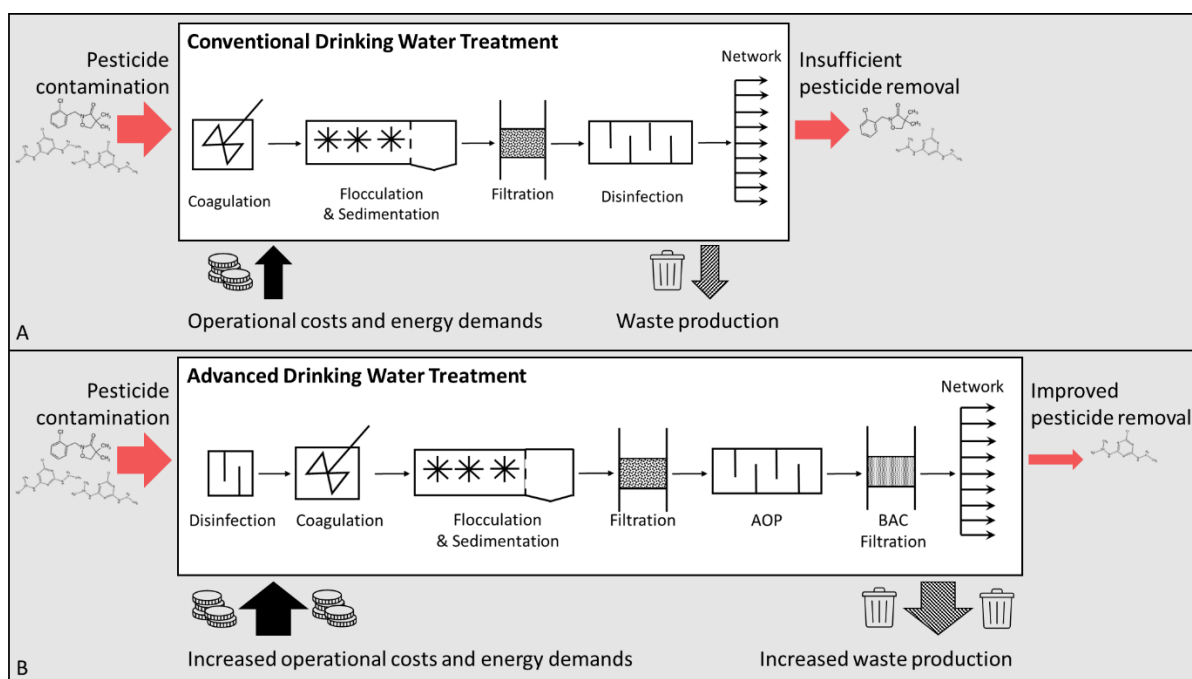


Figure 1 Schematic representation of conventional (A) and advanced (B) drinking water treatment systems.

Physical-chemical treatment

Adsorptive Media / GAC

The three main subjects of recently published research on adsorptive media in general and GAC specifically were identified as:

1. Identification of cheap, sustainable, and locally sourced alternatives to commercial GAC

GAC is the most used and effective adsorbent material for pesticides removal; however, it can be costly. Adsorbent materials derived from agricultural by-products are promising alternatives to conventional GAC; agricultural waste is abundant, cheap, and locally available⁸⁸⁻⁹⁰. Two approaches were suggested: either using agricultural residues directly for biosorption⁹⁰ or as source material for deriving GAC, e.g., from pecan-⁸⁹, bamboo-, or coconut-shells⁸⁸. Adsorption capacity varied and results between insufficient (biosorption)⁹⁰, and comparable to conventional GAC (bamboo and pecan shell-derived GAC)^{88,89} were obtained.

2. Improvement of adsorption performance and filter run time

Greater adsorption capacity of the filter medium ensures better removal performance, longer run time and ultimately reduced maintenance costs^{91,92}. The replacement of GAC by better suited adsorbent material for example, has been discussed in several studies. Powdered activated carbon (PAC) showed improved adsorption capacity for metaldehyde⁹², on the contrary, Bayroxide E33, an iron based granular adsorption medium, commercially available, and originally designed for the removal of arsenic, has proven to be less suitable for pesticide removal⁹³. Lopez et al. (2021)⁹⁴ further found that dosing PAC prior to flocculation and dissolved air flotation (DAF) was most effective for metaldehyde removal from reservoir water, compared to dosing PAC simultaneously or after flocculation⁹⁴.

Improvement of the adsorption capacity has also been studied through combination of adsorption with biosorption. Biofilm formation on the adsorbent surface may increase the surface area for adsorption of the contaminant. The combined adsorption effects potentially reduce reactivation frequency of the GAC, and an improved removal rate for metaldehyde was observed in a batch experimental set-up⁹¹.

3. Combination of adsorption with other treatment technologies

Optimal combination of adsorption steps (GAC, PAC, Biosorption) with existing treatment technologies is of interest to improve the overall treatment process and removal efficiency. The combination of AOPs and GAC for example showed promising results; in an adsorbent-catalyst system, the removal of Metaldehyde from

water by PAC was improved when combined with a UV activated, C-doped TiO₂ nano catalyst⁹⁵. In another study, GAC particles were added to a 3D electrochemical reactor system, combining adsorption and electrochemical oxidation. The effects observed for pesticide removal ranged from additive to synergistic; 28 - 38% in a batch set-up and close to 0 - 70% in a flow cell⁹⁶.

In a pilot-plant set-up, the optimal combination and order of conventional and advanced treatment steps was studied. More than 90% removal of indicator compounds (15 PPCPs including 2 pesticides) was achieved by the three best performing treatment trains; the treatment train identified as most effective was: dissolved air floatation, intermediate ozonation, dual media filtration, and virgin GAC. The effectiveness of treatment trains was proven in this study⁹⁷. It was observed that preliminary removal of natural organic matter (NOM) improved the subsequent treatment of PPCPs and that intermediate ozonation (following NOM removal) was more effective than pre-ozonation⁹⁷.

One of the disadvantages of GAC lies in the limited filter run time, which either requires replacement or reactivation of the GAC upon saturation. GAC reactivation is time-consuming as it is performed off site, which causes high CO₂ emissions and operational costs. A recently published review discusses chemical reactivation of GAC on site as a cost-effective and more sustainable alternative to thermal reactivation⁹⁸.

Oxidation and AOP

Although oxidative water treatment is successful for some micropollutants and pesticides, many are recalcitrant to complete mineralisation. AOPs have gained increasing attention in the last decades, as their amplified oxidation potential results in improved mineralisation rates⁹⁹. Despite these treatment successes, operation, and maintenance of AOPs are cost- and energy-intensive⁵², which poses a major hurdle to overcome in order to apply this technology in low- and middle-income countries, or for them to be a sustainable approach. There is an interest in finding more energy- and cost-efficient alternatives, which is reflected in the results of the literature search. Further, oxidation and AOPs produce potentially hazardous degradation products of concern⁵².

Three main research interests for AOPs were identified in the literature. One or more of these key objectives were addressed in each study:

1. Reduction of toxic degradation or disinfection by-products

One of the shortcomings of using chemical oxidants in water treatments is the potential production of toxic degradation products of contaminants and DBPs⁵². In drinking water, DBPs are formed when disinfection agents react with compounds present in the raw water, which act as DBP precursors. A variety of compounds can serve as DBP precursors, including NOM and anthropogenic contaminants, such as pesticides. DBP formation in drinking water treatment is a cause for concern, as DBPs pose a threat to human health and are classified as emerging contaminants^{37,38}.

Removing DBP precursors prior to disinfection in DWT was suggested as a means to control DBP formation. Lie et al. 2021³⁸ explored the combination of anion exchange resins and chlorination for the removal of organic micropollutants, including 8 pesticides, and NOM from drinking water. Four different resins and their performances were compared. While M80 resin was most effective in the removal of organic micropollutants, its efficiency was compromised in the presence of humic acid, highlighting the challenges in simultaneously addressing organic micropollutants and NOM³⁸.

Pre-chlorination in DWT to control odour and biological risks potentially leads to the oxidation of micropollutants and the formation of oxons; toxic transformation products, as observed for organophosphorus pesticides¹⁰⁰. Alternatives to conventional chlorination showed less reactivity and a lower potential to produce DBPs. For this reason, monochloramine gained popularity as a disinfectant. However, under monochloramine treatment, the herbicide methiocarb showed a pH dependent potential to form degradation products exhibiting an even higher toxicity than the parent compound¹⁰¹.

DBP formation through LED-UV/chlorine treatment of 3 pesticides showed pH dependency too. Consequently, to remove pesticides efficiently but simultaneously keep the formation of DBPs to a minimum a compromise in pH and chlorine dosage is needed¹⁰².

Other studies have explored the possibility to combine oxidation with additional treatment as a remedy to reduce DBP concentrations (i.e., GAC, PAC and nanofiltration). Results varied greatly, with nanofiltration combined with UV photolysis showing high efficiency of both removal of micropollutants and reduction of DBPs^{100,102,103}.

A recently published, systematic literature review provides an in-depth analysis of challenges related to DBP formation in drinking water treatment and an assessment of DBP control processes in advanced drinking water treatment³⁷.

2. Increase of treatment performance

Due to the high costs associated with this technology, increasing its performance has been one of the main drivers of research in the past years.

The coagulation and flocculation processes in DWT generate substantial volumes of water treatment residuals (WTR), which, while non-toxic, are laborious in terms of treatment and disposal. Reuse of WTR might therefore be a promising alternative to treatment¹⁰⁴. Iron-carbon (Fe-C) composites were formed from iron-rich WTR calcinated with PAC. The Fe-C composites were applied in a peroxymonosulfate activation system (Fe-C/PMS system) and effects on atrazine degradation were investigated. The best catalytic performance for atrazine treatment was achieved by the Fe-15%C/PMS system¹⁰⁴.

The combination of UV with a TiO₂ catalyst has shown promising results for effective pesticide treatment, outcompeting other oxidation technologies^{105–107}. Degradation rates of mixed pesticides differed greatly from individual ones¹⁰⁷. But the costs related to energy and UV source maintenance/replacement are hurdles to overcome to apply this technology in low- and middle-income countries.

Nanotechnology to improve AOP performance has gained increasing popularity¹⁰⁸ and nanoparticles may be applied as (parts of) catalysts in DWT, to treat otherwise recalcitrant micropollutants^{99,109}. A range of nanoparticles (structured catalysts, iron nanoparticles (nZVI), and nanoparticles combined with ozonation (O₃/nZVI)) have been proven successful in treating pesticides and either led to complete mineralisation or helped to enhance the mineralisation rate of transformation products. This is crucial to produce safe drinking water since, incomplete mineralisation may form potentially hazardous by-products^{99,110}.

A novel photocatalyst made from FeYO₃ nanoparticles, incorporated with reduced graphene oxide (rGO) ranging from 5 to 20 wt.%, was evaluated. The optimal composition was identified as FeYO₃@rGO-15 wt.%, which achieved enhanced photodecomposition of atrazine under visible light, compared to pure FeYO₃ nanoparticles¹¹¹.

Although nanomaterials showed promising results in DWT, there are still many unknowns in terms of potential hazards when released into the environment and potential risks to human health^{108,109}. To limit the dispersal of nano zinc oxides (ZnO), Doria et al. 2013¹⁰⁹ suggested the application of ZnO incorporated into clay pellets (ZnO/laponite composites), which was found to be a suitable nanotechnology for the treatment of metaldehyde¹⁰⁹. Similarly, the leaching of dissolved cobalt into the environment is of concern. Co₃O₄ was described as alternative catalyst to Co²⁺, showing only little cobalt leaching, reducing environmental risks, while still degrading atrazine¹¹².

Embedding zero valent zinc nanoparticles (ZnNP) into cellulose acetate membranes not only reduces the risk of nanoparticle release to the environment, but also improved the removal efficiency of the herbicides acetochlor and metolachlor from water compared to ZnNP alone¹¹³. Shad et al. 2021¹¹³ further describes cost-effective and eco-friendly ways of producing ZnNP and cellulose acetate membranes from renewable materials.

3. Reduction of costs and increased energy-efficiency

Despite shortcomings due to DBP formation, AOPs have been proven effective in reducing pesticide loads in water treatment. However, AOPs suffer from high energy and economical costs²⁰. Cost-saving alternatives have been proposed by recent studies; MacKul'Ak et al. 2011¹¹⁴ for example proposed the use of Fenton and modified Fenton reactions, which showed the ability to degrade and potentially mineralize atrazine. From a range of UV based AOPs, UV/Fenton achieved the highest degradation and energy-efficiency, leading to complete clopyralid degradation under optimal conditions¹¹⁵. However, sludge production requiring subsequent disposal is a major disadvantage of Fenton-based treatment technologies¹¹⁶.

Recently, TiO₂ has been widely used as photocatalyst for the treatment of many EC¹¹⁷. TiO₂ based nanocomposite powders showed promising results for the degradation of

4-chlorophenol (4-CP), a pesticide of great concern¹¹⁸. Disadvantages of photocatalyst powders include the use of expensive materials and the difficulty of removing them from treated water. Valadez-Renteria et al. 2021¹¹⁸ developed flexible photocatalytic composites from recycled plastic bags and polystyrene, combined with CuS/TiO₂ nanoparticles, achieving maximum 4-CP removal rates of 89 and 100%. The composites provide a low-cost alternative and are easily removed from treated water¹¹⁸.

For locations with many hours of sun, the use of solar radiation for the treatment of drinking water harbours great potential as low-cost treatment method^{119–121}; particularly suitable for small-scale application¹²⁰, in low- and middle-income countries¹²¹ or rural regions¹²². Senthilnathan et al. 2012¹²² emphasise the need for economical yet reliable photocatalysts that perform efficiently under solar radiation. Comparisons of photocatalysts showed most promising performance and suitability for TiO₂¹¹⁹ and N-doped TiO₂¹²² respectively, when tested under solar radiation. A recently published review article discusses the latest advances in the development of visible light responsive photocatalysts and their degradation performance of emerging contaminants¹¹⁷.

Solar-disinfection combined with oxidative processes, harbours the potential to eliminate both, pathogens, and organic contaminants from drinking water^{120,121}. Rodriguez-Chueca et al. 2019¹²¹ achieved increased solar disinfection and micropollutant removal during solar treatment spiked with sulphate radical precursors, and Cheng et al. 2019¹²⁰ observed enhanced degradation of personal care products in real water samples, when generating radicals from free available chlorine (FAC) through solar irradiation.

The research described above is focussed on the reduction of treatment costs of AOPs. However, apart from Wu et al. 2015¹¹⁵ and Rodriguez-Chueca et al. 2019¹²¹, the studies do not give any insight into the costs of the existing AOPs, nor do they provide any statement on the cost reduction achieved by the proposed improved or alternative treatment.

Membrane filtration

An increasing demand for drinking water and legislative pressure on its quality have fuelled research efforts to further develop treatment methods in the past decades, including pressure-driven membrane processes. Membrane treatment became a mainstream technology for removing a variety of contaminants. Commercially established membrane treatments are - reverse osmosis (RO), nanofiltration (NF), ultrafiltration (UF), and microfiltration (MF); which differ in pore sizes. Based on physical properties of most pesticides, NF and RO were suggested for pesticide removal during drinking water treatment¹²³.

Conventional NF or RO treatment plants consist of a pre-treatment stage followed by the membrane filtration process, and a post-treatment. Pre-treatment, although expensive, is of crucial importance since it helps to prevent one of the main shortcomings of membrane treatment: membrane fouling. Membrane fouling leads to decreased process efficiency, shortens the membrane lifetime and requires chemical cleaning, all of which further increase operational costs¹²³.

Although membrane filtration is a promising process, rejection of several pesticides was observed to be poor and overall NF and RO removal performance of micropollutants can vary greatly^{124,125}.

Four main drivers for research on membrane filtration in the past decade were identified:

1. Optimisation of filter performance

Several studies have focussed on understanding how filter membrane performance is influenced by membrane and environmental factors, to improve treatment success of membrane filtration. Membrane and influent water characteristics were identified as key players in membrane filtration, with NOM being one of the main parameters affecting the membrane performance^{125,126}.

The interactions of influent water NOM with pesticides and the filter membrane were identified as influencing pesticide (endosulfan) treatment success¹²⁵. The formation of pesticide-NOM complexes increased the pesticide retention, whereas membrane-NOM interactions enhanced membrane molecular weight cut-off, which led to a lower retention of the studied pesticide. NOM interactions were shown to be pH

dependent, with facilitated pesticide-NOM complex formation under low pH conditions, which increased the pesticide retention¹²⁵. Rejection of target micropollutants during nanofiltration was not affected by different ionic compositions of natural water sources, however size exclusion and hydrophobic interactions were important drivers for micropollutant rejection. Further, rejection performance was not influenced by pre-adsorption of target compounds on the membrane¹²⁷.

It's further important to note that the performance of NF membranes for charged pesticides depended on the pollutant concentrations, and should therefore be evaluated under environmentally relevant concentrations¹²⁶.

2. Optimisation of the overall treatment process

Combining membrane filtration with other treatment methods (e.g., UV photolysis or AOPs) harbours the potential to optimize the overall performance of the treatment process¹²⁸.

NF in combination with low pressure UV radiation performed best, when installed after conventional sand filtration, compared to two other locations within a DWT pilot-plant. Sand filtration reduced turbidity and NOM content of the influent water which prevented the membrane from declined fluxes and fouling¹²⁸. Vice versa, upstream application of membrane filtration enhanced chemical oxidation (ozonation) of atrazine by improving the feed water quality through organic matter retention. Performances of UF, NF and RO in combination with ozonation was shown to depend on pH and type of NOM¹²⁹.

The hydrogen-based membrane biofilm reactor (H_2 -MBfR), designed to simultaneously remove nitrate and pesticides from raw water sources, but initially only tested in wastewater, later proved to efficiently remove nitrate and endosulfan from drinking water¹³⁰.

3. Novel membrane materials

The development of new membrane materials is of interest, as adaptations in filter operation can only achieve limited success; certain disadvantages remain. An ideal membrane material should provide sufficient removal of micropollutants from water streams while avoiding very low permeability¹³¹. The development of an asymmetric polyelectrolyte multilayer (PEM) on a porous membrane for example, yielded

promising results. An improved permeability of 5- to 10-fold, compared to convenient filter materials was achieved, while maintaining a very high (98%) retention of a mix of common micropollutants, which contained the pesticide atrazine¹³¹.

4. Cost- and energy-efficiency

Keeping energy costs down and making (membrane filtration) technology more sustainable is of importance for future development^{132,133}. Membrane filtration was suggested to be coupled to renewable energy sources, which is especially promising for drinking water treatment in rural or off-grid areas¹³². This is particularly relevant in the case of pesticide contamination, as areas with intense agricultural activities are likely to be more affected by this phenomenon⁷².

Biological treatment

Advanced biological micropollutant removal as alternative to existing drinking water treatment methods was proposed and critically assessed in a review by Benner et al. 2013⁵²; the authors focussed on bioaugmentation of biofilters. Bioaugmentation, a sub-category of bioremediation, describes a process in which microbial degradation of contaminants is improved by adding specialized single strain or entire consortia to the system⁵². An alternative approach is biostimulation, which aims at enhancing growth and activity of indigenous bacterial communities by manipulating the physicochemical environment²⁵.

The following section provides an overview of studies on advanced biological treatment, published in the last decade. The key driver for research identified is to improve the pesticide degradation performance of biofilter - different approaches were explored:

1. Biostimulation

Developing an understanding of microbial community functions and micropollutant removal processes in biofiltration is of great interest¹³⁴. Environmental and operational conditions might influence the overall treatment performance and could be controlled as means of bioremediation¹³⁵.

1.1. Environmental conditions – feed water

Water treatment success is potentially influenced by contaminant concentration in the feed water and previous contaminant exposure of the biofilter. Metaldehyde removal rates were shown to be improved; 1. with increased influent water

metaldehyde concentrations and, 2. when the biofilter was previously exposed to high Metaldehyde concentrations¹³⁶. Biofilter acclimation was proposed to significantly decrease the time needed for successful metaldehyde removal¹³⁶.

In a follow-up study, Rolph et al. 2020²⁹ ran lab- and pilot-scale sand bioreactors acclimated for metaldehyde degradation by selective enrichment. They successfully reduced environmentally relevant metaldehyde concentrations from drinking water sources to below the regulatory threshold in a continuous flow bioreactor. Duration of enhanced metaldehyde removal was limited and correlated to the concentration of metaldehyde applied during enrichment²⁹.

Further, the concentration of co-substrates can be crucial for the pesticide removal performance. Bentazone, which is challenging to treat, was observed to be removed in a biofilter fed with methane rich groundwater. Co-metabolic degradation of bentazone was observed in sand filter columns where its removal increased from 31% to 53% in the presence of methane. This knowledge provides a relatively cost-effective way to enhance microbial bentazone degradation by influencing the primary substrate concentration, in this case methane, within the system¹³⁵.

1.2. Filter operational parameters

Filter operational parameters have the potential to shape biofilter communities and therefore their ability to degrade micropollutants. Hydrodynamics, such as superficial velocity, might have a considerable influence on biofiltration performance¹³⁴. As observed in an experimental set-up, higher superficial velocity led to a decrease of superficial biomass but an increase in overall biomass and expanded depth of the biologically active zone. Biotransformation was observed for 11 out of 29 tested micropollutants, and 4 correlated negatively with superficial velocity¹³⁴. The authors pointed out that this indicates a higher influence of EBCT on the removal of those four micropollutants, in comparison to the effect of total biomass¹³⁴.

Besides the flow velocity, the operation mode may influence the biofilter community and, therefore, its performance too. In a pilot-study, the impact of the operation modes, cyclic (operated 8-12h/day) and continuous, were compared. Continuously operated biofilters developed higher biomass density, which was

further enhanced when the EBCT was reduced. However, the enzyme activity measured was higher under cyclic operation, indicating higher microbial activity. In line with this, cyclically operated filters showed a 50% greater reduction of anthropogenic N-nitrosodimethylamine (NDMA) precursors, compared to continuously operated filters¹³⁷. No study has been published so far assessing the impact of operational mode (i.e., cyclic vs continuous) on pesticide degradation in biofilter.

In a different study, operational parameters shaping biofilter communities and their ability to degrade contaminants, were identified as filter media type and pre-ozonation, whereas EBCT was found to have a minor impact¹³⁸. Biofilters based on GAC showed greater microbial activity and treatment efficiency compared to dual anthracite sand media. Pre-ozonation was shown to break down contaminants into smaller molecules which promote microbial growth serving as a growth substrate¹³⁸. The authors argued that water utilities could take advantage of this knowledge to adapt operational parameter and steer water treatment performance in the desired direction¹³⁸. Deepening the fundamental understanding of biofilter processes is essential to optimize operational processes in a systematic way, to improve the micropollutant removal during drinking water treatment production¹³⁴.

2. Bioaugmentation

Bioaugmentation of biofilter harbours great potential for the treatment of targeted contaminants and has already been tested in a pilot-plant set-up. However, the approach showed limitations as the added degrader did not remain in the system and was lost over time, especially during the backwash process¹³⁹. Several studies have tried to address this challenge, but with limited success. Alternative ways of inoculating degrader strains to the system, to prevent rapid loss of cells, were tested; 2,6-Dichlorobenzamide (BAM) degrader cells were incorporated into dried beads¹⁴⁰ or immobilized in an alginate matrix in porous stone¹⁴¹. Both approaches successfully prevented initial cell loss and achieved mineralisation of the contaminant, however, loss and starvation over time ultimately diminished the benefits of immobilized over suspended cells^{140,141}. Most recently Ellegaard-Jensen et al. 2020²⁴ addressed the challenge of BAM degrader cell loss over time in bioaugmented sand filter columns,

by implementing three solution strategies: 1. A novel inoculation process for improved adherence of BAM degrader cells to the filter medium, 2. Reduction of the flowrate, and 3. Increasing the inlet water nutrients by utilizing filter retentate water to prevent the starvation of BAM degraders. Through these modifications, Ellegaard-Jensen et al. 2020²⁴ achieved 100% BAM removal during the entire duration of the experiment, which was 40 days. For comparison, maximal BAM removal efficiency in bioaugmented sand filters of previous studies was 65-80%²⁴.

3. Exploring the potential of combined treatment technologies.

Low contaminant concentration in the influent water presents a challenge for bioaugmentation, which makes it difficult to sustain a degrader community within the biofilter system. Hylling et al. 2019¹⁴² addressed this challenge by proposing a novel hybrid technology combining biological treatment with RO. The concentrated retentate of a RO membrane was fed to bacteria capable of degrading the target contaminant, which ensured high contaminant concentrations and facilitated biodegradation. The results were promising as they showed an enhanced degradation potential in the retentate compared to untreated water¹⁴².

Conclusions

Access to clean and safe drinking water is essential for human health and is included in Goal Number 6 - Clean Water and Sanitation - of the United Nations Sustainable Development Goals. Water supply systems experience increasing pressure caused by population growth, climate change and various forms of pollution^{84,143}. Efficient waste and drinking water treatment are therefore essential. As pointed out earlier in this review, pesticides and other emerging organic contaminants are of great concern as they are not successfully removed or mineralized by conventional treatment methods⁵²⁻⁵⁴. Advanced technologies such as AOPs, GAC, and membrane filtration promise improved drinking water treatment, but their application is limited, especially in low- and middle-income countries, due to high costs^{82,84}. While it is widely known that advanced technologies are cost-intensive, limited data on cost reductions achieved through new and improved treatment technologies is available from the literature. This underscores the need for an in-depth analysis of operational costs associated with new and existing advanced

technologies, to enable evaluation of their suitability for application in low- and middle-income countries.

Conventional technologies such as coagulation-flocculation followed by settling-filtering and disinfection are the main treatment methods for drinking water in Brazil. Combined with intensive agriculture and the resulting pesticide concentrations in surface waters, inadequate treatment poses an increased health risk to the end consumer^{54,144}. As summarised in this review, the presence of pesticides in Brazilian drinking water is widespread, and the legal regulations based on the QCRA principle allow for relatively higher pesticide concentrations in international comparison^{55,56,145}. If more restrictive limits like those of the EU were to be applied, many drinking water suppliers would have to improve their water treatment plants, leading to a significant increase in drinking water production costs.

The *Outcome document of the United Nations Conference on Sustainable Development* emphasises that access to safely managed water resources is crucial for sustainable development of low- and middle-income countries¹³³. Sustainable development is a “*development that meets the needs of the present without compromising the ability of future generations to meet their own needs*”¹⁴⁶, as defined by the Brundtland Commission. Sustainable development is believed to be achieved through the adoption of appropriate technology¹⁴⁷. Appropriate technology was previously described as “*a collection of technologies which requires low investment and capital, highly adaptable and flexible to its environment and easily affordable by the end-users*”¹⁴⁸. Further, “*appropriate technology is always easy to maintain and most importantly environment friendly; its low cost, low usage of fossil fuels and use of locally available resources endear it as a veritable tool for sustainable development*”¹⁴⁷. The availability of energy-efficient and low-emission technologies will play a key role for sustainable development and battling the climate crisis. Furthermore, the safe management of chemicals is of critical importance for human health and the protection of the environment¹³³.

The development of low-cost but effective DWT methods is therefore essential and has been researched intensely in the past decade. Conventional drinking water treatment has not been designed towards the removal of pesticides; thus, pesticide removal is often insufficient. Although advanced physical-chemical treatment technologies often

achieve promising results in pesticide removal, they have some significant drawbacks. The high energy and chemical usage lead to enhanced costs and environmental impact^{52,84}. In line with these drawbacks, the main research interests identified in this review were 1: to maximise treatment performance, 2: to improve energy- and cost-efficiency, and 3: to minimise the formation of degradation by-products / DBPs for conventional and advanced DWT. Many of the studies successfully achieved an improvement of one of these challenges, however, to varying degrees and often at costs in another area. It was noticeable from this literature review, that although cost-effectiveness is an important driver for research, very little actual data on drinking water treatment costs was provided. Consequently, a cost comparison of different DWT methods was not possible and the potential cost reductions for the improved technologies proposed could not be captured.

Despite many advances made for conventional and advanced DWT methods, criteria for appropriate technology, as defined previously, were not met. Enhancements in one aspect of a treatment method often led to other issues remaining unresolved or even being exacerbated. To give an example, the use of solar power for photolysis and disinfection^{119–122} or combining membrane filtration with renewable energy sources¹³² are effective approaches to reduce energy costs and environmental impacts. However, these alternative solutions still present unresolved issues, such as the formation of hazardous by-products and the challenging management of waste streams.

Engineered biofiltration has been proposed as a sustainable and energy-efficient alternative to conventional and advanced drinking water treatment methods⁵². Its low operational costs, lack of additional chemicals, and the possibility of using locally sourced materials fulfil the criteria of an ideal appropriate technology. Engineered biofiltration is ideally suited for rural areas and low—and middle-income countries, where regulations are weaker, but the widespread application of pesticides and the resulting environmental pressures are greater. To fully exploit this potential, however, various challenges must be overcome. The main difficulty identified for bioaugmentation of biofilters, was a rapid loss of added degrader strains, and a subsequent decrease of the treatment efficiency¹³⁹. Despite the fact that degrader cell immobilisation achieved a reduction of initial cell loss, the positive effect was not sustained^{140,141}. Factors favouring

cell loss and aggravating the maintenance of degrader strains over time were identified as physical forces, such as backwashing, limited hydraulic retention times, and high flow rates in rapid sand filtration, but also low and fluctuating concentrations of contaminants and other carbon substrates, leading to competition for resources and starvation of degrader cells^{139–141 25,52,87,149}. An improved understanding of DWTP (operational) parameters and their influence on environmental conditions within biofilters, is crucial to address the challenges identified and to improve and sustain the performance of bioengineered filter units¹³⁴.

Ongoing research and two recently published reviews by Rempel et al. 2021¹⁵⁰ and Thuptimdang et al. 2020¹⁵¹ clearly show that there's strong interest in using biological treatment including bioaugmentation in the drinking water sector, despite existing challenges and knowledge gaps.

In this review, the presence of treatment-resistant pesticides in Brazilian drinking water was highlighted, and the intensive research on enhanced removal methods of these compounds over the past decade was described. However, this research is fragmented, with each study focusing on different pesticides and treatment methods. Consequently, comparing these studies or predicting treatment effectiveness for compounds with different physical-chemical properties is challenging. To address this gap in the future, research should focus on gathering and standardizing data on pesticide removal efficiency to enable the development of predictive models.

To conclude, the development of innovative and sustainable technologies for drinking water treatment is crucial to meet the United Nations Sustainable Development Goals. While biological treatment has been proposed as a sustainable and economically viable alternative to AOPs in drinking water treatment, additional research and supporting evidence to establish its cost-effectiveness and efficacy is required. As pointed out in this review, biological treatment could find its ideal application in low- and middle-income countries, where it holds the potential to serve as a cost-effective, sustainable, and appropriate technology.

Acknowledgements

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Chapter 3 – Supporting Information

Detailed description of the process of systematic literature search and explanation of technical terms.

Method: Systematic Literature review

A systematic literature search based on the PRISMA (Preferred Reporting Items for Systematic reviews and Meta-Analyses)⁷¹ workflow was conducted for gathering the literature presented in this review. Search language, timespan, database, and key search terms are described in sections 1.1. and 1.2.. Duplicates were excluded and results were reviewed and narrowed down by title, followed by abstracts and ultimately full text.

Pesticide occurrence in treated drinking water in Brazil

To gain an understanding of the pesticide occurrence in Brazilian drinking water, the literature search consisted of the key search terms: (drinking water OR potable water) AND (pesticide) AND (Brazil) NOT (parasite OR virus) NOT (antibiotic* OR antibac*). The database searched was Web of Science, the language was set to English and publications between 2010 and 2022 (September) were considered. The search returned 95 publications, of which 16 were included in this review. Studies focussing on method development without examining real water samples, those whose focus was on contaminants other than pesticides and studies solely focussing on ground and freshwater matrixes were excluded. Eleven studies, two meta studies (on Brazilian water quality monitoring data), and three review paper were incorporated in the review. As a comparison, the same search for the EU ((drinking water OR potable water) AND (pesticide) AND (“European Union” OR EU OR Austria OR Belgium OR Bulgaria OR Croatia OR Cyprus OR Czech Republic OR Denmark OR Estonia OR Finland OR France OR Germany OR Greece OR Hungary OR Ireland OR Italy OR Latvia OR Lithuania OR Luxembourg OR Malta OR Netherlands OR Poland OR Portugal OR Romania OR Slovakia

OR Slovenia OR Spain OR Sweden) NOT (parasite OR virus) NOT (antibiotic* OR antibac*)) returned 674 publications.

