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The impacts of airport de-icers on water quality and the growth of
undesirable biofilms in rivers

School of Water, Energy and Environment

PhD

Academic Year: 2019 - 2023

Supervisor: Robert C. Grabowski

Associate Supervisors: Francis Hassard and Angel Medina Vaya

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Abstract

Undesirable river biofilms (URBs, formerly “sewage fungus”) are polymicrobial biofilms that grow in rivers as a response to organic pollution. Despite a continued widespread occurrence, limited research has been conducted in recent decades. Therefore, to reignite research into URBs, this thesis aims to investigate how the delivery of airport de-icer contaminated runoff to receiving waters impacts the growth and composition of URBs. The research conducted can be categorised into four themes: (i) a re-evaluation of “sewage fungus”; (ii) a field study to characterize the composition and drivers of URBs; (iii) microcosm growth experiments to determine how different airport de-icers contribute to URB growth; and (iv) pilot-scale mobilisation experiments to evaluate the mobilisation of airport de-icers in surface runoff.

A bimonthly field study conducted over two winters revealed shifts in the bacterial composition of riverbed biofilms with suspected de-icer discharge. The community became dominated by the genera *Rhodospirillum* (19.5 – 56.7% relative abundance) and *Sphaerotilus* (9.5 – 25.4%) prior to and during a visible URB incident. When aggregated at the family taxonomic level, *Comamonadaceae* (55.7 – 81.4%) emerged as the key indicator taxon of the significant shift in periphyton and has been proposed as a possible organic pollution biosentinel. The URB had a long-term influence on the periphyton, highlighting the importance of preventing their occurrence.

Microcosm experiments of URB taxa growth using common freeze-point depressants as the sole carbon source confirmed de-icers can facilitate URB growth. *Sphaerotilus natans* growth was stimulated by all de-icers, except formate salts, in suspended culture (assessed by turbidimetric microcosms) and total growth was heavily dependent on carbon concentration. There were some significant differences in the growth kinetics between the active ingredients in de-icers (e.g., maximum growth rate). However, there were only negligible differences between pure active ingredients and their commercial products demonstrating that the additives used in commercial de-icers have negligible influence on microbial growth, despite their potential toxicity to aquatic fauna.

Growth experiments with mixed cultures using *S. natans* and *Zoogloea oleivorans* were also tested to explore the interactions between URB taxa, including the novel application of timelapse microscopy in URB growth studies and provide a basis for further research.

Mobilisation of commercial airport de-icers by rainfall was tested after exposure to various meteorological conditions (sun, wind, temperature) to explore the transport pathways of de-icers in runoff. Runoff was significantly affected by weather between de-icer application and a rainfall event. Sunlight and wind caused less de-icer mobilisation than decreases in temperature, attributed to drying effects. This research has considerable implications to inform surface water winter management plans by airport operators.

This research highlights the continued prominence of URBs in rivers, even in countries with advanced wastewater treatment, and has added substantial contributions to knowledge about their composition and growth as a consequence of airport de-icing activities. Through a process-based understanding of de-icer transport and impact, airports can develop more effective and resilient solutions to minimise the ecological impacts in receiving water courses.

Keywords:

Sewage fungus; 16S rRNA sequencing; river periphyton; river pollution; ecological degradation; bioindicator

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List of Abbreviations

AMR	Anti-microbial resistance
BACI	Before-after control-impact
BMWP	Biological monitoring working party
BOD ₅	Biochemical oxygen demand (5-day)
CAD	Commercial airport de-icer
COD	Chemical oxygen demand
CSO	Combined sewer outfall
DO	Dissolved oxygen
DOM	Dissolved organic matter
EPS	Extracellular polymeric substances
HEF	Hyporheic exchange flow
HPLC	High-performance liquid chromatography
IC	Inorganic carbon
LTL	Lowest taxonomic level
maxOD	Maximum optical density at 600 nm
maxROC	Maximum rate of change
MBBR	Moving bed biofilm reactor
MEM	Minimum essential media
M _w	Molecular weight
NGO	Non-governmental organisation
OD ₆₀₀	Optical density at 600nm
ORP	Oxidation-reduction potential
OTU	Operational taxonomic unit
PCO	Principal coordinate analysis (also seen as PCoA)
PERMANOVA	Permutational ANOVA
PFAS	Polyfluoroalkyl substances
PHA	Polycyclic hydrocarbons
qPCR	Quantitative polymerase chain reaction
TC	Total carbon
TDS	Total dissolved solids
TTD	Time-to-detection
URB	Undesirable river biofilm

VOC
WHPT

Volatile organic compounds
Walley Hawkes Paisley Trigg

1 Introduction

1.1 Airport de-icing and its impacts on water quality

Air travel is an integral part of modern society. Over 4.5 billion passengers travelled by air in 2019, which is expected to grow to 10.0 billion by 2040 [1]. However, air travel poses complex and multifaceted environmental pressures, such as greenhouse gas emissions, noise/light pollution and land consumption/habitat loss. One environmental issue that is not generally associated with airports is water quality, including contamination from jet fuels (hydrocarbons), firefighting (PFAS) [2,3], polybrominated diphenyl ethers (PBDEs) [4], and organophosphate flame retardants (OPFRs) [5], in addition to anti- and de-icers (low molecular weight organic chemicals).

In temperate climates, the de-icing of aircraft and runways is crucial for safe winter operations [6–9] with millions of litres used at a large international airport per year [9–11]. Airport anti- and de-icing chemicals are used to prevent the build-up of ice and snow on aircraft surfaces and are primarily comprised of a freeze-point depressant (FPD), water, and various additives to optimise performance [11–13]. The most commonly used FPDs are small, organic chemicals with high proportions of oxygen, and include propylene glycol, ethylene glycol, and acetate or formate salts [14,15]. Historically, urea has been used as an airport pavement de-icer but was phased out and replaced with acetate and formate salts owing to its environmental impacts (e.g., high ammonia concentration in effluents) [16–19]. Whilst these alternatives are seen to be environmentally superior, they still have significant influences on freshwater ecosystems.

The use of airport de-icers has been associated with poor water quality in receiving waters [20,21], most notably a phenomenon called sewage fungus [22–27]. Sewage fungus is a polymicrobial riverbed biofilm, historically used as a bioindicator of organic pollution and saprobic rivers [28–32]. Saprobian rivers describe those rich in organic matter, often with depleted dissolved oxygen levels and low resilience to the impacts of organic pollution [33]. The prolific growth of sewage fungus has both direct and indirect influences on the ecosystem. Direct impacts include the rapid heterotrophic activity of the biofilm which (i) consumes large amounts of dissolved oxygen [34,35]; thereby stressing other, more sensitive aquatic fauna; and (ii) blanketing of the

riverbed, outcompeting aquatic flora and habitat loss for aquatic fauna (e.g., invertebrates and fish spawning). Indirectly, sewage fungus affects ecological connections within the food web. For example, the loss of aquatic flora leads to a reduction in food availability for aquatic fauna, already stressed by multiple compounding pressures. Sewage fungus is an important part of a rivers rapid self-purification system. By consuming excess organic matter, it reduces the length of impact zone, and thus could be seen as a benefit to the watercourse. The growth of sewage fungus microorganisms reduces the organic load in the river and, when their growth is not prolific, they provide an important benefit to water quality. However, their growth is rapid, in response to the very high organic loading, which results in an extensive proliferation of sewage fungus. The resulting 'smothering' or 'blanketing' of riverbeds causes significant and detrimental impacts to freshwater fauna, flora and other river uses.

1.1.1 Knowledge gap and research needs

Sewage fungus remains highly prevalent globally, even in higher-income countries with advanced wastewater treatment [36–41]. Yet there has been negligible research and regulatory focus on sewage fungus for nearly 50-years [34] leading to the first knowledge gap, an outdated and incomplete appreciation of the drivers, microbial composition, and ecological impacts of sewage fungus. Therefore, recent research into biofilms and water quality has been integrated into our existing understanding of sewage fungus to re-evaluate it with a new interdisciplinary understanding and generate a 21st Century understanding. This up-to-date, multidisciplinary review provided the necessary foundation to explore the implications of airport de-icing and its relationship with sewage fungus and environmental impacts. Existing research has identified dominant taxa determined by culture-based enumeration and evaluated the environmental impacts of sewage fungus but had limited investigation on biofilm functions.

The second knowledge gap identified was an outdated appreciation of the changes to the benthic microbiome in response to organic pollution and the environmental drivers that lead to sewage fungus. Common dominant sewage fungus taxa have historically been identified by culturing techniques but has not been thoroughly examined by

newer sequencing approaches. Therefore, a comprehensive field study at a case study airport and river catchment was planned and undertaken. Heathrow Airport (London, UK) is the UK's busiest airport with 80.9 million passengers and over 1.59 million tonnes of cargo transported in 2019 [42]. Surface water from the eastern catchment, with high levels of winter de-icing, is conveyed into the River Crane (London, UK), which has a history of sewage fungus, putatively linked to de-icing at Heathrow Airport [26,27,43], making it an ideal location for study. The findings of this study have significant implications for airport operators [22–27] and contribute substantially to improving our understanding of sewage fungus composition and drivers.

The third knowledge gap addresses the putative link between airport de-icing and sewage fungus. Whilst the two have long been connected [22–27], and even investigated scientifically [20,21], the causality of common FPDs to act as assimilable carbon sources for sewage fungus organisms remains poorly understudied [44]. Therefore, the third phase of this research sought to resolve this gap through a series of highly controlled microcosm growth experiments, generating growth profiles and calculating growth kinetics of sewage fungus taxa in de-icer based media.

Finally, the fourth knowledge gap relates to de-icer transport pathways. The research outlined above contributed significantly to knowledge on the ramifications of airport de-icing (i.e., sewage fungus) and the mechanisms by which common FPDs cause it. However, there is an absence of literature investigating the transport of airport de-icers from application to the discharge of effluents to surface water. The general pathways are understood: when the temperature falls below a threshold temperature, the weather conditions require it, or it is requested by a pilot, de-icers are applied to aircraft using specialist vehicles, where approximately 70% is deposited immediately on the stand where it is mobilised in surface runoff and treated before discharge into surface waters (**Figure 1-1**). The specific temperatures and weather conditions to trigger de-icing vary by airport but are often in the region of 3 – 5 °C and pilots can request de-icing of their aircraft at any time. Approximately 500 L of de-icer is applied to an Airbus A380 and 30,000 L to de-ice a runway [45,46]. Furthermore, commercial de-icer formulations have large biological oxygen demands (BOD₅) (354 g/L for aircraft de-

icers and 270 g/L for runways [9]) resulting in challenging effluents for treatment. Consequently, there is an additional body of research evaluating the treatment processes of airport surface runoff contaminated with de-icers [47–51]. However, the specific mechanisms of transport remain poorly understood. Therefore, to gain new insights into the transportation of de-icers, pilot-scale runoff experiments were designed and performed to investigate the influences of weather and rainfall on de-icer mobilisation.

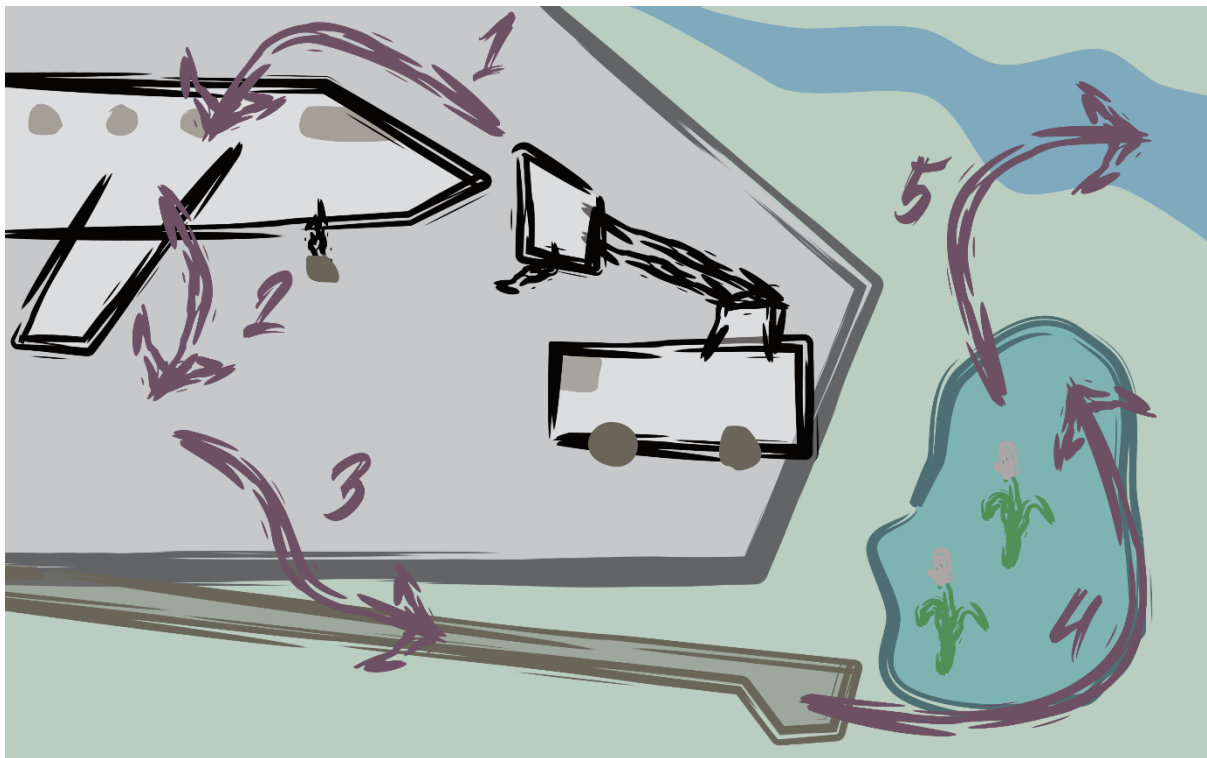


Figure 1-1: A general overview of the transport of airport anti- and de-icers from (1) application on aircraft, (2) deposition on airport stands, (3) mobilisation in runoff, (4) treatment of surface runoff, and (5) discharge of treated effluents into receiving waters.

1.2 Aims and objectives

This thesis aims to investigate how the delivery of airport de-icer contaminated runoff to receiving waters impacts the growth and composition of sewage fungus. It was achieved through four themes (i) a re-evaluation of sewage fungus; (ii) an extensive

field study; (iii) microbial growth experiments; and (iv) pilot-scale erosion experiments (**Figure 1-2**) to meet the following objectives:

Objective 1 – to reassess our scientific understanding of sewage fungus through a critical literature review covering composition, drivers of growth, and continued prevalence, whilst drawing on advancements in biofilm research to address sewage fungus in the 21st Century (Chapter 2).

Objective 2 – to investigate the changes in water quality and microbial composition of river periphyton in a river receiving airport surface runoff during winter (Chapter 3, Chapter 4).

Objective 3 – to evaluate the growth of sewage fungus taxa in airport de-icer based media (Chapter 5, Chapter 6).

Objective 4 – to examine the impact of meteorological conditions on the mobilisation of airport de-icers in surface water runoff (Chapter 7).

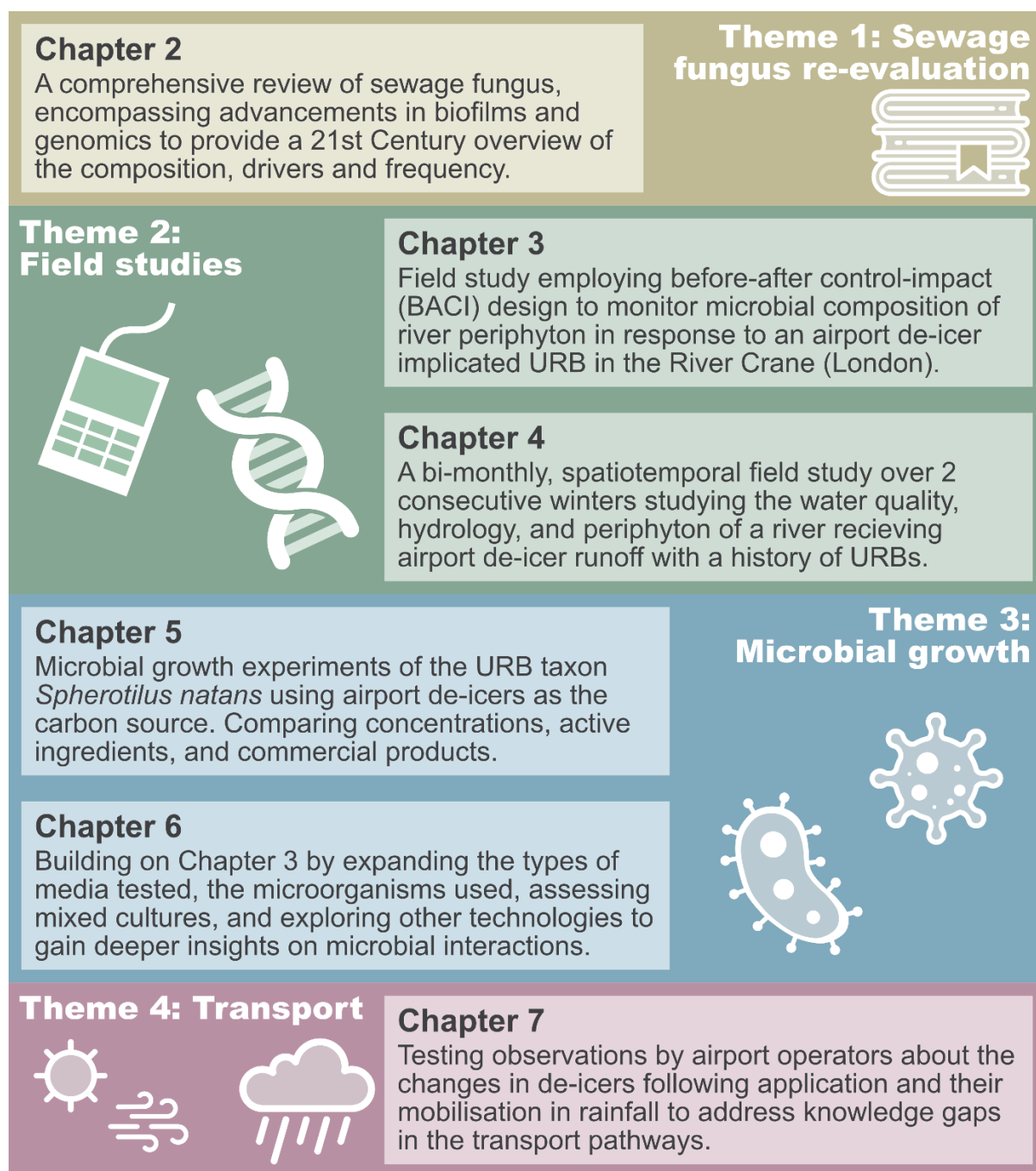


Figure 1-2: A summary of the thesis structure and the links between chapters.

1.2.1 Thesis outline

The thesis has been written in a paper format with the literature review (Chapter 2) written in the style of a critical review and technical chapters (Chapters 3-7) written as

individual scientific papers. Therefore, there are some elements of repetition in describing the underlying literature and methods.

Chapter 2 presents a comprehensive and critical review of sewage fungus literature whilst incorporating advancements made in general biofilm research since the bulk of direct research on sewage fungus (1950 – 1980s) to elucidate a 21st Century, interdisciplinary evaluation of sewage fungus. It contributes significantly to our understanding of sewage fungus whilst highlighting its continued prevalence, even in countries with advanced wastewater treatment, such as the UK. The chapter presents an evaluation of microbial composition from culture-based and sequencing-based approaches; for the first time discusses the biofilm matrix within the context of sewage fungus; and examines the role of nutrient and environmental drivers on sewage fungus growth and morphology. This review also proposes a new, more accurate term to succeed the “misnomer” sewage fungus [28]: undesirable river biofilms (URBs). At the time of submission, Chapter 2 has been submitted to Environmental Indicators (**Table 1-1**).

Chapter 3 investigates an URB event using a BACI (before-after, control-impact) sampling design. High-resolution 16S rRNA (V3-4) sequencing of river periphyton was performed on field samples, in parallel with water quality monitoring, to identify key URB taxa. Several important contributions to knowledge were identified, including reaffirming the role of existing URB taxa, identifying new URB taxa, and establishing a possible new biosentinel of organic pollution. Chapter 3 has been published in Hydrology Research (**Table 1-1**) [52].

Chapter 4 presents a bi-monthly field study over two successive winters, including periphyton sequence data, water quality spot samples, and ongoing water quality, hydrology, and meteorology monitoring datasets for the River Crane and Heathrow Airport (London, UK). The chapter investigates the long-term influence of an URB on river periphyton and the chemical and physical drivers of URB growth. The intention is to submit Chapter 4 for publication.

Chapter 5 reports findings from controlled microcosm growth experiments of a key URB taxon, *Sphaerotilus natans*, using airport de-icers as the sole carbon source. Turbidimetry was used to compare the growth of *S. natans* employing different FPDs,

at several concentrations, and comparing the pure-FPDs to their commercial products (CADs) to evaluate the influence of the additives used to optimise their performance. This study sought to establish if airport de-icers can facilitate the growth of URB taxa, formalising the previously putative link. At the time of submission, Chapter 5 has been submitted to *Frontiers in Microbiology* (**Table 1-1**).

Chapter 6 further expands on microbial growth experiments by adding additional layers of environmental realism. Firstly using river water as a complex but realistic background matrix for URB growth. Second, individually examining additional URB taxa (*Zoogloea oleivorans*) as identified in Chapter 3 and supported by existing literature. Third, using mixed cultures to evaluate interactions between URB taxa. And fourth evaluating the viability of time-lapse microscopy as a novel approach to study the complex interactions between URB species during growth experiments. Together these experiments sought to further improve understanding of URB growth and its role in rivers with poor water quality.

Chapter 7 addresses the transport drivers of airport de-icing compounds by presenting novel mobilisation experiments. This chapter sought to address the mobilisation of CADs in overland flow following exposure to different meteorological conditions (sun, wind, temperature) and subsequent rainfall event. This chapter has direct relevance to airport operators to improve understanding of CAD transport and potentially inform treatment strategies and processes. The intention is to submit Chapter 7 for publication.

Chapter 8 is a combined general discussion and conclusions which summarises the results presented in the previous chapters and discusses them alongside scientific literature to contribute to identifying the underlying processes and drivers that result in URB growth and degraded river ecosystems as a consequence of essential airport de-icing. This chapter also outlines recommendations for future research in the fields of sewage fungus and in managing airport de-icer contaminated runoff.

Table 1-1: Contributions from supervisory team to each chapter and the status of papers submitted for publication. Contributions are categorised as supervision (S), Writing – review and editing (R&E).

Chapter	Status	Journal	R. Grabowski	F. Hassard	A. Medina Vaya
Ch. 2	Submitted	Ecological Indicators	S, R&E	S, R&E	S
Ch. 3	Published	Hydrology Research	S, R&E	S, R&E	S
Ch. 4	-	-	S, R&E	S, R&E	S
Ch. 5	Submitted	Frontiers in Microbiology	S, R&E	S, R&E	S, R&E
Ch. 6	-	-	S, R&E	S, R&E	S, R&E
Ch.7	-	-	S, R&E	S, R&E	S

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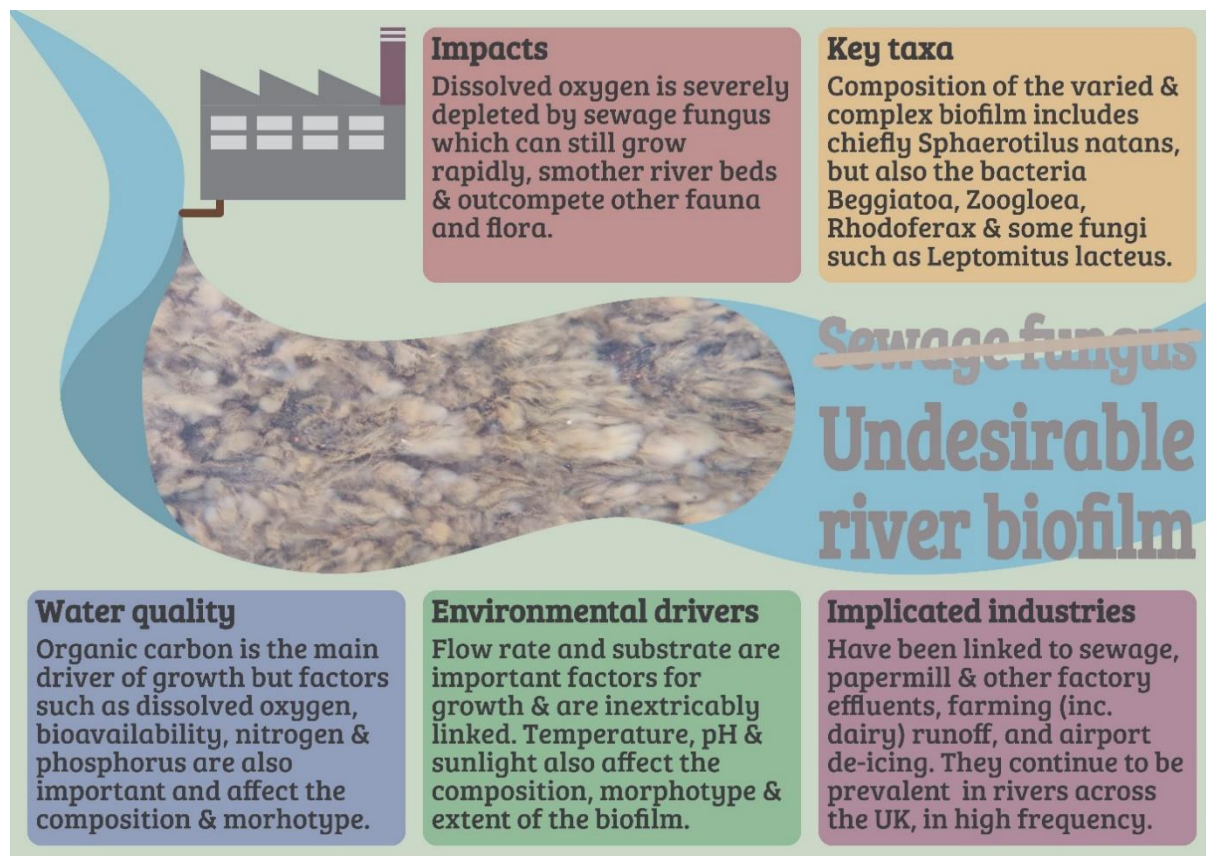
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2 Undesirable River Biofilms: Sewage Fungus in the 21st Century

Sewage fungus has long served as a bioindicator of organic pollution, but our scientific understanding of it has not kept pace with advances in related fields. This multidisciplinary review aims to provide an up-to-date re-examination of sewage fungus to support improved pollution mitigation and holistic river management. First, we argue that a new term, “undesirable river biofilm” (URB), is required to describe the composition, location, and environmental impact of sewage fungus more accurately. Secondly, we use recent genomic research supported by wider studies on stream biofilms to reassess the composition and structure of URBs. Whilst the bacterial taxa *Sphaerotilus*, *Beggiatoa*, and *Zoogloea* have been historically associated with URBs, it is now clear that URBs are polymicrobial consortia, characterized by low ecological diversity, which can be dominated by taxa not previously identified in URBs using culture-based techniques (e.g., *Rhodoferax*). Thirdly, we widen out the review to provide new insights into the environmental factors affecting the growth of URBs, which highlights the importance of water velocity, river substrate, and shading. Fourthly, we present new analyses of government data that documents the widespread and continued presence of URBs in rivers in England (UK); 6025 URB events were recorded by the regulator, in response to pollution investigations, over a 20-year period (2000 – 2020). These observations, though, likely underestimate the scale of the problem, as there is no statutory requirement to record its occurrence. The persistence, or perhaps re-emergence, of URBs in rivers, particularly in countries with robust water quality regulation and wastewater treatment, suggests that water quality limits may be insufficient by themselves to limit the negative ecological impact of organic pollution, and more holistic approaches to assessment that consider river hydrology, geomorphology, and riparian zones are needed.

Graphical abstract



Highlights

1. Sewage fungus, a bioindicator of organic pollution, is still found widely in rivers
2. The term “undesirable river biofilm” (URB) is a more accurate name for sewage fungus
3. Taxa most implicated include *Sphaerotilus*, *Rhodoferax*, *Beggiatoa*, and *Zoogloea*
4. URB occurrence and morphotype are affected by nutrients and environment factors
5. Future research on URBs is required to improve river water quality and ecology

Keywords

Sphaerotilus natans; periphyton; organic pollution; water quality; ecological impacts; bioindicator

2.1 Introduction

Improving biodiversity, which has experienced dramatic declines in recent decades, is a cornerstone of international agreements such as the COP15 biodiversity summit [1] and the sustainable development goals (e.g., SDG 15) [2], and one important aspect of these are efforts to improve water quality. Poor water quality remains a critical issue within freshwater environments globally with recent interest from the public, media [3–6], regulators [7,8], Non-Government Organisations (NGOs), and national governments [9] because of the significant negative impacts poor water quality has on One Health agenda (ecosystems, agriculture and human health nexus) [10–12]. For example, in the UK, a country with robust wastewater treatment, sewage (36%), agriculture (40%) and urban runoff (18%) continue to contribute considerably to poor water quality in rivers [9,13]. In the UK, agricultural activities are responsible for 25% of phosphate, 50% of nitrate, and 75% of sediment loadings in freshwaters [9,14]. Additionally, the continued prevalence of raw sewage discharges from combined sewer overflows (CSOs) creates a myriad of public health impacts and ecological damage in rivers [15]. From this runoff, nutrients, pathogens and micropollutants complex with fine sediments/particles. These particles can foul watercourse interfaces, inhibiting other essential ecosystem services, such as fish spawning, the growth of periphyton, contribute to the loss of keystone species [9,16] and reduce hyporheic exchange flows (HEFs) [17]. In addition to the ecological impacts, poor river water quality compromises waters used for essential human activity including sources of drinking water, agricultural irrigation, and recreational utilisation of freshwaters. While numerous emerging contaminants – such as microplastics, micropollutants nanoparticles and pharmaceuticals – have been detected in water courses [18], investigated scientifically, and placed under scrutiny for regulation [19], many rivers, streams and small watercourses still have a high degree of organic loading [9,20]. In addition to the high organic loading, highly degraded riverine ecosystems with heavy anthropogenic modification create ideal conditions for the growth of sewage fungus – a persisting but often neglected water quality issue.