Advanced and novel Treatment methods

To find advanced or novel DWT methods proposed and investigated in the last decade, the following key search terms were used: (drinking water OR potable water) AND (pesticide*) AND (removal OR (novel* NEAR/5 treatment) OR (alternative NEAR/5 treatment) OR (new NEAR/5 treatment) OR (advanc* NEAR/5 treatment)) NOT (ecotox* OR toxicity) NOT (parasite OR virus) NOT (wastewater) NOT (antibiotic* OR antibac*). The database searched was Web of Science, the language was set to English and publications between 2010 and 2022 (December) were included in the search. A total of 706 publications were found, of which 66 were included in the review. The results were divided into the subcategories physical-chemical treatment (including adsorptive media / GAC (13), oxidation and advanced oxidation processes (AOPs) (19) and membrane filtration (10)) and biological treatment (13). The number in brackets indicates the number of studies included in the respective category. Excluded from the review were studies not focusing on pesticides and studies not discussing a novel or improved aspect of DWT. Key drivers for research were identified and used to organise the collected literature.

Technical Terms

Table 3 provides an overview and description of technical terms used in the review.

Anion exchange resins	“Anion exchange resins are made of an insoluble polymer network to which exchangeable anions are electrostatically bound which can be exchanged with the targeted pollutant anions in solution. ¹⁵² ”
Chemical coagulation and flocculation	Coagulation and flocculation are an essential part of the drinking water treatment process. During coagulation, the charges of the colloidal and suspended particles are neutralised with the help of a metal coagulant (typically aluminium or iron based), which allows aggregation of particles. During subsequent flocculation, these aggregated particles are brought together to form larger clumps, which can then be more easily settled or filtered out. ^{2,153}

Electrochemical oxidation	“Electrochemical oxidation is based on the application of an electric current or a potential difference between two electrodes (anode and cathode), with which hydroxyl radicals or other oxidizing species can be generated, depending on the anode material used and the type of electrolyte of support used. ¹⁵⁴ ”
Endocrine disrupting chemicals	“Endocrine disruptors are chemicals that mimic hormones or have antihormone activity interfering with the functioning of endocrine systems in various living species. ¹⁵⁵ ”
Fenton reaction	“The Fenton reaction is defined as the oxidation of organic contaminants by hydrogen peroxide and Fe (II). ¹⁵⁶ ”
Membrane biofilm reactor	“The membrane biofilm reactor (MBfR), an emerging technology for water and wastewater treatment, is based on pressurized membranes that supply a gaseous substrate to a biofilm formed on the membrane’s exterior. ¹⁵⁷ ”
Ozonation	“Ozonation is a well-known oxidation process generally applied to carry out oxidative degradation of organic contaminants. The solid oxidative properties of the hydroxyl radicals produced during the process mineralize the contaminants and enhance their degradation. ¹⁵⁸ ”
Photo(nano)catalyst	“Photocatalysis is a term that defines a process in which a substance is activated or stimulated under the effect of light. Photocatalyst is the substance that induce this stimulation, it alters the rate of reaction without being involved itself in the chemical reaction. When photocatalyst is in the nano size it is called nano photocatalyst. ¹⁵⁹ ”
Sedimentation	In drinking water treatment, sedimentation is used to remove suspended particles from the water phase by gravity. Sedimentation tanks can be installed before coagulation to reduce the amount of coagulation chemicals required, or after coagulation and flocculation to minimise the concentration of solid particles in the water and thus improving the performance

	of the subsequent filtration stages. Sedimentation is used as a clarification technique before filtration. ^{160,161}
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Chapter 4 – Optimisation of methods for the quantification of microbial communities in biofilters.

Chapter 4.1: Optimisation of DNA extractions from DWTP sand filter

Introduction

To perform molecular analysis of DWTP filter microbial communities, DNA must be extracted from sand and GAC particles. The commercially available FastDNA SPIN Kit for soil (MPbio) was chosen for DNA extractions as it was previously shown to be suitable for the extraction of DNA from DWTP filter samples³⁵. This kit uses bead beating to lyse the cells, but not every lab is equipped with a bead beating grinder and lysis system (FastPrep-24™, MPbio). The original protocol was modified to perform cell lysis by vortexing instead of bead beating, guaranteeing the comparability of DNA extracted by bead beating versus vortexing.

Materials and Method

DNA extraction - Volume: A range of sand volumes was tested to find the ideal volume for DNA extraction using the FastDNA SPIN Kit for soil (MPbio). Sand samples stem from a Scottish DWTP rapid gravity filter (RGF) and were aliquoted to the lysis matrix E tubes provided with the kit. Sand volumes tested in duplicates were 0.3 g, 0.4 g, 0.5 g and 0.6 g, ensuring a minimum of 250 µL head space in each tube prior to cell lysis. DNA extractions were performed following the manufacturer's instructions and eluted in 50 µl DES water provided with the kit. All work was conducted in a sterile biosafety cabinet.

DNA extraction – method and time: In a second step, cell lysis by bead beating was compared to cell lysis using a vortex. Aliquots of 0.6 g of sand were added to the lysis matrix E tubes in duplicates and either processed by bead beating following manufacturer's instructions (40s, 6.0 m/s), or vortexed on maximum speed for 8, 10, or 12 minutes. All DNA extractions were performed following manufacturer instructions thereafter.

Quantification: DNA extracts were quantified by Qubit 2.0™ using the Invitrogen™ Qubit™ dsDNA HS Assay Kit. DNA quantification was performed following

manufacturer's instructions, using 190 μL of working solution and 10 μL of sample. Each sample was quantified in triplicates.

Statistical analysis: Statistical analysis was performed using RStudio (2022.07.2 Build 576 and RStudio 2024.12.1 Build 563) for windows. Kruskal-Wallis test was used to analyse differences between treatments, with significance defined as $p < 0.05$.

Results and Discussion

DNA extraction - Volume: DNA extracts from sand volumes ranging from 0.3 g to 0.6 g showed that a higher volume of sand yielded a higher amount of DNA (Figure 2). The lowest amount of DNA ($10.31 \pm 0.49 \text{ ng}/\mu\text{L}$) was extracted from 0.3 g of sand, while the highest amount of DNA ($29.75 \pm 5.55 \text{ ng}/\mu\text{L}$) was extracted from 0.6 g of sand.

Differences in DNA yield extracted from different sand volumes were not significant (Kruskal-Wallis test, p -value: 0.1038). However, statistical power is limited due the small sample size. 0.6 g of sand sample yielded the most DNA, while being the maximal volume of sand that can be added to the lysis matrix E tube, while still ensuring a headspace of 250–500 μL as required by the manufacturer's instructions. 0.6 g of DWTP RGF sand sample was chosen as the optimal volume of sand for DNA extractions and used for all DNA extractions performed within the scope of this PhD project.

DNA extraction – method and time: After determining the optimal sand volume for DNA extractions, an alternative to cell lysis using a bead beating homogeniser (MPbio) was tested (Figure 3). Extracting DNA from 0.6 g of sand, following the original protocol yielded $22.6 \pm 0.8 \text{ ng}/\mu\text{L}$ DNA. In comparison, replacing bead beating by 8, 10, or 12 minutes of vortexing yielded 25.1 ± 0.7 , 16.67 ± 6.73 (1 outlier included) and $23.65 \pm 0.35 \text{ ng}/\mu\text{L}$ DNA. The amounts of DNA extracted by the different extraction methods and times did not differ significantly (Kruskal-Wallis test, p -value: 0.2039). Bead beating can therefore be replaced by 8 minutes of vortexing at maximal speed while still ensuring comparability of results.

Conclusion

The DNA extraction protocol using the FastDNA SPIN Kit for soil (MPbio) was optimised to yield the maximum amount of DNA from DWTP sand filters. DNA extractions for this PhD project were performed using 0.6 g of DWTP sand samples. Further, the optimised

protocol allowed to extract DNA from DWTP sand samples taken in Brazil, using a vortex, allowing for comparability between DNA extracted by vortexing and bead beating.

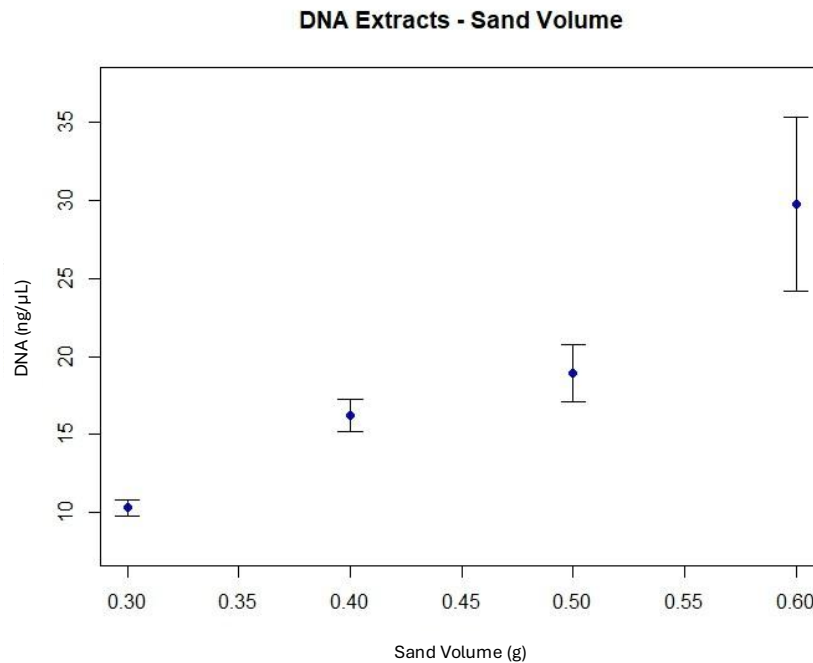


Figure 2 DNA (ng/μL) extracted from different volumes of sand (0.3 g, 0.4 g, 0.5 g, 0.6g) using the FastDNA SPIN Kit for soil (MPbio). Error bars represent standard deviations of biological duplicates.

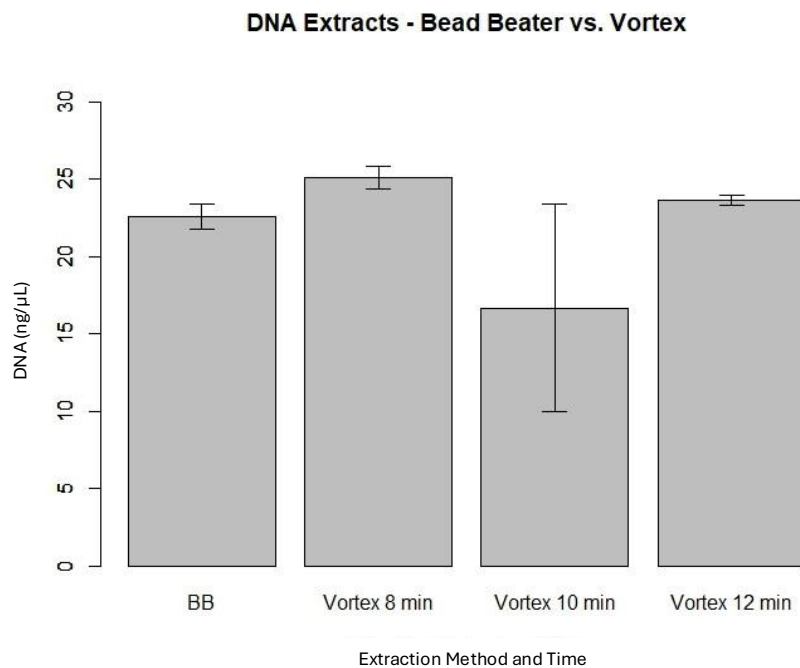


Figure 3 DNA (ng/μL) extracted from 0.6 g of sand by bead beating (BB) and vortexing for 8, 10, or 12 minutes, using the FastDNA SPIN Kit for soil (MPbio). Error bars represent standard deviations of biological duplicates.

Chapter 4.2: Method optimisation for the quantification and characterisation of microbial communities in sand filter from drinking water treatment plants

Introduction

Sand and GAC filtration are common treatment steps in conventional drinking water treatment. Fine particles and soluble organic materials are removed from the influent water that passes through the porous filter bed²⁰. Sand and GAC filters naturally harbour highly diverse and complex microbial communities and favour the establishment of biofilms^{17,18,162}. If not prevented by disinfection, this leads to the development of biofilters^{163,164}, also referred to as biologically active filters (BAF) or biologically active carbon filters (BAC) when GAC is used as porous material. Drinking water treatment plant (DWTP) microbial communities feed on organic and inorganic nutrients present in the system^{164,165} and have been shown to influence filter performance and drinking water quality^{39,42,47,163–165}. Filter operational practises fostering biological activity have been adapted to deliberately improve drinking water quality through biofiltration¹⁶⁶. Engineered biofiltration offers numerous potential benefits. For instance, lab and pilot-scale studies have demonstrated that bacterial communities in biofilters can degrade micropollutants that are otherwise persistent in DWTP systems^{19,52,86,167}.

The abundance of intact microbial cells in water of DWT systems is as high as 10^5 - 10^6 cells/mL⁸⁵. Reliable quantification of microbial cells in the DWT system allows for an improved understanding of the dynamics that drive biofilm formation, growth, and function¹⁶⁸. In recent years, flow cytometry (FCM) has gained popularity as a tool to quantify microbial cells quickly and reliably, offering an alternative to conventional enumeration methods such as heterotrophic plate counting (HPC)^{169–172}. FCM surpasses HPC by providing rapid detection of all cells, including those that are non-culturable, thus enabling a more accurate reflection of the total microbial load in drinking water^{170,173}. FCM offers distinct advantages, including rapid analysis times of less than 3 minutes per sample, high sensitivity, and reliable reproducibility of results. It further supports various staining and labelling techniques for comprehensive single-cell analysis^{171,172}. FCM has become a widely accepted and valuable tool for the detection, quantification, and characterisation of aquatic microbial communities. A

growing number of water service providers and regulatory bodies are investigating and considering the potential advantages of FCM for water quality assessment¹⁷⁴. Scottish Water, which has conducted extensive research in this area, was among the first in Europe to apply and develop FCM techniques for full-scale DWTPs¹⁷⁴. Recent advances in FCM include RT-FCM^{175,176}, online FCM^{177,178}, and community profiling through fingerprint analyses^{179–182}.

FCM is a high-throughput, single cell technology, that individually guides suspended cells through a laser beam inside the flow cell (Figure 4), enabling an optical description of each particle based on scatter and fluorescent information. The scatter signal depends on the cell size and morphology, while the fluorescence signal is caused by autofluorescence or fluorescent cell staining^{179,181,183,184}. The most common downstream analysis of microbial FCM data is the enumeration of cells, quantified as cells/mL¹⁷². FCM further allows for cultivation-independent viability assessments, through fluorescent staining. Numerous fluorescence stains for viability exist, targeting different cellular compartments. SYBR Green, in combination with Propidium iodine, a fluorescent stain used to assess cell membrane integrity, is widely used to quantify total and intact cell counts (TCC, ICC)¹⁷². A more detailed description of the fluorescence staining process and the FCM cell quantification mechanism is given in the introductory chapter of this thesis.

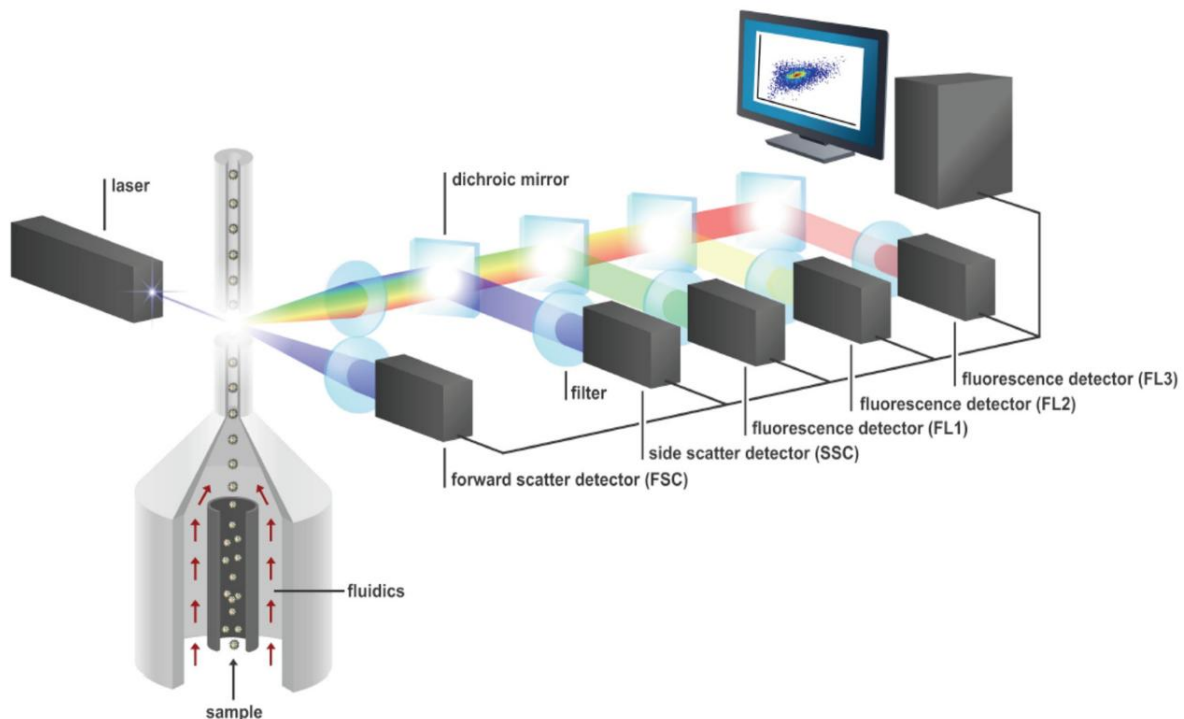


Figure 4 The scatter (forward scatter (FSC) and side scatter (SSC)) and fluorescence signals (FL 1-3) from each cell are collected by several detectors. Fluorescence is recorded across various wavelength ranges (three in this example). The electronic signals from these detectors are then converted into digital signals. This schematic was published by Rubbens & Props, 2021¹⁷⁹.

The single cell aspect of FCM provides information on cell size, density, and nucleic acid content, enabling a more in-depth downstream analysis of FCM data, beyond standard enumeration of total and intact cell counts¹⁷². A review by Rubbens and Props, 2021¹⁷⁹ provides an insight into existing data processing pipelines tailored towards FCM data analysis in microbial ecology.

The Phenoflow^{180,183} data processing pipeline “fits bivariate kernel density functions to phenotypic parameter combinations of an entire microbial community and concatenates them to a single one-dimensional phenotypic fingerprint”¹⁸⁰. From this fingerprint the phenotypic diversity index can be calculated, and microbial community dynamics can be quantified^{183,185}. The phenotypic diversity index derived from fingerprint analysis and the 16S rRNA taxonomic diversity of microbial communities from various environments have previously been shown to strongly correlate^{180,183}. FCM fingerprint analysis provides a fast and robust analysis for microbial community profiling and the phenotypic diversity index may serve as a proxy to capture shifts in microbial community diversity in engineered systems¹⁸³.

While the quantification and analysis of microbial cells in suspension is less challenging and methods are well established, FCM analyses of cells embedded in biofilms or attached to solid matrixes pose a challenge and their application is still limited. Novel and rapid protocols for the extraction and flow cytometric quantification of microbial cells from soils, sediments, sludge and DWTP biofilters have recently been published^{168,186–188}. To analyse microbial cells on the FCM, they must be separated mechanically or optically from solid matrixes, that potentially interfere with the FCM signal¹⁸⁶.

Vignola et al. 2018¹⁶⁸ assessed the detachment efficiency of various chemical and physical extraction methods to quantify total microbial cells associated with DWTP biofilters. They evaluated five chemical dispersants, mechanical pre-treatments, and two fixation methods. The final protocol put forward suggests the combination of physical and chemical extraction methods to address the challenges of not only detaching cells from the solid matrix but also dispersing cell clusters and extracellular polymeric substances (EPS), into individual cells to allow reliable flow cytometric quantification¹⁶⁸. The highest yield of detached cells was achieved when treating the samples with a combination of a surfactant (Tween 80 (5% v/v)) and an ionic dispersant (sodium pyrophosphate (10 mM)) (Tween 80 PP) and exposure to sonication for a total of four rounds¹⁶⁸. Vignola et al. 2018¹⁶⁸ attributed the high extraction efficiency to the combination of two dispersants with different mechanisms of action. Non-ionic surfactants, such as Tween 80, may interfere with the hydrophobic interactions responsible for crosslinking the biofilm matrix¹⁸⁹. Sodium pyrophosphate on the other hand functions as a chelating agent, destabilizing biofilm structures by capturing bivalent cations like Ca^{2+} and Mg^{2+} , which cross-link the negatively charged groups on the surface of the EPS components^{168,190,191}.

A challenge faced when detaching microbial cells from solid matrixes is the lysis of cells during the harsh extraction processes, which dissolves not only the EPS but also cell membranes. Surfactants disrupt lipid-lipid and lipid-protein interactions, leading to the dissolution of lipids that form the cell membrane^{168,192}. To maintain the morphological cell structures during the extraction process, Vignola et al. 2018 pre-treated samples with a biological fixative. Among the two tested fixatives – Ethanol and

Glutaraldehyde, Glutaraldehyde 1% v/v performed best, resulting in significantly higher total cell counts retrieved after the extraction process¹⁶⁸. Glutaraldehyde finds its traditional use as a fixative for electron microscopy¹⁹³ but has previously been suggested as a suitable fixative for flow cytometric analyses¹⁹⁴. Glutaraldehyde belongs to the group of additive fixatives and preserves cells by creating covalent chemical bonds between the proteins of the cell membrane, forming an outer cell cross-link structure^{195,196}. These outer cell cross-link structures ensure that cells do not burst during the extraction process, allowing the quantification of total cell counts on the FCM¹⁶⁸. Glutaraldehyde 1% v/v, however, could not prevent the dissolution of the lipid membranes, creating pores in the cell membrane through which the PI stain could penetrate, making it impossible to quantify intact cell counts present in the system after the extraction process¹⁶⁸. In summary, the protocol put forward by Vignola et al. 2018¹⁶⁸ ensures thorough detachment of total cells from biofilter medium, but intact cells cannot be quantified due to the membrane-dissolving properties of the surfactant used.

Gaining an insight into the ratio of dead to live cells within biofilters is crucial to understanding and predicting the water quality produced in any given DWTP. Decay rates are high in these systems³⁹; microbial cells might be present, but they are potentially inactive or damaged. It is therefore important to find an alternative to Tween 80 PP that does not dissolve cell membranes, allowing the quantification of intact cells. Ouyang et al. 2021¹⁸⁷ combined physical blending using a laboratory blender with chemical treatment (Tween 20), and centrifugation with 80% Nycodenz to extract viable⁽¹⁾ microbes from soil. No study has yet evaluated the use of an alternative method for quantifying both ICC and TCC in DWTP sand filters.

Storage conditions of soil samples have previously been shown to impact the flow cytometric quantification of viable cells¹⁸⁷, as well as the microbial community composition characterised by 16S rRNA analysis¹⁹⁷. To ensure accurate quantification of TCC and ICC, it is crucial to understand and minimize biases introduced by improper

⁽¹⁾ The extraction protocol published by Ouyang et al. 2021¹⁸⁷ uses the term “viable cells”. However, there is an ongoing debate among the scientific communities whether this term should be used. In this thesis the term “intact cells” is used preferably. The term “viable cells” is only used in context with the extraction protocol published by Ouyang et al. 2021.

sample storage. DWTPs are often remote, and biofilter samples may not be processed in a laboratory within a few hours after sampling. Samples might need to be stored for a prolonged period before processing or shipped overseas for analysis. To my knowledge, the influence of storage conditions and storage time on the quantification and FCM fingerprinting of microbial cells from DWTP sand filters has not been assessed previously.

The overall goal of this study was to improve the quantification method for the detection of intact cells in samples of biofilters and to assess the effect of different storage conditions on the microbial quantification and the FCM fingerprinting signal.

In more detail, the following questions were addressed in this study:

1. Should cell extracts be fixed individually or cumulated after four rounds of sonication?

Vignola et al. 2018¹⁶⁸ quantified TCC after each of four rounds of sonication, a process that increases both the number of analyses and the time required; hence, an alternative method where cell counts are measured after the final sonication round is desirable to streamline the process. Microbial cells extracted from sand and collected in a sterile glass bottle may be affected by the chemical dispersant while the outstanding rounds of extraction and sonification are performed. To determine whether the time passed between cell extraction and fixation impacts cell counts, an experiment compared cell extracts fixed with 1% v/v Glutaraldehyde immediately after each round of sonication to those fixed only after completing all four rounds. This comparison helped determining whether there is a significant difference in cell counts between the two fixation timings.

2. How does Glutaraldehyde 1% v/v pre-treatment influence total and intact cell count?

Glutaraldehyde 1% v/v is expected to have a preservative effect on cell structures^{194,196}. To confirm and make sure, Glutaraldehyde 1% v/v does not cause any unexpected damage to cell structures, a control experiment was performed.

3. How does the choice of (chemical) dispersant influence total and intact cell counts?

Two chemical dispersants and one buffer solution were tested for their suitability to extract and quantify both total and intact cell counts from sand samples. Tap water served as a control solution, against which the recovery of cells from the biofilm was measured.

4. How do sample preparation and storage conditions influence total and intact cell counts recovered over time?

The effect of four different storage conditions on the total and intact cell counts recovered from sand samples, was analysed for storage times ranging from 1 day to 6 months. In addition, the efficacy of Glutaraldehyde 1% v/v pre-treatment as a protective measure for both total and intact cells during sample storage was tested.

5. How do sample preparation and storage conditions influence the FCM fingerprinting signals over time?

In addition to assessing the impact of storage conditions on total and intact cell counts over time, FCM fingerprinting signals were analysed. FCM fingerprinting offers valuable insights into changes in community composition caused by experimental treatment conditions, allowing for a more in depth analysis of the effects of storage conditions on sample analysis.

This study describes the development of an optimised extraction protocol for detaching and analysing both total and intact cells from DWTP sand filter samples. We tested alternatives to Tween 80 PP that do not dissolve cell membranes, facilitating the accurate quantification of intact cells. The study found that not only the cell extraction process but also the preparation of sand samples, as well as the storage time and conditions, influence the total and intact cell counts. A flowchart summarising all optimisation steps is provided in figure 5.

Materials and Method

Preparation

Sand samples: Sand samples were collected from rapid gravity filters (RGF) from two different DWTP located in Scotland operated by Scottish Water. Filter samples were taken from the top layer of the RGFs, before backwashing. Sample collection was

performed by the plant operator. After collection, the sand was transferred into sterile 500 mL sampling containers and transported to the laboratory at room temperature within a few hours. The sand was stored in the dark at 4 °C until the experiments were performed. Sand samples were kept in the refrigerator for 6 to 15 weeks before starting the different treatments of the storage experiment. To ensure consistent results and allow for accurate cross-comparison of dispersants, all cell extractions to evaluate dispersants were performed on the same day, minimising discrepancies from varying storage times. While the origin of samples varied between different optimisation experiments, it remained consistent within each individual optimisation experiment.

Chemical preparation: 0.22 µm filtered Glutaraldehyde 1% v/v in 18.2 M Ohm deionised carbon-free water (Milli-Q / UP) was made from Glutaraldehyde 25% aqueous solution (Sigma Aldrich) and served as fixative to protect microbial cells from disruption during storage and extraction processes. Glutaraldehyde 1% v/v was batch made at the beginning of the experiment and stored in 50 mL falcon tubes at -20 °C. Throughout the experiment, the Falcon tubes were thawed as required and subsequently kept in the refrigerator until empty.

Cell extraction performances of two dispersants were tested against UP water and 1x Phosphate Buffered Saline (Fisher BioReagents™) (PBS); Polyethylene glycol sorbitan monooleate (TWEEN® 80, Sigma Aldrich) 5% v/v in a 10 mM solution of the ionic dispersant sodium pyrophosphate decahydrate (Sigma Aldrich) (Tween 80 PP)¹⁶⁸, and Polyethylene glycol sorbitan monolaurate (TWEEN® 20, Sigma Aldrich) 0.5% v/v in a 1x solution of PBS (PBS Tween 20)¹⁸⁷. Except for Tween 80 and Tween 20, all solutions were autoclaved prior to use.

Experimental set-up

Extraction Protocol: This section describes the detailed protocol followed during cell extractions from DWTP biofilter samples. The protocol was adapted from Vignola et al. 2018¹⁶⁸ and modified to allow the enumeration of intact cells as well as total cells. Modifications are described in detail in the following sections and illustrated in figure 5.

1. 2 g wet weight (WW) of sand samples were aliquoted in triplicates into sterile specimen containers (Kartell™), fixed with Glutaraldehyde 1% v/v (if applicable)

at a ratio of 1:1 mL of fixative to gWW of sand, and left to act for 30 minutes at room temperature, prior to cell extractions.

2. Microbial cells were extracted from fixed or non-fixed sand samples by four rounds of sonication. In each round, 50 mL of chemical dispersant (or UP water / PBS, according to the experiment set-up) were added to the sand and exposed to high energy sonication for 30 seconds at 40 W (Sonic Dismembrator (FB-50), Fisher Scientific). The exhausted dispersant (or UP water / PBS) containing the extracted cells was transferred into a sterile glass bottle and the extraction process was repeated three more times.
3. Immediately after four rounds of sonication, an aliquot of 2 mL was taken from the cumulated cell extracts collected in the glass bottle and fixed with Glutaraldehyde 1% (v/v) at a ratio of 1:1 v/v fixative to cell extracts. Fixed cell extracts were stored at room temperature and analysed by FCM within 2 hours, as described below. All cell extracts were analysed in technical duplicates.
4. An incinerated (500 °C, 4h) sand sample serving as abiotic control was extracted and processed by FCM alongside each sample, following the same protocol. As an additional control, a sample of Glutaraldehyde 1% (v/v) and the chemical dispersant (or UP water / PBS) was processed by FCM to detect potential biological contamination of the chemical solutions.

Individual vs. cumulative fixation of cell extracts: After each round of sonication, 2 mL aliquots of cells extracted with Tween 80 PP were collected and immediately fixed in a sterile falcon tube with Glutaraldehyde 1% (v/v) at a 1:1 ratio (v/v). Following four rounds of sonication, additional 2 mL aliquots of the cumulative cell extracts were taken, as described in extraction protocol step 3. The experiment was performed in biological triplicates. Aliquots taken individually after each round of sonication were pooled and mixed by inversion prior to cell quantification by FCM. To test if immediate fixation with Glutaraldehyde 1% (v/v) is necessary to prevent significant loss of intact and total cells, cell counts of individually fixed and pooled cell extracts were compared to those of cumulative cell extracts.