Sewage fungus is a historical reference used to describe brown or white filamentous growths located on aquatic substrates in watercourses and has been primarily associated with watercourses subject to significant organic pollution located

downstream of point source sewage discharge in rivers [21] (**Figure 2-1**). Before water quality monitoring in rivers was frequently conducted, sewage fungus was readily used as a bioindicator of organic pollution [22] owing to its rapid appearance in saprobic rivers and its decay after pollution ceased [23]. Sewage fungus is the net effect rather than the cause of poor water quality; however, their growth further exacerbates ecological damage in rivers as they overgrow natural periphytons [24], prevent natural riverbank filtration [25,26] and their impacts persist long after the pollution event has finished. There is also data to support the role of key sewage fungus taxa influencing poor wastewater treatment performance through the phenomenon of bulking sludge in wastewater treatment [27–29]. During the 20th century, the advent of improved wastewater treatment reduced the loading of point-source municipal and industrial sources of organic material being discharged into watercourses and improved inland water quality [30] in the context of middle and higher-income countries. These measures have the effect of reducing the extent and severity of sewage fungus. However, sewage fungus continues to represent a persistent problem with prevalence most associated with environments receiving high organic loading beyond the carrying capacity of the fluvial environment (**Frequency of URBs detection in England (UK), Table 2-1**), including countries/regions with well-established wastewater conveyance and treatment systems.



Figure 2-1: Sewage fungus (i.e., undesirable river biofilm (URB)) growth in the River Crane, London (UK) linked to the use of de-icers at an adjacent airport from (a) the entire channel width (approximately 7.5 m), to (b) a small section of the channel, to (c) a close-up of the sewage fungus (approximately 30 cm width).

Sewage fungus has direct and indirect ecological interactions with natural fluvial microorganisms and higher biota, such as invertebrates and fish [31–33]. It is primarily an aerobic heterotrophic microbial consortium, therefore dissolved oxygen (DO) is required for growth [34–37]. However, during periods of low DO concentrations, it remains able to thrive and readily outcompetes other benthic organisms [33,38–40]. Sewage fungus rapidly overgrows the riverbed and associated periphyton, substantially degrading the benthic habitat for vital benthic organisms (e.g., invertebrates) [41–43] and ecosystem services such as fish spawning [32,44,45]. Sewage fungus utilises 10-20 fold more DO than is typically produced by aquatic macrophytes of the same biomass [46], consequently the high respiration rate of sewage fungus adds to localised DO depletion in rivers during organic pollution events. Furthermore, although the growth rate of sewage fungus in fluvial systems is similar to other microbial heterotrophs, the biomass yield of sewage fungus is substantial and has significant impacts to ecosystem functions. Sewage fungus is also reported to accumulate heavy metals (e.g., Zn^{2+} , Cd^{2+} , and Ni^{2+}) [47,48], other toxic substances (e.g., erythromycin, ethyl succinate, acetaminophen, acidic pharmaceuticals, steroidal hormones, and 4-nonylphenol) [49] and deleterious non-polar substances (such as benzene and its derivatives) [50] within its matrix. This leads to the entrapment and subsequent removal of deleterious substances from aquatic systems, thereby protecting surrounding fauna and flora. Nevertheless, the bioaccumulation of these substances may contribute to an increased loading within the watercourse upon the disintegration of the biofilm, resulting in ecological impacts throughout the food web. One important but unexplored aspect of sewage fungus ecology is their role in shaping the fate of antibiotic residues or antimicrobial resistance (AMR) genes. In other fluvial biofilms, they have been detected [51] and notable sewage fungus taxa [52,53]. Contaminants accumulate in the biofilm and can be released during periods of slough resulting in pollutant pulses during periods of sewage fungus decay [50]. Sewage fungus impacts are persistent in that they exhibit secondary consequences, in some cases including further degradation and suppression of natural ecosystems, beyond the time organic pollution load is lessened or removed [54], creating a further loss in biodiversity [55] and other biomass (flora [56] and fauna [57]) in the river. The ecological impact of poor water quality, especially

visible manifestations of this (such as sewage fungus), influences public perception and interaction with rivers – such as a loss of recreation (e.g., angling, dog walking, wild swimming, and paddle sports) [21,24].

Sewage fungus is a bioindicator of organic pollution and occurs in rivers where there is excessive organic loading. However, there remains a data dearth on the specific composition and morphology of sewage fungus, especially in relation to the type of available nutrients and environmental factors governing growth. There also remains a limited appreciation for the current extent of sewage fungus and implicated industries. Whilst these industries can still cause the growth of sewage fungus if poorly treated effluents are discharged or leak into rivers, a reliance of historic literature and reports of issues by local media groups is required to indicate these implicated industries (**Figure 2-2**). Currently, there are not established clean-up or treatment practices for affected rivers. Therefore, preventing pollution represents the primary control strategy in our rivers in terms of water quality and anthropogenic altering of the watercourses.

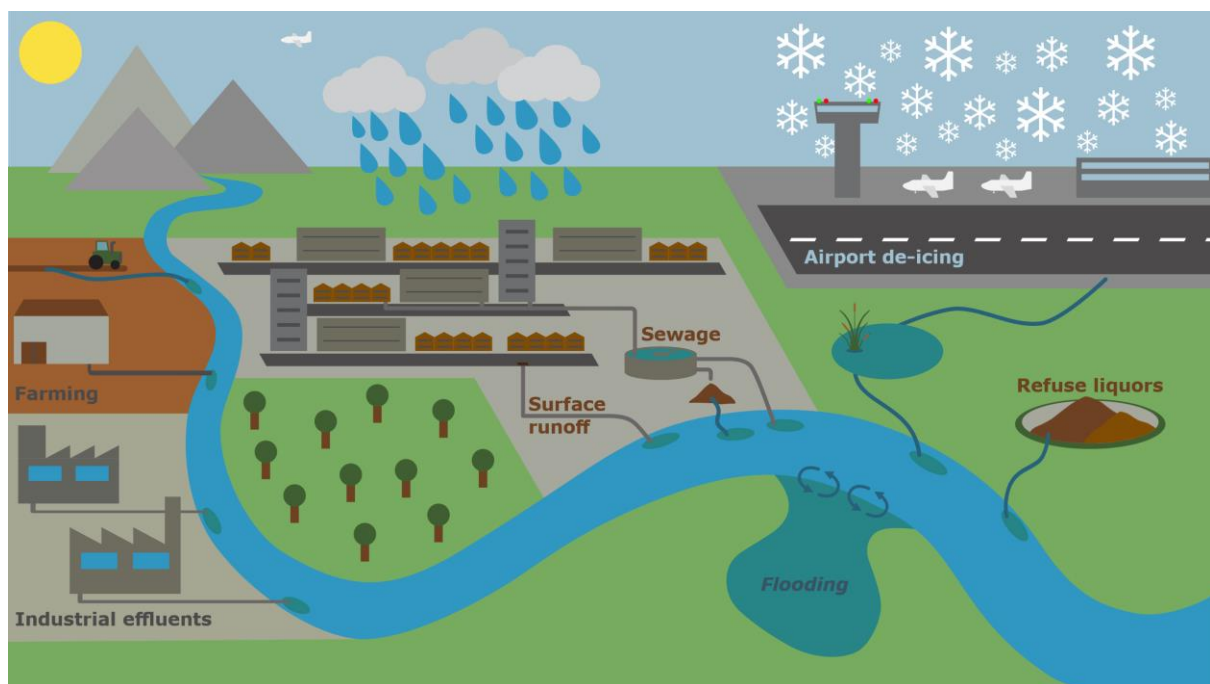


Figure 2-2: An overview of the industries and sources of pollution frequently implicated in causing sewage fungus.

Accordingly, this review aims to reassess our scientific understanding of sewage fungus including: (i) to propose a new, more accurate name for sewage fungus; (ii) to summarise and reinterpret sewage fungus using advancements in biofilm research and genomics; (iii) to explore the triggers of sewage fungus outbreaks, including nutrient availability and environmental factors; (iv) evaluate the effects and environmental impacts of sewage fungus proliferation; and (v) to examine industries and events which have been linked with sewage fungus outbreaks to support recovery in aquatic ecosystems.

This multidisciplinary review involved (i) the collation and critical review of research on sewage fungus and related topics (e.g. stream biofilms) and (ii) the visualization of sewage fungus occurrence data provided by a national regulator to provide new evidence of its continued presence in rivers at national scale.

Literature was identified using keyword searches in online databases, which was expanded through a manual search of cited references in key papers, which was particularly important to find older articles. The initial literature search was conducted in Scopus with the search term “sewage fungus”, further literature was identified using Google Scholar as some of it pre-dates accurate Scopus discoverability. Most of the research on sewage fungus was conducted prior to 1990 so, to advance our understanding of URBs, additional literature on biofilms was incorporated to review URBs as a biofilm. The search was expanded to include the search terms “stream biofilm” and “river biofilm”, filtered to exclude literature pertaining to organismal biology or medicine, which provided a foundation for studying sewage fungus as a biofilm. The search was not limited to any specific period, and research articles, books, technical papers, and review papers were included. Finally, highly cited papers on the “biofilm matrix”, limited to the years 2002 – 2022, were reviewed to provide insights into the biophysical structure of sewage fungus.

To illustrate the continued presence of sewage fungus in rivers, even in a country with strong wastewater treatment regulation, data on the recorded incidences of sewage fungus in England were analysed and visualised. Occurrence data were obtained from the Environment Agency (EA, England) via a standard data request. Whilst sewage fungus is not formally monitored by the EA, environment officers log a record if it is

present when they attend a pollution incident [58]. The EA provided records of all recorded incidents of sewage fungus for the years 2000 – 2020. For data protection reasons, no identifying information was included with the dataset (i.e. pollution source, river or administrative district); it listed solely the date and coarse spatial location (10x10 km grid). A heatmap was constructed by plotting coordinates and frequency of occurrences and subsequent analysis was performed in Microsoft Excel.

2.2 History of sewage fungus - an undesirable river biofilm (URB)

At the outset, we propose herein, that the term “sewage fungus” should not be used and henceforth renamed as “undesirable river biofilm” (URB) as this better reflects its composition, environmental context, and impacts. The best available evidence is discussed within the context of (i) examining the origins of the term “sewage fungus”; (ii) detailing why the commonly used term should be replaced, and (iii) defining and rationalising a new term.

The term “sewage fungus” was first defined in 1932 by R.W. Butcher to describe filamentous growths on the beds of polysaprobic and eutrophic rivers, despite stating it was a misnomer [59]. “Sewage fungus” is inaccurate for two important reasons. Firstly, it is not largely comprised of fungi but represents a diverse polymicrobial biofilm bound within a matrix of extracellular polymeric substances (EPS) [24,46]. Secondly, whilst “sewage fungus” was, and still can be, found near sources of untreated and poorly treated sewage [20,21,23,60,61], its occurrence is not limited to sewage outlets. Other industries and sources of organic pollution have also been implicated, notably airport de-icer and agricultural runoff (**Table 2-1**). Historically, other terms for sewage fungus have been used interchangeably, though inconsistently, including heterotrophic biocoenosis [62]; slime infestation [23]; heterotrophic slime [24]; and biological floc [63]. However, these alternative terms still do not appropriately describe and convey the composition, and ecology of sewage fungus. Similarly, other general terms for benthic microbial assemblies – such as river periphyton, microbial mats, or benthic biofilms – could be used, but they are not specific enough to sewage fungus. Therefore, we propose a new name that accurately reflects these polymicrobial assemblies that grow in organically enriched conditions in rivers, streams and other watercourses.

We propose the term “undesirable river biofilm” (URB) to replace “sewage fungus”, as it (i) addresses the common misunderstanding about the dominant biological community, (ii) identifies the ecosystem in which they are found, (iii) describes their morphotype; and (iv) that although biofilms, including URBs, are near-ubiquitous in nature [23,32], they become problematic and potentially ecologically damaging as their growth is rapid and blankets large sections of the river bed.

Firstly, “undesirable”. Biofilms can be categorised as beneficial, desirable, undesirable or disastrous [64,65], and whilst URBs perform an important ecological function, consuming excess dissolved organic carbon, they cause considerable damage to ecosystems with their prolific and dominating growth. Therefore, the term “undesirable” is most appropriate. Secondly, “river”. URBs are only found in flowing waters [23], as they require a minimum water velocity for oxygen supply and nutrient uptake (**Environmental**), and are a type of “stream biofilm” but are a specific consequence of excess organic loading. Thus URBs are often not found in upland streams nor in large estuaries where the water has too much energy, hence the more accurate categorisation of “river” biofilm. Finally, “biofilm”. URBs are fundamentally a biofilm, a “matrix-enclosed microbial population adherent to each other and/or to surfaces or interfaces” [66] (e.g., a riverbed or outfall apron), so the inclusion of “biofilm” was essential.

The appearance of an URB (i.e., colour and morphology) can vary greatly, depending upon its unique environment (i.e., water chemistry, hydrology, riverbed substrate, etc.) and specific microbial composition. For example, some URB outbreaks have been described as being like “cotton wool” (**Figure 2-1c**) whereas others are more like a “slime” and in a variety of colours (grey, brown, white, and sometimes with pink or yellow tints). The varied appearance of URBs has been widely documented [58], but how it relates to environmental conditions, the type of organic pollution, and ultimately the microbial community composition, EPS matrix composition and structure has not been well researched. Therefore, to gain a greater scientific understanding of URBs, findings from across a range of disciplines must be evaluated and integrated.

2.3 The composition and structure of URBs

The first step in our reassessment of URBs is to revisit their composition and structure so that their complexity and variability are better understood and considered in future research. In this section we (i) summarise the existing understanding of the microbial composition of URBs; (ii) examine how research using genetic approaches further explains the microbial composition of URBs; (iii) introduce the structure and functions of a biofilm (such as the biofilm matrix) in the context of URBs; (iv) discuss how advancements in biofilm research should be used to improve our understanding of URBs; and (v) illustrate the competitive and survival advantages of URBs.

2.3.1 What microorganisms create URBs?

The perceived microbial composition of URBs comes from work done between the 1950s and 1980s using culturing techniques [32,63,67–73]. Several genera were identified during these early studies, however, significant advances in microbiological and genetic approaches in recent decades should be leveraged to gain greater insights into the URB community dynamics [24,74]. In this section, we summarize the earlier research that has most influenced our perceptions of URB composition, counterbalancing it with recent findings that suggest a richer and more varied microbial community than early studies reliant on culture-based enumeration.

Sphaerotilus spp., especially *Sphaerotilus natans*, has been the keystone species in URBs, to such an extent that, in many studies, it is synonymous with “sewage fungus” [32,63], despite URBs being a diverse assembly of microorganisms. *S. natans* is a filamentous bacterium with rod-shaped, non-sporing, Gram-negative cells with rounded ends [46] embedded in a firm sheath [23,28]. However, if released from the sheath, *Sphaerotilus* cells are motile using their subpolar flagella [75]. Carbon sources available to *Sphaerotilus* spp. include alcohols, organic acids and sugars, whilst using ammonium and nitrates as nitrogen sources. *Sphaerotilus* spp. are strictly aerobic but can grow at very low DO concentrations (<0.1 mg/L) [75] highlighting their importance in the URB when DO levels are depleted in the water course. It is often found in organically enriched river waters [21,31,46,76–78] and is itself an indicator of high levels of organic pollution [24,79]. *S. natans* has been observed to be a particularly important contributor to URB events with high inputs of mono- and disaccharides, low

molecular weight (M_w) organic acids, low M_w alcohols, glycerol's and amino acids [23,46,80,81], specifically, glucose and acetate [45]. Other *Sphaerotilus* species, such as *S. tenue* and *S. montanus* have also been reported as minor components of URBs [24,79].

The genera *Zoogloea* and *Beggiatoa* have also been reported as dominant taxa in URBs [34,58]. *Zoogloea* are rod-shaped bacteria with a single polar flagellum and readily form a gelatinous biofilm known as *Zoogloeeae* [82]. These biofilms are also found in freshwaters that have been organically polluted and are usually restricted to slower-flowing waters [58], such as exposed surfaces in wastewater treatment plants [82,83]. The metabolism of *Zoogloea* is strictly aerobic and can utilise a range of organic carbon sources and are not nutritionally particularly fastidious [82,84]. The main carbon sources used by *Zoogloea* are organic acids, alcohols, and various aromatic salts. Its nitrogen requirements can be satisfied by a broad range of organic nitrogen (such as dicarboxylic amino acids) and ammonia but not nitrate [82]. *Beggiatoa* often consist of long filaments (5-10 cm) and freshwater strains often have cell diameters $<5.0\ \mu\text{m}$. *Beggiatoa* are found on sediments at the interface between anoxic and oxic zones and are typically found in waters containing sulfur due to their ability to oxidise H_2S as an energy source [85]. *Beggiatoa*, like *Sphaerotilus*, is aerobic but can persist during microaerophilic conditions. *Beggiatoa* is more selective and oxidises a smaller pool of carbon compounds than *Sphaerotilus* or *Zoogloea*, but it is reported to grow on C_{2-4} organic acids and sometimes amino acid equivalents [85]. Both freshwater and marine strains of *Beggiatoa* can fix N_2 and utilise nitrate, nitrite, ammonia, and certain amino acids as nitrogen sources [85]. Other bacterial species *Thiothrix* II, *Flavobacterium* spp., and *Flexibacter* spp., have also been associated with URBs [58] but knowledge of their abundance, distribution and contribution to URBs in rivers with organic pollution is limited.

The microbial composition of URBs has more recently been tested using next-generation sequencing approaches in the context of airport de-icer contaminated runoff: Nott *et al.* (2020) used PhyloChip G3 DNA microarrays with whole genome performed on certain samples [86]; and Exton *et al.* (2023) used targeted amplicon sequencing of 16S rRNA, V3-4 region [87]. Both studies reaffirmed that *Sphaerotilus* represents a keystone genus, accounting for 14.0% of URB taxon based on relative

abundance [87]. Nott *et al.* (2020) performed metagenomic analysis on select samples and isolated a *Sphaerotilus* strain that was identified as a new strain of *S. montanus*. Other taxa previously associated with URBs have also been detected for example Nott *et al.* (2020) found the genera *Thiothrix* but not *Leptothrix* whereas Exton *et al.* (2023) found that members of the family *Comamonadaceae* (64.1% of relative abundance) including *Zoogloea* were most frequently associated with URBs. Within this family, the genus *Rhodoferrax* (32.8% relative abundance) was identified in high abundance for the first time, suggesting the need for further research using genomic techniques to better understand the composition of URBs, especially for taxa which may have previously been underrepresented by culturing techniques [87]. *Rhodoferrax* are typical freshwater bacteria [88] especially those suffering from organic enrichment [89] but were not associated with URBs in prior works. Some species within *Rhodoferrax* are facultative photoheterotrophs [89], some facultative anaerobes [90], and some fully anaerobic in the light [89] so organisms may not feature in aerobic cultures which explains their absence in those studies. Further genomic studies are needed to better elucidate how URB influences and is influenced by the natural streambed microbiome.

Other taxa of microorganisms may also be important constituents of URBs, but, at present, there is limited empirical evidence of their abundance and roles. Fungi have been found in URBs, for example, *Leptomitium lacteus* [58,91] (“water mould” [68]). This is a branching fungal hyphae with a “cotton wool” appearance [92]. Found in freshwaters, *L. lacteus* is an indicator species of water contaminated with organic matter, such as sewage and sugar factory refuse [67,91,93] and can utilise low Mw organic acids but not sugars, which are typically more bioavailable [67]. However, *L. lacteus* is less studied compared to *Sphaerotilus*, *Zoogloea*, and *Beggiatoa*. This species can proliferate in acidic waters and grows well using high Mw organic nitrogen compounds (such as amino acids but not ammonium, nitrate or nitrite) [23,67]. Other associated fungi include *Geotrichium candidum*, *Fusarium aquaeductuum*, and *Achlya* spp. [58]. Algae are naturally present in river periphyton with some species, such as *Cladophora glomerata* [58], also found to be important components of URBs. For example, algal strands have been reported to provide excellent support for *S. natans*’ growth [22]. However, the role of algae in URBs largely remains poorly understood. In stream biofilms, algae can produce a significant amount of organic substrate for

heterotrophic biofilm microorganisms [94] and may also play a role in the structure and organization of the biofilm matrix [95]. Archaea and protozoa (e.g., *Carchesium polypinum* [58]) are a minor component of benthic stream biofilms taxonomy [96,97], and viruses have been detected within stream biofilms but are not thought to impact URB growth [97].

The microbial composition of an URB is therefore more diverse than early studies suggest. The water quality factors influencing URB growth are reviewed in **Water quality** and the environmental drivers in **Environmental drivers of growth**. However, knowledge gaps as to how factors, such as source water microbiome, available nutrients [98] and environmental conditions [74,99], determine the specific and unique URB microbiome remain.

2.3.2 The biofilm matrix in the context of URBs

The matrix in an URB is produced by microorganisms, including bacteria [47], microalgae [100], and fungi [101,102], with the extracellular polymeric substances (EPS) accounting for over 90% of biofilm mass [47]. Within the URB biofilm matrix, distinct microhabitats form with differing physical properties [103–107] (such as oxygen and substrate gradients) influencing the types of metabolic activity within [103–110]. As a result, URBs have a high nutrient utilisation [111–113], permitting URBs to quickly establish and dominate the river habitat. Established biofilms can often use their matrices and lysed cells to sustain themselves after nutrient sources have disappeared [111–113]. The role of intermittent organic pollution events and the persistence of URBs during periods of low or intermittent organic loading remain to be investigated.

Biofilms are highly responsive and adaptive to environmental stimuli, offering the constituent organism(s) several strong competitive and survival advantages. For example: using their phenotypic plasticity (the ability of an organism to change its behaviour, morphology, and physiology in response to a unique environment) to quickly adapt [66]; be tolerant to desiccation [114–116]; be resistant to attack from bacteriophages and vortex-feeding protozoa [66]; and the ability for active dispersal of the biofilm by partial matrix degradation [117]. Thus indicating that the highly organised biofilm is not an end-point but rather undergoes continuous remodelling [118]; it adapts

and evolves to maintain ecological competitiveness. Therefore, URBs must not be considered static entities, their matrix structure and microbial composition can quickly react to changing environments (including available nutrients and environmental/hydrological factors). The wide-ranging, unique features of biofilms give them strong, competitive advantages which make tackling URB occurrences and the resulting impacts in natural, aquatic ecosystems even more challenging.

2.3.3 Implications of advancements in biofilm analyses for URBs

In recent years, there have been a number of key advancements in studying biofilms, allowing for a better understanding of their composition and functions [119], including next-generation sequencing [120–122] (some studies have begun to use these, as described above [86,87]); measuring growth kinetics [119]; EPS measurements; microscopy techniques; fluorescence in situ hybridisation techniques; and biofilm structure [123,124]. These advancements, summarised by Azeredo *et al.* (2017) [119], should be implemented in the context of URBs to give a much richer understanding of how these environmental biofilms initiate growth, proliferate and impact local environments, utilise available nutrients and/or pollutants, and how we can reduce their impact, severity and ecological damage.

2.4 Factors affecting URB growth

The conditions that affect where URBs reside and their morphology include (i) water quality, especially organic carbon in addition to other macro- and micro-nutrients required for microbial growth, and (ii) the various environmental factors that can affect the growth of URBs (**Figure 2-3**).

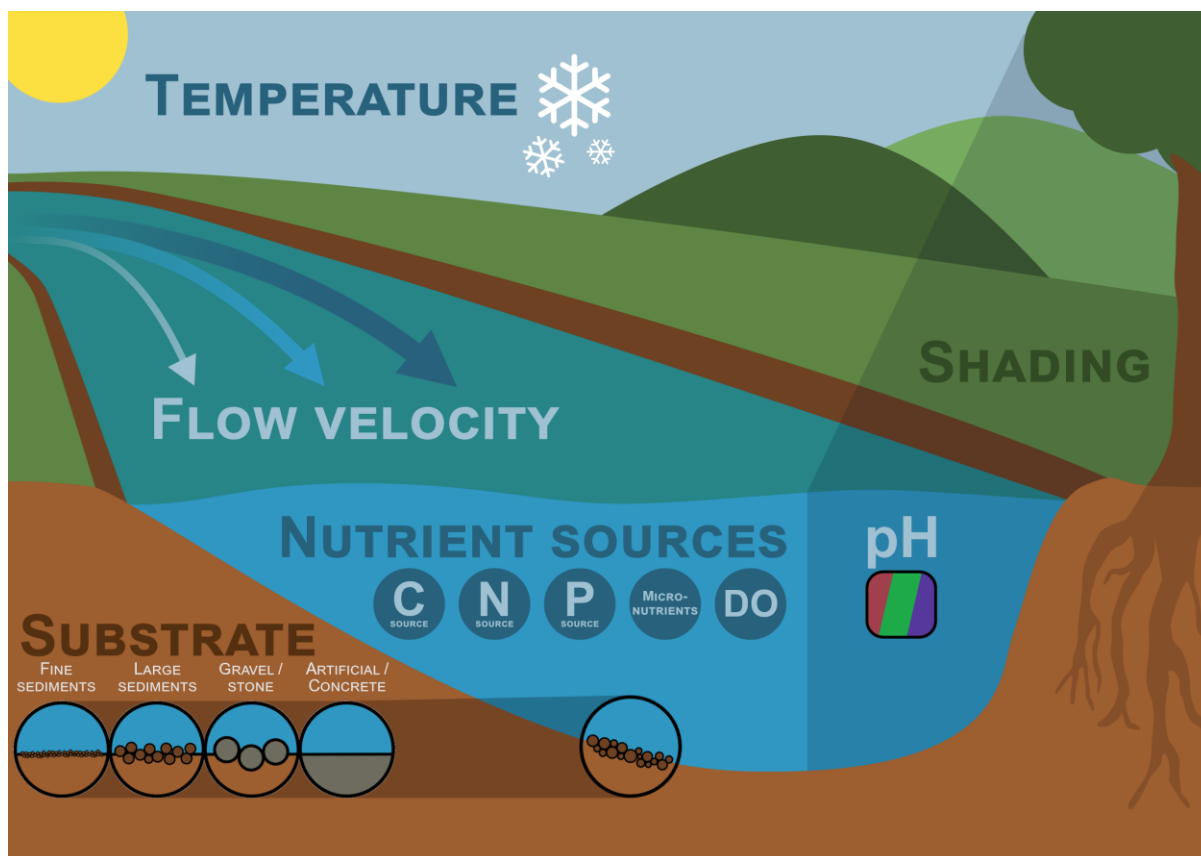


Figure 2-3: An overview of the environmental and water quality drivers affecting the growth and morphotype of undesirable river biofilms (URBs).

2.4.1 Water quality

URBs are natural biological indicators of organic pollution [22,125], therefore their growth and composition are most associated with the availability of organic carbon [24,98]. Previous research has identified some of the carbon sources that certain URB organisms can utilise, particularly the range of organic compounds found in sewage. However, the bioavailability of organic carbon remains a complex issue [126] and is still not fully understood, especially in the context of URBs. There has been limited research on the specific carbon sources different URB taxa can utilise and the preferential orders of substrate utilisation in a diverse and complex media [127,128]. Methods to examine bioavailability and nutrient preference are established [129–131] but have not yet been applied in the study of URBs. Furthermore, ratios between nutrients (e.g., C:N:P ratio) is important to the amount of and composition of biofilms,

however has not been directly studied in URBs [132,133]. Future work should aim to examine nutrient utilisation in URB taxa and common pollutants associated with their growth.

Nitrogen is an essential macronutrient for URB growth [71,134] and can be utilised for growth in many forms, more broadly as organic nitrogen, ammonium, nitrate, and nitrite. *Sphaerotilus* can use ammonium and nitrate [75] but the role of vital nutrients like nitrogen and phosphorus is less important than the demand for organic carbon [83]. Available sources of nitrogen are generally found in adequate amounts for URB proliferation in natural freshwaters [21]. However, specific industries can have an impact on the concentration of nutrients, such as nitrogen. For example, dairy factory effluents have been recorded as being slightly nitrogen deficient to support URB growth [135] but, depending on the extent of the pollution, background water quality may offset this deficiency from becoming limiting. Whereas effluents from sources such as slaughterhouses have been reported to generally contain sufficient nitrogen sources to prevent any limitation [136,137].

The supply of phosphorus in most freshwaters (usually in the form of phosphate) is, like with nitrogen requirements, only rarely limiting for URB growth – a phosphate content of only 0.05 mg/L was found to support heavy URB growths [45]. Some studies have reported that phosphate concentrations of <0.04 mg/L may limit growth [138,139], while others noted that at 0.01 mg/L growth was not supported [140], with different studies finding that increasing phosphate concentrations above 0.05 mg/L did not result in enhanced growth [21]. *S. natans* can reportedly be a dominant species over a wide range of phosphate concentrations of 0.01-1.5 mg/L, encompassing the concentrations of phosphate found in most rivers in the UK [140–142]. It has also been reported, however, that only a small proportion of phosphorus is incorporated into URB biomass [45].

Specific micronutrients are important for the growth of URB taxa, for example, calcium is reportedly an important micronutrient requirement for *S. natans* to develop its sheath, in turn allowing filamentous growth to become a biofilm [46]. Vitamin B₁₂ or methionine has also been reported as important for *S. natans* growth [23], though other studies suggest that vitamins are rarely at levels where they inhibit URB

development [21,71]. Certain chemical conditions in the river environment can also promote certain taxa, for example, high sulfide concentrations results in an URB that is dominated by *Beggiatoa* sp. [78,143].

2.4.2 Environmental drivers of growth

Whilst the availability of nutrients, especially dissolved carbon, is highly important to microbial growth and composition, environmental factors have a strong influence on the presence and growth rates of URBs and influence the microbial composition and morphotype of the biofilm. These factors include flow velocity, substrate types, exposure to sunlight, temperature, and pH.

Water velocity and discharge are an important factor influencing URB presence and is the most significant factor in defining the structure and morphotype of URBs [95]. URBs require a minimum velocity to replenish nutrients and DO (which are rapidly consumed by URBs) [22,32,63], and, thus, are not found in stagnant waters [46]. The minimum water velocity to support filamentous growth of *Sphaerotilus* spp. has been reported as 0.19 m/s [46]. Velocities below this threshold only support single-cell forms of the bacteria which can manifest as degraded ecosystems, not URB growth [139]. The maximum water velocity that URBs can support before being scoured away has been reported at 0.6 m/s [22,23,46]. It is hardly surprising that the biomass of benthic biofilms decreases drastically following a flood event [144] but this does cause the burial of parts of the biofilm [145] so taxa are still in the ecosystem. The resulting increased organic loading that often results from flooding, or storm water overflows after periods of heavy rainfall [146], could therefore help to facilitate prolific URB growth once flows decline. Minimum and maximum thresholds likely vary according to the dominant taxa and morphology of the URB, but additional research is needed in this area to confirm. However, thresholds are inextricably linked to the substrate to which the URB is adhered, where lower maximum thresholds exist due to the mobilisation of the substrate.