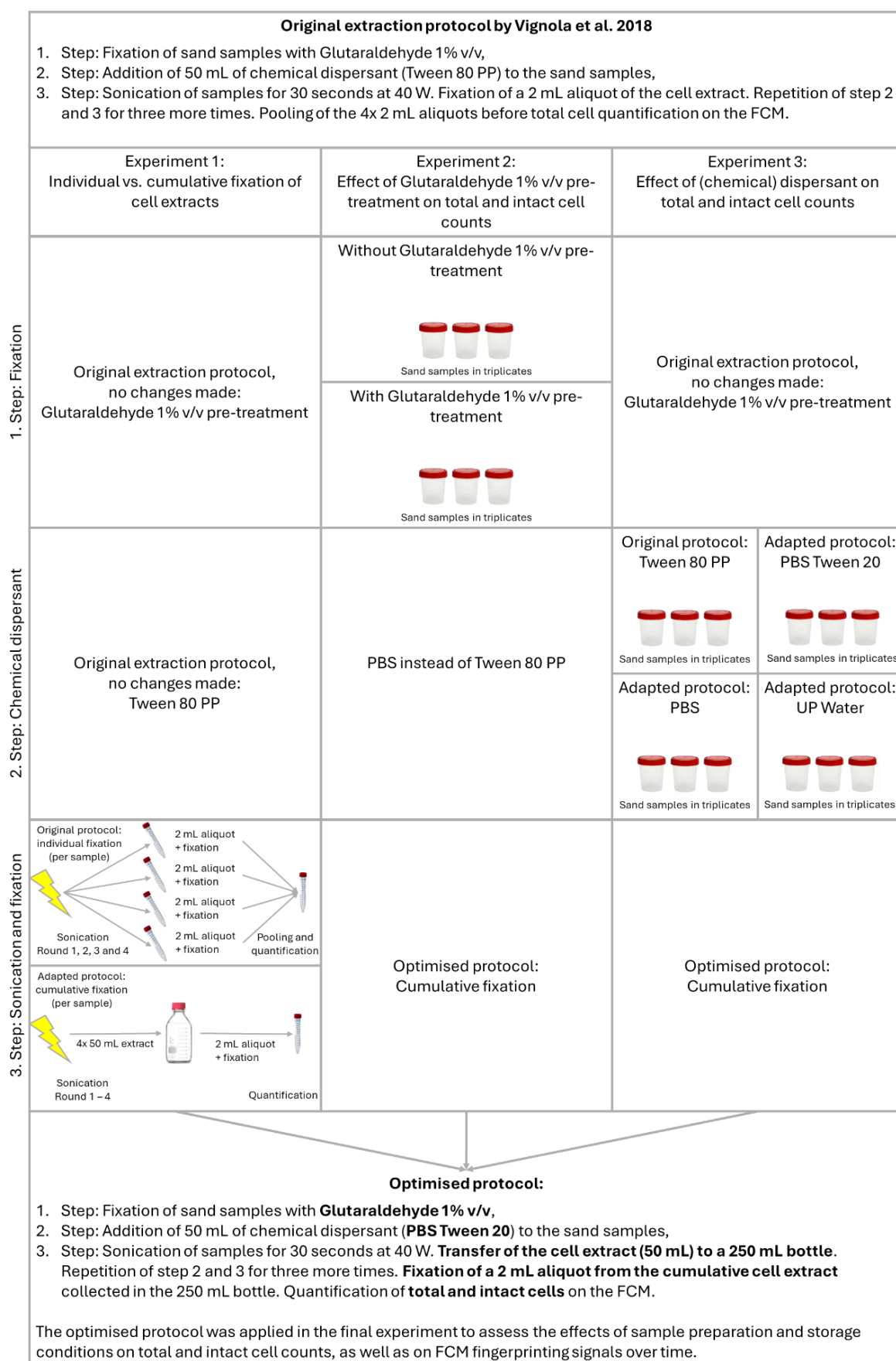


Figure 5 Flowchart providing an overview of the optimisation experiments performed to adapt the existing extraction protocol¹⁶⁸ to enable the quantification of both total and intact cells from biofilter sand samples.

Effect of Glutaraldehyde 1% v/v pre-treatment on total and intact cell counts: The effect of sample pre-treatment with Glutaraldehyde 1% v/v on total and intact cells recovered after extraction was evaluated. Two grams of sand samples, in triplicates, were either pre-treated with two millilitres of Glutaraldehyde 1% v/v (left to act for 30 minutes) or left untreated before extraction of cells. The modified extraction protocol as described above (steps 1. to 4.) was followed, using PBS instead of a chemical dispersant. Total and intact cells were quantified by FCM, as described in extraction protocol step 3.

Effect of (chemical) dispersant on total and intact cell counts: The cell extraction protocol proposed by Vignola et al. 2018¹⁶⁸ includes the application of a chemical surfactant that helps detach cells from sand and prevents cell clusters. In this study, extraction performances of two chemical dispersants, Tween 80 PP¹⁶⁸, and PBS Tween 20¹⁸⁷, were tested and compared. Further, the extraction performance of 1x PBS, a buffer solution that does not display any cell membrane destroying properties, as an alternative to chemical dispersants, was assessed. UP water was used as a control to evaluate the effectiveness of the extraction solutions by comparing the number of cells they extracted against those extracted with UP water. For each of the four treatments, experimental triplicates were processed. Cells were extracted following the modified extraction protocol as described above and all samples were pre-treated with Glutaraldehyde 1% v/v. Total and intact cells were quantified by FCM, as described in extraction protocol step 3.

Effect of storage conditions on total and intact cell counts: The effects of sand sample preparation, storage condition and storage time on total and intact cell counts were tested; an overview is given in Figure 6.

2 g of sand samples were either exposed to Glutaraldehyde 1% v/v at a ratio of 1:1 v/v before or after storage, to evaluate the protective effect of Glutaraldehyde 1% v/v during sample storage. Samples were stored under four different conditions; 4 °C, -20 °C, -80 °C, and flash freezing with liquid Nitrogen and subsequent storage at -80 °C. Total and intact cells were quantified prior to storage (day 0) and after 1 day, 3 days, 6 days, 4 weeks, 10 weeks, and 6 months of storage, following the extraction protocol optimised previously.

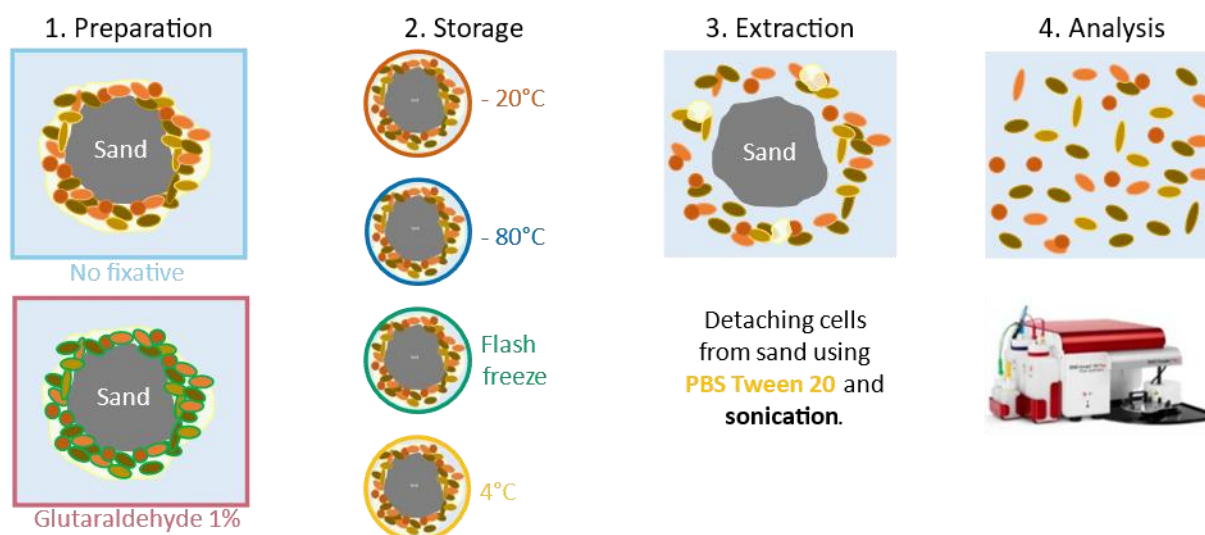


Figure 6 Four different storage conditions; 4 °C, -20 °C, -80 °C, and flash freezing with liquid Nitrogen and subsequent storage at -80 °C. Quantification of TCC and ICC after 0, 1, 3, 6 days, 4, 10 weeks, and 6 months of storage. All cells were extracted from sand using PBS Tween 20 as a chemical dispersant and four rounds of sonication. Cell extracts were quantified on the FCM. Each treatment was performed in biological triplicates, FCM cell counts were established in technical duplicates.

PBS Tween 20 was used as a chemical dispersant for all cell extractions. Sand furnaceed at 500 °C for four hours served as an abiotic negative control. Controls were processed along with samples for each storage condition and time point. Total and intact cells were quantified by FCM, as described in extraction protocol step 3.

Sample Analysis

FCM cell quantification: Total and intact cells extracted from the filter medium were quantified using a BD Accuri™ C6 Plus (Becton, Dickinson and Company, BD Biosciences, CA, USA) equipped with an automatic sampler (CSampler Plus) and a 488 nm laser. Microbial cells were stained using the fluorescent stains SYBR Green I® (SG) to quantify total cells and SG combined with propidium iodide (SGPI) to quantify intact cells. Working stock solutions for SG were prepared by diluting 10 µl of SYBR Green I® stock solution (10'000 x in DMSO, Sigma Aldrich, Darmstadt, Germany) in 1 mL of 0.22 µm filtered 10 mM Tris-EDTA buffer (pH 8.0, Sigma Aldrich, Darmstadt, Germany); working stock solutions for SGPI were prepared by mixing 10 µL of SYBR Green I® stock solution (10'000 x in DMSO) with 400 µL Propidium Iodide stock solution (1.6 mM, Sigma Aldrich, Darmstadt, Germany) and 600 µL of 0.22 µm filtered 10 mM Tris-EDTA buffer (pH 8.0, Sigma Aldrich, Darmstadt, Germany), resulting in a final concentration of 0.6 mM (PI) and 100x (SG) respectively. All working stock solutions were prepared in

autoclaved Eppendorf tubes, protected from light, and stored in the fridge. Microbial cells were stained separately, mixing either 5 μ L of SG or 5 μ L SGPI with 0.5 mL of sample. Stained samples were protected from light and incubated at 37 °C for 15 minutes, after which they were immediately analysed on the FCM. Technical duplicates were run on the FCM for each sample. Sample readings were performed and analysed using the BD Accuri™ C6 Plus software version 1.0.23.1, with the following settings: run limit of 50 μ L, medium flow rate (35 μ L/min), thresholds set to 200 reads for FL1-H and 150 reads for FL3-H respectively, and PerCP-A (675 nm) displayed on the y-axis and FITC-A displayed (533/30 nm) on the x-axis. Electronic gating was applied to distinguish between microbial cells and background reads, consisting of inorganic and organic particles (Figure 7).

To help with electronic gating and to separate microbial cells from background noise, abiotic control samples (ash control) were run. 2g aliquots of WW DWTP sand samples were first dried at 105 °C overnight and then exposed to 500 °C for 4 h in a muffle oven (CARBOLITE GERO, Neuhausen, Germany) the following day. This process burned off all organic material present in the sample. The ash control samples were prepared and run on the FCM alongside biotic samples and allowed to clearly distinguish between abiotic background noise and microbial cells on the FCM fingerprints.

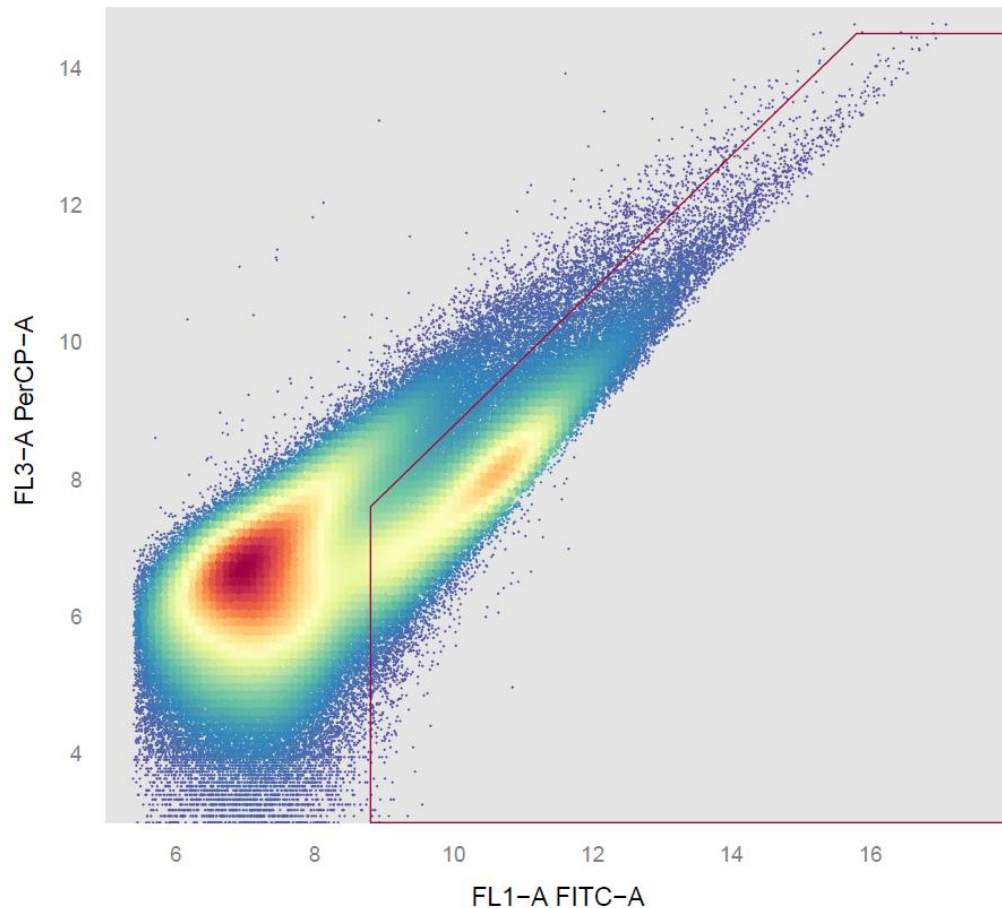


Figure 7 This figure presents a density dot plot generated by the Phenoflow data processing pipeline for a DWTP sand sample at timepoint zero, prior to storage. The y-axis displays FL3-A PerCP-A fluorescence (675 ± 12.5 nm), while the x-axis shows FL1-A FITC-A fluorescence (533 ± 30 nm). Each signal is represented by a dot, with intensity levels depicted as a colour gradient ranging from dark blue (low intensity) to red (high intensity). An electronic gate was manually drawn to differentiate between background particles (outside the gate) and microbial cells (inside the gate). This gate was consistently applied across all sand samples, irrespective of storage conditions or timepoints, to ensure comparability of results.

FCM fingerprint analysis: Flow cytometric standard (FCS) files were exported from the BD Accuri™ C6 Plus software and imported into RStudio (2022.07.2 Build 576), where fingerprint analysis was performed using the PhenoFlow pipeline and its dependencies^{180,183}. The PhenoFlow pipeline converts the raw FCM data into normalised fingerprints, which allow for detection of phenotypic differences between samples¹⁸⁰. Standardised electronic gating of the microbial community was used during preprocessing of the data, to filter out background noise and to allow comparability of results between samples¹⁸¹. An example is given in figure 7. The polygon gate was applied to the FL-A (area) dataset, from which total cell counts were extracted after denoising. When creating and exporting the “flowBasis”, green and red fluorescence signals (FL1-A, FL3-A) were accounted for and number of bins was set to 400. The

“flowBasis” table exported from the PhenoFlow pipeline was used as an abundance table for downstream statistical analysis.

Statistical analysis: Statistical analysis was performed using RStudio (2022.07.2 Build 576 and RStudio 2024.12.1 Build 563) for windows. Beta diversity of FCM fingerprints was calculated using the Vegan R package. The script for assessment of beta diversity and calculating and visualising the Bray-Curtis dissimilarity is available from [R code for ecological data analysis \(gla.ac.uk\)](https://github.com/gla.ac.uk/ecoDataAnalysis)¹⁹⁸. The distance matrix created from Bray-Curtis dissimilarity values was analysed using Dunn’s test, and p-values were adjusted for multiple comparisons using the Benjamini–Hochberg procedure to control the false discovery rate.

Results

Individual vs. cumulative fixation of cell extracts: TCC and ICC of extracts fixed with 1% v/v Glutaraldehyde immediately after each round of sonication were compared to those fixed cumulatively immediately after all four rounds of sonication were completed. The extraction yields varied greatly across different dispersants - UP water, PBS, PBS Tween 20, and Tween 80 PP - with Tween 80 PP achieving the highest yield of total cells. Results are displayed in figure 8 (TCC) and figure 9 (ICC). Comparison of cell extracts fixed with Glutaraldehyde 1% v/v immediately (individual fixation) or after completion of four rounds of sonication (cumulative fixation) showed no significant difference for TCC extracted with UP water and ICC extracted with UP Water, PBS, PBS Tween 20, and Tween 80 PP. TCC extracted with PBS, PBS Tween 20 and Tween 80 PP showed slightly significant differences between individually and cumulatively fixed extracts, with higher cell counts for cumulatively fixed samples. All p-values and statistical tests are given in table 4. Since cell counts of cumulatively fixed extracts were either the same as or higher than individually fixed extracts, the cumulative extraction protocol was chosen for this thesis, in order to simplify and streamline the extraction process.

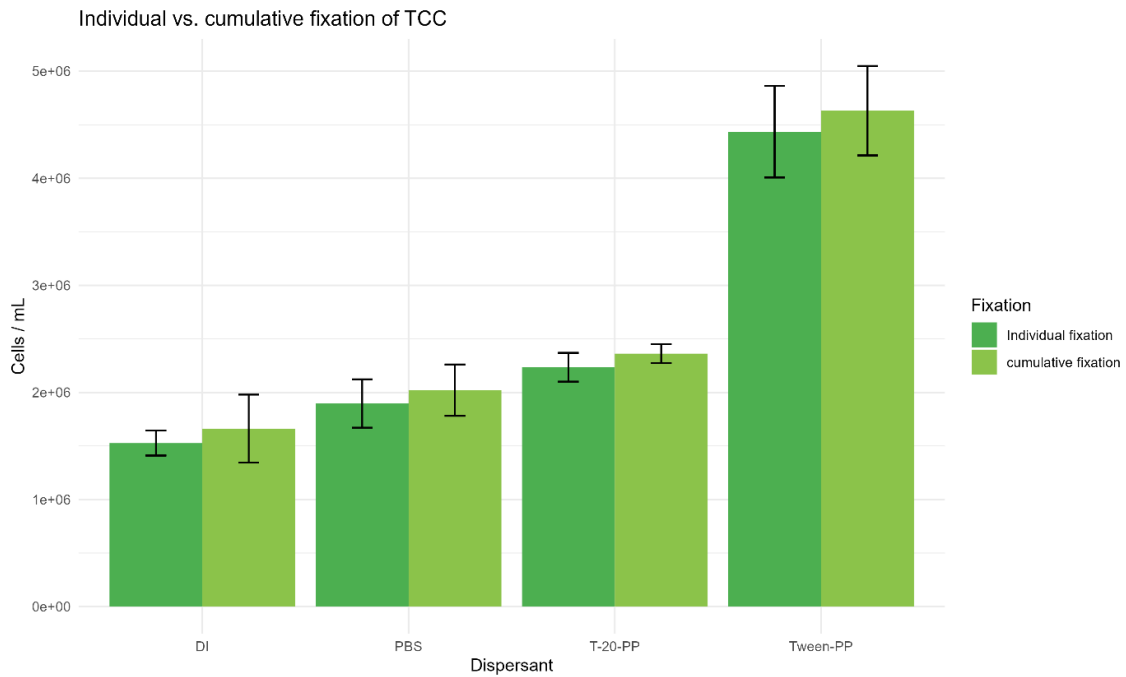


Figure 8 displays total cell counts (TCC) for both, individually and cumulatively fixed cells extracted with UP water (DI), PBS, PBS Tween 20 (T-20-PP), and Tween 80 PP (Tween-PP). Each treatment was performed in biological triplicates, FCM cell counts were established in technical duplicates. Total cell counts extracted with UP water and PBS Tween 20 did not differ significantly between individually and cumulatively fixed cell extracts. Total cell counts extracted with PBS and Tween 80 PP did show a statistically significant difference between individually and cumulatively fixed cell extracts, with higher cell counts for cumulatively fixed samples. All *p*-values are listed in table 4. Cell counts are shown as mean $\pm$ 95% confidence intervals.

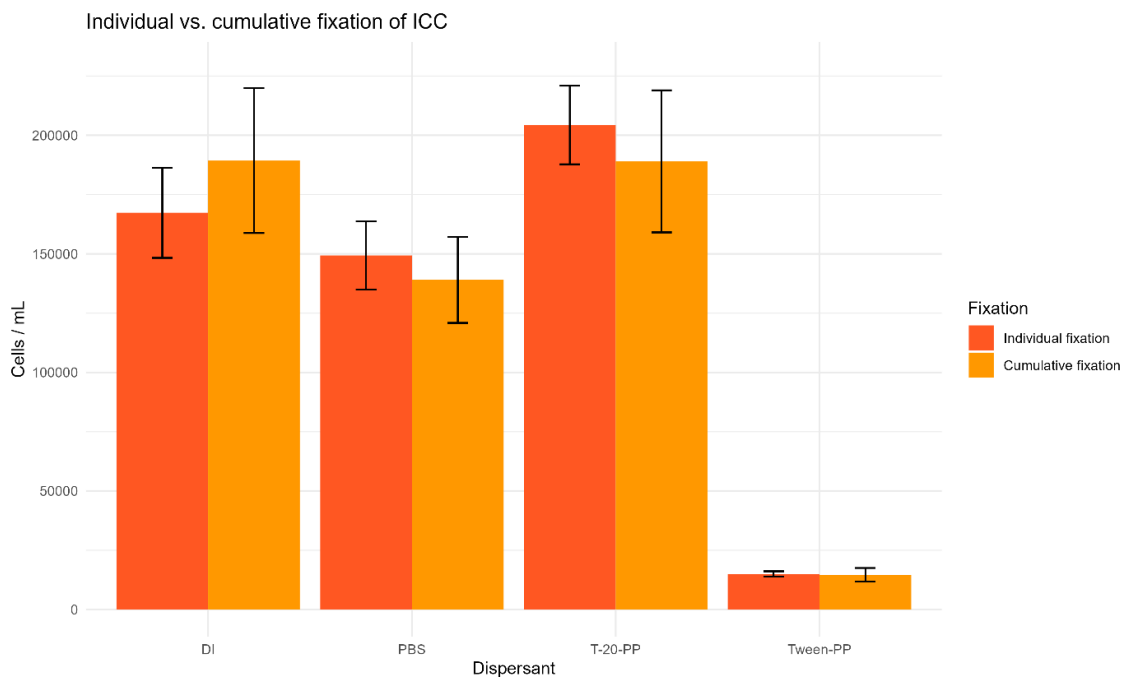


Figure 9 displays intact cell counts (ICC) for both, individually and cumulatively fixed cells extracted with UP water (DI), PBS, PBS Tween 20 (T-20-PP), and Tween 80 PP (Tween-PP). Each treatment was performed in biological triplicates, FCM cell counts were established in technical duplicates. No significant difference in intact cell counts was observed between individually and cumulatively fixed cell extracts. All *p*-values are listed in table 4. Cell counts are shown as mean $\pm$ 95% confidence intervals.

Table 4 This table presents p-values derived from statistical tests (paired t-test or Wilcoxon test) of differences in cell counts from individually vs. cumulatively fixed cell extracts, sorted by (chemical) dispersant.

Total Cell Counts		Intact Cell Counts	
Individual vs. cumulative fixation		Individual vs. cumulative fixation	
UP Water	p-value = 0.476 (paired t-test)	UP Water	p-value = 0.201 (paired t-test)
PBS	p-value = 0.00999 (paired t-test)	PBS	p-value = 0.174 (Wilcoxon test)
PBS Tween 20	p-value = 0.0323 (paired t-test)	PBS Tween 20	p-value = 0.396 (paired t-test)
Tween 80 PP	p-value = 0.002 (paired t-test)	Tween 80 PP	p-value = 0.84 (paired t-test)

Effect of Glutaraldehyde 1% pre-treatment on total and intact cell counts:

Glutaraldehyde 1% v/v is expected to preserve cell structures. To confirm it does not cause any unexpected damage, the effects on cells during extraction were evaluated. PBS instead of the aggressive Tween 80 PP was used to extract TCC and ICC. Pre-treatment of sand samples with Glutaraldehyde 1% v/v did not influence total cell counts significantly (p-value = 0.097, t-Test), confirming that Glutaraldehyde 1% v/v doesn't adversely affect cell counts. However, Glutaraldehyde 1% v/v pre-treatment protected intact cells from disruption during the harsh extraction process, resulting in significantly (p-value = 0.006, t-Test) higher intact cell counts, compared to untreated samples. Results are shown in figure 10.

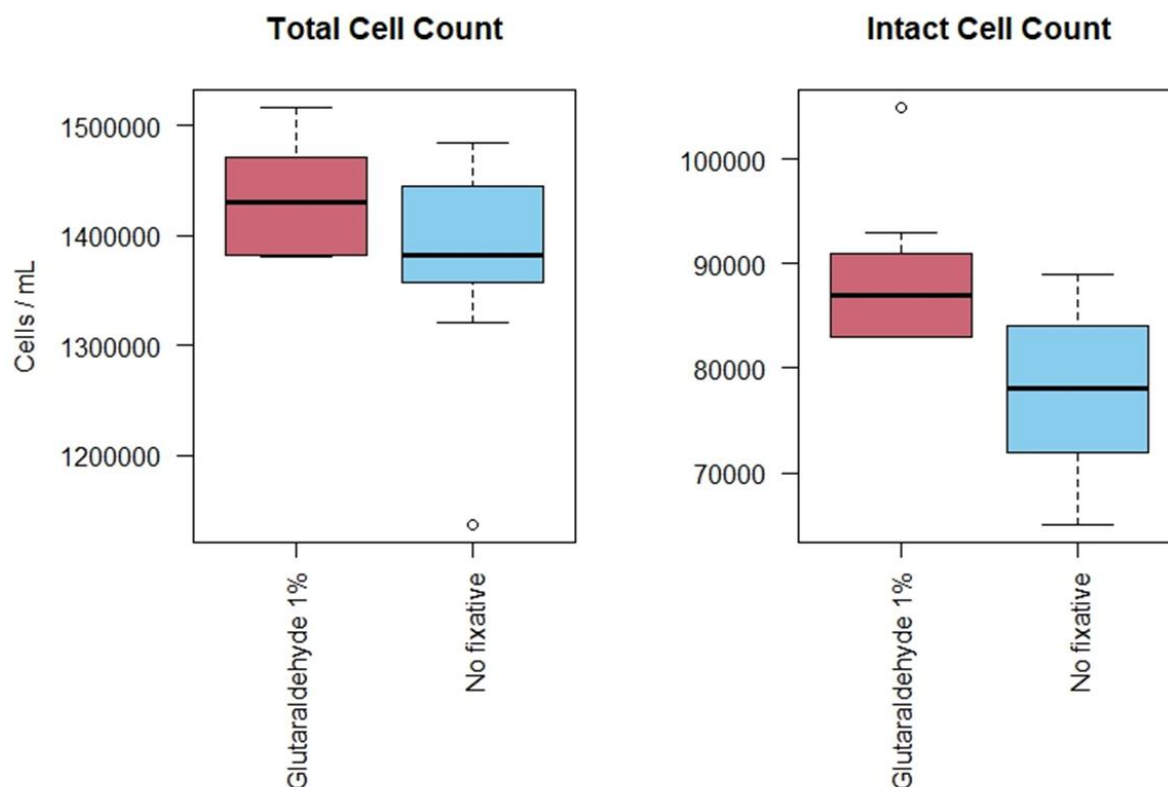


Figure 10 Total (left) and intact (right) cell counts extracted from 2.00 g of sand samples (cells / mL of extract). Samples were either pre-treated with a fixative (Glutaraldehyde 1% v/v) vs. no fixative. Each treatment was performed in biological triplicates, FCM cell counts were established in technical duplicates. TCCs of Glutaraldehyde 1% vs. no fixative did not differ significantly (p -value = 0.097, t -Test), but ICCs did (p -value = 0.006, t -Test).

Effect of (chemical) dispersant on total and intact cell counts:

Two chemical dispersants, one buffer solution (PBS) and UP water were assessed for their suitability to extract both total and intact cells from DWTP sand filters. All samples were pre-treated with 1% v/v Glutaraldehyde, regardless of the (chemical) dispersants used. Tween 80 PP performed distinctively better than UP water, PBS, and PBS Tween 20 when considering total cells extracted from sand samples (figure 11), but intact cells extracted were much lower, in comparison with the other extraction methods. The second highest number of total cells was extracted by PBS Tween 20, followed by PBS and UP water. Most intact cells were extracted with PBS Tween 20, followed by UP water and PBS. The highest ratio of total to intact cell counts was achieved by PBS Tween 20. Extraction performances of all four dispersants for TCC and ICC are shown in figure 11.

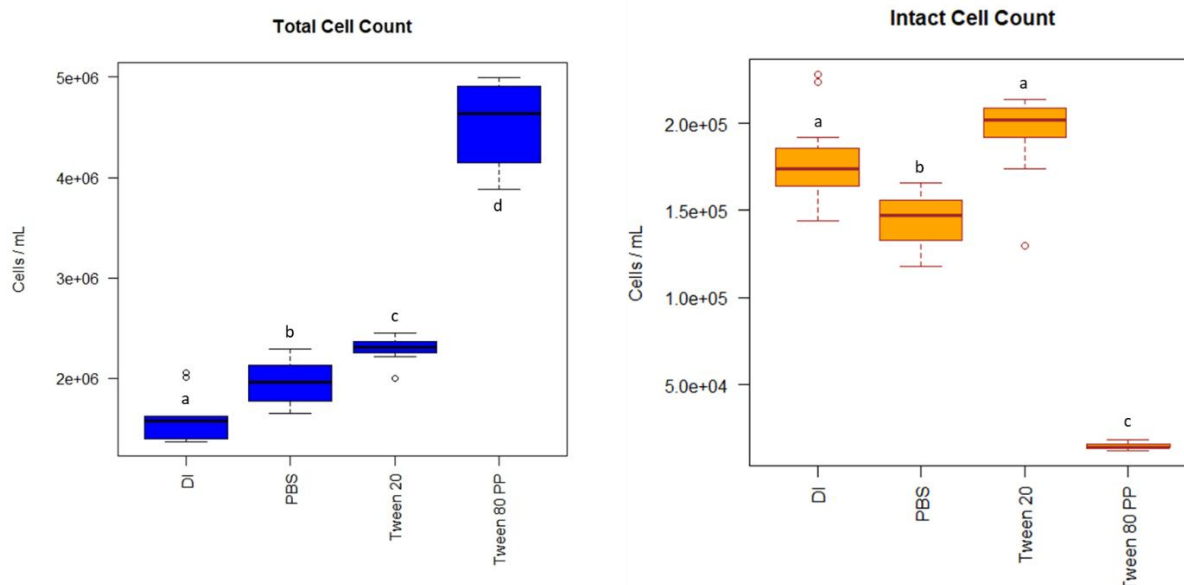


Figure 11 Extraction performance for total cell counts (left) and intact cell counts (right) extracted from 2.00 g of sand samples (cells / mL of extract), for the four (chemical) dispersants UP water (DI), PBS, PBS Tween 20 (Tween 20), and Tween 80 PP. Each treatment was performed in biological triplicates, FCM cell counts were established in technical duplicates. Same letters denote no significant difference, different letters denote significant differences between treatments.

Effect of storage conditions on total and intact cell counts:

The impact of four different storage conditions on TCC and ICC from DWTP sand samples was assessed for storage durations of 1 day to 6 months. Furthermore, the effectiveness of a 1% v/v Glutaraldehyde pre-treatment in protecting both total and intact cells during storage was examined. To allow the quantification of both, TCC and ICC, PBS Tween 20 was used as chemical dispersant. Storage time and condition influenced TCC and ICC over time (figure 12).

Intact cell counts: Storing the samples at -20 °C caused an immediate and statistically significant loss of intact cells, irrespective of Glutaraldehyde 1% pre-treatment (p-value = 0.001861, t-test) or not (p-value = 0.002295, t-test). ICC of samples that were stored at -20 °C and not fixed with Glutaraldehyde 1% further diminished until reaching zero after a storage duration of six months (figure 12). ICC of samples stored at -80 °C and flash frozen with subsequent storage at -80 °C declined over time (figure 12), irrespective of pre-treatment, resulting in a loss of ICC of 39.2% (-80 °C, with pre-treatment), 86.3% (-80 °C, without pre-treatment), 79.4% (flash freezing, with pre-treatment) and 73.1% (flash freezing, without pre-treatment), after 6 months of sample storage.

Performing an One-way-ANOVA followed by a Tukey HSD post-hoc test identified, at which timepoints ICC of samples stored in the fridge (5 °C) might differ from T0. A statistically significant difference of ICC was observed after 3 days of storage (T2) for both, samples pre-treated with Glutaraldehyde 1% (p-value = 0.0139, Tukey HSD) and non-fixed samples (p-value = 0.0139, Tukey HSD). However, this was the only timepoint that differed from T0. No other timepoint until the end of the experiment after 6 months of storage showed a statistically significant difference from ICC at T0. It needs to be reported that, for non-fixed samples stored at 5 °C two of the three replicates have dried completely after 6 months, indicating that the storage container wasn't fully airtight, which might have influenced the results obtained.

Total cell counts: Total cell counts were reduced significantly within 24h hours of storage (T0 vs. T1) of non-fixed sand samples stored at -20 °C (p-value = 0.003233, t-test), -80 °C (p-value = 0.003116, t-test), and flash frozen (p-value = 0.003953, t-test). Glutaraldehyde 1% pre-treatment did not protect TCC of sand samples stored at -20 °C from significant loss of cells within the first day of storage (T0 vs. T1) (p-value = 0.02876, t-test). However, Glutaraldehyde 1% pre-treatment protected sand samples stored at -80 °C (p-value = 0.08086, Wilcoxon test) and flash frozen (p-value = 0.2428, t-test) from immediate loss of cells upon storage (T0 vs. T1). Over time, TCC of samples stored at -80 °C and flash frozen with subsequent storage of -80 °C declined, even when pre-treated with Glutaraldehyde 1%. 25.4% and 24.8% of TCC were lost over the experiment duration of six months, resulting in a statistically not significant difference (p-value = 0.05159, t-test) and a statistically significant difference (p-value = 0.0152, t-test) when comparing TCC at the start and end of the storage experiment at -80 °C and flash frozen respectively. TCC of sand samples stored in the fridge (5 °C) did not show statistically significant differences within the first 10 weeks of storage (T0 vs T6) for samples pre-treated with Glutaraldehyde 1% (p-value = 0.07353, t-test) and non-fixed samples (p-value = 0.2708, t-test). After 6 months of storage, non-fixed samples showed high variance of cell counts between replicates, being attributed to completely drying out of these samples.

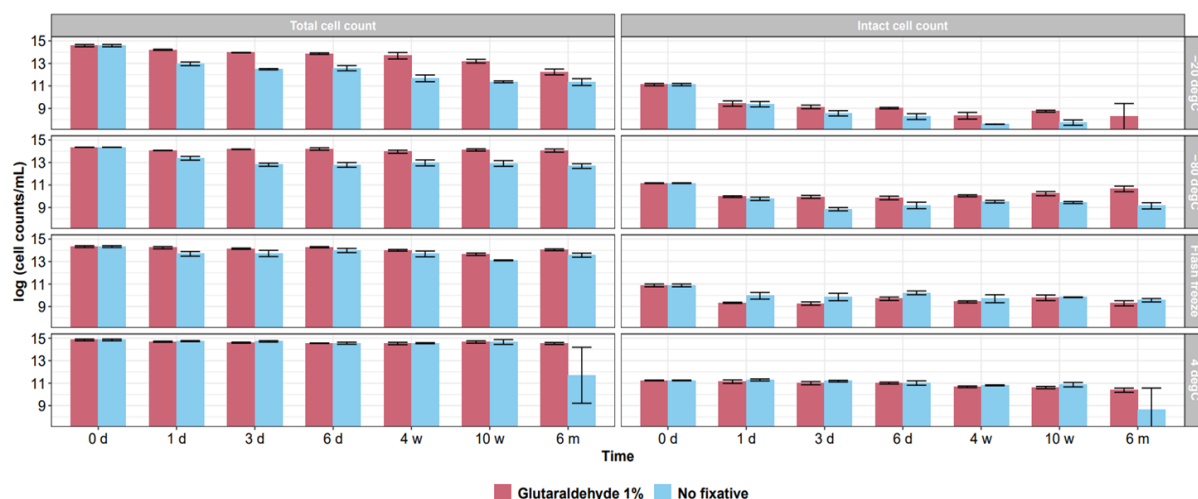


Figure 12 Effects of four different storage conditions (-20 °C, -80 °C, flash freezing, and fridge (4 degC) on total (left) and intact (right) cell counts / mL of cell extract recovered over time (0, 1, 3, 6, 28 days, 10 weeks, and 6 months). Samples were either pre-treated with Glutaraldehyde 1% (red) prior to storage or stored without any pre-treatment (blue). Each treatment was performed in biological triplicates, FCM cell counts were established in technical duplicates.

FCM fingerprint analysis:

Along with the quantification of cell counts, the microbial community composition, visualised by FCM fingerprinting, was assessed. The Bray-Curtis dissimilarity index was applied to compare microbial community compositions derived from FCM fingerprinting across samples subjected to different pre-treatments, storage temperatures, and storage durations. Results are displayed in figures 13 to 16.

Storage time affected FCM fingerprint composition for all pre-treatments and storage conditions (figures 13 -16); between 40% to 63% of variation can be explained by the duration of sample storage (PERMANOVA). Every sample regardless of pre-treatment and storage condition became significantly different from T0 during the span of the experiment. This happened at different times during the experiment for different storage conditions, but at the very latest between T4 (4 weeks) and T5 (10 weeks) of sample storage (figure 16).

2.7% to 11.8% of variance can be attributed to the pre-treatment of sand samples (PERMANOVA). Figure 13 provides a visual representation of the Bray-Curtis dissimilarity values of all samples pre-treated with Glutaraldehyde 1% versus all non-fixed samples (regardless of their storage conditions) over time. The graph shows an overlap between glutaraldehyde-treated and non-fixed samples at the start of the

experiment. As storage time progresses, the groups diverge more noticeably, with greater separation displayed between them (figure 13). Although the two groups overlap visually in figure 13, they become significantly different from each other within the first 24 hours of the storage experiment ($p\text{-value} = 4.96 \times 10^{-17}$, Dunn's test).

Figures 14 to 16 illustrate the effect of storage conditions over time, for both, glutaraldehyde-treated and non-fixed samples. Sand samples pre-treated with Glutaraldehyde and stored at $-20\text{ }^{\circ}\text{C}$ did not exhibit significant differences of Bray-Curtis dissimilarity values when compared to T0 until after 6 days of storage (T3). Non-fixed samples stored at $-20\text{ }^{\circ}\text{C}$ showed a significant change in Bray-Curtis dissimilarity values within the first 24 hours of the experiment (figure 16). Bray-Curtis dissimilarity of samples stored at $-20\text{ }^{\circ}\text{C}$ was strongly impacted by pre-treatment and increased with time (figure 15a, figure 16). In a similar way, Bray-Curtis dissimilarity values of sand samples pre-treated with Glutaraldehyde and stored at $-80\text{ }^{\circ}\text{C}$ did not differ significantly from T0 until after 4 weeks of storage (T4). Dissimilarity values of sand samples stored for 4 weeks differ significantly from sand samples stored for 10 weeks (graph not shown), but remain stable between 10 weeks and 6 months of storage. Non-fixed samples stored at $-80\text{ }^{\circ}\text{C}$ showed a significant change in Bray-Curtis dissimilarity values within the first three days of storage (figure 16). Sand samples that have been flash frozen (FF) prior to storage at $-80\text{ }^{\circ}\text{C}$ showed a significant change in Bray-Curtis dissimilarity values within the first 24 hours of the experiment, for both, glutaraldehyde pre-treated and non-fixed samples (figure 16). A different result was observed for sand samples stored in the fridge ($5\text{ }^{\circ}\text{C}$). Bray-Curtis dissimilarity values for samples pre-treated with Glutaraldehyde became significantly different from T0 after just one day of storage (T2), whereas non-fixed samples did not differ significantly from T0 until after three days of storage (T3) (figure 16). Bray-Curtis dissimilarity values remained constant after 3 days of storage until the end of the experiment after 6 months, for both, pre-treated and non-fixed sand samples. Outliers after six months of storage (T6) for non-fixed samples are attributed to drying of samples as described previously (figure 15d, figure 16).

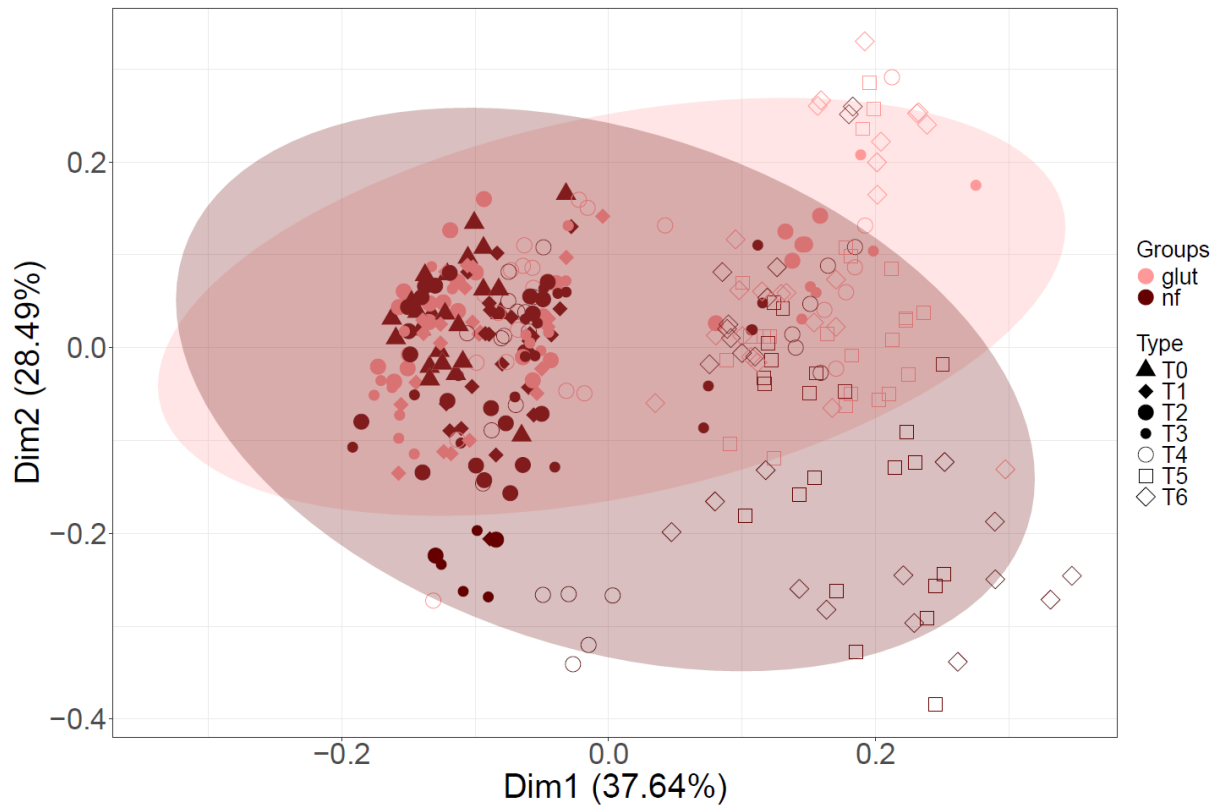


Figure 13 The Bray-Curtis PCoA visualises the fingerprints of all samples analysed during the storage experiment. Samples were grouped and visualised according to two key factors: the pre-treatment they received (either Glutaraldehyde 1% v/v (glut) or no fixative (nf)) and the different time points at which they were sampled (0 days (T0), 1 day (T1), 3 days (T2), 6 days (T3), 28 days (T4), 10 weeks (T5), 6 months (T6)).

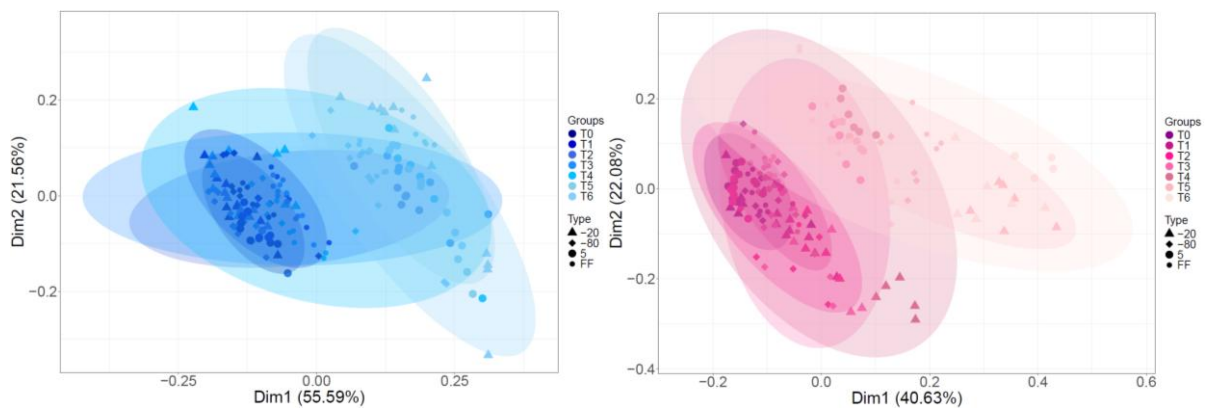


Figure 14 presents two Bray-Curtis PCoAs that illustrate the impact of different storage conditions on microbial community structure over time. In panel 9a, all samples were fixed with Glutaraldehyde 1% v/v, with colours representing the progression from T0 to T6. The shapes of the points in the plot distinguish between the four different storage conditions. Panel 9b, on the other hand, displays the results for non-fixed samples, also distinguished by time and storage conditions.

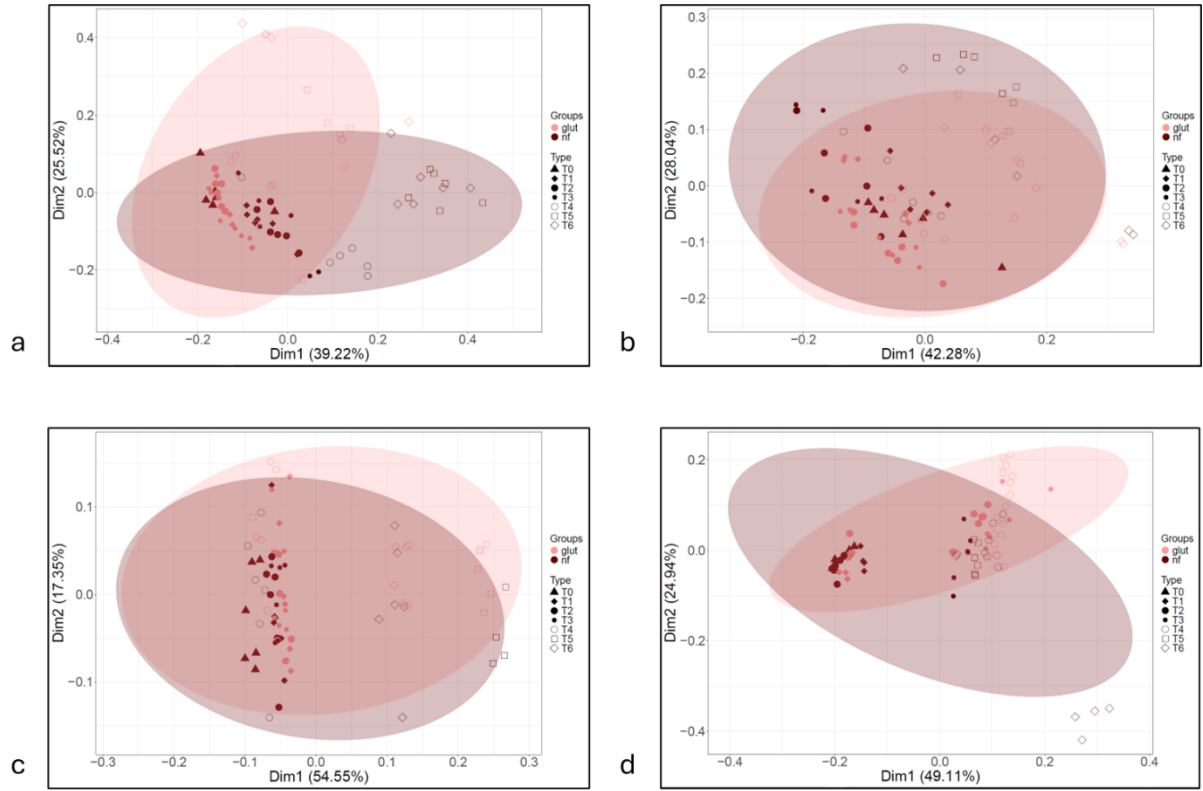


Figure 15 displays Bray-Curtis dissimilarity of non-fixed samples (nf) and samples pre-treated with glutaraldehyde 1% v/v (glut) stored at -20 °C (10a), -80 °C (10b), flash frozen (10c), and 5 °C (10d) over the course of 6 months of storage (0 days (T0), 1 day (T1), 3 days (T2), 6 days (T3), 28 days (T4), 10 weeks (T5), 6 months (T6)).

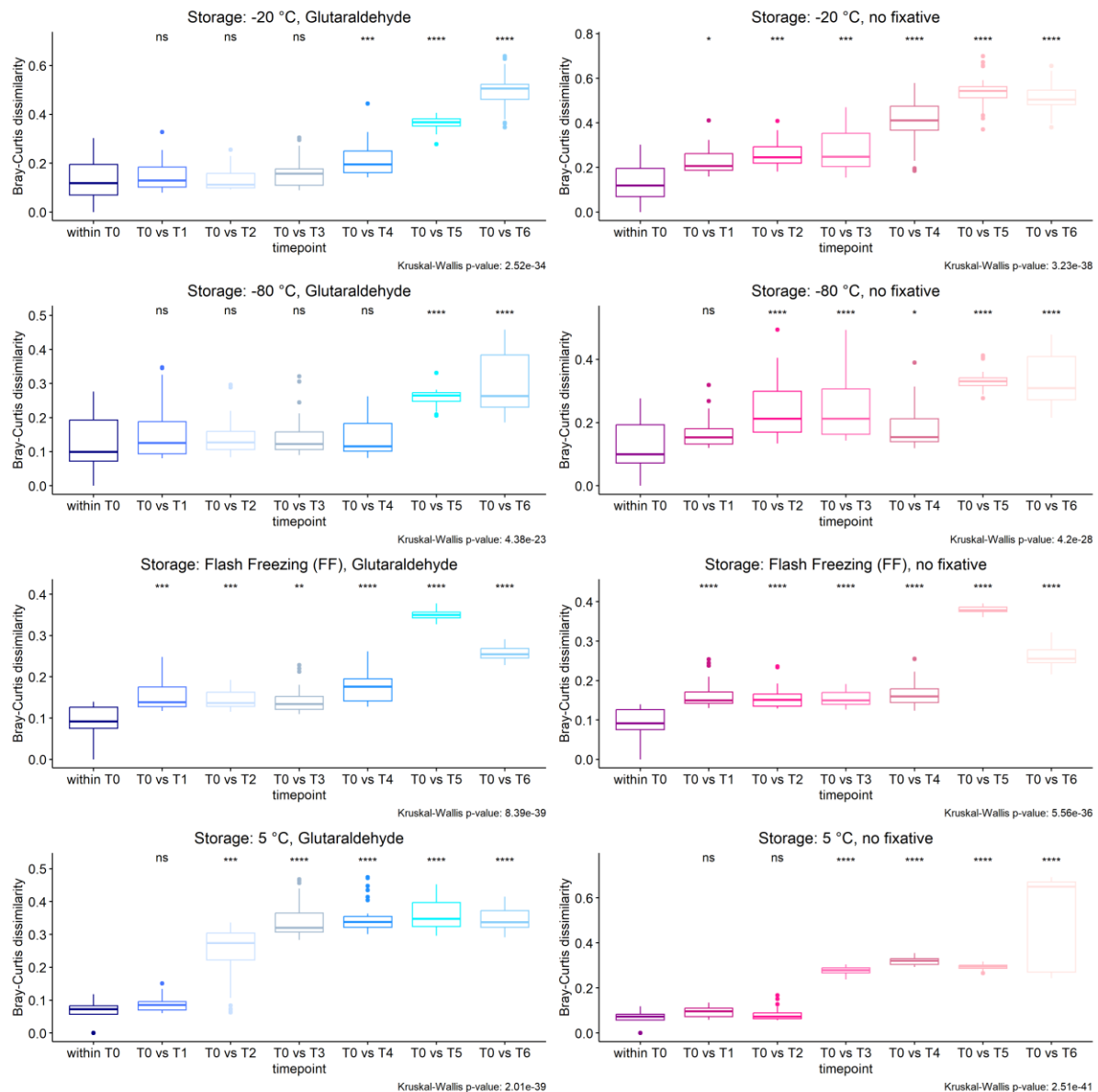


Figure 16 compares Bray-Curtis dissimilarity values of sand samples either pre-treated with Glutaraldehyde 1% (left, blue) or not (right, pink) for four different storage conditions (-20 °C, -80 °C, flash frozen, and 5 °C) over time. Bray-Curtis dissimilarity values are displayed on the y axis of each graph. Each box compares either Bray Curtis dissimilarity values within T0 or compares T0 to Tn. Tn = 0 days (T0), 1 day (T1), 3 days (T2), 6 days (T3), 28 days (T4), 10 weeks (T5), 6 months (T6). P. Values are calculated with Dunn's Test.

Discussion

Reliable quantification of microbial cells in drinking water treatment systems is essential to provide insight into the complex dynamics of microbial communities. Microbes developing on filter media can significantly enhance drinking water quality by breaking down pollutants present in the influent water^{19,39,42,47,52,86,166,167}. Understanding the underlying dynamics enables the implementation of appropriate operational parameters to promote the establishment of optimal microbial communities. However,

achieving this requires a comprehensive understanding of the key drivers influencing microbial communities and their abundance. By accurately quantifying microbial cells, we can better understand these dynamics and study the factors that support optimal populations. This knowledge can then be used to manipulate relevant factors, ultimately leading to improved water treatment processes and water quality.

While the quantification of total and intact cells suspended in water is well established and facilitated by time-efficient and reliable FCM analysis^{169–172}, quantifying cells attached to solid matrices remains a challenge¹⁶⁸. To enumerate microbial biofilm on particles by FCM or microscopy, cells must be detached from surfaces and dispersed into single cells¹⁶⁸. Previous research on cell extraction methods for single cell analysis mainly focused on the efficient recovery of microbial cells from soil samples^{186,187,199}. While most studies were interested in the total amount of cells present in the system, Ouyang et al. 2021¹⁸⁷ recently proposed a protocol for the extraction of viable microbial cells from various soil samples. However, little research has been performed on the challenges of extracting microbial cells from DWTP sand biofilters¹⁶⁸, and to our knowledge, no protocol for the reliable extraction of intact microbial cells has been proposed to date.

Vignola et al. 2018¹⁶⁸ put forward a protocol for the extraction and quantification of total cells from DWTP biofilter. This study is limited by the harsh extraction process, not allowing the quantification of intact cells present in the system. Based on their protocol, we performed a study that addressed the following questions:

1. Should cell extracts be fixed individually or cumulatively after four rounds of sonication?
2. How does Glutaraldehyde 1% v/v pre-treatment influence total and intact cell count?
3. How does the choice of (chemical) dispersant influence total and intact cell counts?
4. How do sample preparation and storage conditions influence total and intact cell counts recovered over time?
5. How do sample preparation and storage conditions influence the FCM fingerprinting signals over time?

The aim of this project was to improve the ability to quantify intact cells from DWTP sand samples and to understand the biases that arise in both TCC and ICC due to different storage conditions and times.

To determine whether the time passed between cell extraction using a (chemical) dispersant and fixation negatively impacted cell counts, we compared cells fixed with 1% v/v Glutaraldehyde immediately after each round of sonication to those fixed after completing all four rounds of sonication. The results showed that ICC did not significantly differ for extracts fixed individually or cumulatively (p-values in table 4). TCC showed slight significance in cell counts from extracts fixed immediately compared to extracts fixed after four rounds of sonication, with slightly higher TCC when extracts were fixed with Glutaraldehyde 1% v/v after completion of all four rounds of sonication. It is important to note that this experiment was conducted with triplicates, which limits the statistical power of the results. Performing four rounds of sonication takes about 10 minutes. The cumulative extracts were fixed with Glutaraldehyde 1% v/v immediately after these four rounds of sonication. An experimental set-up that requires more time might not be suitable for cumulative fixation, as cells exposed to chemical dispersants are more likely to be damaged with extended exposure. Additionally, we tested only one type of DWTP biofilter sand sample, and results from other DWTP biofilters may differ. However, given the results obtained and the need to streamline the extraction process, I chose to perform the fixation of cumulative cell extracts in all the subsequent experiments.

We further found that 1% v/v Glutaraldehyde pre-treatment of DWTP biofilter samples had no negative effect on TCC and ICC recovered. On the contrary, fixation protected intact cells during the extraction process, resulting in significant (p-value = 0.006, t-Test) higher ICC for fixed compared to non-fixed samples. These results align with previous research that found that glutaraldehyde helps to maintain the morphological cell structures^{168,196}, making it indispensable for the extraction of total and intact cells from DWTP biofilters.

When evaluating the impact of four different (chemical) dispersants on the extraction of TCC and ICC from DWTP biofilter samples, we found that Tween 80 PP outperformed UP water, PBS, and PBS Tween 20 in extracting TCC from sand samples. This result was

anticipated and is consistent with previously reported findings¹⁶⁸, further confirming that the method is effective across different types of samples. Tween 80 PP has proven to be the preferred dispersant for quantifying total cells. However, intact cells extracted with Tween 80 PP are low, confirming the membrane destroying properties of Tween 80 PP. Tween 20, a milder surfactant compared to Tween 80, was hypothesized to exert less stress on cell membranes during the extraction process while still effectively facilitating the detachment of cells from sand and EPS. The second-highest number of total cells was extracted using PBS Tween 20, with PBS and UP water following in order. In terms of intact cells, PBS Tween 20 was the most effective, followed by UP water and PBS. PBS Tween 20 further produced the highest ratio of TCC to ICC and showed a smaller variance of data, compared to Tween 80 PP. Cell extractions performed with PBS Tween 20 offer a quick and reliable method to compare ICC between samples, assuming the recovery is similar for different sample types. PBS Tween 20 was chosen as chemical dispersant for subsequent experiments, replacing Tween 80 PP, allowing for comparison of ICC in storage experiments. Although choosing PBS Tween 20 over Tween 80 PP as a chemical dispersant introduces a bias in TCC quantification, opting for the less harsh treatment resulted in lower variability and enabled the simultaneous extraction of both TCC and ICC. It is important to note that each method introduces its own biases, which must be carefully considered and understood in the context of the study's objectives.

Based on these findings, the protocol proposed for the extraction of total and intact cells from DWTP biofilter sand samples is Glutaraldehyde 1% v/v pre-treatment, four rounds of sonication and chemical dispersion with PBS Tween 20.

Ideally, DWTP biofilter samples should be processed shortly after collection. However, because many DWTPs are remote and the cell extraction process is time-intensive, these samples often cannot be analysed within a few hours of sampling. This raises the question of the best way to store DWTP biofilter samples before processing. The storage experiment provides evidence that both storage condition and time can introduce a relevant bias to total and intact cell counts recovered. In summary, storage of DWTP biofilter samples at -20 °C, -80 °C and flash frozen led to an instant loss of intact cells within the first day of storage, which was even more pronounced when

samples were not pre-treated with Glutaraldehyde 1% v/v prior to freezing. The results suggest optimal storage of DWTP biofilter samples pre-treated with Glutaraldehyde 1% v/v at 5 °C for short and long-term storage, of 10 weeks and potentially up to 6 months, without a statistically significant loss of total (p-value = 0.07353 at 10 weeks, t-test) and intact cells (p-value = 0.839 at 10 weeks, Tukey HSD). In general, however, the earlier the samples are analysed, the better.

Freezing and thawing cause strong physiological stresses for microbial cells²⁰⁰; at low temperatures, lipid cell membranes may solidify²⁰¹, and ice crystals can cause them to rupture²⁰². It has previously been shown that freeze-thawing significantly changes soil microbial biomass and community composition^{203,204}. Freeze-thawing events kill substantial portions of the soil microbial biomass, despite the fact, that soil microorganisms possess adaption strategies to (near) freezing temperatures^{203,205}. This die-off may result from the freezing process itself or from the physiological stress caused by the subsequent thawing of soils, during which soil microorganisms must quickly reverse their physiological adaptations to avoid cell rupture²⁰⁰. In this study, frozen sand samples were thawed at room temperature, which potentially contributed to the immediate loss of cells observed after 24 hours of storage in unfixed samples stored at -20°C, -80°C, and flash frozen. In the future, it may be more effective to thaw samples in the fridge to slow down the thawing process and reduce physiological stress on microbial cells.

For FCM analysis of microbial cells, water samples are typically flash-frozen in liquid nitrogen and stored at -80 °C until analysis¹⁹⁴. Kamiya et al. 2007¹⁹⁴ observed that the addition of a combination of 1% paraformaldehyde and 0.01% glutaraldehyde as fixatives for seawater samples did not significantly impact bacterial counts. However, they noted that flash-freezing and storage at -80 °C led to a decrease in counts, with a rapid decline occurring within the first 3 days, followed by a more gradual reduction over time. These results are consistent with the effects of fixation and storage conditions on cell counts from DWTP sand samples observed in this study. No negative effect of glutaraldehyde 1% v/v on cell counts was detected, but freezing of samples led to an immediate and drastic loss of both, TCC and ICC.

Freezing of cells with liquid nitrogen minimizes ice crystal formation, helping to preserve the integrity of the cells, making flash freezing more effective in comparison with freezing at other temperatures, however, ICC declined upon sample storage in the freezer, irrespective of freezer temperature and pre-treatment. For the extraction of intact cells, sample storage in the freezer should be avoided. If samples must be frozen, flash freezing and subsequent storage at -80 °C is preferred as it causes the least amount of cell loss.

Although Glutaraldehyde 1% v/v demonstrated a protective effect on intact cells during the cell extraction process and sample storage, it did not fully prevent cell rupture during freeze-thawing events. This may have been due to inadequate distribution of glutaraldehyde within the sample, or insufficient penetration and fixation of cells before freezing. Glutaraldehyde is known to react with cells relatively fast, within minutes to hours²⁰⁶. Although glutaraldehyde 1% v/v was allowed to react for 30 minutes before freezing, extending this time or increasing the concentration of glutaraldehyde used for fixation might enhance sample preservation in the future. Further, it is important to note that glutaraldehyde, while effective for fixation, is not typically used as a cryoprotectant^{207,208} and may not fully protect cells from the mechanical stresses associated with freezing. Glutaraldehyde alone may not be sufficient to protect cells during the freezing process and the addition of a cryoprotectant should be considered in the future.

Samples fixed with Glutaraldehyde 1% v/v and stored at 5°C exhibited the least reduction in ICC and TCC over time, making this storage condition optimal for both long- and short-term storage. Contrary to expectations, non-fixed samples stored at 5 °C did not exhibit any cell growth over time. In a similar experiment (data not shown), water samples without Glutaraldehyde 1% v/v pre-treatment showed microbial growth during storage at 5 °C. One possible explanation for the lack of growth in the sand samples might be that sand had been stored in the refrigerator for several weeks before the storage experiments started, potentially allowing the microbial community to reach equilibrium. This could explain why no further cell growth was observed over the course of the experiment.

Non-fixed sand samples stored at 5 °C showed a sudden and drastic loss of intact cells after 6 months of storage, as well as a high variance of cell counts between samples. It is important to note, that this was likely caused by the fact that two of the three samples have dried completely after 6 months, indicating that the storage container wasn't fully airtight. If prolonged storage of samples is intended, additional sealing of containers e.g., with parafilm is recommended.

In addition to quantifying total and intact cells, understanding the changes in microbial community composition caused by storage time and conditions is of great importance. In this study, we used FCM fingerprinting analysis as a proxy for changes in community composition. Previously, a strong correlation between the phenotypic diversity index obtained from FCM fingerprint analysis and the 16S rRNA taxonomic diversity of microbial communities across different environments was observed^{180,183}, justifying this approach.

Beta diversity analysis was performed on community composition information derived from FCM fingerprints. Beta diversity was substantially influenced by storage time of sand samples, irrespective of pre-treatment and storage conditions, emphasizing the importance of processing samples as soon as feasible and to refrain from storing samples whenever possible. Sand samples pre-treated with Glutaraldehyde 1% and stored at either -20 °C or -80 °C did not show a significant change in Bray-Curtis dissimilarity until after 6 days of storage (T3) and 4 weeks of storage (T4) respectively. In view of maintaining consistent communities during the storage period, Glutaraldehyde 1% pre-treatment and subsequent storage in the freezer should be preferred over other storage conditions tested. Flash freezing of sand samples resulted in immediate and significant changes in beta diversity and is not recommended. Interestingly, samples stored in the fridge (5 °C) did show a significant change in Bray-Curtis dissimilarity after the first day of storage when pre-treated with Glutaraldehyde 1%, but only after three days of storage for non-fixed samples. TCC and ICC of samples stored at 5 °C stayed constant during this period, indicating a change in cell morphology rather than loss or gain of cells. This change might have been brought about by the exposure to Glutaraldehyde 1%, which has previously been shown to change the morphology of cells²⁰⁹ that could have influenced the FCM signal. After the initial change in beta

diversity, presumably caused by the mode of action of Glutaraldehyde, Bray-Curtis dissimilarity values stayed constant for the remaining duration of the experiment.