URBs grow on submerged surfaces in rivers, such as the riverbed (e.g., cobble, gravel and sand), artificial beds and banks, and other objects, of natural and anthropogenic origins (e.g., branches, plastics, rubble and litter) which are often present alongside organic pollutant sources. Immobile substrates such as submerged tree branches,

large anthropogenic-derived litter and concrete-lined channels are excellent facilitators of URB growth where the limiting factor becomes the scouring force. Fine sediments are mobilised much more easily than larger substrates and are becoming more prevalent in urban rivers [147–149]. However, the EPS of biofilm matrices plays a major role in the biostabilisation capacity of riverbed sediments [145,150,151] – i.e., the cohesion of sediments and biofilm to the substratum [152] – thus making the bed less affected by higher flows when there is an established biofilm [153,154]. The biofilm also changes the hydraulic properties of the bed [154–156]. Whilst this literature relates to general river biofilms, sediment stabilisation and river hydraulics have not been studied in direct relation to URBs. Therefore, we have extrapolated from broader research about river biofilms to summarize that both the river velocity and substrate play vital roles in enabling and facilitating URB growth and, although immobile substrates best facilitate URBs, the biofilm can stabilise more readily mobilised sediments to better facilitate growth.

URB forming species can grow over a relatively wide temperature range [157]. For example, *S. natans* has been reported to grow between 5 and 40 °C [63,158,159], but other studies suggest a narrower range of 15 to 40 °C [160], with an optimum growth between 25 to 30 °C [32]. Various URB species have similar temperature ranges. For example, *Zoogloea ramigera* grows between 9 and 37 °C with an optimum range of 20 to 25 °C [161] and *Beggiatoa* spp. can grow between 0-40 °C [85]. These temperature ranges suggest that URBs might not proliferate quickly enough to establish and persist in winter. However, URB growth has been observed in temperate rivers receiving airport de-icer contaminated runoff in cold winters (**Table 2-1**), for example in the River Crane (London, UK) where winter water temperatures generally range from 2.5 – 7.5 °C [162]. The differences observed between laboratory studies and environmental observations reflect the remarkable resilience of biofilm growth in harsh or extreme conditions [74,163,164].

A biofilms matrix can buffer pH changes by establishing steep, stable gradients (e.g., by heterotrophic metabolism) providing localised habitats in the biofilm and protecting constituent cells, even early in the biofilm lifecycle [50]. Consequently, URBs are able to tolerate a wider pH range than other more sensitive organisms [165]. The pH of

poorly buffered rivers is also further lowered by the enhanced heterotrophic activity of URB proliferation, by the production of CO₂ – in turn affecting other aquatic flora and fauna in the river, causing a further loss in biodiversity [24]. However, common URB taxa (as described above), are not described as nitrifiers which can lower pH by the oxidation of ammonia to nitrate [166].

There can be considerable variation in the length, both spatially and temporally, of URB outbreaks. Some outbreaks are seasonal, or may recover better in certain seasons [46]. For example, sunlight has a strong effect on URB composition as the ratio of chemical nutrients to sunlight determines the abundance of heterotrophs to phototrophs in the URB [167]. This is affected by factors such as seasonal light variations, but also shading from overhanging vegetation, river depth, and turbidity [168]. It is unlikely that increased light contributes to increased URB growth as URBs are inherently a response to high organic loading. Nonetheless, both heterotrophs and phototrophs exist within URBs and the proportion of them are affected by light and nutrient availability. Fundamentally, URB outbreaks remain as long as sufficient nutrient availability and appropriate environmental conditions remain [46]. Whilst environmental conditions are undoubtedly important to the composition and proliferation of URBs, there is contention over the contributions of genetics, nutrient, and environmental conditions to the development of biofilm structure [169,170] – but this should not imply that any are mutually exclusive.

2.5 Frequency of URBs detection in England (UK)

URBs remain a problem in rivers and streams globally, despite improvements in wastewater treatment and environmental and water quality regulation, but their reporting remains poor, even in countries with robust environmental reporting practices. With a renewed interest in water quality impacts in Europe, a renewed interest in URBs must also emerge. In the UK, the continuous discharge of treated effluent and intermittent discharge of raw sewage (i.e., from CSOs) have been raised as significant issues affecting the ecological status of river water bodies and public health [7,20]. Therefore, in this section, we use England (UK) as a case study where some recording of URB incidents still occur. The frequency of URB events is reviewed from primary data to (i) provide new empirical evidence on the widespread occurrence

of URBs, (ii) examine what industries are implicated; and (iii) explore in more detail an industry and activity that is frequently linked with URB outbreaks, commercial aviation, and airport de-icing.

Official records from the Environment Agency, the environmental regulator for England, identified 6025 incidents of URBs across all counties in England between 2000-2020 at pollution investigations (**Figure 2-4**). The number and the spatial distribution of cases, although this is likely to be a substantial underestimate due to underreporting and reliance on monitoring surveys and citizen science reports. The Environment Agency officers record the presence of URBs, as “sewage fungus” as a criterion for the identification of an unsatisfactory storm overflow [171] and whilst the presence of “sewage fungus” is noted when attending a pollution incident (using their field guide) [58], it is not something that regulators (such as the Environment Agency) monitor or investigate. It is also probable that spatial distribution is a reflection of meticulousness of different field officers and is not necessarily a true representation of URB distribution. The underrepresentation of the problem is evident from the lack of data at locations for which there have been repeated confirmed URB events from observations by members of the public, the authors, reports by local news sites, and communications with several Environment Agency officers. Given the increasing awareness of the levels of organic pollution in UK rivers, particularly from sewage and farming, this further indicates the likely significant underestimation of URB occurrences [9,20,172,173]. Furthermore, additional information about each occurrence, such as the implicated industry, was not provided and no community analyses were performed.

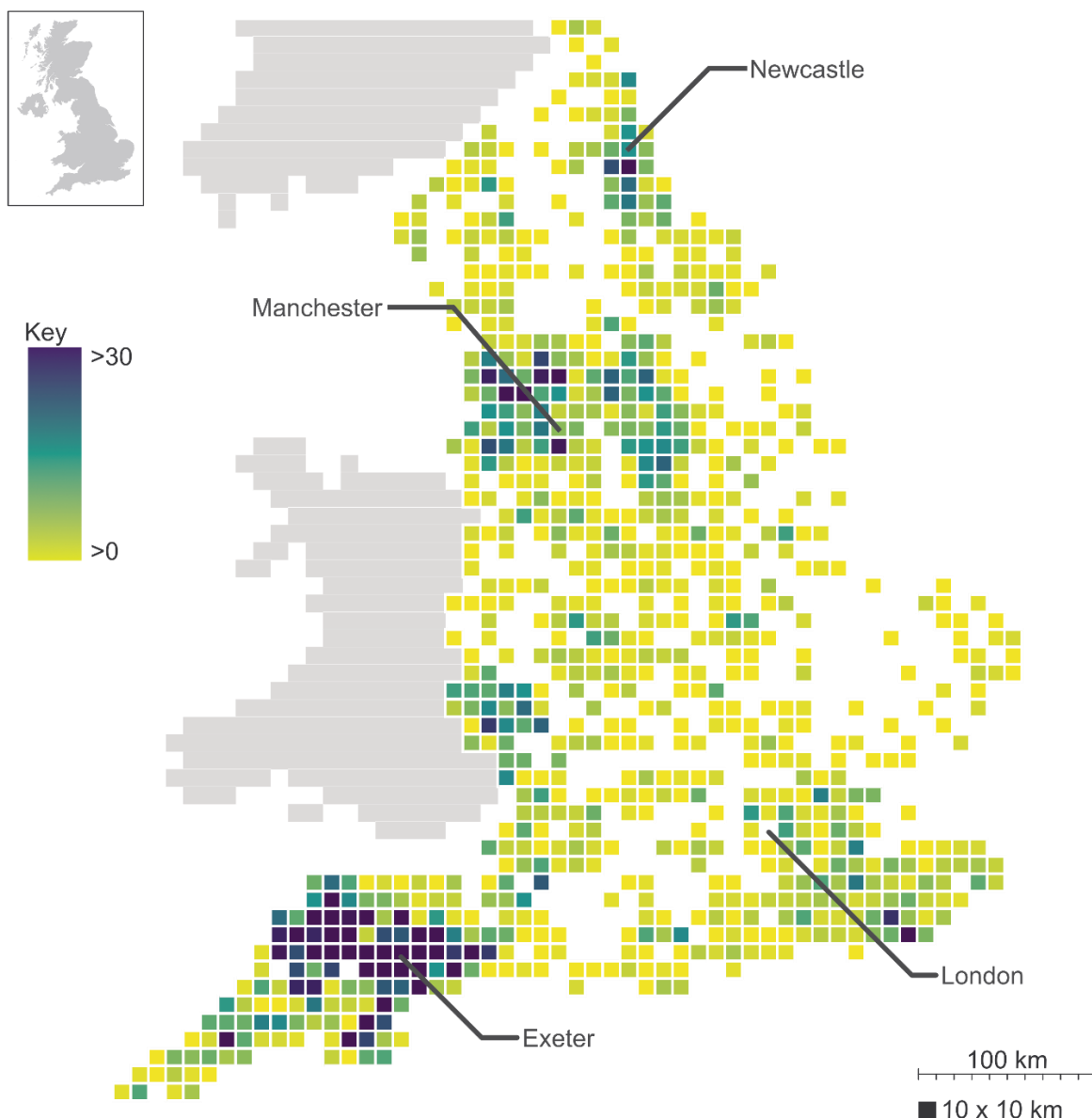


Figure 2-4: Heatmap of undesirable river biofilm (URB) occurrences per 10x10 km² in England between 2000-2020 recorded by the Environment Agency. Using data provided with permission by the Environment Agency.

Attributing the cause of URB growth to specific industries and activities remains difficult, especially for diffuse pollution (**Figure 2-2**). Due to the nature of the pollution and water quality data recording and management in England, observations of “sewage fungus” (as presented in **Figure 2-4**) are not linked to a suspected cause. Equally, whilst the likely source of a pollution incidence is recorded when it is

investigated by environment officers, the evidence used to assess the event, which includes “sewage fungus”, is not recorded. There remains a need for data that links URB incidences and sources so better, more informed mitigation approaches can be developed.

Therefore, here we rely on historic literature, national and local news, community groups and Government press releases to find pollution sources linked to URBs (**Table 2-1**). Existing scientific literature details implicated industries including farming (especially dairy effluents), wastewater, power plants, refuse liquors, and a range of industrial effluents (**Figure 2-2**). However, with improving wastewater treatments and tighter regulations, these industries causing URBs may have changed in recent years. Therefore, leveraging observations by members of the public, community groups and reports by local news outlets in the UK, we compiled a list of currently implicated industries in **Table 2-1** to help build a better, current understanding of industries causing URB outbreaks. Whilst direct causation has not been proven, high levels of correlation between certain industries, effluences and URBs has been well documented over many years (**Table 2-1c**). Whilst many industries historically associated with URBs, such as agriculture, industrial effluents and untreated sewage remain prevalent, an industry with increasingly extensive association is the runoff from airport de-icing [86,87,174].

Table 2-1: Reports of “sewage fungus” outbreaks showing the putatively implicated pollutants in (a) UK news, (b) UK Government reports, and (c) scientific literature.

(a) UK media coverage	Implicated pollutant	River	Date (of article)	Link
Fermanagh business fined for water pollution offences	Chicken shed washings (agriculture)	unnamed tributary of the Arney River, Northern Ireland	17 Feb 2023	[175]
Thames Water’s real-time map confirms raw sewage discharges	Raw sewage	Colwell brook (River Windrush), Witney, UK	23 Jan. 2023	[176]
England’s rivers pay the price for hollowed-out Environment Agency	Airport de-icer	River Trent (& its tributaries), UK	04 Nov. 2022	[5]
Giant fungus spreading into rivers near East Midlands Airport being investigated	Airport de-icer	River Trent (& its tributaries), UK	09 Jun. 2022, 01 Jun. 2022	[177–179]
Water firm fined £240,000 over County Durham sewage discharges	Raw sewage	Coundon Burn, Bishop Auckland, UK	19 Jan. 2022	[180,181]
Sewage fungus sighted in West Oxfordshire after heavy rain	Raw sewage	Colwell Brook (River Windrush), UK	06 Jan. 2021, 13 Jan. 2021,	[182–184]

21 Apr. 2021				
Sewage warning for Witney stream after heavy rainfall	Raw sewage	Colwell Brook & Queen Emma's Dyke (River Windrush), Witney, UK	26 Dec. 2019	[185]
River Almond polluted by de-icer from Edinburgh Airport	Airport de-icing	River Almond, Edinburgh, UK	05 Dec. 2019	[186]
Fungal blooms on the River Crane may be caused by pollution from Heathrow outfall	Airport de-icing	River Crane, Hounslow, UK	19 Feb. 2019	[187]
Farmer fined after 'sewage fungus' contaminated stream which feeds 'important' Somerset fishery	Agricultural wastes	Sedgemoor Old Rhyme, Stoke St Gregory, UK	04 Jan. 2019	[188]
Stoborough Heath sewage spill: Wessex Water pays £35,000 for restoration works	Raw Sewage	Stoborough Heath, UK	23 Jan. 2018 (pollution occurred)	[189]
(b) UK government reports	Implicated pollutant	River (UK)	Date (of event)	Link
Somerset supplier to national supermarkets fined for polluting watercourse	Farm manure runoff (agriculture)	<i>tributary of the Congresbury Yeo near Cheddar</i>	28 Jun. 2019	[190]

Negligent farmer fined for fouling SSSI	Agricultural runoff (inc. cattle waste)	<i>tributary of the River Great Ouse, Salcey Forest</i>	April 2018	[191]
Wessex Water pays for polluting Dorset nature reserve	Sewage discharges	<i>ditch in Stoborough Heath, Dorset</i>	23 Jan. 2018	[192]
Somerset farmer ordered to pay nearly £4,000 for stream pollution	Farm silage & slurry (agriculture)	<i>stream near Stoke St. Gregory, Somerset</i>	04 Dec. 2017	[193]
Company admits polluting Fenland watercourses	Silage liquor	<i>Little Racy Drain, Emneth Hungate, Norfolk</i>	07 Feb. 2017	[194]

(c) Journal articles	Implicated pollutant(s)	River	Year published	Reference
Polybacterial shift in benthic river biofilms attributed to organic pollution – prospect of a new biosentinel?	Airport de-icers	<i>River Crane, UK</i>	2023	[87]
Stream pollution causes aggregation of wintering insectivorous birds through increased aquatic emergence	Sewage	<i>Vedeggio, Switzerland</i>	2022	[195]
Detection of untreated sewage discharges to watercourses using machine learning	Sewage	<i>(anonymous) UK</i>	2021	[20]

Acute riverine microplastic contamination due to avoidable releases of untreated wastewater	Untreated urban wastewater	River Tame, UK	2021	[196]
Advanced biofilm analysis in streams receiving organic deicer runoff	Airport de-icers	Edgerton Channel & Wilson Park Creek, Milwaukee, Wisconsin, USA	2020	[86]
River biofilm community changes related to pharmaceutical loads emitted by a wastewater treatment plant	Pharmaceuticals (from WWTP)	Arve River, France	2018	[61]
Understanding Microbial Biofilms in Receiving Waters Impacted by Airport De-icing Activities	Airport de-icing effluents	Kinnickinnic River & Thornapple River	2014	[174]
Morphological and Biochemical Properties of a <i>Sphaerotilus</i> sp. Isolated From Paper Mill Slimes	Paper mill effluent	(<i>unknown</i>)	1999	[157]
The impact of livestock-farming on Welsh streams: The development and testing of a rapid biological method for use in the assessment and control of organic pollution from farms	Livestock farming (agriculture)	(various) West Wales	1993	[197]
Assessment and Control of Farm Pollution	Farm pollution (agriculture)	Eastern Cleddau catchment, west Wales	1992	[198]