It's important to state that every method of storage has its own advantages and disadvantages which have to be carefully evaluated with regard to the objective of the respective study.

While this study provides valuable insights, several limitations should be acknowledged. First, the number of experimental replicates was limited, which constrains the statistical power of the findings. Findings should be interpreted with caution when considering their applicability to broader contexts. Additionally, the experiments were conducted using only one type of sand sample. To make more generalisable conclusions, future research should include sand samples from different DWTPs to determine whether the observed results are consistent across varied environments. Another limitation relates to the use of fingerprinting as a proxy for microbial community analysis. Although previous studies have shown correlations between fingerprinting and DNA-based methods, such as 16S rRNA sequencing^{180,183}, the research in this area is not yet comprehensive. In this study, we discuss changes in fingerprint patterns with the assumption that they reflect microbial community shifts, which could also be detected through 16S rRNA analysis. However, without performing 16S rRNA analysis, this assumption cannot be fully validated. Due to time and resource constraints, 16S rRNA analysis was not conducted within the scope of this experiment, but it is recommended for future studies to strengthen the conclusions drawn from fingerprinting data.

Conclusion

To conclude, engineered biofiltration harbours great potential for improved drinking water quality. To enhance biofilter performance, a better understanding of biofilm dynamics in the drinking water treatment plant system is required. The existing method for the quantification of total cells from biofilter was optimised for intact cells, and a recommended protocol for biofilter sample preparation, storage, and cell extraction for accurate quantification of intact cells, is provided.

In summary, the main questions of this chapter can be answered as follows:

1. Cell extracts do not have to be fixed with Glutaraldehyde 1% v/v individually after each round of sonication. Cell extracts can be fixed cumulated after four rounds of sonication without introduction of a significant bias to total and intact cells quantified.
2. Glutaraldehyde 1% v/v does not adversely affect cell counts. In contrary, it protects intact cells from disruption during the extraction process. *Sample pre-treatment with 1% v/v Glutaraldehyde prior to extraction is recommended when quantifying both total and intact cells from DWTP sand filters.*
3. PBS Tween 20 is the chemical dispersant of choice when extracting and quantifying microbial cells from DWTP sand filter. PBS Tween 20 allows for the simultaneous extraction of both total and intact cells, with the caveat that some total cell counts may be lost due to the less harsh extraction process, compared to Tween 80 PP.
4. The recommended storage condition for DWTP sand filter is 5 °C, which showed stable total and intact cell counts for the duration of 10 weeks up to 6 months, when pre-treated with the biological fixative Glutaraldehyde 1% v/v.
5. Beta diversity was strongly influenced by storage time of sand samples, irrespective of pre-treatment and storage conditions, highlighting the importance of processing samples as soon as feasible. If samples must be stored and the studies main interest lays on FCM fingerprinting analysis of total cells, sand samples pre-treated with Glutaraldehyde 1% v/v should best be stored at either -20 °C or -80 °C, which did not cause a significant change in Bray-Curtis dissimilarity until after 6 days of storage and 4 weeks of storage respectively.

Chapter 5

Chapter 5.1: Evaluation of primer sets for the quantification of atrazine and MCPA biodegradation genes in DWTP biofilter samples

Introduction

The aim of this work was to identify the most relevant functional genes in the process of microbial atrazine and MCPA degradation and to quantify those genes through qPCR techniques in biofilter samples from full scale DWTPs. Being able to quantify the pesticide biodegradation potential within biofilter microbial communities of DWTPs is of great importance to predict the fate of these pollutants in the system and helps to understand which factors control the biodegradation rate of pesticides. In a systematic literature search, primer sets tailored to selected functional genes were gathered. Notably, most of these primer sets were initially published around 20 years ago and designed based on single microbial strains, primarily intended for application in soil samples. In response to this, the chapter focuses on selecting and optimizing primer sets tailored explicitly to DWTP RGF and GAC samples. The primer sets selected for five key functional genes (three for atrazine and two for MCPA) were then used for qPCR analyses of DWTP biofilter samples from Brazil and Scotland.

Atrazine biodegradation

The biodegradation pathway of atrazine is divided into an upper pathway (atrazine to cyanuric acid) and a lower pathway (cyanuric acid to ammonia and carbon dioxide), as shown in figure 17. The upper pathway can be accomplished through three different routes of which one is hydrolytic (atrazine to hydroxyatrazine to N-isopropylammelide to cyanuric acid), and two are oxidative-hydrolytic pathways (1. atrazine to deethylatrazine to deisopropyl-deethylatrazine to 2-chloro-4-hydroxy-6-amino-1,3,5-triazine to ammelide to cyanuric acid, and 2. atrazine to seoisopropylatrazine to seoisopropyl-hydroxyatrazine to N-ethylammelide to cyanuric acid)²¹⁰.

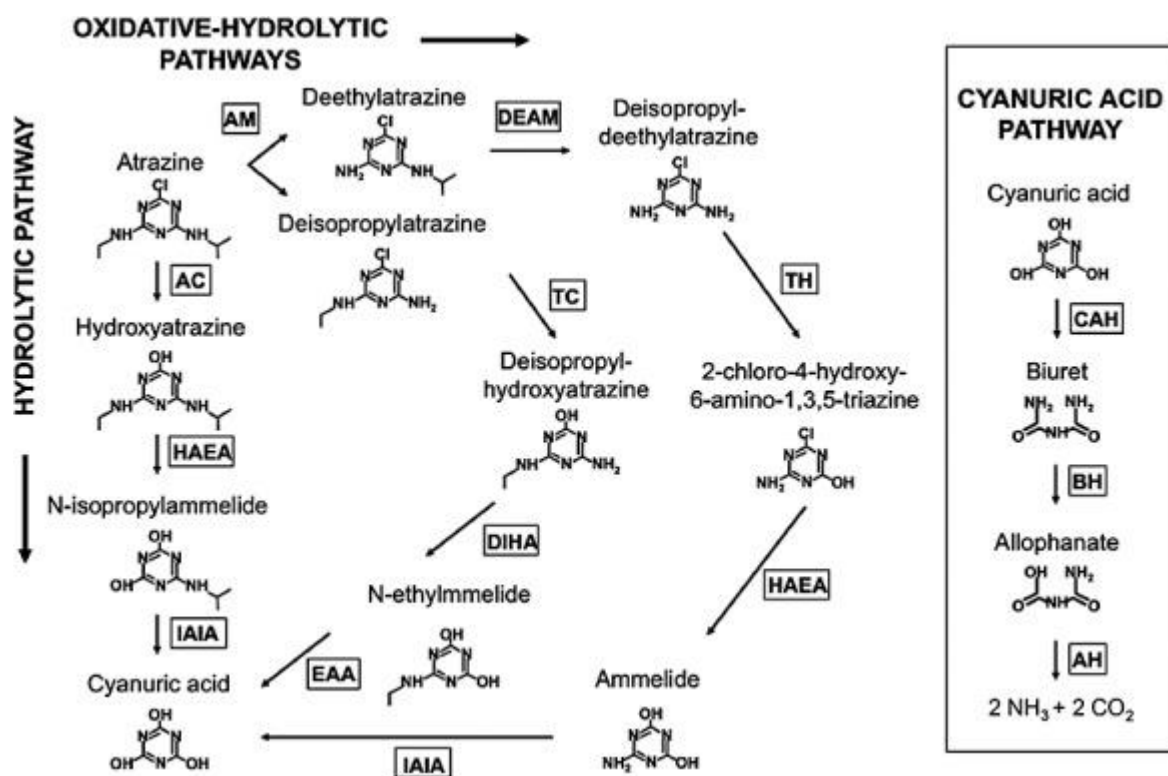


Figure 17: Atrazine degradation pathways, published by Govantes et al., 2009²¹⁰. The left panel illustrates the enzymatic steps converting atrazine to the common intermediate, cyanuric acid. The right panel shows the cyanuric acid degradation pathway. (AC: atrazine chlorohydrolase, HAEA: hydroxyatrazine ethylaminohydrolase, IAIA: N-isopropylammelide isopropylamidohydrolase, TC: s-triazine chlorohydrolase, AM: atrazine monooxygenase, DEAM: deethylatrazine monooxygenase, DIHA: deisopropylhydroxyatrazine amidohydrolase, EAA: N-ethylammelide amidohydrolase, TH: s-triazine hydrolase, CAH: cyanuric acid hydrolase, BH: biuret hydrolase, AH: allophanate hydrolase.)

Initially it was believed that the oxidative-hydrolytic pathway was the dominant degradation pathway in soil, due to the detection of the dealkylated metabolites Desethylatrazine and Desisopropylatrazine in soil samples. It was further believed that chemical rather than biological processes were responsible for atrazine dechlorination and the production of hydroxyatrazine in soil²¹¹. No single bacterium able to perform the full oxidative-hydrolytic degradation pathway of atrazine to cyanuric acid has been identified^{210,211}, until recently²¹². In contrary, various bacterial strains enabling hydrolytic dechlorination of atrazine to hydroxyatrazine and cyanuric acid have been identified in the 1990s from geographically widespread samples^{213–215}, shifting the paradigm regarding the significance of the hydrolytic biodegradation of atrazine in the environment²¹¹. More recently, metagenomic analysis indicated that the hydrolytic pathway is predominant in ecological wastewater treatment²¹⁶.

With this understanding, the project exclusively focused on the hydrolytic biodegradation pathway, not taking into consideration other pathways.

Among the many atrazine biodegrading microorganisms identified, *Pseudomonas sp.* ADP and *Arthrobacter aureescens* TC1 have been studied intensively and serve as model strains for the biodegradation of atrazine^{217–219}. *Pseudomonas sp.* ADP harbours the *atzA,B,C,D,E,F* set of genes, enabling complete atrazine mineralisation through the hydrolytic pathway²²⁰.

Besides the *atzA,B,C* genes²²¹, *Arthrobacter sp.* has been found to harbour the alternative genes *trzN-atzB,C*^{217,222,223}. Atrazine biodegradation to hydroxyatrazine is catalysed by atrazine chlorohydrolase, which is encoded by *atzA* or *trzN*. This initial biodegradation step of the upper hydrolytic pathway is followed by the biodegradation of hydroxyatrazine to N-isopropylammelide catalysed by hydroxyatrazine ethylaminohydrolase, which is encoded by *atzB*. The subsequent biodegradation to cyanuric acid is catalysed by N-isopropylammelide isopropylamidohydrolase, encoded by *atzC*^{210,217}. All degradation steps and associated enzymes are presented in Figure 17. *Arthrobacter* FM326, was recently observed to completely mineralise atrazine to carbon dioxide and ammonia via the hydrolytic pathway, expressing *trzN*, *atzB* and *atzC*²¹⁷. While *atzB* and *atzC* are found in nearly all known atrazine degraders and show high similarity across different bacterial strains, indicating high conservation²¹⁷, *trzN* functions as a substitute for *atzA* in certain Gram-positive strains^{210,217}. In a recent study, three atrazine-degrading isolates were examined. One isolate, identified as *Solibacillus*, demonstrated higher atrazine degradation rates across a wide range of temperatures and pH conditions, and was found to harbour the *trzN* degradation gene²¹². Three major gene combinations can be identified from atrazine degrading bacteria; *trzN-atzB,C*, *atzABC-trzD* and *atzABCDEF*²²⁴, however, the *atzABCDEF* gene combination was found to be the most common^{217,223,225}.

Cyanuric acid is a common s-triazine biodegradation metabolite^{210,226,227}, but also occurs in the environment naturally, independent from the presence of s-triazines²²⁷. It can be mineralised by enzymes widespread in soil microbial communities^{218,228,229}. Cyanuric acid biodegradation in many bacterial strains is encoded by the *atzD,E,F* operon^{220,230}. Occasionally *trzD*, *trzE*, and *trzF* substitute for *atzD*, *atzE*, and *atzF*^{227,231–233}.

The *atzA,B,C,D,E,F* genes of *Pseudomonas sp.* ADP can be divided into two groups, both of which are situated on the catabolic plasmid pADP-1. Given the relatively recent

introduction of atrazine to the environment, it is assumed, that *atzA,B,C* genes have evolved and disseminated recently. This theory is supported by the finding that *atzA,B,C*, which encode broad-specificity enzymes, are dispersed in an unstable region of the plasmid, and flanked by transposons, indicating their novelty^{220,234}. In contrary, *atzD,E,F* are clustered in an operon and positioned in a stable region of the pADP-1 plasmid, encoding enzymes with a narrow specificity²²⁰. Cyanuric acid is frequently present in nature which has potentially driven the assembly and evolution of the *atzD,E,F* genes. It is hypothesized that this occurred prior to the acquisition of *atzA,B,C* genes through transposition or recombination events²¹⁰.

Given that cyanuric acid is readily biodegraded and the *atzD,E,F* genes are widespread in the environment, making them unsuitable as indicators of atrazine biodegradation potential, the research focus was set on the upper hydrolytic atrazine degradation pathway. The first degradation step from atrazine to hydroxyatrazine (encoded by *atzA* or *trzN*) and the third degradation step of N-isopropylammelide to cyanuric acid (encoded by *atzC*) were targeted with qPCR to identify hydrolytic biodegradation potential of atrazine in samples from DWTP porous filter beds.

MCPA biodegradation

The first biodegradation step of the two phenoxy herbicides 2,4-dichlorophenoxyacetic acid (2,4-D) and MCPA is catalysed by Fe(II)/ α -ketoglutarate-dependent dioxygenases encoded by *tfdA*-like genes²³⁵. Alternative genes such as *cadA*, *rdpA*, and *sdpA*, which are associated with the degradation of 2,4-D and MCPA via the same pathway as *tfdA*-like genes, were not studied further in the course of the project due to their relatively low abundance in the environment^{236–239}. After the initial degradation step (figure 18), which is considered to be rate limiting, complete MCPA mineralisation is encoded by *tfdB,C,D,E,F* genes or homologs, which appear to be relatively common in the environment^{236,239–242}. The initial step of MCPA biodegradation catalysed by *TfdA*-like enzymes is assumed to be rate limiting²³⁹ and has therefore been focused on in the course of this study.

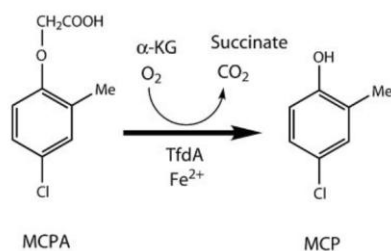


Figure 18 shows the first and rate-limiting step of MCPA biodegradation, catalysed by Fe(II)/ α -ketoglutarate-dependent dioxygenases. Figure: Baelum et al. (2006)

tfdA-like genes are divided into three sub-groups; group one *tfdA* found in *beta*- and *gamma*-*proteobacteria* and groups two and three *tfdAa* found in *alpha*-*proteobacteria*^{243–246}. Canonical *tfdA* genes are well studied and further divided into three classes (I, II, and III)^{243,245,247}, which was first suggested by McGowan et al. 1998²⁴⁵. Similarities between classes were established to be between 76% and 80%. Baelum et al. 2010²⁴⁸ sequenced full lengths coding sequences (CDS) of *tfdA* genes and confirmed the division of *tfdA* genes into three distinct phylogenetic classes. They further suggested establishing two subclasses for class I *tfdA* genes; class I-a and I-b, based on the difference in amino acid sequences. Class I showed 76% and 78% identity with class II and III respectively. Class II and III showed 92% identity, with class II *tfdA* genes exclusively found in *Burkholderia* sp. Class I-a, I-b and class III genes were identified in various families, indicating horizontal gene transfer²⁴⁸. Baelum et al. 2006, 2008^{242,249} provided strong evidence, that *tfdA* class III harbouring bacteria are the main drivers of MCPA biodegradation, as opposed to strains harbouring *tfdA* class I and II, which did not show enhanced gene expression in the presence of MCPA^{242,249}.

Unlike initially believed, *tfdAa* genes found in *alpha*-*proteobacteria* encode Fe(II)/ α -ketoglutarate-dependent dioxygenases²⁴³, which catalyse a biodegradation pathway similar to that mediated by *tfdA* genes. *tfdA* genes found in *beta*- and *gamma*-*proteobacteria* and *tfdAa* genes found in *alpha*-*proteobacteria* showed 56 to 60% similarity, indicating a common ancestor and subsequent divergent evolution²⁴³. *tfdA* genes of classes I, II and III are more similar to each other than to *tfdAa* genes, which form a distinctive cluster²⁴³.

tfdAa genes are widely found in the environment and have been isolated from pristine soils not previously exposed to phenoxy herbicides, indicating an original function of

the *tfdAa* genes in the environment other than phenoxy herbicide degradation^{243,244}. Taking into account the properties of TfdAa Fe(II)/ α -ketoglutarate-dependent dioxygenases, which exhibit specificity for non-chlorinated compounds, Itoh et al. 2002²⁴³ proposed the hypothesis that *tfdA* genes evolved from ancestral *tfdAa* genes and ultimately gained enhanced specificity for the highly chlorinated phenoxy herbicide 2,4-D. Zapras et al. 2010²⁴⁴ detected highly diverse *tfdAa* gene sequences in soils, including soils not previously enriched with phenoxy herbicides. *tfdAa* genes outnumbered *tfdA* genes by several orders of magnitude, in line with the hypothesis that phenoxy herbicide degradation is not the primary function of *tfdAa* genes²⁴³.

Materials and Methods

This project aimed to identify and optimize qPCR primer sets targeting functional genes essential for the biodegradation of atrazine and MCPA in sand and GAC samples collected from DWTPs in Brazil and Scotland, known to be historically exposed to one or both of the two pesticides. The research was structured into four work packages: the first three packages concentrated on selecting optimal primer sets, and in the final step, the selected primer pairs were applied to environmental samples (figure 19).

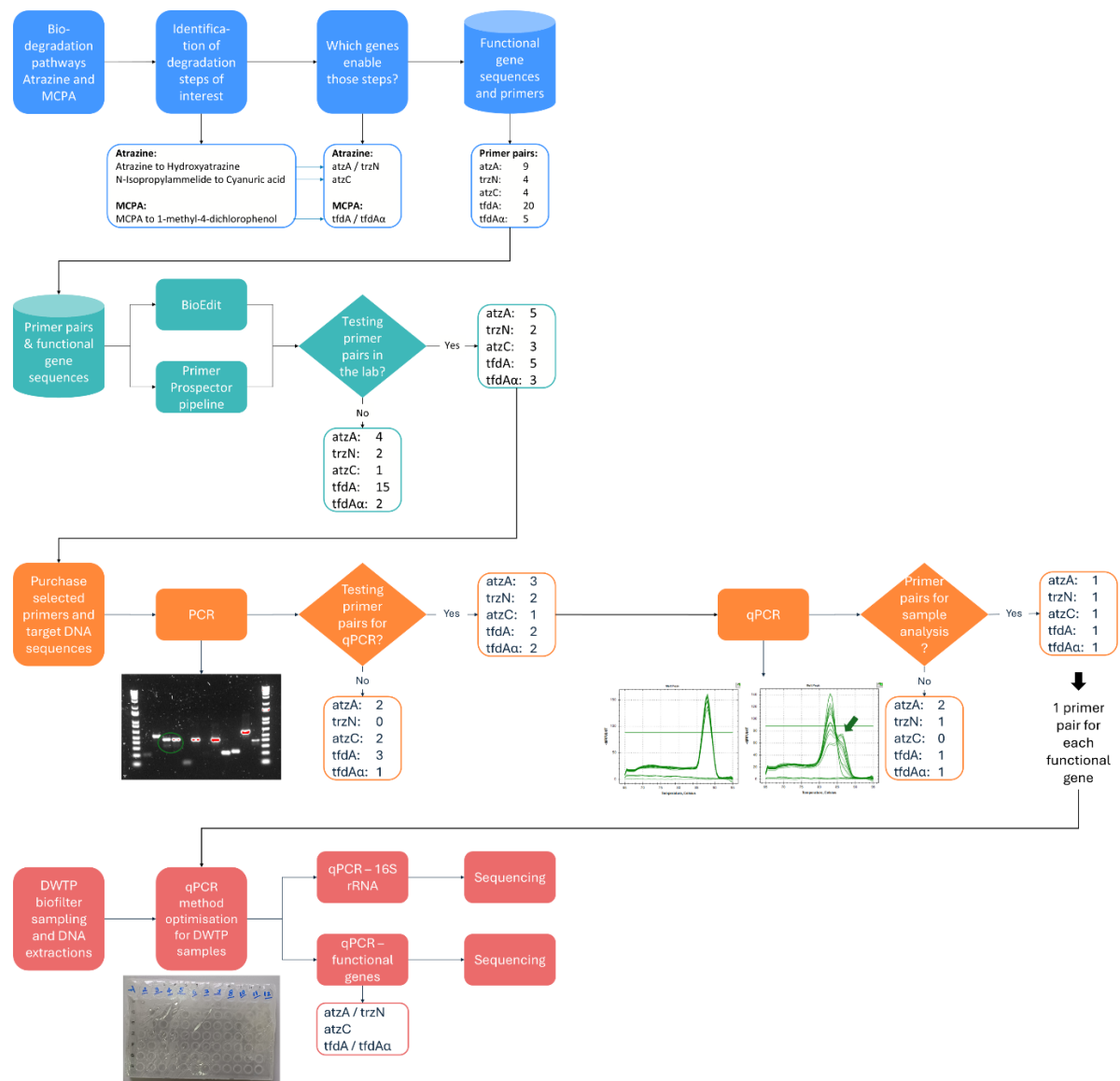


Figure 19 shows the primer optimisation workflow. The process can be divided into 4 packages, whereby the first three focus on the selection and optimisation of primers. In the last step, the selected primers are applied to environmental samples.

Systematic literature review

A systematic literature review was performed to retrieve atrazine (*atzA*, *trzN*, *atzC*) and MCPA (*tfdA*, *tfdAa*) primer sets from peer-reviewed publications. The **Web of Science™** database was searched using the key search terms (*atzA* OR *atzC* OR *trzN* OR *tfdA*) AND (primer OR PCR). The timespan covered publications from 01.01.1980 to 31.03.2021.

Reference gene database curation

Reference gene sequences were used to evaluate primer performance in-silico.

Despite their relevance in the environment and the associated research interest, no curated gene sequence databases were available for *atzA*, *trzN*, and *atzC* (status 2021). Full length CDS for *atzA*, *trzN*, and *atzC* were retrieved from the National Centre for Biotechnology Information (NCBI), a resource for molecular biology information. Reference genes were used as a proxy for the in-silico evaluation of the performance of selected primer sets with Primer Prospector.

The Functional Gene Pipeline and Repository (FunGene) provided databases of numerous common ecofunctional genes and proteins²⁵⁰. As of 2021, it contained 60'000+ entries for *tfdA*-like genes, of which 1'424 (*tfdA*) and 386 (*tfdAa*) gene sequences were identified as relevant for primer evaluation. These sequences form the basis for the in-silico evaluation of the corresponding primer sets. The FunGene database is no longer online; information as of July 2024.

In silico primer evaluation

Collected primer sets were evaluated in silico using the BioEdit Sequence Alignment Editor²⁵¹ version 7.2.5 and Primer Prospector, a pipeline for the design and analysis of PCR primers²⁵². The primer analysis module (*analyze_primers.py*, standard settings) of the Primer Prospector pipeline evaluates primer performances against a reference gene database (unaligned, FASTA file). Primers are individually aligned against each target sequence in the database and a weighted score is determined, based on the number of mismatches and gaps identified. The weighted score is based on a penalty-system, with higher penalties given for mismatches and gaps in the 3' end (first 5 nucleotides) of the primer. The standard penalties are the following: + 0.4 per non-3' mismatch, + 1.0 per 3' mismatch, + 3.0 for a final base mismatch, + 1.0 per non-3' gap, and + 3.0 per 3' gap. The sum of all penalties determines the weighted score of the primer, with a high weighted score indicating poor compatibility of the primer and the reference gene sequence. A weighted score of 0 equals perfect alignment.

Primer pairs might align in reverse order, resulting in the reverse primer to align in front of the forward primer (5' to 3'), preventing a PCR product to form. The primer binding orientation of each primer pair was tested for selected reference gene sequences, identifying primer pairs with incorrect primer-binding orientation. Each primer was aligned to a selected reference gene sequence, given in table 6, using the ClustalW

Multiple alignment application²⁵³ in BioEdit with standard settings. The alignment position of each primer was identified. The information on primer binding orientation, amplicon length, and the mean weighted score were used to select most promising primer sets for validation in the laboratory.

PCR and qPCR primer evaluation

Following the in-silico evaluation, the selected primer sets were assessed in the laboratory using endpoint PCR. Oligonucleotide primers were synthesized by Eurofins Genomics Europe Synthesis and reconstituted in DNase/RNase-Free Distilled Water (Invitrogen™, Thermo Fisher Scientific Inc., UK) as described by the producer. *atzA*, *trzN*, *atzC*, *tfdA* and *tfdAa* reference gene sequences (table 6) were purchased from Integrated DNA Technologies (IDT) U.K., Ltd. and reconstituted as specified. Reference gene sequences were amplified by PCR, which was carried out in a total volume of 25 µL using the HotStarTaq DNA Polymerase kit (©QIAGEN). The reaction volume was composed of 19.8 µL Water, 0.5 µL forward primer (10 µM), 0.5 µL reverse primer (10 µM), 0.5 µL dNTPs (10 µM), 0.2 µL HotStarTaq, 2.5 µL 10x PCR buffer, and 1.0 µL DNA. Thermal cycling conditions were chosen based on manufacturers recommendations as follows: initial denaturation at 95 °C for 15 min, followed by 25 cycles of denaturation at 94 °C for 30 sec, annealing at 55 °C for 30 sec, and extension at 72 °C for 30 sec. A final extension step was performed at 72 °C for 10 min. PCR products were visualized with gel electrophoresis (figure 21) to test primer performance and to confirm expected amplicon sizes. The 1% agarose gel was made by melting 0.4 g agarose in 40 mL boiling TAE 1x buffer. After letting the liquid agarose cool for a few minutes, 4 µL SYBR safe was added, the liquid agarose was poured into a running tray and a well comb was added. After letting the agarose cool and solidify for 30 minutes, gel electrophoresis was performed at 90 V for 40 minutes. A 1 Kb Plus DNA Ladder (Invitrogen™, Thermo Fisher Scientific Inc., UK) was run alongside PCR amplicons. Primer sets producing double bands were excluded from further testing.

Following PCR, most promising primer sets were tested for qPCR, which was performed using a Bio-Rad CFX96 Touch Real-Time PCR Detection System and the QuantiTect™ SYBR Green PCR Kit (©QIAGEN). Reactions were carried out in a total volume of 50 µL composed of 25 µL QuantiTect™ SYBR Green PCR Master Mix, 1.5 µL forward primer (10

μM), 1.5 μL reverse primer (10 μM), 20 μL water, and 2 μL DNA. Thermal cycling conditions applied were initial denaturation at 50 °C for 2 min and 95 °C for 15 min, followed by 40 cycles of denaturation at 94 °C for 15 seconds, annealing at 60 °C for 30 seconds, and extension at 72 °C for 30 seconds. Fluorescence data was collected at the end of each cycle, and the threshold cycle (Ct) values were determined automatically by the Bio-Rad CFX Manager 3.1 software. Melt curve analysis was performed at the end of the qPCR run, ranging from 65 °C to 95 °C with 0.5 °C increments every 5 seconds to verify assay specificity (single peak melt curve).

Stock solutions of synthetic IDT reference genes (table 6) were diluted to a 10 mM working solution in DNase/RNase-Free Distilled Water. Gene copy numbers were calculated using ([Copy Number Calculator | Technology Networks](#)), and the working solutions were diluted to a concentration of 10⁸ copies/μL, followed by a 10-fold serial dilution (10⁸ to 10² copies/μL). Each concentration was amplified by qPCR in triplicates and Ct values were used to construct the standard curve. Standard curves for all primer sets and their corresponding reference gene sequences were constructed the same way. Standard curve parameters (efficiency, slope, y-intercept and R²) are reported in table 7. Final primer sets for the analysis of environmental samples were chosen based on standard curve parameters.

Application of selected primers to samples from Scottish and Brazilian DWTP filter medium

Samples of filtration medium of two Scottish (DWTP A and DWTP B) and two Brazilian (DWTP C and DWTP D) DWTPs were taken between December 2022 and August 2023. DWTPs C and D are located in rural areas of Minas Gerais State, and the area around DWTP D in particular is characterised by agriculture. For DWTPs A, C and D, filter samples were taken from the top and bottom of each RGF in triplicate, corresponding to six samples per plant. From DWTP B three RGF and three GAC samples were taken. All samples were collected in sterile sampling containers and stored in the refrigerator as soon as arriving in the laboratory and prior to DNA extraction.

DNA extractions

DNA was extracted from all DWTP biofilter samples using the FastDNA™ SPIN Kit for Soil (MP Biomedicals), following the extraction protocol optimised and described in chapter

3.1., using a bead beater (Scotland) or a vortex (Brazil). DNA extraction in Brazil was performed by Bruna Coelho Lopes, who is a post-doctoral resident at the Federal University of Minas Gerais. Extracted DNA was stored at -80 °C until shipment to the UK. DNA samples were shipped on dry ice and stored at -20 °C upon arrival and prior to sample evaluation in Scotland. DNA concentrations were quantified using the Qubit™ dsDNA HS Assay Kit (Invitrogen™).

qPCR optimisation – based on 16S

Prior to testing DNA extracts for the presence of the targeted functional genes, the ideal dilution factor of DNA extracts was determined. The presence of molecules that affect the in vitro DNA polymerisation or the fluorescence signal may negatively affect the qPCR performance. These molecules are called PCR inhibitors, and can stem from the sample matrix, target cells, or the reagents used while preparing the samples²⁵⁴. A common strategy to mitigate the negative effects of these PCR inhibitors is dilution of the DNA extracts. At ideal dilution, the concentration of the PCR inhibitors is small enough to no longer interfere with the qPCR, while the amount of target DNA remains high enough to be amplified and quantified²⁵⁵.

DNA extracts from biofilter samples from Scotland and Brazil were diluted 1:1, 1:5, 1:10, 1:50, and 1:100 (v/v) with DNase/RNase-Free Distilled Water. Then bacterial 16S rRNA qPCR was performed using the QuantiTect™ Probe PCR Kit (©QIAGEN). The final reaction volume of 20 µL consisted of 10 µL QuantiTect™ Probe PCR Master Mix, 1.8 µL forward primer (1369F²⁵⁶, 10 µM), 1.8 µL reverse primer (1492R²⁵⁶, 10 µM), 0.4 µL probe (1389P²⁵⁶, 10 µM), 4 µL water, and 2µL DNA. The qPCR thermal cycle applied was initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 94 °C for 30 seconds, and combined annealing/extension at 60 °C for 30 seconds.

Quantification of atzA, trzN, atzC, tfdA and tfdAa genes from environmental samples

atzA, *trzN*, *atzC*, *tfdA*, and *tfdAa* genes were quantified from RGF and GAC samples from four different DWTPs, two in Scotland and two in Brazil, using the selected qPCR primer sets (*atzA* 01F & R, *trzN* 03F & R, *atzC* 02 & 01R, *tfdA* 03F & 18R, and *tfdAa* 01F & 04R) and qPCR conditions described in the section above. Brazilian samples were diluted 01:10 with DNase/RNase-Free Distilled Water, whereas Scottish samples were diluted 01:05 to achieve optimal qPCR results, as established beforehand. Five rounds

of qPCR were performed (one for each targeted gene) and each plate consisted of a calibration curve in duplicates, all DNA extracts from environmental samples (n=24 (8x biological triplicates)) in technical duplicates and two no template controls (NTC). It must be noted, that DNA extracts shipped from Brazil unfortunately evaporated an unknown amount of water during storage or transport, which prevented the accurate quantification of targeted genes within these samples. The only statement that can be made is whether or not the targeted gene was present in the samples.

Results

Published qPCR primer sets targeting selected functional genes that are required for atrazine and MCPA biodegradation were evaluated. Optimal primers were chosen for the quantification of target genes in environmental samples collected from a total of four DWTPs in Scotland and Brazil.

Collection of primer sets from the literature

The systematic literature search returned 204 publications, from which 79 unique primers were identified within the main text or supplementary materials. Table 5 lists all primer sets acquired from the literature. A total of 18 (*atzA*), 6 (*trzN*), 9 (*atzC*), 38 (*tfdA*), and 8 (*tfdAa*) individual primers were gathered and evaluated. Probe based primers (n=3) were excluded from analysis, as the aim of the project was to perform dye based (SYBR Green) qPCR. Table 6 lists the reference gene sequences selected for primer alignment in BioEdit and to test selected primer sets in the laboratory.

Table 5 List of primers collected from peer reviewed literature and their Weighted Scores generated with the Primer Prospector pipeline.

Name	Ref.	Sequence 5 -> 3	Weighted Score
Atz A01f	257,258	ACGGGCGTCAATTCTATGAC	4.70
Atz A01r	257	CACCCACCTCACCATAGACC	4.97
Atz A02f	213	CCATGTGAACCAGATCCT	3.68
Atz A02r	213	TGAAGCGTCCACATTACC	3.50
Atz A04f	259	GCACGGACGTCAATTCTA	4.41

Atz A04r	259	CGCATTTCCTTCAACTGTC	3.90
Atz A05f	260	GGCGATGAGACCGGAGGACG	4.87
Atz A05r	260	TGAAGCGTCCACATTACC	3.50
Atz A06f	261	GTTAGCCACCATGTTGCCCC	5.10
Atz A06r	261	TGCAAACGCTCAGCATCCAG	5.00
Atz A07f	262	TTGCGGTGCAGGTTTTTCGATG	4.70
Atz A07r	262	TGCAGCAACGGCGTCATTTTC	4.40
Atz A08f	263	AATTCTATGACTGGCTGTTC	4.54
Atz A08r	263	CGCACAATACAACCTCAC	3.57
Atz A09f	264	CCGAGCTCATGCAAACGCTCAGCA	5.00
Atz A09r	264	AATCTAGACTAGAGGCTGCGCCAAGC	6.00
Atz A11f	265	GCACGGACGTCAATTCTAGTACGGTCATCATCTGACAC	9.70
Atz A11r	265	CGCATTTCCTTCAACTGTCAATACATCAAACGCCGCGAC	9.90
trzN 01F	266	CACCAGCACCTGTACGAAGG	0.22
trzN 01R	266	GATTCGAACCATTCCAAACG	0.00
trzN 03F	267	GCGACGGGAAGTTCGGTC	0.00
trzN 03R	267	CGAGCGTCATCGATGACCT	0.00
trzN 04F	262	ATGATCCTGATCCGCGGACTGA	0.00
trzN 04R	262	CTACAAGTTCTTGGGAATGAGTG	0.00
AtzC 01R	257	TCCCCCAACTAAATCACAGC	4.61
AtzC 02F	213	GCTCACATGCAGGTACTCCA	4.96
AtzC 02R	213	GTACCATATCACCGTTTGCCA	5.08
AtzC 03F	268,269	AGTCAGCGAAGGGCGTAGGTATCA	6.43
AtzC 03R	268,269	GACAAATCCGGGAGACACAAGGTT	6.42
AtzC 04F	260	AATCCTTGGATATGGG	3.38

AtzC 04R	260	AAATCTCTGATATTGTCCGAAG	5.66
AtzC 05F	258	GCTCACATGCAGGTACTCCA	4.96
AtzC 05R	258	TCCCCCACTAAATCACAGC	4.61
tfdA 01F	242	GCATACGACGCGCTGCCTCG	5.29
tfdA 01R	242	CTTCGGCCACCGGAAGGCCT	4.83
tfdA 02F	242	GAGCACTACGCRCTGAAYTCCCG	3.23
tfdA 02R	242	CTTCGGCCACCGGAAGGCCT	4.83
tfdA 03F	247	GAGCACTACGCRCTGAAYTCCCG	3.23
tfdA 03R	247	SACCGGMGGCATSGCATT	3.38
tfdA 07F	248	GTGAGCGTCGTCGCAAAT	5.65
tfdA 07R	248	GCATCGTCCAGGGTGGTC	4.84
tfdA 08F	248	TGAGCATCAATTCCGAATACC	5.93
tfdA 08R	248	AAGACTGACCCCGTGGACT	4.99
tfdA 09F	248	TGAGCATCACTTCCGAATACC	5.34
tfdA 09R	248	ACAGCGTCGTCCAACGTC	4.96
tfdA 10F	247	GAG CAC TAC GC(AG) CTG AA(CT) TCC CG	3.23
tfdA 10R	247	GTCGCGTGCTCGAGAAG	3.46
tfdA 12F	270	CAG CA(G/A) ATC GCC TT(T/C) GCG	3.74
tfdA 13F	271	CGACACCGAGTTCTGC gayatgmngc	3.89
tfdA 13R	271	CGCAGCGGTTGtcccanatnac	3.29
tfdA 14F	272	AAC GCAGCG(G/A)TT(G/A)TCCCA	5.07
tfdA 14R	272	ACGGAGTTCTG(C/T)GA(C/T) ATG	3.76
tfdA 15F	243	AC(C/G)GAGT TCTG(C/T)GA(C/T) ATG	3.28
tfdA 15R	243	(A/G)ACGCAGCG(G/A)TT(G/A)TCCC A	4.63
tfdA 16F	273	GACGATCACGTTGGGATGCAGC	4.58
tfdA 16R	273	AAAAGAG GATG AAGGGGATTGCGA	7.23

tfdA 17F	274	CTAGACGACGGCATCGTCCAG	5.30
tfdA 17R	274	GTGAGCGTCGTCGCAAATCC	5.36
tfdA 18F	275	CGAAGACATCGACCTTCGAG	5.67
tfdA 18R	275	GCC GAT GAA GAG AAA CTT GC	3.65
tfdA 19F	275	CGTTCGTAGTGCAGGTGC	4.80
tfdA 19R	275	GGTGCGTACATCTTGTGG	5.65
tfdA 20F	244	CCTTCATCCWCTTTTCGYCG	4.81
tfdA 20R	244	GCTCGGCGMAGYTCACGC	3.35
tfdA 21F	244	CTSTGGCATTSCGACAGYTC	3.58
tfdA 21R	244	CSGSGTGSGTICGVYCAG	NA -contains Inosin
tfdA 22F	235	AGCGATGGCGGTACACGAACTGGCG	5.99
tfdA 23F	236	TGCGTCCGCGGTGAAGA	5.33
tfdA 23R	236	TGCGTCCGCGGTGAAGA	4.11
tfdA 24F	236	TTTCAAGAAGGAACTGCAGGG	4.15
tfdA 24R	236	AAACTTCGGTTGCGTCGCAT	4.15
tfdAa 01F	243	AC(C/G)GAGTTC(G/T)(C/G)CGACATGCG	1.65
		OR	
		ACSGAGTTCKSCGACATGCG	
tfdAa 01R	243	(A/G)ACGCAGCG(G/A)TT(G/A)TCCC A	2.00
		OR	
		RACGCAGCGRTTRTCCCA	
tfdAa 02F	244	GGCGTCGATCTGCGCAAGCC	5.44
tfdAa 02R	244	GTTGACGACGCGCGCCGACA	5.26
tfdAa 03F	244	ACSGAGTTCGSIGAYATSC	NA -contains Inosin

tfdAα 03R	244	CAGCGGTTGTCCCACATCAC	1.97
tfdAα 04F	244	ACSGARTTCKSIGACATGC	NA -contains Inosin
tfdAα 04R	244	AGCGGTTGTCCCACATCAC	1.91

Table 6 lists the reference gene sequences used to test primer alignments. Full-length sequences are provided in Table 10 (Appendix, Chapter 5)

Gene	Accession (NCBI GenBank)	Organism
<i>atzA</i>	U55933.1	<i>Pseudomonas</i> sp.
<i>trzN</i>	FJ161692.1	<i>Pseudomonas</i> AD39
<i>trzN</i>	DQ989289.1	<i>Arthrobacter</i> sp. AD 25
<i>atzC</i>	KF453509.1	<i>Arthrobacter</i> sp. (DNS10)
<i>tfdA</i>	EU827465.1	<i>Pseudomonas</i> sp. TFD39
<i>tfdAα</i>	AB074492.1	<i>Alpha-Proteobacterium</i> HW13

In-silico primer evaluation

The in-silico analysis of primer pairs collected from publications was performed using the primer analysis module of the Primer Prospector pipeline²⁵². This function establishes a weighted score for each primer individually, evaluating their compatibility with the targeted gene sequence. Weighted scores are given in table 5.

atzA

atzA primers were tested in-silico against a set of 29 reference gene sequences. Weighted scores ranged from 3.5 (*atzA* 02R) to 9.9 (*atzA* 11R) (table 5). In a first step, primers that performed poorly in comparison with the rest of the primers were removed from further analysis. An initial line was drawn at a weighted score of five or higher, which excluded *atzA* 06F&R, *atzA* 09F&R, and *atzA* 11F&R from further analysis. Primer pair *atzA* 07F&R spans almost the entire gene, which would produce an amplicon too big for qPCR analysis, which is why it was not taken into consideration any further. This

left atzA 01F&R, atzA 02F&R, atzA 04F&R, atzA 05F&R, atzA 08F&R for evaluation in the laboratory.

trzN

trzN primers were evaluated in-silico against 12 reference gene sequences. Reference gene sequences showed high similarity when aligned with BioEdit, with many conserved regions across all 12 reference genes. These conserved regions and only minor variations between sequences are ideal for the development of qPCR primers. All except one primer showed perfect alignment with a weighted score of 0 and 0.22 respectively for *trzN* 01F (table 5). *trzN* 04F&R were designed to span the entirety of the *trzN* functional gene, producing an amplicon of 1360 bp, which isn't ideal for qPCR. *trzN* 04F&R has not been analysed in the laboratory, but *trzN* 01F&R and *trzN* 03F&R were taken to the next step.

atzC

The in-silico evaluation of primers targeting *atzC* gene sequences against 59 reference gene sequences revealed, that weighted scores for all primers ranged between 3.38 and 6.42 (table 5). The weighted score of *atzC* 04F was the lowest, indicating fewer nucleotide mismatches with reference gene sequences and better suitability for PCR and qPCR quantification of the targeted gene. *atzC* 03F&R showed the highest weighted score and were also found to bind in reverse orientation relative to the gene sequence. Incorrect orientation of the primers prevents the amplification of a PCR product, and the primer pair has been removed from further analysis in the laboratory. Similarly *atzC* 05F&R, although having a lower weighted score, did bind reversely for some targeted genes and has been removed from further analysis. PCR and qPCR performances of *atzC* 02F&01R, *atzC* 02F&02R and *atzC* 04F&R were tested in the laboratory.

tfdA

A total of 38 individual primers were tested in-silico against 1088 *tfdA* reference gene sequences. Weighted scores ranged from 3.23 (*tfdA* 03F) to 7.23 (*tfdA* 16R) (table 5). In an initial step, primers with weighted scores above 4 were excluded from further evaluation. Two exceptions were made for *tfdA* 15R (WS 4.63) and *tfdA* 02R (WS 4.83), based on alignment results in BioEdit. A few more primers were excluded from further analysis, even though their weighted score was below 4, for the following reasons: *tfdA*

14R (WS 3.76) showed poor alignment in BioEdit, tfdA 10R (WS 3.46) could not be purchased from the supplier because the sequence was too short for synthesis, and tfdA 20R (WS 3.35) would generate amplicons that are too long for qPCR, regardless of which forward primer it would be combined with. The following primer pairs were thought to be most promising for PCR and qPCR analysis in the laboratory: tfdA 03F&02R, tfdA 03F&R, tfdA 12F&15R, tfdA 13F&R, tfdA 15F&R, tfdA 03F&18R.

tfdAa

Four primer sets targeting the *tfdAa* gene sequence were run against 386 reference gene sequences, using PrimerProspector. Weighted scores are listed in table 5. Primer tfdAa 03F and tfdAa 04F both contain Inosin (I), which could not be processed by the PrimerProspector pipeline. These two primers were therefore excluded from further analysis. Of the remaining primers, tfdAa 02F&R returned relatively higher weighted scores (WS 5.44 and 5.26), excluding them from further analysis too. Primers tfdAa 01F&R, 03R, and 04R returned relatively similar weighted scores. The primer set tfdAa 01F&R was chosen for analysis in the laboratory, as it was the initially published primer set. Primers tfdAa 03R and tfdAa 04R, were designed based on tfdAa 01R, binding the target sequence in the same region, but having slightly adjusted nucleotide composition. tfdAa 03R and 04R differ only in 1 nucleotide, and tfdAa 03 was chosen for further analysis, forming a second primer set with tfdAa 01F.

PCR and qPCR primer evaluation

Following the in-silico analysis of primers collected from literature, the most promising primer sets identified were tested in the laboratory. Endpoint PCR was performed to confirm amplicon length and to exclude primer sets that amplify non-target DNA fragments, followed by qPCR.

atzA

Based on in-silico analysis, five primer sets (atzA 01F&R, atzA 02F&R, atzA 04F&R, atzA 05F&R, atzA 08F&R) were chosen for PCR evaluation. Of those selected primer sets, atzA 04F&R and atzA 05F&R produced double bands when amplifying the reference gene (*Pseudomonas sp.*, table 6) using PCR (figure 21). They were excluded from further evaluation steps. Evaluation of qPCR performance of the three remaining primer sets (atzA 01F&R, atzA 02F&R, atzA 08F&R) revealed that atzA 01F&R performed best. atzA

01&F showed high efficiency (94.8%) and produced an amplicon of 187 bp, which is generally considered an ideal lengths for qPCR and sequencing (table 7). atzA 01F&R was selected for the analysis of environmental samples.

trzN

Both primer sets (trzN 01F&R and trzN 03F&R) tested against two reference genes (*Pseudomonas* AD39 and *Arthrobacter* sp. AD25, table 6) amplified the target gene sequences without showing double bands when performing PCR (figure 21). Given the shorter amplicon length (204 bp) and slightly higher qPCR efficiency of 82%, trzN 03F&R was selected for the analysis of environmental samples (table 7).

atzC

Three primer sets (atzC 02F&01R, atzC 02F&02R and atzC 04F&R) were selected to amplify the reference gene (*Arthrobacter* sp. (DNS10), table 6) using PCR. atzC 02F&02R and atzC 04F&R produced double bands (figure 21) and were excluded from further evaluation. atzC 02F&01R achieved an efficiency of 89% during qPCR and was selected for the analysis of environmental samples (table 7).

tfdA

Six primer sets (tfdA 03F&02R, tfdA 03F&R, tfdA 12F&15R, tfdA 13F&R, tfdA 15F&R, tfdA 03F&18R) were selected for PCR amplification of the reference gene (*Pseudomonas* sp. TFD39 TfdA, table 6). tfdA 03F&02R, tfdA 03F&03R and 03F&18R did not show double bands and produced shorter amplicons that are better suited for qPCR (figure 21). However, when performing qPCR, tfdA 03F&R amplified an unwanted product as visualised by a double peak in the melt curve (figure 20). This primer set was excluded from further evaluations. The remaining primer sets tfdA 03F&R and tfdA 03F&18R performed equally well, both achieving very high efficiencies of 100.4% and 102.8% respectively (table 7). tfdA 03F&02R was initially chosen for the analysis of environmental samples, however this primer set amplified a lot of background DNA fragments that weren't the targeted gene sequence. qPCR analysis of environmental samples was repeated using the tfdA 03F&18R primer set instead.

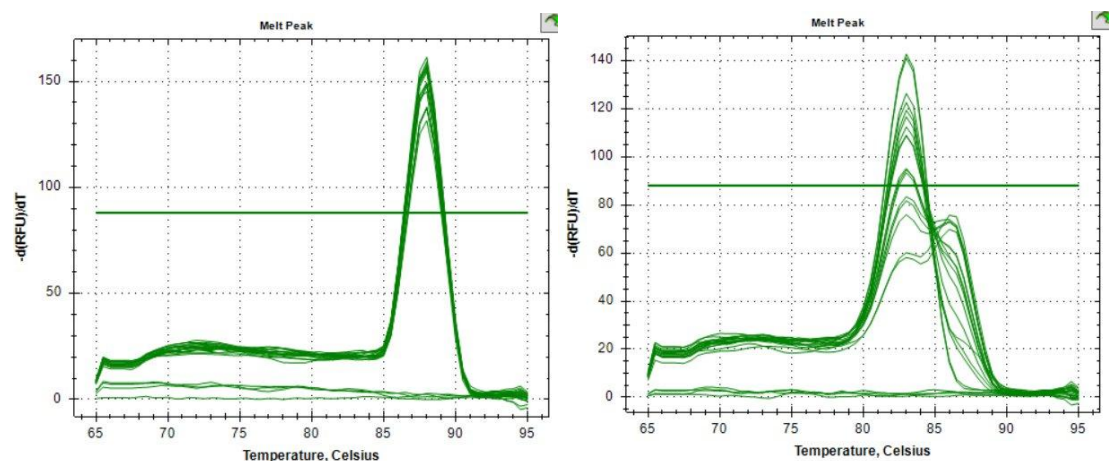


Figure 20 shows two images of different qPCR runs. The image on the left depicts an ideal qPCR melt curve with one distinct peak, whereas the image on the right depicts the melt curve with a double peak that was created when using the primer set *tfdA* 03F&R to amplify the reference sequence. The double peak indicates the amplification of an unwanted product.

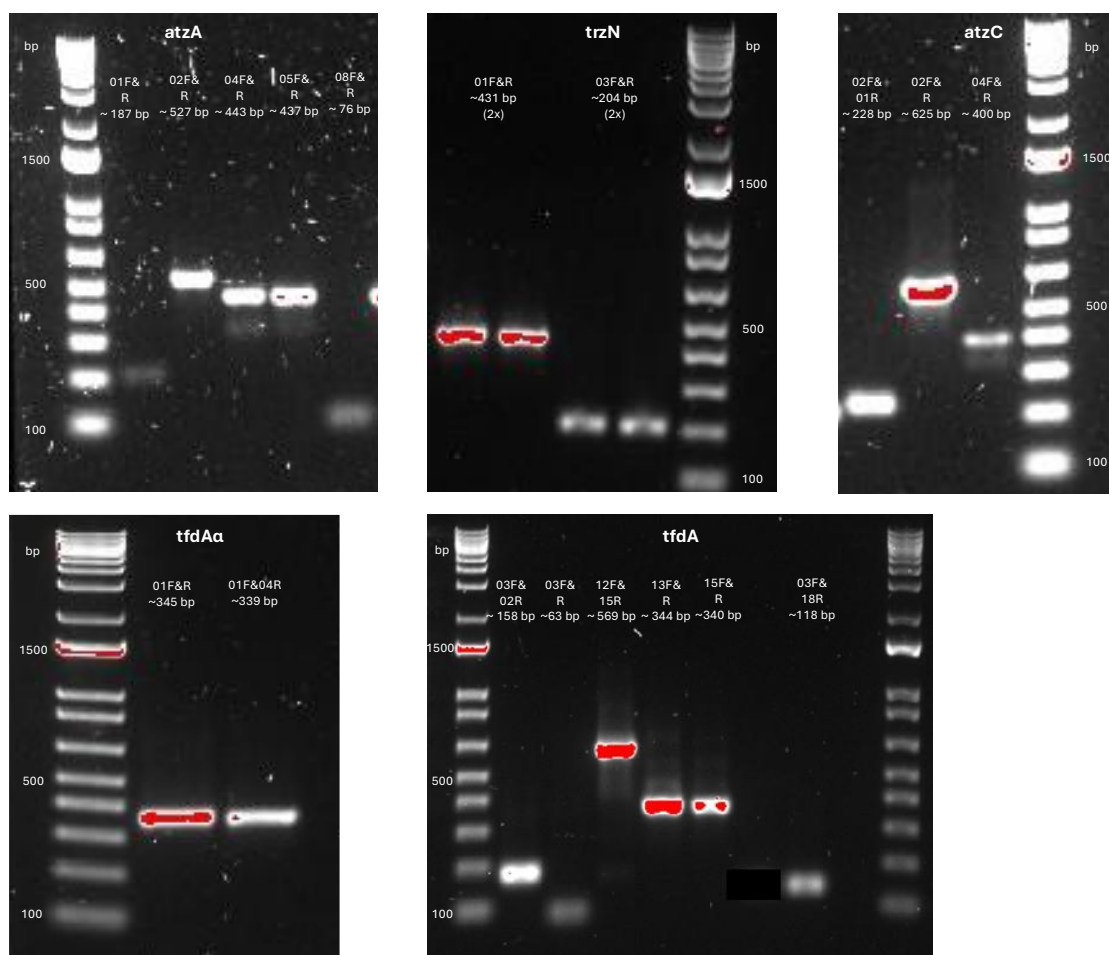


Figure 21 shows images of gels created by gel electrophoresis of PCR amplicons. Primer pairs are named within the images and expected amplicon sizes are given.

tfdAa

Two selected primer sets (tfdAa 01F&R and tfdAa 01F&04R) performed equally well during PCR and qPCR amplification of the reference gene (*Alpha-Proteobacterium* HW13, table 6) (figure 21), with qPCR efficiencies being 89.5% and 90.9% (table 7). Both primer sets were applied for the analysis of environmental samples.

Table 7 provides an overview of qPCR performances of selected primer pairs.

	Ct averaged										
	trzN 01F&R	trzN 03F&R	atzA 01F&R	atzA 02F&R	atzA 08F&R	atzC 02F&01R	tfdA 03F&02R	tfdA 03F&R	tfdA 03F&18R	tfdAa 01F&R	tfdAa 01F&04R
1.00E+08	8.6	6.5	6.9	13.1	8.5	6.0	N/A	N/A	N/A	13.3	7.4
1.00E+07	12.9	11.4	10.9	17.3	13.1	9.4	8.0	9.2	8.8	N/A	N/A
1.00E+06	16.9	15.3	14.7	21.6	17.1	13.9	12.0	12.8	12.5	20.5	15.2
1.00E+05	20.9	18.9	17.8	24.7	20.1	17.2	15.5	16.1	15.9	24.0	18.6
1.00E+04	24.7	22.5	21.3	29.8	23.7	20.6	18.8	19.4	19.1	27.3	22.1
1.00E+03	28.6	26.1	24.5	33.3	27.0	23.8	22.0	22.5	22.1	31.1	25.8
1.00E+02	32.1	30.2	28.1	37.7	30.8	27.9	25.3	25.9	25.3	34.6	28.8
1.00E+01	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	38.9	32.5

Amplicon length	431	204	187	527	76	228	158	63	118	345	339
Meltcurve	OK	OK	OK	OK	OK	OK	OK	Double Peak	OK	OK	OK
Efficiency %	79.7	82.0	94.8	76.3	89.9	89.0	96.8	100.4	102.8	89.5	90.9
R2	0.9993	0.9977	0.9979	0.9908	0.9928	0.9972	0.9978	0.9989	0.9976	0.9921	0.9987

qPCR optimisation

To mitigate the potential negative effects of PCR inhibitors, different DNA sample to water ratios were tested, aiming to dilute the PCR inhibitors to a concentration that is small enough, to no longer interfere with the qPCR signal. The ideal dilution was found to be 01:05 for Scottish samples and 01:10 for Brazilian samples.

*Quantification of *atzA*, *trzN*, *atzC*, *tfdA* and *tfdAa* genes from environmental samples*

Following selection and optimisation, the best performing primer sets were applied to quantify targeted genes in environmental samples that were collected from four DWTPs, two in Brazil and two in Scotland.

Of the atrazine degrading functional genes, neither *atzA* nor *atzC* were amplified during qPCR in any of the environmental samples. A qPCR product, however, was found to be present in all six samples collected from the RGF of DWTP D (figures 22 and 23). The melt curves and melt peaks shown in figure 23 reveal two distinct peaks, one around 87 °C and another around 90 °C. The left-hand peak corresponds to the positive control (standard), while the right-hand peak was only observed in biofilter samples from DWTP D. This suggests that a qPCR product was amplified in the environmental samples, but with a different melting temperature compared to the standard.

Of the two functional genes encoding MCPA degradation, *tfdA* was not detected in any of the samples, but *tfdAa* was amplified from each of the 24 samples, from both, Scottish and Brazilian DWTPs (figure 22). Biofilter samples of DWTP A were taken from the top ($4.3\text{E}+05$ *tfdAa* gene copies/sample) and the bottom ($2.6\text{E}+05$ *tfdAa* gene copies/sample), showing higher quantity of *tfdAa* functional gene copies at the top versus the bottom of the biofilter. Since the samples from Brazil evaporated an unknown amount during either transport or storage, functional genes present in Brazilian samples could not be quantified accurately. The data is presented in the Appendix.

	Atrazine			MCPA		Exposure History	
	atzA	trzN	atzC	tfdA	tfdAa	Atrazine	MCPA
DWTP A (SCOT) 6 samples	✗	✗	✗	✗	✓	N/A	< 0.002 to 0.218 µg/L
DWTP B (SCOT) 6 samples	✗	✗	✗	✗	✓	N/A	N/A
DWTP C (BR) 6 samples	✗	✗	✗	✗	✓	< 0.01 µg/L	N/A
DWTP D (BR) 6 samples	✗	✓	✗	✗	✓	< 0.01 µg/L	N/A

Figure 22 provides an overview of samples taken from four different DWTPs, two in Scotland (DWTP A and B) and two in Brazil (DWTP C and D) and whether or not one of the functional genes *atzA*, *trzN*, *atzC*, *tfdA*, or *tfdAa* was detected in the respective samples. It further provides information on the exposure history of these DWTPs to the two pesticides atrazine and MCPA.

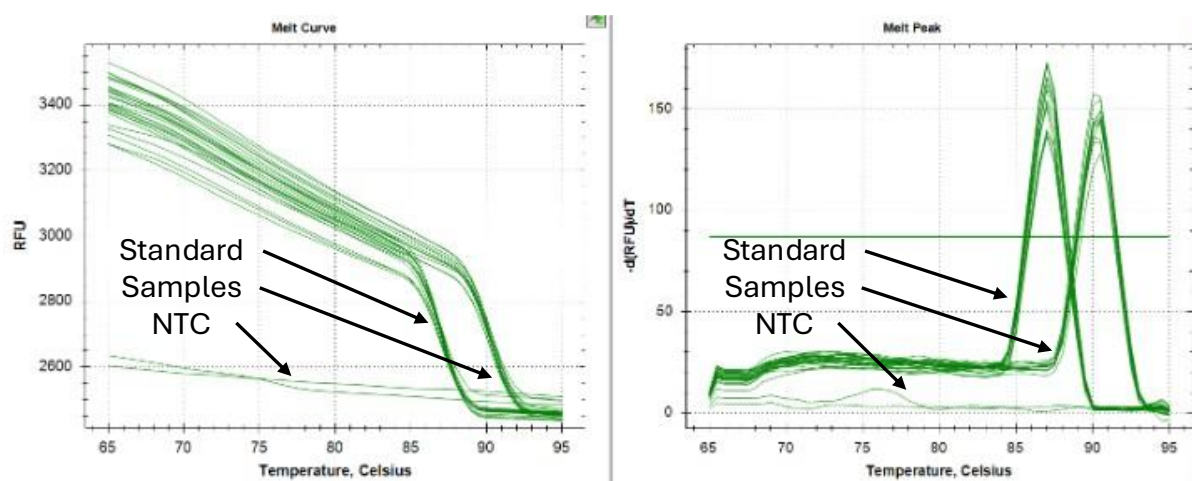


Figure 23 qPCR melt curves and melt peaks show no template controls (NTC), *trzN* standards in concentrations ranging from 10^8 to 10^2 gene copies/µL (Standard), and *trzN* functional genes amplified from environmental samples (Samples) from DWTP D.

Discussion

To establish the biodegradation potential of selected pesticides in biofilters of DWTPs, quantification of relevant functional genes within the microbial community is essential. For atrazine, most relevant functional genes of the hydrolytic biodegradation pathway were established to be *atzA* and *trzN* which encode the first degradation step of atrazine to hydroxyatrazine, and *atzC* which encodes the third degradation step of N-isopropylammelide to cyanuric acid. The mineralisation of cyanuric acid is widespread in the environment and is therefore not suitable as an indicator for the biodegradation of atrazine. The first biodegradation step of MCPA is rate limiting and catalysed by Fe(II)/α-ketoglutarate-dependent dioxygenases encoded by *tfdA*-like genes. The *tfdA*-

like genes *tfdA* and *tfdAa* were chosen as proxies for the biodegradation potential of MCPA within biofilter microbial communities.

A total of 79 individual PCR and qPCR primers targeting the selected functional genes (*atzA*, *trzN*, *atzC*, *tfdA*, or *tfdAa*) were collected from the literature. Most primer pairs were first published around 25 years ago and intended for application in soil samples. The suitability and quality of primer pairs determine the specificity and sensitivity of any PCR assay, making them the most critical component of this powerful technique. Validating and optimising primer pairs and qPCR conditions is essential before working on precious samples, to avoid inconsistent experimental results or failed qPCR runs²⁷⁶. Primer pairs collected from the literature were systematically evaluated both in-silico and in the laboratory, and finally, qPCR conditions were optimised for the analysis of DWTP samples from Scotland and Brazil. Primer pairs selected for the quantification of targeted functional genes from biofilter samples were *atzA* 01F&R²⁵⁷ for *atzA*, *trzN* 03F&R²⁶⁷ for *trzN*, *atzC* 02F&01R^{213,257} for *atzC*, *tfdA* 03F&18R^{247,275} for *tfdA*, and *tfdAa* 01F&R²⁴³ and *tfdAa* 01F&04R^{243,244} for *tfdAa*.

Of the targeted functional genes essential for the degradation of atrazine, only a product potentially corresponding to *trzN* was amplified during qPCR of environmental samples. Neither *atzA* nor *atzC* functional genes were found in any of the samples. The potential presence of *trzN* functional genes indicates the presence of certain Gram-positive bacterial strains, such as *Arthrobacter* sp.^{217,222,223} or *Solibacillus*²¹², in which *trzN* replaces *atzA*^{210,217}. Although the potential presence of *trzN* indicates the biodegradation of atrazine by Gram-positive bacteria, no *atzC* functional genes were detected. This could reflect incomplete degradation of atrazine and the presence of intermediate degradation products inside the DWTP biofilter or the effluent water. The build-up of such degradation products is problematic as they are potentially more toxic and some are even more difficult to remove from drinking water than their parent compounds²⁷⁷. To obtain certainty about the presence of such compounds, the pesticides and their degradation products would have to be quantified using liquid chromatography in combination with mass spectrometry. In addition to qPCR analysis, this would provide a complete picture and is recommended for future analyses.

In a study completed from 2021 to 2023, 46 pesticides, including atrazine, and 8 intermediate degradation products, three of which were degradation products of atrazine, were continuously monitored twice a week in surface and drinking water in Québec²⁷⁷. One of the atrazine degradation products measured was atrazine-2-hydroxy (ATZ-OH, hydroxyatrazine), which is the first degradation product of atrazine via hydrolytic biodegradation. It is formed by the enzymatic degradation encoded by *atzA/trzN* functional genes. Hydroxyatrazine concentrations in surface water and drinking water did not differ significantly, which shows that the atrazine degradation product hydroxyatrazine is not effectively removed by conventional drinking water treatment²⁷⁷. These observations go hand in hand with the circumstance described in this chapter that no enzymes appear to be present for further degradation of the initial atrazine degradation products, within the DWTP systems tested in these studies.

Atrazine has been banned from agricultural use in the EU for over 20 years, but is still applied in Brazil. Atrazine is known to be very persistent, potentially remaining in the environment for decades^{278,279}. It can still be detected in ground and surface water to this day²⁸⁰. Historical exposure data of Scottish DWTPs A and B influent water of the three years prior to sampling did not show any contamination with atrazine above the limit of detection (<0.003 µg/L). The absence of the targeted functional genes in the biofilter samples of these two DWTPs could therefore be explained by no or very low atrazine exposure in the past few years. DWTPs sampled in Brazil are both located in rural areas and DWTP D in an area where agriculture is the predominant economic sector. DWTP filter microbial communities are therefore likely to have been exposed to atrazine. However, historical exposure data did not quantify any atrazine concentrations above the limit of detection (0.01 µg/L, figure 22) in the past year before sampling.