Effects of slaughterhouse and dairy factory wastewaters on epilithon: A comparison in laboratory streams	Slaughterhouse & dairy wastewaters (agriculture)	Manawatu River, New Zealand	1989	[37,199,200]
Sewage fungus growth in rivers receiving paper mill effluent	Paper mill effluent	(<i>unknown</i>) UK	1977	[201]
The occurrence of sewage fungus in rivers in the United Kingdom	Industrial effluents, food & drinks industries, domestic sewage	(<i>various</i>) UK	1971	[21]
Slime Infestation: Literature Review	Various industrial wastewaters	(<i>various</i>)	1958	[23]

One industry that has received recent and well-reported attention regarding URB proliferation in recent decades is aviation [86,87,174]. URB outbreaks are commonly observed during winter months in watercourses that receive surface runoff from airports, which has been linked to the use of chemical anti- and de-icers [86,87,174]. Numerous incidences have been reported at airports in the USA and UK, including Milwaukee Mitchell Airport (Wisconsin, USA), Airport (Michigan, USA); Bishop International Airport (Michigan, USA), Gerald R. Ford International Des Moines International Airport (Iowa, USA), Cincinnati/Northern Kentucky International Airport (Kentucky, USA), Pittsburgh International Airport (Pennsylvania, USA), T. F. Green Airport (Rhode Island, USA), Heathrow Airport (London, UK), Edinburgh Airport (Edinburgh, UK), and East Midlands Airport (Leicestershire, UK) [86,174,177,179,186,187,202,203]. Required for safe operation during cold months [204], aircraft and other airport surfaces are applied with millions of litres of anti- and de-icers annually [205]. Anti- and de-icing fluids include propylene glycol, ethylene glycol, or acetate/formate based, resulting in high organic loading in surface runoff which is treated (most commonly by aerated wetlands or moving-bed biofilm reactors) [206–208] before ultimately being discharged within permitted consents into receiving waters but potentially causing URB growth (**Figure 2-1**). However, the mechanisms (e.g., thresholds of de-icer discharged, dominant taxa, nutrient utilisation/growth kinetics, and the influence of environmental factors) by which these chemical anti- and de-icers trigger URB growth is unclear. To address these critical gaps in our scientific understanding of URBs and the water quality and environmental conditions that trigger URBs a renewed focus needs to be placed on this persistent issue. Future research should, therefore (i) draw on the significant advances that have been made in related fields in biofilm research, and (ii) recognise the opportunity airport de-icing can offer as a more temporally predictable cause of URB growth so that effective management solutions can be developed.

2.6 Conclusions and future direction

Bacterial communities and biofilms are integral components of rivers [23,32,209]. URBs are a natural part of aquatic life, appearing at times and locations of excess organic loading [24,46]. As a component of the self-purification processes in rivers,

their presence on the riverbed is a sign that the watercourse remains aerobic and excess organic load is being processed. However, URB growth often and quickly becomes extensive or persistent and has profound impacts on the river ecosystem. URBs considerably deplete DO as they rapidly grow, which, in turn, blankets the channel causing habitat loss for aquatic plants, invertebrates and fish – resulting in lasting impacts throughout the food web along with potential for recreation and livelihoods continuing long after the pollution event and URB.

The relevance of URBs is clearly demonstrated by the findings presented here and the pervasiveness of URBs in UK rivers is evident. URBs have significant ecological impacts within rivers by substantially depleting DO, outcompeting other more sensitive species, and smothering riverbeds, undoubtedly having a profound effect on the biosphere. The microbial composition of URBs needs further investigation but the presence of *S. natans* is prominent. The specific microbial composition and morphotype of an URB are defined by the nutrients present (most significantly organic carbon) and environmental conditions (e.g., flow rate, substrate, temperature, and other water chemistry) for each occurrence. Improving wastewater treatment has undoubtedly improved the quality of our rivers and reduced the frequency and extent of URBs. However, **Figure 2-4** highlights, yet still underestimates, the extent of URBs in UK rivers and indicates that a renewed focus is required so that we can address pollution at source and improve other self-purification mechanisms (e.g., hyporheic zone) [210].

This review provides the basis for a renewed focus on URBs using developments in more general biofilm research to improve our understanding of sources of pollution, the diversity and composition of URBs, how water chemistry and physical processes affect the microbial composition, and how this can be used to better address organic river pollution. Specifically, future research needs to address:

- Genomic data on URBs across different continents, seasons and in response to various point and diffuse pollution sources.
- Detailed work on the physiological and ecological mechanisms that cause the shift from a stream biofilm to an URB and back.

- Growth kinetics for a wide-range of URB taxa and a wider range of organic compounds.

In this UN Decade on Ecosystem Restoration, emphasis should be placed on addressing the underlying reasons for the decline in natural ecosystems. Rivers are hotspots of biodiversity and essential to ecosystem functioning and recovery. However, they are still suffering from the old, persistent problem of organic pollution. By better understanding the water quality and environmental conditions that trigger URB growths in rivers, we can support the development of more effective, integrated ecosystem management and restoration.

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Data access statement

No new data were created or analysed for this article.

Conflicts of interest

The authors declare there are no conflicts of interest for this research.

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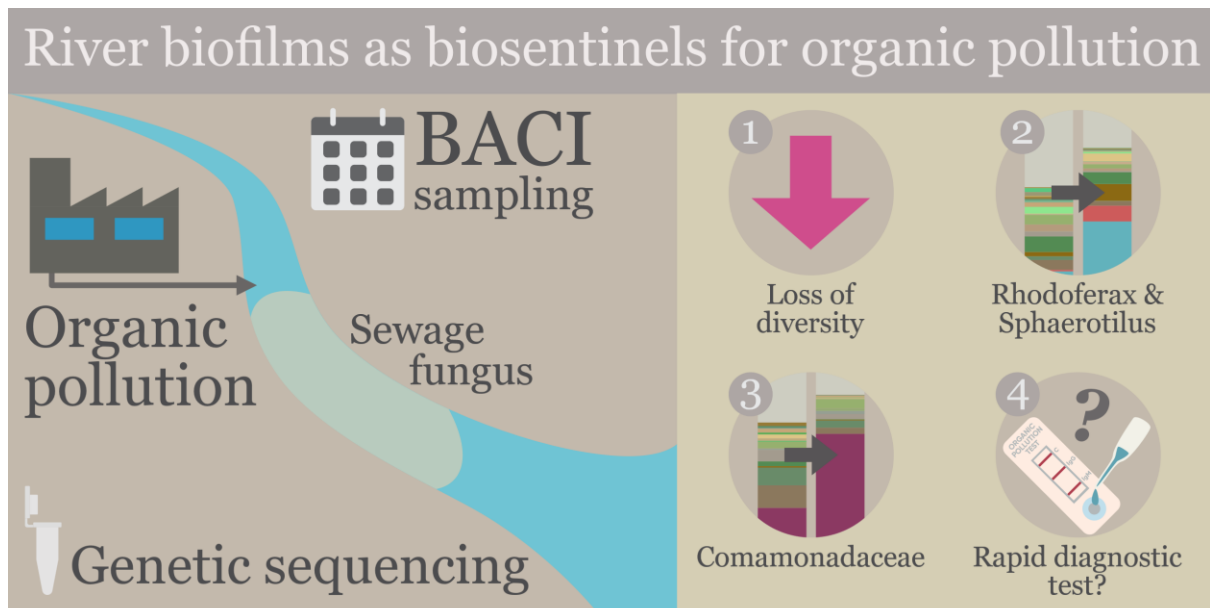
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3 Polybacterial Shift in Benthic River Biofilms Attributed to Organic Pollution – Prospect of a New Biosentinel?

Abstract

Organic pollution continues to contaminate river water and degrade aquatic ecosystems worldwide. In highly altered river systems with high organic loading, sewage fungus, a heterotrophic biofilm, can form on the riverbed. The aim of this study was to determine how the polybacterial community of riverbed biofilms changes prior to and during a sewage fungus outbreak to inform the development of novel biomonitoring approaches. Riverbed biofilm samples were collected from a site that experienced sewage fungus outbreaks previously and an upstream control, following a BACI (before-after control-impact) design. The polybacterial community was characterized using targeted amplicon sequencing (16s rRNA). The results indicate that the community became dominated by two bacterial genera prior to and during the sewage fungus outbreak, *Rhodoferax* and *Sphaerotilus*, which accounted for 32.8 and 14.2% of the relative abundance. When aggregated at a higher taxonomic level, the genetic data show that the community was comprised largely of bacteria from a single family, *Comamonadaceae*, totalling 64.1% of the relative abundance. Statistically significant differences in the polybacterial community over time and between impact and control sites provide initial evidence that genetic-based fingerprinting could be a promising biosentinel approach to identify organic pollution inputs and monitor their ecological impact.

Visual abstract



Highlights

1. Riverbed biofilm community was monitored using 16s rDNA in a BACI design
2. Polybacterial community was dominated by the genera *Rhodoferrax* and *Sphaerotilus* during a visible sewage fungus outbreak.
3. A single-family, Comamonadaceae, accounted for 64.1% of the relative abundance during the outbreak
4. Significant changes in polybacterial community occurred before the sewage fungus appeared.
5. Potential for riverbed biofilm to act as bio-sentinels for organic river pollution.

Keywords

16s rRNA amplicon analysis, bioindicator, *Rhodoferrax*, sewage fungus, *Sphaerotilus*, water quality.

3.1 Introduction

Organic river pollution is resurfacing as a major water management problem, even in countries with robust environmental regulation and advanced wastewater treatment [1]. Policy-makers, regulators and the public have become more aware of the lack of progress towards meeting ecological objectives for surface waters, which has been compounded by changing societal expectations for the use of rivers (e.g., bathing and wild swimming) [2]. Currently, the renewed attention has focused on untreated discharges of wastewater (e.g., combined sewer overflows, CSOs) [3], which have increased in frequency due to: (i) a lack of wastewater storage capacity, (ii) increased population density in many areas, (iii) ageing infrastructure, and (iv) more intense and unpredictable rainfall attributed to climate change [4,5]. However, a range of diffuse and point sources of organic pollution are also linked to poor water quality and ecological status, including agricultural runoff, industrial effluents, and effluents (treated and untreated) from wastewater treatment works [6–8]. A key challenge for monitoring and assessment is the development of sensitive indicators of organic pollution that can be applied robustly in river systems to identify the location, precise timing, assess severity, and evaluate the ecological impact of organic pollution.

Organic pollution is assessed in rivers via several means, but none are easily applicable to detect pollution events from the wide range of potential sources. Water quality determinants are monitored in many rivers to assess organic pollution (e.g., biological oxygen demand (BOD₅) and ammonia) and risks to public health (e.g., bacteria indicator organisms), but they may not capture (i) the organic compounds driving ecological degradation and (ii) intermittent or event-based pollution events, meaning elevated levels or organic pollutants may not be captured through water quality surveys [9,10]. Furthermore, whilst numerous biological indicators (i.e., bioindicators) [11,12] and indices have been developed to evaluate water quality pressures based on the absence or dominance of key aquatic taxa (e.g., saprobic index, Biological Monitoring Working Party (BMWP) indices, Walley Hawkes Paisely Trigg (WHPT) indices) [9], they are difficult to apply at high frequency and spatial resolutions to identify all organic pollution sources due to their reliance on taxonomic identification, culture-based enumeration, and a heavily-impacted ecological community [10,11].

Sewage fungus is a bioindicator of poor water quality which has been documented extensively [13]. It is a polymicrobial biofilm that forms on the bed of extremely degraded river channels with high loadings of organic pollution [7,14–16]. It represents an ecological endpoint, where the sewage fungus community outcompetes other benthic microorganisms to thrive in the high organic load and low dissolved oxygen (DO) environment [17–20], with wider ecological impacts [21–30]. Thus, whilst the presence of sewage fungus is a useful visual bioindicator of severe ecological degradation linked to organic pollution, other more sensitive approaches are needed to identify pollution earlier, prior to widespread ecosystem decline.

Biosentinels are part of an emerging ecological toolkit for aquatic monitoring studies, already applied comprehensively in monitoring pollution such as indoor air quality [31], micropollutants (e.g., pesticides) [32] and faecal material in coastal waters [33,34]. They are differentiated from bioindicators in that they are the initial organism(s) to respond to changes in ecosystem quality [33,34], rather than simply providing information on “the quality of the environment” [11]. Both bioindicators and biosentinels can be used to track changes in environmental quality over time (i.e., biomonitoring) [11], but the biosentinel should identify pressures earlier than bioindicators providing data as a leading indicator prior to substantial (usually deleterious) changes to ecological communities. Previous research on sewage fungus, saprobic indices, and the microbial community in biological wastewater treatment has identified dominant taxa associated with organic enrichment (e.g., *Sphaerotilus natans*, the genus *Zoogloea*, *Beggiatoa alba*; and *Leptomitopsis lacteus* [14,35,36]), which could serve as biosentinels in the riverbed biofilm for organic pollution monitoring and assessment.

The aim of this study was to assess changes in the polybacterial community in riverbed biofilms using targeted amplicon sequencing technology in a structured before-after-control-impact (BACI) survey to identify possible biosentinels for organic pollution. Amplicon sequencing has been widely used for monitoring studies [37] and to assess the environmental microbiome [38], but there has been a limited application to sewage fungus [39] where most of our existing understanding of composition comes from culture-based approaches [14,36]. The specific objectives are to (i) quantify the diversity and abundance of the bacterial community in riverbed biofilms before, during and after exposure to organic pollution; (ii) determine the taxonomic composition of

sewage fungus; and iii) identify the impact of (i) on (ii) with aim of developing a framework for early detection of pollution events (e.g., overflows and diffuse pollution). By directly determining the response of the polybacterial community to organic pollution events, in consideration of an upstream control and seasonal changes, this research can support the development of modern genomic approaches that use microbial biosentinels to improve the detection and mitigation of organic pollution in rivers.

3.2 Methods

3.2.1 Study location

To examine the impacts of organic pollution on the periphyton community, a location on the River Crane (London, England) was identified (**Figure 3-1**) as somewhere outbreaks have been observed by local action groups and the environmental regulator regularly in winter [40–42]. The Crane catchment covers 57.1 km² and has a mix of land uses (mostly urban, suburban and grassland with some broadleaf woodland and arable/horticulture land cover), and receives a major contribution from the Yeading Brook (49.2 km²) a heavily modified river with primarily suburban land cover with some grassland, urban and broadleaf woodland. A prominent feature of the Crane catchment is Heathrow Airport, with surface runoff from its eastern catchment draining into the River Crane via the Eastern Balancing Reservoir and treatment systems.