In contrary to atrazine, MCPA is still regularly used as an effective herbicide in both, Scotland and Brazil. No historical exposure data of DWTP B, C, and D was available for MCPA, however, historical exposure data of DWTP A was available and showed exposure of the plant to MCPA concentrations in the raw water ranging from < 0.002 to 0.218 µg/L (figure 22) in the past three years before sampling. Scottish DWTP A and B

were specifically chosen for sampling as they reportedly had MCPA contamination in the raw water (personal communication, 2023).

The initial and rate limiting biodegradation step of MCPA is enabled by Fe(II)/ α -ketoglutarate-dependent dioxygenases encoded by *tfdA* and *tfdAa* genes. While *tfdA* could not be amplified by qPCR in any of the DWTP samples from Brazil nor Scotland, *tfdAa* genes were found to be present in all of the samples. Highly diverse *tfdAa* genes sequences have previously been found in soils, including pristine soils not previously exposed to phenoxy herbicides²⁴⁴. It was further observed that *tfdAa* genes outnumbered *tfdA* genes by several orders of magnitude, providing evidence that phenoxy herbicide degradation may not be the primary function of *tfdAa* genes²⁴⁴. Baelum et al. 2006, 2008^{242,249} proposed that *tfdA* classIII genes are the main drivers of MCPA biodegradation, as they observed enhanced gene expression in the presence of MCPA^{242,249}. *tfdA* genes were not amplified from any of the DWTP sand samples from Brazil and Scotland, which suggests that the concentration of phenoxy herbicides in the system was not sufficient to exert selection pressure for this gene. The presence of *tfdAa* in all of the samples does not confirm the presence of phenoxy herbicides, as the gene can also be found in pristine environments²⁴⁴. However, phenoxy herbicide degradation may still occur. Quantification of chemical compounds is essential to be able to draw a final conclusion.

Conclusion

The aim of this chapter was to select and optimise qPCR primer sets targeted at functional genes essential for the biodegradation of two selected pesticides, atrazine and MCPA. Key biodegradation steps were identified as atrazine to hydroxyatrazine and N-isopropylammelide to cyanuric acid for atrazine and MCPA to MCP for MCPA, encoded by *atzA/trzN*, *atzC*, *tfdA*, and *tfdAa* genes. The following primer sets were chosen for qPCR of environmental samples from DWTP sand filter from Brazil and Scotland: *atzA* 01F&R²⁵⁷ for *atzA*, *trzN* 03F&R²⁶⁷ for *trzN*, *atzC* 02F&01R^{213,257} for *atzC*, *tfdA* 03F&18R^{247,275} for *tfdA*, and *tfdAa* 01F&R²⁴³ and *tfdAa* 01F&04R^{243,244} for *tfdAa*.

qPCR analysis of biofilter samples from Scotland and Brazil did not amplify *atzA*, *atzC*, or *tfdA* in any of the samples, *trzN* potentially in all of the samples from the DWTP D and *tfdAa* in all of the samples tested. The lack of *atzC* degradation genes might indicate

insufficient atrazine biodegradation in DWTP D and the potential presence of hazardous biodegradation intermediates. To gain comprehensive insight into the processes taking place within these systems, chemical analyses to quantify the presence of pesticides and their metabolites is recommended for future work. Further, the analyses performed in this work are DNA based, which means, that even if a specific gene is present in the system, it might not be expressed. To further study the biodegradation potential of samples mRNA analyses are recommended.

Chapter 5.2: Analysis of *trzN* and *tfdAa* functional genes amplified from environmental samples

Introduction

In the previous chapter, the relevant biodegradation steps of atrazine and MCPA were identified. Subsequently, qPCR primer sets that target the selected functional genes were identified from peer reviewed literature and evaluated *in-silico* as well as in the laboratory. Finally, selected primer sets for each of the 5 targeted functional genes were applied to environmental samples collected from a total of four DWTP biofilters in Scotland and Brazil. *trzN* was potentially amplified in all six samples collected at DWTP D, and *tfdAa* was amplified from all 24 samples that were taken in total from four different DWTPs. This chapter focuses on analysing the two functional genes *trzN* and *tfdAa* that were amplified from DWTP biofilter samples using the primer pairs *trzN* 3F&R and *tfdAa* 1F&R / *tfdAa* 1F&4R respectively.

The overall aim of this chapter was to confirm that the amplified genes are, in fact, the targeted functional genes. This was achieved in multiple steps. Firstly, the samples in which the targeted functional genes had already been detected were selected. The functional genes were then amplified by PCR, extracted, multiplexed, pooled and subsequently sequenced. Downstream analysis following sequencing and demultiplexing is explained in detail in this chapter.

In a recent publication, Cholet et al. 2022 proposes the amplicon sequence variant (ASV) approach as the preferred method for amplicon reconstruction of raw sequencing data of functional genes, rather than the *de facto* approach of generating operational taxonomic units (OTUs), well established for 16S rRNA/18S rRNA sequencing analyses.

In contrast to OTU construction, ASVs do not require user-defined similarity thresholds, which makes ASVs more suitable as a standardized method for functional genes²⁸¹. The authors further found that the ASV approach retained a higher number of raw reads compared to the OTU approach and that the choice of amplicon reconstruction method impacts the results of downstream ecological analyses²⁸¹. Based on these insights, ASVs were chosen for the reconstruction of *trzN* and *tfdAa* functional gene amplicons.

The two pipelines most commonly used for ASV construction are DADA2²⁸² and Deblur²⁸³. The DADA2 pipeline merges both the forward and the reverse reads, whereas Deblur relies on forward reads only to create the ASVs^{282,283}. This might be advantageous when amplicons cannot be merged properly due to their length, or when the reverse reads are of poor quality²⁸⁴. Both pipelines were used in this work. Following ASV construction, BLAST searches were performed to confirm the identity of the amplicons.

Studying functional genes is of great interest, as they grant an insight into microbial processes that are taking place within biogeochemical cycles and enable linking microbial diversity directly to ecosystem processes^{281,285}.

Materials and Method

Illumina MiSeq Sequencing

Illumina amplicon sequencing library preparation was performed by Dr Xiang Shi, who at the time was a postdoctoral research associate at the University of Glasgow. The Nextera® XT DNA Library Preparation Kit (Illumina, United Kingdom) was used for library preparation and after pooling at equimolar concentrations, the library was submitted to “Molecular Analysis – NGS” at the University of Glasgow, United Kingdom for Illumina MiSeq sequencing (2x250bp on a V2 500 cycle nano cartridge).

ASV construction

Amplicon Sequence Variants (ASVs) were created in RStudio (Version 2024.12.1+563) following the DADA2 Pipeline Tutorial (1.16), published at <https://benjjneb.github.io/dada2/tutorial.html>. First, read quality profiles were generated using `plotQualityProfile()`. These served as basis for decisions on quality trimming, performed by `filterAndTrim()`. Parameters for `trimRight` and `trimLeft` were tailored to each target gene, to ensure removal of primer binding sites and low-quality

bases at the start and end of the sequencing reads. Details are given in table 8. A minimal overlap of 12 bp between the forward and reverse reads was maintained for all functional genes. Given that the amplified gene sequence of *trzN* is short, an overlap of more than 30 bp was possible. In the next step, the `learnErrors()` function was used to generate error models against the trimmed and filtered forward and reverse reads, followed by inferring the ASVs using the `dada()` function. Forward and Reverse reads were merged using the `mergePairs()` function, followed by the construction of a sequence table using `makeSequenceTable()`. Finally, chimeras were removed with `removeBimeraDenovo()`, before exporting the remaining ASVs for downstream analysis and construction of a count table. The R script used to create the ASVs is available in the supplementary material of Chapter 5.

Table 8 summarises the number of bases trimmed from both the forward and reverse reads and how many bases overlapped between the forward and reverse reads.

trzN 3F&R	trimLeft=c(18,19)	trimRight=c(80,100)	Overlap: 33 bp
tfdAα 1F&R	trimLeft=c(20,18)	trimRight=c(25,85)	Overlap: 12 bp
tfdAα 1F&4R	trimLeft=c(20,19)	trimRight=c(25,84)	Overlap: 12 bp

Phylogenetic tree construction

Phylogenetic trees were constructed using the QIIME 2 pipeline (version 2019.7). ASVs were aligned using MAFFT²⁸⁶ with default parameters. Midpoint-rooted phylogenetic trees were inferred using FastTree²⁸⁷, and exported in NEWICK format. The trees were visualised in RStudio (Version 2024.12.1+563) using the ape package. The script used to create the phylogenetic trees is available in the supplementary material of Chapter 5.

Amplicon read quality check

Quality control was carried out to confirm the reliability of the constructed ASVs. This consisted of two steps. First, ASV sequences, from which primer sequences have been removed as previously described, were submitted to a BLASTx search with standard settings against the standard nr database. BLASTx searches the protein database using translated nucleotide queries, meaning that it first translates the input ASVs into proteins, which are then blasted against the protein database. BLASTx of *tfdAα* ASVs, amplified by two different sets of primers, returned the expected protein and no further

quality testing was carried out. BLASTx of *trzN* ASVs did not return the expected protein and further analysis was performed. As a first measure, the Deblur pipeline was used to test whether the ASVs created in this way differed from the ASVs already created using DADA2 and whether the BLASTx search produced a different result. ASVs could not be constructed using the Deblur pipeline. For this reason, Expasy (<https://web.expasy.org/translate/>), an online tool that enables the translation of a nucleotide sequence into a protein sequence, was used to determine the reading frames of DADA2 ASV nucleotide sequences. Each DNA sequence is translated into six protein reading frames, of which three are 3' to 5' frames and three are 5' to 3' frames. All six protein reading frames for each ASV were then submitted to a BLASTp search. BLASTp compares input protein sequences against the ClusteredNR database of known protein sequences. Standard settings were chosen.

Results

Construction of ASVs

Conversion of raw reads to ASVs was successful for 56.06% of *trzN* sequences and for 82.9% and 94.09% of *tfdAa* sequences amplified by *tfdAa* 01F&R and *tfdAa* 01F&04R respectively (table 9). Primer set *tfdAa* 01F&04R therefore amplified over 3'000 more gene sequences than primer set *tfdAa* 01F&R from the same environmental samples. In addition, a larger percentage of the raw reads generated with primer set *tfdAa* 01F&04R passed through the DADA2 pipeline. For these reasons, only the ASVs created by primer set *tfdAa* 01F&04R were considered in the subsequent work.

For *trzN* 03F&R, a total of 60 ASVs were generated from six environmental samples of DWTP D. For *tfdAa* 01F&04R a total of 876 ASVs were generated from 24 environmental samples from all four sampled DWTPs A, B, C and D. To facilitate the subsequent analyses, a sub-dataset was created for *tfdAa* 01F&04R, consisting of 30 randomly selected ASVs.

Table 9 shows the total number of reads for *trzN* and *tfdAa* amplicons at each step of the DADA2 pipeline. The number of reads retained throughout the pipeline is given in %.

Name	Input [total number of reads]	Filtered [number of reads]	DenoisedF [number of reads]	DenoisedR [number of reads]	Merged [number of reads]	Nonchim [number of reads]	Input reads retained [%]
<i>trzN</i> 03F&R	10'476	10'475	9'869	9'321	5'873	5'873	56.06
<i>tfdAa</i> 01F&R	263'400	263'399	259'263	252'898	233'018	218'357	82.9
<i>tfdAa</i> 01F&04R	296'342	296'337	292'979	291'295	283'795	278'821	94.09

ASV quality check

A quality check of the ASVs was performed to confirm the reliability of the amplified sequences. Firstly, ASVs constructed from raw reads were submitted to a BLASTx search, in which ASVs were automatically translated to protein sequences and subsequently blasted against a protein database. ASVs of *trzN* submitted to BLASTx, did not return the target protein TrzN. Instead, ASVs aligned significantly with a chemotaxis protein called CheA, covering up to 100% of the ASV lengths (query coverage). Creating a phylogenetic tree consisting of *trzN* ASVs and *trzN* reference genes (described in chapter 4.1 Reference gene database curation), showed two distinct clusters without overlapping (figure 24). Figure 25 shows a phylogenetic tree constructed with *trzN* ASVs and 12 *cheA* reference sequences (sequences are listed in table 11 in the supplementary material of chapter 5). In contrary to the first phylogenetic tree, this second tree shows overlap of *trzN* ASVs and reference sequences. Notably, 3-3 *trzN* 22 and *cheA*12 are closely related in the phylogenetic tree. Further, a close cluster is formed out of *cheA*3, *cheA*9, *cheA*11 and 3-3 *trzN* 20, 3-3 *trzN* 36, and 3-3 *trzN* 58. Another cluster is formed by *cheA*4 and *cheA*5 together with 3-3 *trzN* 31, 3-3 *trzN* 38, 3-3 *trzN* 39, and 3-3 *trzN* 57.

Since the ASVs constructed with the DADA2 pipeline did not return the targeted protein TrzN in the BLASTx search, ASVs were also constructed with the Deblur pipeline in order

to test whether these ASVs returned a different result in the BLASTx search. However, no ASVs could be generated using the Deblur pipeline as none of the trimmed sequences passed the deblur denoising step. Since none of the standard reference sequence databases could be used for functional genes, the manually compiled collection of reference sequences (chapter 4.1 Reference gene database curation) was used instead. The fact that no trimmed sequences passed the deblurring step against the custom reference sequences was another indicator that the amplicons, in fact, differ from the targeted functional gene *trzN*. In the following step, DADA2 ASVs were translated into protein sequences individually using Expasy, an online tool to translate DNA or RNA sequences into protein sequences. Based on the phylogenetic trees (figure 24), ASV sequences least distant from the reference sequences were chosen, namely: ASV 01, 04, 11, 13, 19, 21, 26, 33, 42, 46. Selected ASVs were translated into protein sequences by Expasy, producing six frames per ASV. All protein frames were subjected to a BLASTp search in order to gain a more detailed insight compared to the BLASTx batch search. None of the protein frames aligned with TrzN within the top 100 alignments returned by the BLASTp search. Together, these results suggest that the gene sequences amplified from DWTP D biofilter samples were not *trzN*, but likely represent entirely different genes, possibly *cheA*, as further supported by the presence of a second, distinct melt peak in figure 23. Finally, a BLASTn search of a full length *cheA* gene sequence from *e. coli* against the forward and reverse *trzN* primers showed 67% (*trzN* 03F) and 74% (*trzN* 03R) query cover.

BLASTx search of ASVs created from *tfdA* 01F&04R raw reads returned a range of protein sequences belonging to the TauD/TfdA family dioxygenase, with some protein sequences being classified as Fe(II)/ α -ketoglutarate-dependent dioxygenases specifically. The primer set amplified the targeted functional gene from the DWTP biofilter samples. This is also reflected in the phylogenetic tree, which shows that ASVs and reference sequences do not form distinct clusters, but overlap (figure 26).

trzN ASVs and reference sequences

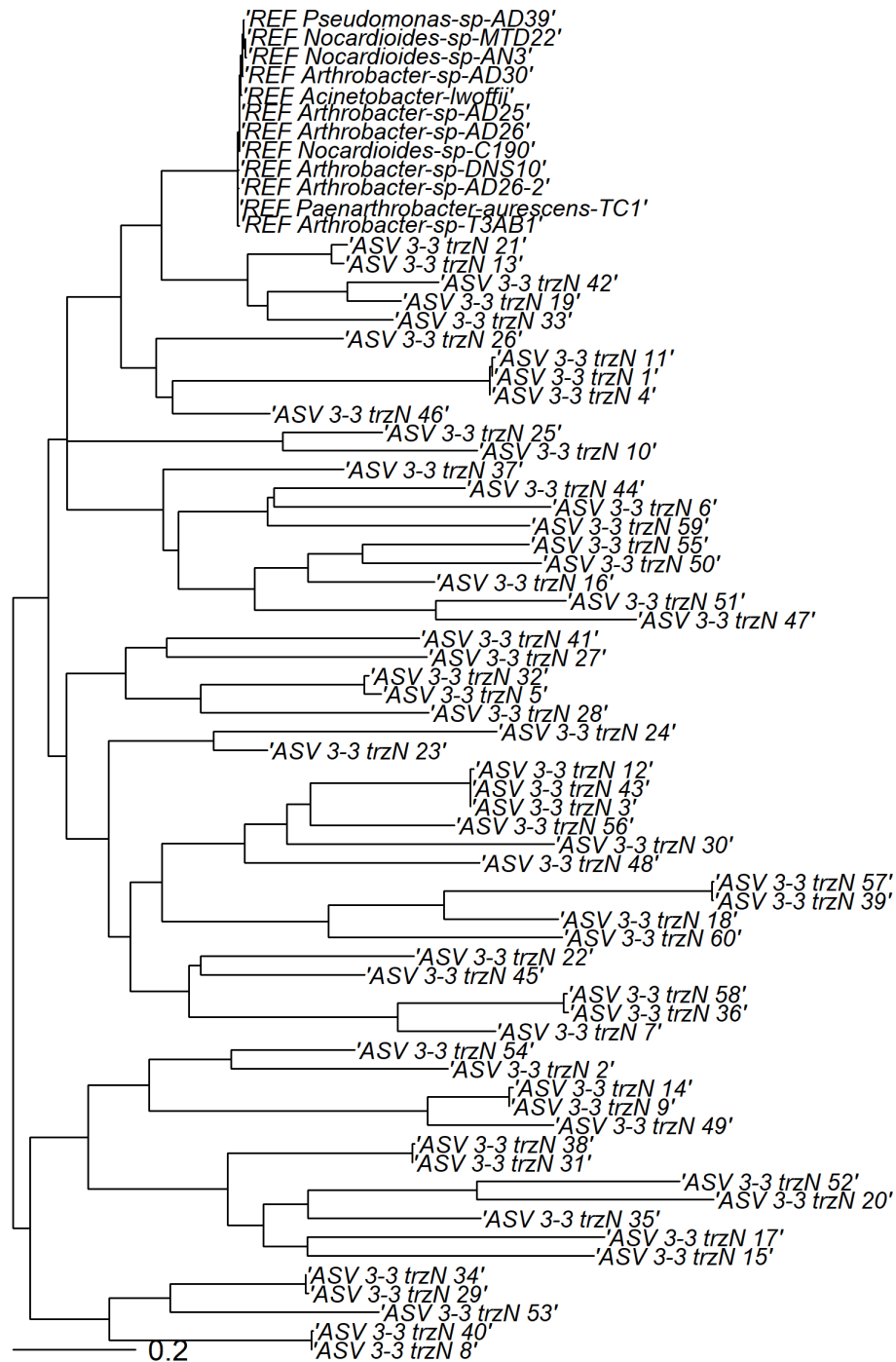


Figure 24 shows a phylogenetic tree (midpoint-rooted) constructed of *trzN* ASVs (ASV 3-3 *trzN*) and *trzN* reference gene sequences (REF). Reference gene sequences form a distinct cluster, clearly separated from the *trzN* ASVs.

trzN ASVs and cheA reference sequences

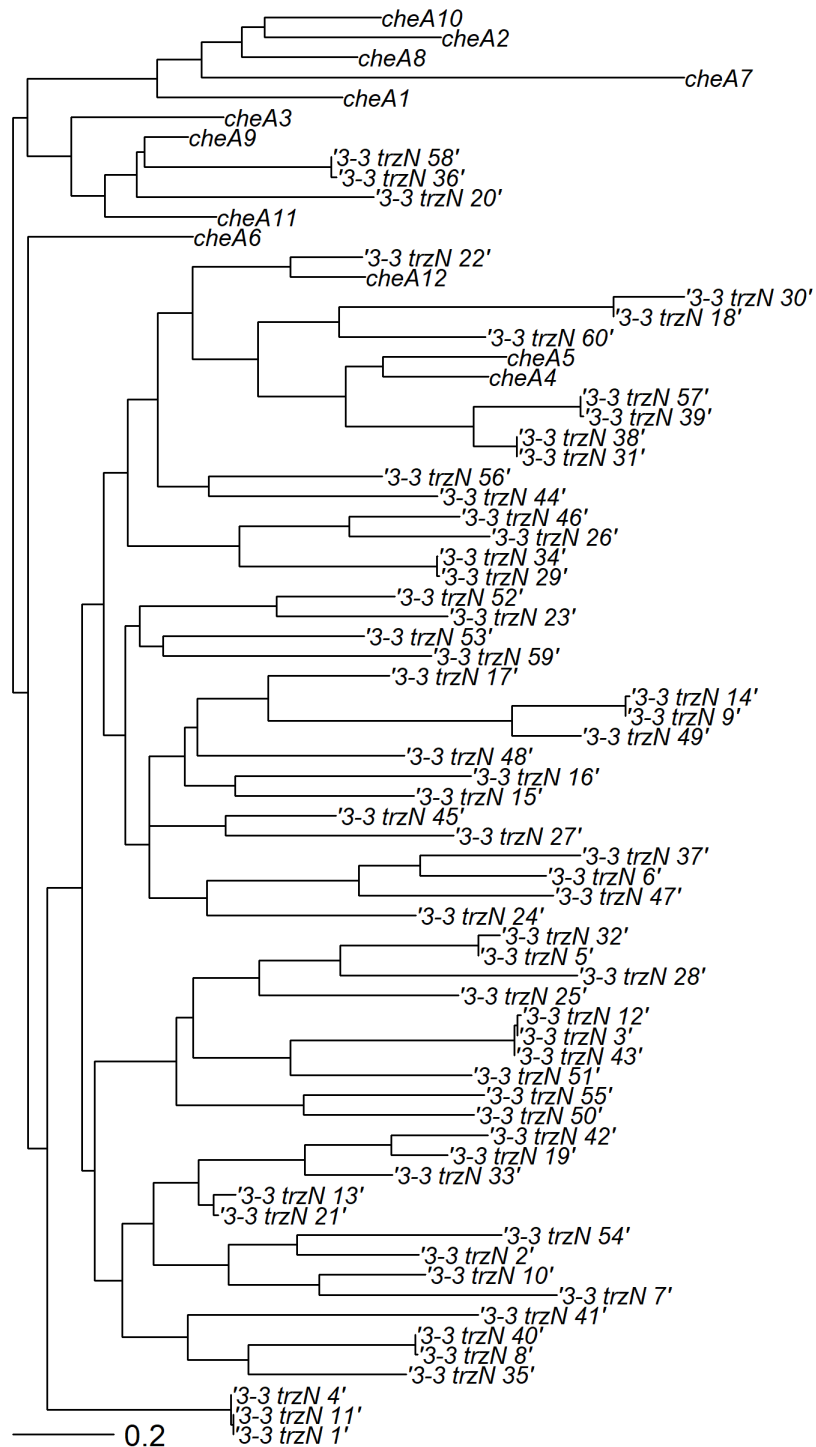


Figure 25 shows a phylogenetic tree (midpoint-rooted) constructed of *trzN* ASVs (ASV 3-3 *trzN*) and *cheA* reference gene sequences (*cheA* 1 - 12). *cheA* reference sequences are not clearly separated from the *trzN* ASV sequences.

tfdAα 30 ASV and 30 reference sequences

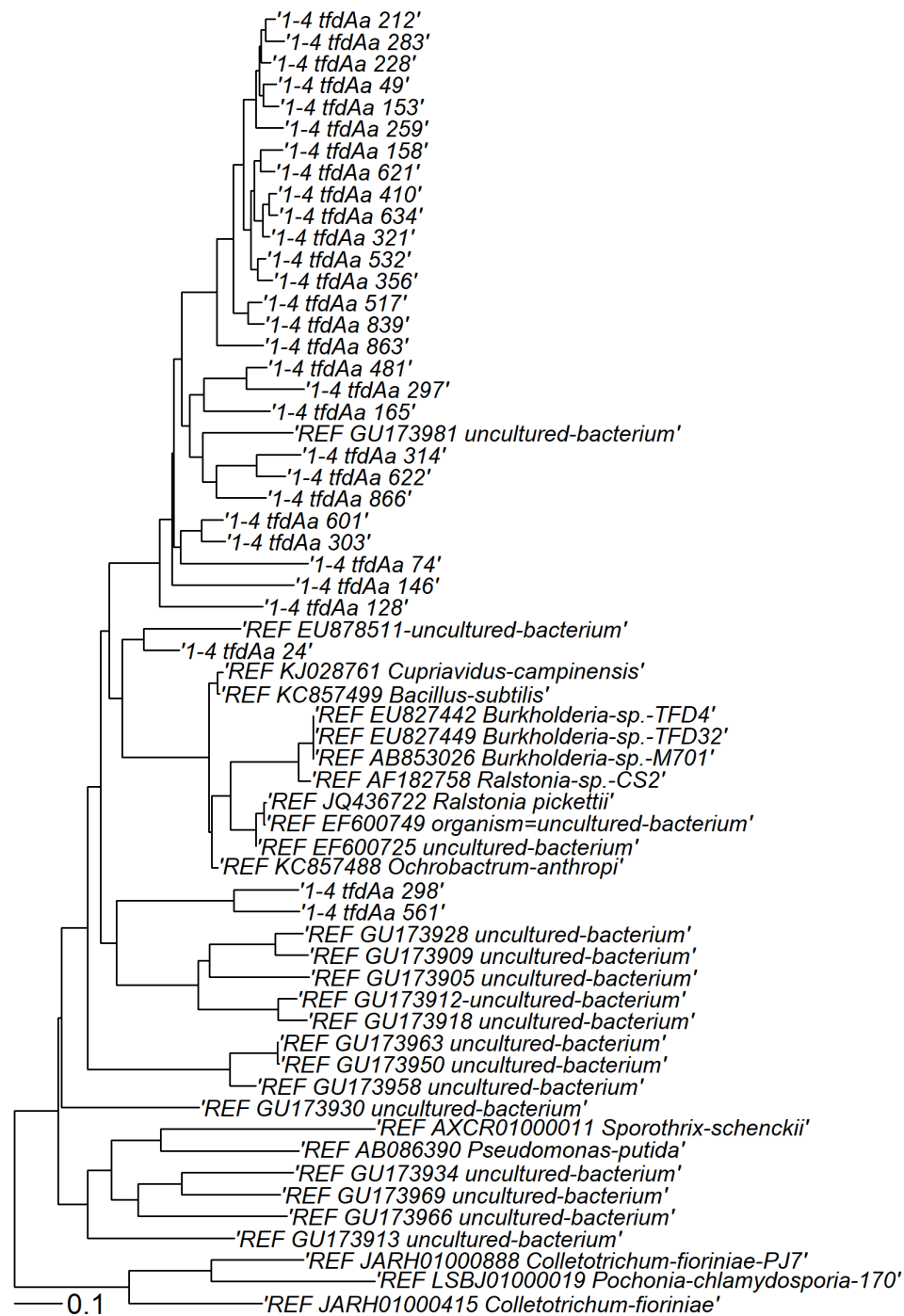


Figure 26 shows a phylogenetic tree (midpoint-rooted) constructed of 30 randomly selected tfdAα ASVs (1-4 tfdAα) and 30 randomly selected tfdAα reference gene sequences (REF). Reference gene sequences do not form distinctive clusters from ASVs.

Discussion and conclusion

The two most promising primer sets to amplify *trzN* based on in-silico assessment were *trzN* 01F&R and *trzN* 03F&R. Both primer sets were tested against two reference gene sequences in the laboratory and amplified the targeted sequence without any troubles (chapter 4.1). The primer set *trzN* 03F&R produced a shorter amplicon and achieved a slightly higher qPCR efficiency compared to *trzN* 01F&R, which led to the selection of this primer set for the application in environmental samples collected from DWTP biofilters. However, sequencing and BLAST search of the sequences amplified by *trzN* 03F&R from DWTP D biofilter samples did not confirm that the amplified sequence was truly the target functional gene *trzN*. It is more likely that the amplified gene sequence encodes the protein CheA. CheA is a histidine protein kinase that plays a key role in bacterial chemotaxis, by controlling signal transmission to CheY, which controls the flagellar motor^{288,289}. *cheA* has been detected in various environments, like soil²⁹⁰, maize rhizospheres²⁹¹, and subseafloor sediments²⁹². To my knowledge, it has never been identified from drinking water treatment systems, but it is widespread in the environment and therefore feasible that it was found in all six samples of DWTP D, taken from both the top and bottom of the biofilter. The result indicates that the primer set *trzN* 03F&R, despite being sensitive, lacks specificity. Primer set *trzN* 03F&R returned false positives and is not suitable for the analysis of environmental samples, specifically DWTP biofilter samples. Analysis of samples should be repeated using the *trzN* 01F&R primer set instead.

The two primer sets *tfdA* 01F&R and *tfdA* 01F&04R performed equally well during PCR and qPCR optimisation (chapter 4.1) and were therefore both applied to biofilter samples of different DWTPs. Primer set *tfdA* 01F&R was designed and first published by Itoh et al., 2002²⁴³. This primer set was later adapted by Zapras et al., 2010²⁴⁴ as a part of a set of five novel and modified primer pairs targeting *tfdA*-like genes. The authors aimed at creating primer sets that are specific to target all major groups of *tfdA*-like genes and to establish qPCR assays based on these primer sets for different soil samples in order to assess the diversity of *tfdA*-like genes in soil²⁴⁴. In comparison to the original primer set, the modified primer set *tfdA* 01F&04R has amplified 12.5% more gene sequences from DWTP biofilter samples. Further, a higher percentage of raw

reads (94.09% vs 82.9%) amplified with *tfdAα* 01F&04R primers passed the DADA2 pipeline compared to *tfdAα* 01F&R, indicating higher quality of the raw reads. Primer set *tfdAα* 01F&04R is well suited for the amplification of *tfdAα* functional genes from DWTP biofilter samples and is recommended for application in these systems. To my knowledge, this primer set has not previously been applied to DWTP systems and *tfdAα* genes have not been quantified from biofilter samples previously.

Chapter 6 – Conclusions and Recommendations

6.1 Restatement of research aim and objectives

The aim of this thesis is to lay a robust foundation for the reliable quantification of biological activities, specifically biodegradation in sand filters, by further developing the necessary methods and adapting them for use in the context of drinking water biofiltration. The thesis is also dedicated to the biodegradation of two selected pesticides, namely atrazine and MCPA, and the identification of the functional genes required for this in environmental samples from DWTPs in Scotland and Brazil.

To reach the aim of this thesis, the following objectives were addressed:

1. To highlight the challenge posed on drinking water safety by widespread use of pesticides in combination with incomplete removal of these contaminants during drinking water treatment, illustrated by the example of Brazil
2. To analyse and assess alternative and improved drinking water treatment methods researched in the past decade, evaluating them for their suitability to be applied as sustainable technology in low- and middle-income countries.
3. To establish a protocol for the extraction and quantification of intact microbial cells from biofilter samples, based on an existing protocol that quantifies total microbial cells from biofilter samples.
4. To assess how the storage of biofilter samples impacts the quantification of total an intact cell counts, to minimise the introduction of bias. This is of special importance for samples that are collected from remote areas or that must be shipped overseas prior to analysis.
5. To identify functional genes essential for the biodegradation of atrazine and MCPA and to select suitable primer sets to target those genes to quantify their presence in DWTP biofilters.
6. To amplify and sequence target functional genes relevant for atrazine and MCPA biodegradation identified in biofilter samples collected from Scottish and Brazilian DWTPs.

6.2 Summary of key findings of chapters 3 to 5

6.2.1 Chapter 3 – Review on Pesticide Contamination and Drinking Water Treatment in Brazil: The Need for Improved Treatment Methods

Chapter 3 discusses the impact of pesticide pollution on drinking water safety and highlights the importance of improved drinking water treatment methods, that are cost effective and environmentally sustainable. This study centred on Brazil – a country with intensive use of pesticides in agriculture and DWTPs that are not fully equipped to remove these substances effectively. Two separate systematic literature searches were performed to identify: 1. the occurrence of pesticides in Brazilian drinking water, and 2. the advanced or novel drinking water treatment methods researched and proposed in the past decade.

The analysis of scientific publications revealed that pesticides are widely present in Brazil's natural waters, which exerts great pressure on drinking water quality and safety. Pesticides have further been identified in drinking water in concentrations frequently exceeding maximum permissible concentrations according to EU regulations (0.1 µg/L). EU regulations are based on the precautionary principle and more stringent in international comparison⁶³. If such regulations were to be applied in Brazil, water supply companies would have to retrofit their existing DWTP at great expense. This understanding emphasises the need for cost-effective and sustainable drinking water treatment methods that remove pesticide from drinking water effectively.

The second part of chapter 3 presents the intensive research that has been performed in the past decade to improve the removal of pesticides from drinking water. While advances in physical-chemical drinking water treatment technologies achieved promising results regarding pesticide removal, they were not without drawbacks. In chapter 3, the main research questions in this area were identified as: 1. to maximise treatment performance, 2. to improve energy- and cost-efficiency, and 3. to minimise the formation of degradation by-products / DBPs for conventional and advanced DWT. Chapter 3 further highlights, that the research on these subjects is fragmented with key focusses on different pesticides and treatment methods, which does not allow a comprehensive comparison of novel and advanced treatment methods. In future research, data collection on effectiveness of pesticide removal should be

standardised, to enable the development of predictive models. This chapter highlights also, that although cost-effectiveness is an important driver for research, very little actual data on drinking water treatment costs was provided. A comparison of the cost effectiveness of suggested drinking water treatment enhancements could therefore not be conducted.

Finally, chapter 3 discusses engineered biofiltration and its suitability as a sustainable and economically viable alternative to physical-chemical drinking water treatment. The chapter concludes, that engineered biofiltration meets all criteria of appropriate technology and would find its ideal application in rural areas and low-and middle-income countries. Chapter 3 identifies main challenges that need to be addressed in order to fully exploit the potential of engineered biofiltration, which are: 1. the rapid loss of bioaugmented degrader strains from DWTP filters²¹, even when cells were immobilised^{22,23}, and 2. gaining an improved understanding of the influence DWTP operational and environmental parameters have on the performance and persistence of beneficial microbial communities within the biofilter²⁶. The chapter finally highlights that additional research on the cost-effectiveness of engineered biofiltration is required.

6.2.2 Chapter 4 – Optimisation of methods for the quantification of microbial communities in biofilters.

Chapter 4 puts forward a simplified and improved protocol that allows the extraction and flowcytometric quantification of both, total and intact microbial cells from DWTP biofilters. Adaptations were made based on a previously published extraction protocol²⁹³. The original protocol applies a harsh extraction method, that, while being very effective at detaching cells from the filter medium, damages cell membranes, making it impossible to quantify the intact cells present in the system.

Four different (chemical) dispersants were tested for their extraction performance of total and intact cells from DWTP biofilter samples. As anticipated, Tween 80 PP outperformed UP water, PBS, and PBS Tween 20 when extracting total cell counts, confirming the effectiveness of the previously published extraction protocol²⁹³. PBS Tween 20, a milder surfactant, was most effective at extracting intact cells from biofilter samples, while maintaining the highest ratio of TCC to ICC and smaller data

variance, compared to Tween 80 PP. The extraction protocol developed in chapter 4 suggests pre-treatment of samples with Glutaraldehyde 1% v/v, four rounds of sonication and chemical dispersion using PBS Tween 20. With the proposed protocol, intact and total cells can be quantified quickly and reliably. It provides insight into the relative proportion of intact cells within the system, which was not possible previously.

In Chapter 4, the question of how samples from DWTP are best stored was also addressed, as DWTPs are often remote and it is not guaranteed that samples can be analysed in the laboratory within a few hours. The aim was to understand biases that are introduced to both, TCC and ICC by different storage conditions. DWTP sand samples were stored for 6 month at four different storage conditions, either with Glutaraldehyde 1% v/v pre-treatment or without. The results suggest that optimal storage of DWTP biofilter samples is pre-treated with Glutaraldehyde 1% v/v and subsequent storage at 5 °C for short and long-term storage of 10 weeks and potentially up to 6 months. Ideally, however, samples should always be analysed as soon as possible. Freezing the samples led to an instant and ongoing loss of cells, irrespective of pre-treatment and freezer temperatures.

6.2.3.1 Chapter 5.1 – Evaluation of primer sets for the quantification of atrazine and MCPA biodegradation genes in DWTP biofilter samples

In chapter 5.1 the most relevant functional genes for the microbial biodegradation of the pesticides atrazine (*atzA*, *trzN*, and *atzC*) and MCPA (*tfdA* and *tfdAa*) were identified. qPCR primer sets targeting those functional genes were collected in a systematic literature search, and evaluated for their suitability to be applied to DNA extracts from DWTP biofilter samples.

A total of 79 unique primer sets were identified from the literature for the selected functional genes *atzA* (18 primer sets), *trzN* (6 primer sets), *atzC* (9 primer sets), *tfdA* (28 primer sets), and *tfdAa* (8 primer sets). Of those, *atzA* 01F&R^{257,258}, *trzN* 03F&R²⁶⁷, *atzC* 02F&01R^{213,257}, *tfdA* 03F&18R^{247,275}, and *tfdAa* 01F&04R^{243,244} were selected for application in environmental samples from DWTP biofilters.

Selected primer sets were used to determine whether the target genes were present in any of the DNA extracts from DWTP biofilter samples from Brazil and Scotland, using

qPCR. Of the atrazine degrading genes only the *trzN* functional gene was amplified. *trzN* was found in all six samples collected from DWTP D in Brazil. In contrary, *tfdAa* was amplified from all 24 samples collected in total from Brazil and Scotland. The *tfdA* functional gene was not amplified at all.

6.2.3.2 Chapter 5.2 – Analysis of *trzN* and *tfdAa* functional genes amplified from environmental samples

Chapter 5.2 focuses on the analysis of the functional genes *trzN* and *tfdAa* that have been amplified from DWTP biofilter samples, using the selected primer pairs *trzN* 03F&R and *tfdAa* 01F&R / *tfdAa* 01F&04R respectively. After sequencing and demultiplexing, ASVs were created using the DADA2 pipeline²⁸².

tfdAa 01F&04R produced amplicons of higher quantity and quality, compared to *tfdAa* 01F&R. Primer set *tfdAa* 01F&04R amplified over 3'000 more gene sequences than primer set *tfdAa* 01F&R from the same DNA extracts. Additionally, a higher percentage of raw reads passed the DADA2 pipeline and were converted to ASVs – 82.9% of reads amplified by *tfdAa* 01F&R vs. 94.09% of reads amplified by *tfdAa* 01F&04R. Only ASVs created by the primer set *tfdAa* 01F&04R were analysed further.

BLASTx and BLASTp searches were performed to confirm the identity of the amplified *trzN* sequences. The results indicated that the amplified sequences do not correspond with the targeted functional gene *trzN*. Instead, the amplified gene sequences appear to be an entirely different sequence, possibly encoding the chemotaxis protein CheA.

BLASTx search of ASVs created from *tfdAa* 01F&04R raw reads matched a range of protein sequences of the TauD/TfdA family dioxygenase. Some protein sequences were classified as Fe(II)/ α -ketoglutarate-dependent dioxygenases. The selected primer set has therefore successfully amplified the targeted functional gene sequences from DWTP biofilter samples.

6.3 Challenges, conclusion and recommendations for future research

This thesis presents an adapted protocol for the extraction and flow cytometric quantification of total and intact cells from DWTP biofilter samples. The results suggested that sample pre-treatment with Glutaraldehyde 1% v/v, four rounds of sonication, and cell extraction using PBS Tween 20 should be recommended for the

quick and reliable quantification of intact cells habituated on sand taken from a DWTP RGF. However, it is important to note, that the statistical power of this experiment is limited, as the amount of replicates that could be processed per sample was limited to three. Further, intact cells have only been extracted from one type of sand. In the future, this experiment should ideally be repeated, using filter medium from different locations, including sand as well as GAC, in order to confirm applicability of these results to a wide range of DWTP biofilter samples.

A portion of the biofilter samples analysed within this thesis was collected in Brazil. One of the questions that arose was: what's the impact of storage conditions and time on the biofilter microbial community and how can the introduction of bias be minimised? A six months storage experiment revealed, that ideally, biofilter samples are pre-treated with Glutaraldehyde 1% v/v prior to storage at 5 °C, to prevent the sudden loss of cells that was observed when freezing the samples. Storage of pre-treated samples at 5 °C showed stable total and intact cell counts for short and long term storage. Although these insights are highly useful for future sampling campaigns, it must be noted that there were limitations in the study design. The amount of replicates were limited to three per storage condition and time point, limiting the statistical power of the experiment. Further, as already described above, experiments, including the storage experiment were only performed on one type of biofilter sample. For future experiments it would be of great interest, to repeat the storage experiment with different DWTP biofilter samples. There were also two small handling issues during the experiment that would ideally be adapted in the future: 1. For long time sample storage in the fridge, it must be made sure, that the storage containers are fully airtight. Two of the samples within this experiment dried completely by six months of storage. 2. Frozen samples should be thawed in the fridge rather than at room temperature to minimize cell rupture.

Total and intact cells were quantified by FCM over the 6 months storage period. Following the experiment, the FCM data was also used to perform community profiling through fingerprint analysis. Phenotypic diversity indexes can be calculated based on FCM fingerprints, which have previously been shown to strongly correlate to 16S rRNA taxonomic diversity of microbial communities^{180,183}. Phenotypic diversity indexes

derived from FCM fingerprints were used as a proxy for 16S rRNA taxonomic diversity within this thesis. It must be stated however, that to my knowledge, there has not been a study in the past that compared 16S rRNA data with phenotypic diversity indexes derived from FCM fingerprints of DWTP biofilter samples. In the future, the storage experiment should ideally be repeated with the addition of taking sand samples for DNA extraction and subsequent molecular analysis for each storage condition and timepoint. This would a) strengthen the results of the storage experiment, and b) be an interesting study in itself, as biofilter media has not previously been used to compare diversity indexes derived from FCM fingerprints to those derived from 16S rRNA taxonomic diversity data.

Chapter 5 presents the selection, optimisation and application of qPCR primer sets targeting selected functional genes relevant for the biodegradation of the pesticides atrazine and MCPA. Of the 5 selected functional genes, two were amplified from DWTP biofilter samples. To my knowledge, these functional genes have never been targeted in DWTP systems. qPCR primer sets described within this chapter were mostly designed on single strains and applied to soil samples only. It was observed that the selected primer pair trzN 03F&R did not amplify the targeted functional gene but a different gene entirely. This emphasises the importance of confirming the identity of any amplicons from environmental samples by sequencing analyses. Primer set trzN 01F&R performed equally well as primer set trzN 03F&R during primer evaluation stages but binds the targeted gene sequence in a different location. It is recommended to test the environmental samples again, using this alternative primer set.

Further, it is recommended to also perform mRNA analyses in future studies to gain an insight into the activity of functional genes in addition to knowing their presence within the DWTP biofilter. And finally, it is recommended to also perform chemical analyses of DWTP biofilter samples to know whether the pesticide of interest or its biodegradation products are present.

This thesis presents a robust set of validated methods for analysing microbial communities in the context of drinking water treatment, which can serve as a basis for further experiments. Future studies could design an experiment in which the developed methods are jointly applied and further validated. For example, a controlled batch

biodegradation experiment could be performed, in which biofilter sand samples from various DWTPs could be enriched with atrazine or MCPA. The decrease in pesticide concentration, shifts in microbial community composition and the expression of relevant functional genes could be analysed over time. The question of whether the environmental or operating conditions of the respective DWTP plant have an influence on the biodegradation efficiency would be of particular interest. For example, it could be analysed whether biofilter material from DWTPs that have previously been exposed to one of these pesticides shows faster and more efficient biodegradation in the batch experiment.

Appendix – Chapter 5

Table 10 lists the full length functional gene sequences used to test the alignment of primer pairs, including accession numbers (NCBI GenBank).

Gene	Full length sequence	Accession (NCBI GenBank)	Organism
<i>atzA</i>	CCATGTGAACCAGATCCTCCTGCGCGGAGGGCCCTCGCAC GGGCGTCAATTCTATGACTGGCTGTTCAACGTTGTGTATCCGG GACAAAAGGCGATGAGACCGGAGGACGTAGCGGTGGCGGT GAGGTTGTATTGTGCGGAAGCTGTGCGCAGCGGGATTACGA CGATCAACGAAAACGCCGATTGCGCCATCTACCCAGGCAAC ATCAGAGGCCGCGATGGCGGTCTATGGTGAGGTGGGTGTGAG GGTCGTCTACGCCCCGATGTTCTTTGATCGGATGGACGGGC GCATTCAAGGGTATGTGGACGCCTTGAAGGCTCGCTCTCCC CAAGTCGAACTGTGCTCGATCATGGAGGAAACGGCTGTGGC CAAAGATCGGATCACAGCCCTGTCAGATCAGTATCATGGCAC GGCAGGAGGTCTGATATCAGTTTGGCCCGCTCCTGCCACTA CCACGGCGGTGACAGTTGAAGGAATGCGATGGGCACAAGC CTTCGCCCCGTGATCGGGCGGTAATGTGGACGCTTCA	U55933.1	<i>Pseudomonas sp.</i>
<i>trzN</i>	CACCAGCACCTGTTGGAAGGCGCAATGCGGGCGATTCCCCA ACTCGAGCGGTACGATGGCCAGTTGGCTCGAAGGCGTCCT GACCAGAAGCGCAGGATGGTGGCGCGACGGGAAGTTCGGT CCAGATGTCATCCGGGAGGTTGCGCGCGCCGTCTCTCTCCA GTGCTCCTCGGTGGCATCACACGGTCGCCGACCAGCAC CTATTCTTCCCCGGTGCCACGGCCGATAGCTATATCGACGCA ACCATCGAGGCCGCCACCGACCTCGGGATCCGGTTCCATG CGGCAAGGTCATCGATGACGCTCGGCAAGAGCGAAGGTGG CTTCTGCGACGACCTGTTCTGTCGAGCCCGTCGACAGAGTCG TCCAGCACTGTCTGGGTCTCATCGACCACTACACGAGCCG GAACCGTTTGAATGGTTCGAATC	FJ161692.1	<i>Pseudomonas AD39</i>
<i>trzN</i>	CACCAGCACCTGTACGAAGGCGCAATGCGGGCGATTCCCC AACTCGAGCGGGTACGATGGCCAGTTGGCTCGAAGGCGTC CTGACCAGAAGCGCAGGATGGTGGCGCGACGGGAAGTTCG GTCCAGATGTCATCCGGGAGGTTGCGCGCGCCGTCTCTCTC GAGTCGCTCCTCGGTGGCATCACACGGTCGCCGACCAGC ACCTATTCTTCCCCGGTGCCACGGCCGATAGCTATATCGACG CAACCATCGAGGCCGCCACCGACCTCGGGATCCGGTTCCA TGCGGCAAGGTCATCGATGACGCTCGGCAAGAGCGAAGGTG GCTTCTGCGACGACCTGTTCTGTCGAGCCCGTCGACAGAGTC	DQ989289.1	<i>Arthrobacter sp. AD 25</i>

	GTCCAGCACTGTCTGGGTCTCATCGACCAGTACCACGAGCC GGAACCGTTTGAATGGTTCGAATC		
<i>atzC</i>	GCTCACATGCAGGTAATCCATGGGACTTTATACCCCGGACC CATGTAGATGTAGATTGAGTTGCTAAAACAAAAGCAGTGGAAG CAGTTTTAGAAGCCAAGGAAGAGTTAAAGGATCTTATCGATATA CAAGTCGTAGCCTTTGCACAGAGTGGATTTTCGTTGATTGGA ATCTGAATCATTGATTAGAAAATCCTTGGATATGGGCTGTGATT AGTTGGGGGAGTTGATCCTGCTACGCGGGAAAATAATGTTGA GGGTTCTTTAGACCTATGCTTTAAATTAGCAAAGGAATACGATG TTGATATCGACTATCACATACATGATATTGGAAGTGTGGAGTA TATTCGATAAATCGTCTTGCCCAAAAGACAATTGAAAATGGGTA TAAGGGTAGAGTAAGTACGAGTCATGCCTGGTGTTCGAGAT GCTCCGTCCGAATGGCTCGATGAGGCAATCCCATTGTACAAG GATTCGGGTATGAAATTTGTTACCTGTTTTAGTAGTACACCGCC TACTATGCCGGTGATAAAGCTGCTTGAAGCTGGCATCAATCTT GGCTGTGCTTCGGACAATATCAGAGATTTTGGGTTCCCTTTG GCAACGGTGATATGGTAC	KF453509.1	<i>Arthrobacter sp.</i> (DNS10)
<i>tfdA</i>	CAGCAGATCGCCTTCGCGCGCAATTCGGGCCACTCGAAGG CGGTTTCATCAAGGTCAATCAAAGACCTTCGAGATTCAAGTAC GCGGAGTTGGCGGACATCTCGAACGTCAGTCTCGACGGCAA GGTCGCGCAACGCGATGCGCGCGAGGTGGTCGGGAAGTTC GCGAACAGCTCTGGCACAGCGACAGCTCCTTTCAGCAACC TGCTGCCCCGCTACTCGATGCTCTCCGCGGTGGTGGTTCCGC CGTCGGGCGGGGACACCGAGTTCTGCGACATGCGTGCGGC ATACGACGCGCTGCCTCGGGACCTCCAATCCGAGTTGGAAG GGCTGCGTGCCGAGCACTACGCACTGAACTCCCGCTTCCTG CTCGGCGACACCGACTATTCGGAAGCGCAACGCAATGCCAT GCCGCCGGTCAACTGGCCGCTGGTTCGAACCCACGCCGGC TCCGGGCGCAAGTTTCTTTCATCGGCGCGCACGCGAGCCA CGTCGAAGGCCCTCCGGTGGCCGAAGGCCGGATGCTGCTT GCGGAGCTTCTCGAGCACGCGACACAGCGGAATTCGTGTA CCGGCATCGCTGGAACGTGGGAGATCTGGTGATGTGGGACA ACCGCTGCGTT	EU827465.1	<i>Pseudomonas sp.</i> TFD39
<i>tfdAa</i>	GGCGTCGATCTGCGCAAGCCGCTGACGAAGCAGGAAGCGA TCGATATCGAGGCCGGCATGGACAAATACGCCGTGCTCGTC TTCCACGGCCAGGACATTTAGACGACCAGCAGATGGCGTT CGCGCTGAACTTCGGCAAGCGCGAGAACGCGCGCGGGCGG CACGGTGACCAAGAAGGAAGACTACCGCCTCTCCTCCGGG CTCAACGATGTCTCAATCTCGGCAAGGACGGCAAGCCGCT GCCGAAGGATCACCGCACCCACCTGTTCAATCTCGGCAACT	AB074492.1	<i>Alpha-Proteobacterium</i> HW13

	GCCTGTGGCACTCCGACAGCTCGTTCCGGCCGATCCCGGC GAAATTCTCGCTGCTGTCGGCGCGCTCGTCAACCCGAAGG GCGGCAACACCGAGTTCGCCGACATGCGCGCTGCCTATGA CGCGCTCGACGACGAGACCAAGACCGAGATCGACGACATG ATCTGCGAGCACTCGCTGATGTATTGCGCGGGCTCGCTCGG CTTCCTCGACTACACCGACGAGGAGAAGGAGATGTTCAAGC CGGTGCTGCAGCGGCTGGTGCGCACCCATCCGGCGCACG GCCGCAAGTCGCTGTATTTGCCTCGCATGCCGGCGCCATC AGGGGCATGAGCATGCCGGAGGCGCGGCTGTTGCTGCGCG ACCTGACCGAGCATGCGACGCAGCCGAATTCGTCTATGTG CACAAATGGACGGTGCATGACCTCGTGATGTGGGACAACCG CCAGACC		
--	---	--	--

Table 11 lists *cheA* genes 1 to 12 and their respective accession numbers for the NCBI GenBank

Gene	Accession (NCBI GenBank)	Gene	Accession (NCBI GenBank)
<i>cheA1</i>	U30501.1	<i>cheA7</i>	AMSD01000001.1
<i>cheA2</i>	X76170.1	<i>cheA6</i>	CP011802.1
<i>cheA3</i>	CP022115.1	<i>cheA9</i>	CP005094.1
<i>cheA4</i>	LR730402.1	<i>cheA10</i>	DQ150110.1
<i>cheA5</i>	NIBR01000001.1	<i>cheA11</i>	JFOK01000021.1
<i>cheA6</i>	X80027.1	<i>cheA12</i>	CWHS01000002.1

Quantification of functional genes

Biofilter samples of three DWTPs A (Scot), C (Brazil), and D (Brazil) were taken from the top (n=3) and bottom (n=3) of the biofilter. In DWP B, samples were taken from the RGF (n=3) and the GAC filter (n=3). DNA extracts from Brazil unfortunately evaporated an unknown amount of liquid during either storage or shipment to the UK. The quantification of gene copy numbers per sample are therefore not reliable, but displayed in figures 27 (*tfdAa*) and 28 (*trzN*)

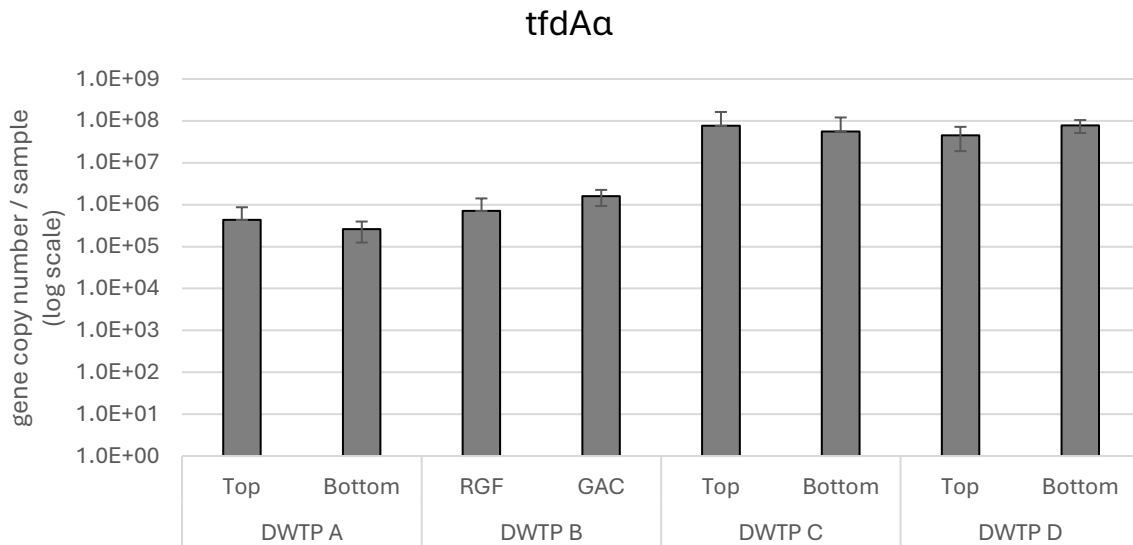


Figure 27 shows gene copy numbers / sample of *tfdAa* for samples taken from the top and bottom of a biofilter from DWTP A (Scot), C (Br), and D (Br). For DWTP B samples were taken from the rapid gravity filter (RGF) and the granular activated (GAC) filter.

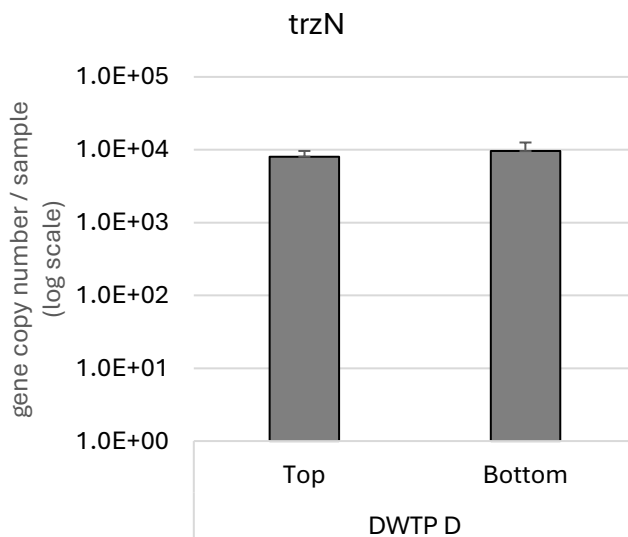


Figure 28 shows gene copy numbers / sample of *trzN* for samples taken from DWTP D (Br).

ASV construction – DADA2 Pipeline

```
#----GETTING READY----
```

```
library(dada2); packageVersion("dada2")
```

```
# trzN 3_3:
```

```
path <- "C:/.../clean_3_3" #adjust
```

```
list.files(path)
```

```
#----STRING MANIPULATION----
```

```
# Forward and reverse fastq filenames have format: SAMPLENAME_R1_001.fastq and  
SAMPLENAME_R2_001.fastq
```

```
fnFs <- sort(list.files(path, pattern="_R1_001.fastq", full.names = TRUE)) #adjust
```

```
fnRs <- sort(list.files(path, pattern="_R2_001.fastq", full.names = TRUE)) #adjust
```

```
# Extract sample names, assuming filenames have format: SAMPLENAME_XXX.fastq
```

```
sample.names <- sapply(strsplit(basename(fnFs), "_"), `[`, 1)
```

```
#----INSPECT READ QUALITY PROFILES----
```

```
plotQualityProfile(fnFs[3:6]) #adjust
```

```
plotQualityProfile(fnRs[3:6]) #adjust
```

```
#----FILTER AND TRIM----
```

```
# Place filtered files in filtered/ subdirectory
```

```
filtFs <- file.path(path, "filtered", paste0(sample.names, "_F_filt.fastq.gz"))
```

```
filtRs <- file.path(path, "filtered", paste0(sample.names, "_R_filt.fastq.gz"))
```

```
names(filtFs) <- sample.names
```

```
names(filtRs) <- sample.names
```

```
# Added 04.05.2025 to check raw read counts
```

```
file.info(fnFs)$size
```

```
file.info(fnRs)$size
```

```

# ----3_3 Data:----

out <- filterAndTrim(fnFs, filtFs, fnRs, filtRs, trimLeft=c(18,19), #adjust
trimRight=c(80,100), rm.phix=TRUE) # On Windows set multithread=FALSE #adjust

head(out)


#----LEARN THE ERROR RATES----

errF <- learnErrors(filtFs, multithread=TRUE)

errR <- learnErrors(filtRs, multithread=TRUE)

plotErrors(errF, nominalQ=TRUE)


#----Sample Inference----

dadaFs <- dada(filtFs, err=errF, multithread=TRUE)

dadaRs <- dada(filtRs, err=errR, multithread=TRUE)

dadaFs[[1]]


#----MERGE PAIRED READS----

mergers <- mergePairs(dadaFs, filtFs, dadaRs, filtRs, verbose=TRUE)

# Inspect the merger data.frame from the first sample

head(mergers[[1]])


#----CONSTRUCT SEQUENCE TABLE----

seqtab <- makeSequenceTable(mergers)

dim(seqtab)

# Inspect distribution of sequence lengths

```

```
table(nchar(getSequences(seqtab)))
```

```
#----REMOVE CHIMERAS----
```

```
seqtab.nochim <- removeBimeraDenovo(seqtab, method="consensus",  
multithread=TRUE, verbose=TRUE)
```

```
dim(seqtab.nochim)
```

```
sum(seqtab.nochim)/sum(seqtab)
```

```
#----TRACK READS THROUGH THE PIPELINE----
```

```
getN <- function(x) sum(getUniques(x))
```

```
track <- cbind(out, sapply(dadaFs, getN), sapply(dadaRs, getN), sapply(mergers, getN),  
rowSums(seqtab.nochim))
```

```
# If processing a single sample, remove the sapply calls: e.g. replace sapply(dadaFs,  
getN) with getN(dadaFs)
```

```
colnames(track) <- c("input", "filtered", "denoisedF", "denoisedR", "merged",  
"nonchim")
```

```
rownames(track) <- sample.names
```

```
head(track)
```

```
#----ASV Renaming, FASTA Export, and Count Table (for functional gene analysis)----
```

```
# Load required library
```

```
library(seqinr)
```

```
# Custom dataset label
```

```
dataset_label <- "3-3_trzN" #adjust
```

Step 1: Extract ASV sequences and generate readable names

```
asv_seqs <- colnames(seqtab.nochim)
```

```
asv_headers <- paste0(dataset_label, "_", seq_len(length(asv_seqs)))
```

```
# e.g., 3-3_trzN_1, 3-3_trzN_2, ...
```

Step 2: Write representative sequences to a FASTA file

```
write.fasta(sequences = as.list(asv_seqs),
```

```
            names = asv_headers,
```

```
            file.out = paste0("ASVs_", dataset_label, ".fasta"))
```

```
# Output: ASVs_3-3_trzN.fasta
```

Step 3: Rename ASVs in the count table to match FASTA labels

```
asv_tab <- t(seqtab.nochim) # Transpose to make ASVs rows
```

```
rownames(asv_tab) <- asv_headers
```

Step 4: Write count table

```
write.table(asv_tab,
```

```
            file = paste0("ASV_counts_", dataset_label, ".tsv"),
```

```
            sep = "\t", quote = FALSE, col.names = NA)
```

```
# Output: ASV_counts_3-3_trzN.tsv
```

ASV construction – Deblur Pipeline

STEP 1: Import the sequences

```
d="/home/.../DEBLUR/";
```

```
export PATH=/home/opt/miniconda2/bin:$PATH
```

```
source activate qiime2-2019.7
```

STEP 2: Run Deblur - Comment: None of the sequences passed quality control initially - > manual trimming of the first 20 bases (very poor quality) of each sequence and quality control afterwards.

```
mkdir trimmed_fastq; for fq in /home/.../DEBLUR/*_R1_*.fastq; do name=$(basename $fq .fastq); cutadapt -u 20 -o trimmed_fastq/${name}_trimmed.fastq $fq; done
```

```
(echo -e "sample-id\tabsolute-filepath"; for fq in /home/.../DEBLUR/trimmed_fastq/*_trimmed.fastq; do sample=$(basename "$fq" | cut -d'_' -f1); echo -e "$sample\t$fq"; done) > se-33-manifest-trimmed
```

```
qiime tools import --type 'SampleData[SequencesWithQuality]' --input-path se-33-manifest-trimmed --output-path demux-trimmed.qza --input-format SingleEndFastqManifestPhred33V2
```

```
qiime quality-filter q-score --i-demux demux-trimmed.qza --o-filtered-sequences demux-trimmed-filtered.qza --o-filter-stats demux-trimmed-filter-stats.qza --p-min-quality 20
```

For control (optional):

```
qiime metadata tabulate --m-input-file demux-trimmed-filter-stats.qza --o-visualization demux-trimmed-filter-stats.qzv
```

Open demux-filter-stats.qzv at <https://view.qiime2.org>

For control (optional):

```
qiime demux summarize --i-data demux-trimmed-filtered.qza --o-visualization demux-trimmed-filtered.qzv
```

Open demux-trimmed.qzv at <https://view.qiime2.org>

➔ *It looks good now! Poor quality reads at the beginning of the sequences were successfully cut and a reasonable amount of sequences passed the quality filter step.*

STEP 3: Deblurring

Standard reference sequences are meant for 16S analyses and cannot be used for functional gene analysis - Import custom reference genes:

```
qiime tools import \  
  
  --input-path trzN_12_reference_sequences.fas \  
  
  --output-path trzN_reference.qza \  
  
  --type FeatureData[Sequence]  
  
qiime deblur denoise-otus \  
  
  --i-demultiplexed-seqs demux-trimmed-filtered.qza \  
  
  --i-reference-seqs trzN_reference.qza \  
  
  --p-trim-length 115 \  
  
  --o-representative-sequences rep-seqs-deblur.qza \  
  
  --o-table table-deblur.qza \  
  
  --p-sample-stats \  
  
  --o-stats deblur-stats.qza \  
  
  --p-min-size 0 \  
  
  --p-min-reads 1 \  
  
  --verbose
```

➔ No reads pass the filter. ASVs and reference sequences don't match!

Phylogenetic Tree construction

```
export PATH=/home/opt/miniconda2/bin:$PATH
```

```
source activate qiime2-2019.7
```

```
qiime tools import --type 'FeatureData[Sequence]' --input-path ASVs_3-3_tfdAa.fasta --  
output-path ASVs_3-3_tfdAa.qza
```

```
unset MAFFT_BINARIES
```

```
qiime phylogeny align-to-tree-mafft-fasttree --i-sequences ASVs_3-3_tfdAa.qza --o-  
alignment aligned-rep-seqs.qza --o-masked-alignment masked-aligned-rep-seqs.qza --  
p-n-threads 0 --o-tree unrooted-tree.qza --o-rooted-tree rooted-tree.qza
```

```
qiime tools export --input-path rooted-tree.qza --output-path output
```

```
qiime tools export --input-path aligned-rep-seqs.qza --output-path output
```

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