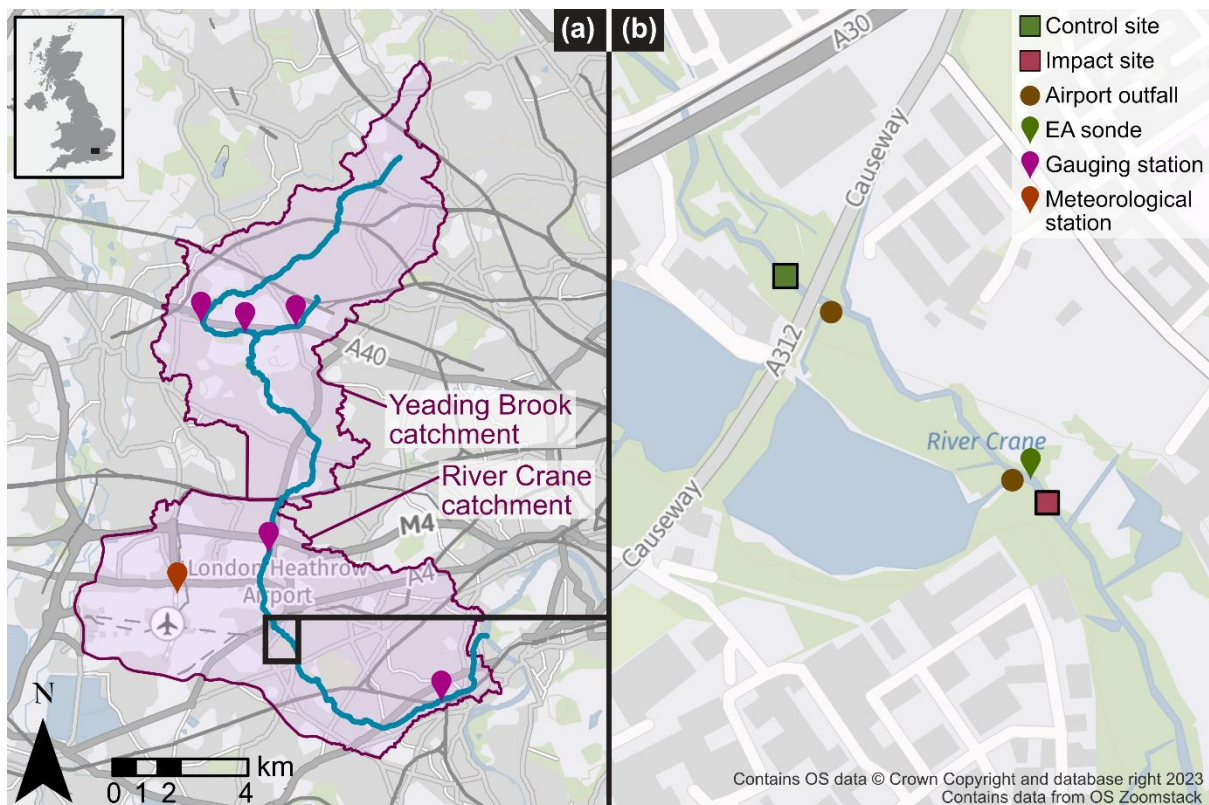


Figure 3-1: Annotated map of the (a) catchment and (b) study area (River Crane, London, UK). Water quality data in this study was provided by the Environment Agency (EA sonde), river gauging data by the National River Flow Archive Gauging station), and meteorological data from Centre for Environmental Data Analysis (CEDA) (Meteorological station).

Sewage fungus outbreaks in the River Crane have frequently occurred in the winter and have been attributed to anti- and de-icing activity at the adjacent airport [40,43–45]. Commonly applied freeze-point depressants (the active ingredient) in commercial airport de-icers include propylene glycol (aircraft), ethylene glycol (ground) and formate/acetate salts (ground) [46,47]. The riverbed periphyton were sampled at two sites (control vs impact) over the 2020-21 winter period (October 2020 – April 2021). The sites were located upstream (control, TQ 10605 75527) and downstream (impact, TQ 10958 75226) of an outfall that drains surface water runoff from the eastern catchment of the airport. This surface water runoff is subjected to several forms of treatment, including a treatment wetland system and later a moving bed biofilm reactor (MBBR) facility, before permitted discharge into the River Crane [48–50]. Aerated

wetland and reed bed systems are commonly used to treat airport de-icer contaminated runoff and are effective nature-based solution to reduce biological oxygen demand (BOD) [51–54]. Whereas, MBBS's are fixed-biofilm systems used to remove a variety of industrial pollutants and have, more recently, been applied in treating de-icer contaminated runoff [55–57].

The study adopted a BACI design; however, as the timing of the intermittent organic pollution discharge could not be predicted, a combination of regular and event-based sampling was conducted. The regular sampling was conducted bimonthly during the study: 19/10/2020, 07/12/2020, 16/02/2021, and 20/04/2021 (termed Oct-20, Dec-20, Feb-21, and Apr-21, respectively) with an additional “event” sample collected during a putative sewage fungus outbreak which occurred after the Feb-21 sampling on 24/02/2021 (referred henceforth as “SF” in results).

3.2.2 Sampling methodology

Riverbed biofilms were sampled using UK and EU guidance (UKTAG River DARLEQ2 [58] and EU COST Action DNAqua-Net [59]). Coarse gravel- and cobble-sized sediment grains were collected from the riverbed at each location, placed into pre-labelled, resealable polythene bags, transported on ice to the laboratory within 24 hours and refrigerated (4 °C). Subsequently biofilm and surface deposits (e.g., periphyton) were sampled aseptically from the rock samples using a single-use toothbrush. The samples were rinsed and resuspended in filtered (0.22 µm), sterile de-ionised water, then vortex homogenised and centrifuged (3800 rpm, 12 minutes) at 20 °C to pellet microbial biomass. The supernatant was decanted, and samples were frozen at -20 °C for short-term storage until defrosted for DNA extractions (Macherey-Nagel NucleoSpin® Soil kit) with quality quantified using NanoDrop and concentration by Qubit fluorometer using DNA HS kit. DNA extracts were then stored at -20 °C prior to amplicon sequencing.

3.2.3 Genetic sequencing and taxonomic assignment

Sequencing of 16S rRNA of the V3-V4 region was performed by Novogene Co., LTD and amplification was performed using forward Primer: 5'-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG-5' [341] and reverse primer 5'-GTC TCG TGG GCT

CGG AGA TGT GTA TAA GAG ACA G-3' [785R] for the hypervariable regions V3–V4 of 16S rRNA. Amplicons were processed with Nextera XT DNA Library Prep Kit to obtain fragments of > 300 bp. 16S rRNA DNA amplicons were sequenced on an Illumina MiSeq instrument (Illumina Inc, USA) using 250-bp paired-end sequencing. PCR and purification steps were as follows: (i) amplification of V₃-V₄ regions from 16S rRNA genes were amplified from genomic DNA using the specific 515F-806R primer; (ii) loading buffer was then mixed with PCR products for electrophoresis on 2% agarose gel, samples with 400-450bp were chosen; and (iii) PCR products were mixed and purified followed by analysis. Forward and reverse Illumina reads were joined using fast-qjoin. QIIME (1.7.0 pipeline [60]) was used for Operational Taxonomy Unit (OTU) picking for the 16S rRNA gene amplicons. OTUs were clustered against UPARSE (v7.0.1090) [61] and reads failing to hit the reference were subsequently clustered de novo at the 97% similarity level using UCLUST [62]. OTU sequences were aligned using PyNAST [63]. OTU taxonomy was determined using the naïve Bayesian RDP classifier for the Mothur method, SSUrRNA database from SILVA [64–66].

3.2.4 Statistical analysis

Taxa richness, diversity and evenness were calculated at the genus level of taxonomical classification using Microsoft Excel. Shannon's diversity index [67] was used for diversity and evenness as it handles highly diverse samples better than other diversity indices and is commonly used in other studies [67]. Relative OTU abundance ($\geq 2\%$) was plotted in R Studio [68] using ggplot2 [69] to compare both spatial and temporal changes. Principal coordinate analysis (PCO) was performed for all sites surveyed further up- and downstream in the River Crane as part of a wider monitoring project to examine the dissimilarities among microbial community assemblages after square root transformation of relative abundances and subsequent construction of resemblance matrix using Bray-Curtis distances in PRIMER7 with the PERMANOVA+ add-on [70]. Square root transformation was performed to minimise variance between samples and mitigate the influence of highly abundant taxa. Pearson Correlation coefficients between taxa and each PCO axis were extracted to determine relationships between taxa, sampling locations, and sampling dates.

3.2.5 Water chemistry and meteorological analysis

Water quality data were obtained from an Environment Agency multi-parameter sonde located downstream of the airport outfall into the River Crane (TQ 109 753) (**Figure 3-1b**). Weather data were obtained from the CEDA (Centre for Environmental Data Analysis) archive dataset at the greater-london/00708_heathrow station (UK hourly weather observation data) (TQ 076 766) (**Figure 3-1a**).

3.3 Results and Discussion

Throughout the winter season (October 2020 – April 2021), there were substantial changes to the taxonomic composition, abundance, and diversity, especially in the sampling immediately preceding and during the sewage fungus incident (**Figure 3-2**). At the control site upstream of the surface water discharge, the community composition shifted over the sampling period, but there were no substantial changes in diversity (4.303 – 4.555) or evenness (0.675 – 0.721) (**Table 3-1**). Whilst the relative abundance of the taxa (represented as lowest taxonomic unit) changed over time, no single taxa was dominant; for example, greatest relative abundance of OTUs based on lowest taxonomic unit was 7.1% (**Figure 3-2a**). The impact site had similar diversity, richness, and evenness to the control during early winter (Oct-20 and Dec-20). However, from Dec-20 to Feb-21, there was a reduction in diversity by 42%, from 4.375 to 3.204, and the evenness also reduced by 38.7%, from 0.693 to 0.501 (**Table 3-1**). Diversity and evenness remained consistently low during the sewage fungus incident but increased to values comparable to the control in the Apr-21 sampling suggesting a recovery of the periphyton community in terms of evenness and richness.

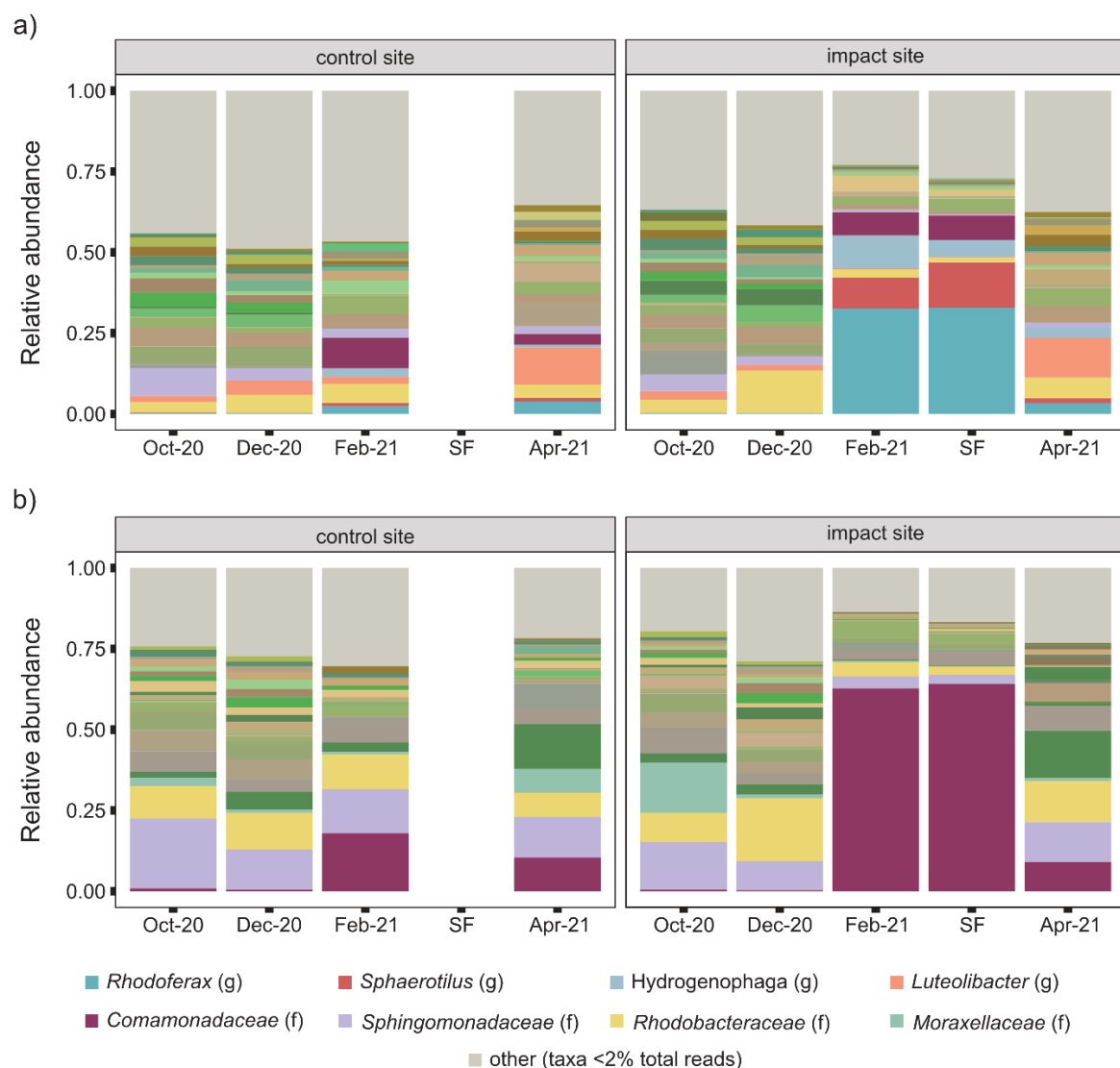


Figure 3-2: Relative abundance of periphyton at control and impact site over time at (a) lowest taxonomic level and (b) family taxonomic levels. The minimum threshold for inclusion was set at 2% relative abundance, with taxa below this grouped as “other”. “SF” denotes the date the visible sewage fungus event was sampled (24/02/2021).

Table 3-1: Ecological diversity, richness, and evenness of microbial biofilm taxa at control and impact sites over time. “SF” denotes an occurrence of visible sewage fungus sampled in late Feb-21 (24/02/2021).

	Control site					Impact site				
	Oct-20	Dec-20	Feb-21	SF	Apr-21	Oct-20	Dec-20	Feb-21	SF	Apr-21
Shannon's diversity	4.417	4.544	4.555	-	4.303	4.336	4.375	3.204	3.237	4.235
<i>Richness</i>	492	545	700	-	584	465	550	597	549	581
<i>Evenness</i>	0.713	0.721	0.695	-	0.675	0.706	0.693	0.501	0.513	0.665

Interrogation of the chemical water quality monitoring data (**Figure 3-3**Figure 3-2b) collected near the site suggested that no detectable organic pollution event occurred in January or February 2021 to trigger the growth of sewage fungus. There were fluctuations in DO in the weeks preceding the SF event, but they are inversely related to water temperature, as expected for this parameter. Calculated percent saturation of DO (**S3-3**) also closely follows the trends in DO concentration. Percent saturation was higher in early January 2021 (81 – 117%) and decreased as the water warmed throughout the second half of January (80 – 104%) before slowly rising during February 2021 (37 – 115%) except where it again plateaued during the visible sewage fungus towards the end of February. Moreover, ammonia concentrations were consistently low in the river for the weeks preceding the event, which does not suggest untreated sewage was the cause either. Low concentrations of airport de-icers in the treated surface water may have been a contributing factor. In the weeks preceding the sewage fungus outbreak, temperatures were low (< 5°C, the trigger for de-icing) and both snowfall and rainfall were recorded (CEDA archive [71]), suggesting that de-icers were used and mobilized through the drainage system (**Figure 3-3a**), despite the airports surface water discharge being monitored for compliance within environmental permitting. An important factor in triggering sewage fungus growth is the altered state of the river, especially the stable riverbed material and moderate flow velocities which create ideal conditions for the growth of sewage fungus [8,72,73]. Consequently, the compounding effects of these pressures and altered states suggest the utility of a bioindicator over chemical water monitoring for identifying when and where organic pollution may have an ecological impact. However, a bioindicator is needed that provides an earlier warning and is more sensitive to organic pollution than sewage fungus.

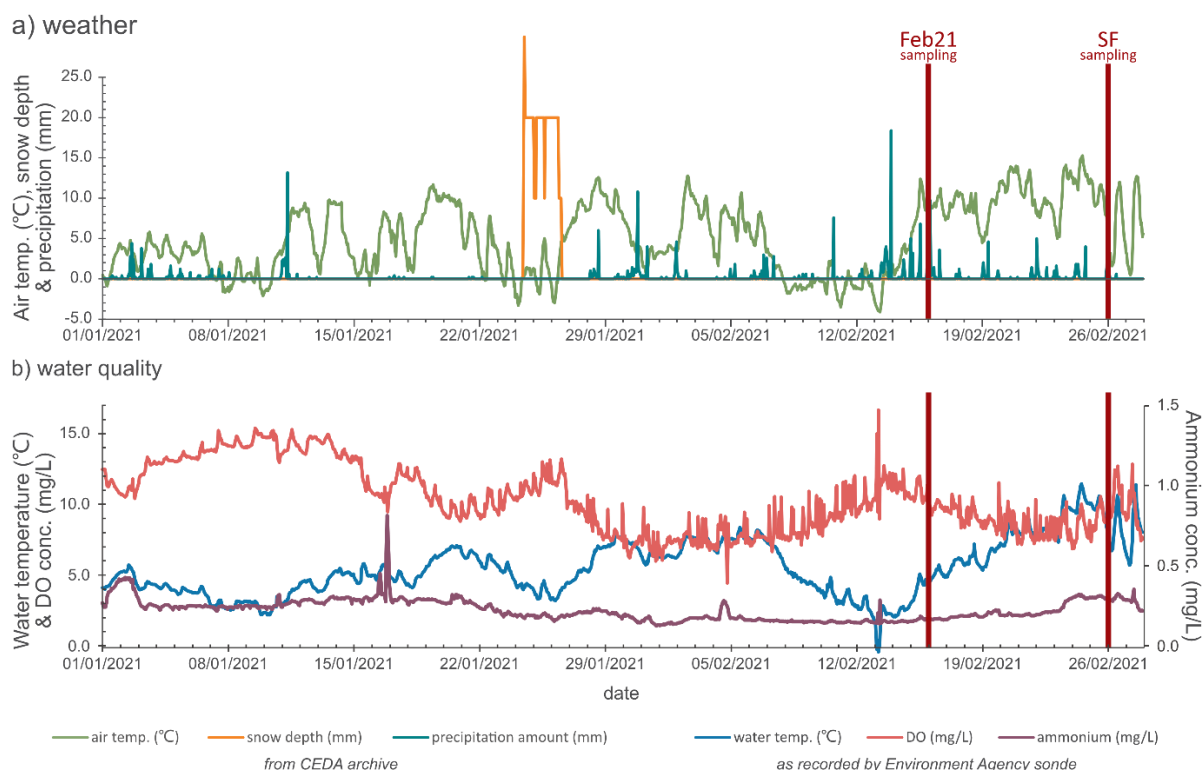


Figure 3-3: Weather and water quality preceding Feb-21 and SF sampling. Weather data was obtained from CEDA archive at Heathrow weather station, and water quality data was obtained from the Environment Agency sonde in the River Crane downstream of outfalls from the neighbouring airport.

The apparent loss of diversity which occurred prior to the Feb-21 and SF event appears to be impacted by the establishment and dominance of two genera, *Rhodoferax* and *Sphaerotilus*, alongside an overall polybacterial shift to the riverbed biofilm (**Figure 3-2a**). The dominant taxon contributing to this change was *Rhodoferax* (32.8% relative abundance). Whilst this genus is described in the literature as a “typical freshwater bacteria” [74] that has been isolated from ditch waters and activated sludge [75,76], this study is the first to identify the genus directly from a sewage fungus-like sample. Most species within the *Rhodoferax* genus are facultative anaerobes [77] and some could be non-culturable, which would have been harder to have identified using the culture-based approaches of earlier studies. The presence of *Rhodoferax* in the samples could be driven by anoxic conditions within the riverbed biofilm or more generally within the river [19,78]. *Sphaerotilus* sp. is the species most commonly

associated with sewage fungus [17,35] and accounted for 14.2% of the relative microbial abundance in the sewage fungus event at the impact site (SF, **Figure 3-2a**). *Zoogloea* and *Hydrogenophaga* were the next most abundant taxa within the sewage fungus. Of the taxa identified here, only *Sphaerotilus* and *Zoogloea* have previously been identified from sewage fungus using culture-based enumeration [17,79,80] but were not found in a recent study using sequencing approaches in the USA [39]. Additional studies would be needed to validate whether there is redundancy in terms of key taxon associated with sewage fungus both spatially and temporally. There are strengths and shortcomings of both sequencing and culturing approaches (for example, detection of genome fragments of environmental bacteria does not always correlate to viable bacteria; whereas culturing underrepresent the abundance of key non-culturable taxa). Our justification for using a sequencing approach is that sewage fungus communities likely have significant populations of non-culturable or difficult to culture organisms and therefore community level assessments of impact of pollution and sewage fungus has yet to be fully realised.

The shift polybacterial community at the impact site became even more pronounced when the taxonomic data was aggregated to the family level. In the Feb-21 and SF events, the community was dominated by a single family, *Comamonadaceae*, which increased significantly from <0.9% relative abundance in Dec-20 to 64.1% in Feb-21 (PERMANOVA, $P < 0.01$; **Figure 3-2b**). The bacterial community did not differ significantly between Feb-21 and SF (PERMANOVA, $P = 0.081$), thus the shift to a riverbed biofilm community dominated by *Comamonadaceae* occurred prior to the formation of a visible sewage fungus, highlighting the potential of this family as a leading indicator of impact caused by pollution events, prior to ecosystem degradation. These results are supported Nott *et al.* (2020) which identified *Comamonadaceae* in river biofilms receiving airport de-icer runoff [39]. In our study, significant differences between the bacterial community were observed between sample events (PERMANOVA, $P < 0.01$), despite the similar biodiversity (Shannon's diversity, **Table 3-1**). This result suggests that the periphyton polybacterial community varies with season. Therefore, it is important to resolve pollutant level impacts on the microbial population from the dynamic baseline which is influenced by season (water temperature, rainfall, sunlight, etc). *Comamonadaceae* was also identified at the

control site in Feb-21, but at a much lower abundance than at the impact site (18.0% vs. 62.7%, respectively; **Figure 3-2b**), with most of this difference attributed to reduced relative abundances of the genera *Rhodoferax* and *Sphaerotilus* (**Figure 3-2a**). The appearance of *Comamonadaceae* at the control site may be related to other sources of organic pollution in the highly urbanized catchment, different pathways for aircraft de-icers (e.g., aerial deposition) and/or other environmental factors. The significant increase in *Comamonadaceae* at the impact site suggests that whilst these taxa may have been present upstream (**Figure 3-2b**), the additional organic loading downstream of airport outfalls contributed to the considerably shifted periphyton composition and the manifestation of a visible sewage fungus. Additional research is required to determine the specific triggers for sewage fungus in lowland rivers comprising multiple water quality, hydrological and geomorphological pressures.

Taxa of note at the control (before and after) and impact (before) sites include the families *Sphingomonadaceae* and *Rhodobacteraceae* (**Figure 3-2**). The relative abundances of these taxa were greatly reduced in Feb-21 and SF event (2.9% and 2.6% in SF-impact compared to 12.4 – 21.6% and 10.0 – 11.5% at the control site), especially when aggregated at the family taxonomic level (**Figure 3-2b**). The genus *Luteolibacter* was reduced to negligible abundance during the sewage fungus event (Feb-21 and SF) but had returned in Apr-21 to a much higher abundance (**Figure 3-2a**). Whereas the genus *Hydrogenophaga* (also within the family *Comamonadaceae*), a taxon not previously associated with sewage fungus, became a prominent taxon in the sewage fungus event (10.2% and 5.3% in Feb-21 and SF).

The significant changes in the polybacterial community that occurred prior to the appearance of a visible sewage fungus outbreak suggest several possible candidates for biosentinels. At the genus level, the appearance of the genera *Rhodoferax* and *Sphaerotilus* within the riverbed biofilm community could be a threshold for suspected organic pollution (**Figure 3-2a**). Equally, the appearance or dominance of the family *Comamonadaceae* could also serve as a biosentinel and would require less reliance on and analysis of high-resolution taxonomic data (**Figure 3-2b**). Group average CLUSTER analysis on the resemblance matrix at the family level shows that the polybacterial community forms three distinct clusters (at 70% similarity): (i) Oct-20 and Dec-20, (ii) Feb-21 (impact) and SF, and (iii) Feb-21 (control) and Apr-21 (**Figure 3-4**,

S3-1). There are several important observations from these results that support the potential development of a biosentinel approach. First, the Feb-21 and SF samples at the impact site negatively correlate with PCO1 and PCO2, which account for 66.3% and 13.4% of total variation respectively (**Figure 3-4**). Whereas Oct-20 and Dec-20 samples have a strong positive correlation with PCO1 and little correlation along PCO2, and compared to Feb-21 (control site) and Apr-21 samples which correlate weakly in a negative direction to PCO1 but positively with PCO2. Thus, the biofilm communities sampled immediately prior to and during the visible sewage fungus outbreak had significantly different bacterial composition from samples collected earlier in the winter. With a >92.5% correlation threshold, the only family to correlate to the impact samples (Feb-21 impact and SF) is *Comamonadaceae*, suggesting the utility of the *Comamonadaceae* as a biosentinel. At genera level *Rhodoferrax*, *Sphaerotilus* and *Zoogloea* correlated negatively to PCO1 (−69.9%, −54.4%, and −64.5%) and PCO2 (−61.6%, −45.4%, and −58.4%) confirming that, for the riverbed biofilm sampled, these taxa are most associated with the transition to a sewage fungus-like riverbed biofilm.

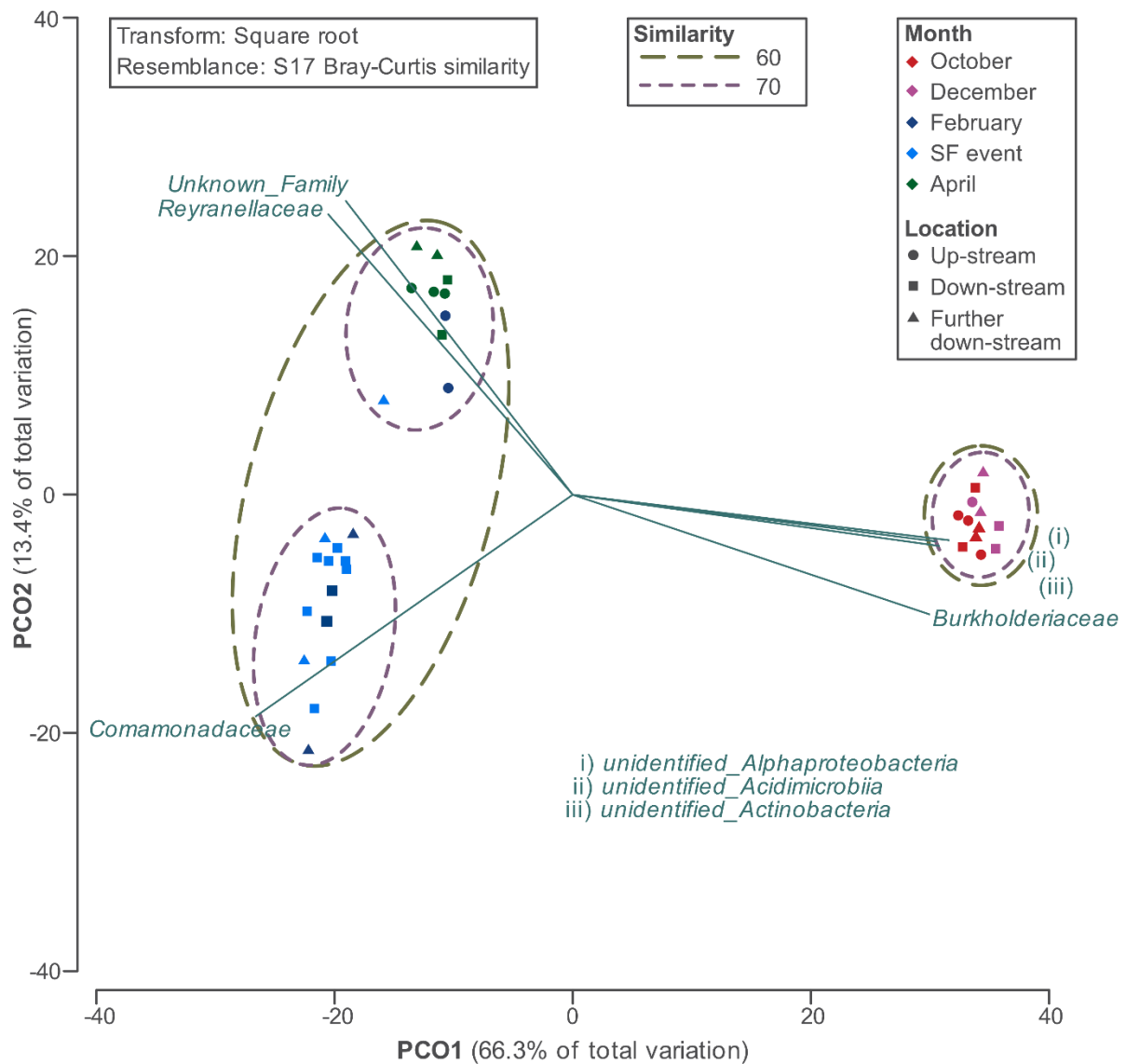


Figure 3-4: PCO plot constructed of all winter 2020-21 samples using a Bray-Curtis based resemblance matrix of square root transformed relative abundance at the family taxonomic level. Colour denotes sampling event and shape the location as per figure legend.

Second, the Feb-21 and SF samples at the impact site are grouped within the same cluster. Thus, the changes to the polybacterial community associated with the transition to a sewage fungus occurred prior to the formation of a visible biofilm, suggesting the possibility of early warning of potential ecological impact. Third, the control and impact sites for the Feb-21 sampling are assigned to different clusters

(70% similarity) (**Figure 3-4**). Thus, they have significantly different polybacterial communities, despite the minor abundance of *Comamonadaceae* at the control site, suggesting a genomic fingerprinting-based approach could potentially discriminate from point and diffuse sources of organic pollution. Finally, although contained within the same 60% cluster showing some remaining resemblance, the polybacterial community composition at the impact site in Feb-21 and SF was significantly different from the Apr-21 sampling. Thus, the biofilm community rapidly begins recovery from exposure to organic pollution (i.e., within less than 2 months) suggesting that intermittent or event-based pollution events could be identified. This is further supported by extracting the Pearson correlation from the PCOs (**S3-2**). Of the noteworthy families mentioned in this study, *Comamonadaceae* is the only family to have both a negative Pearson correlation with PCO1 and PCO2. Whereas families noted in **Figure 3-2** as having moderate relative abundance before the suspected pollution event (*Rhodobacteraceae*, *Sphingomonadaceae*, *Moraxellaceae* – Pearson correlation of 72.0%, 74.3%, and 65.0% in PCO1) correlate similarly with Burkholderiaceae, (86.8% in PCO1) and the three unidentified families positively along PCO1 (97.1%, 97.8%, and 96.9%) but differ along PCO2 (**S3-2**).

The statistically significant differences in the polybacterial community of the riverbed biofilm prior to and during a sewage fungus outbreak appear to be related to the dominance of the family *Comamonadaceae*. Further monitoring and assessment work should be done with amplicon sequencing and metagenomic approaches in several watercourses to validate these findings. If confirmed, the shift to a single dominant taxon could underpin the development of a rapid diagnostic tool based on quantitative PCR, which is easier, more rapid to conduct, and less expensive than genomic sequencing. The development of a low-cost, genetic-based biosentinel approach based on riverbed biofilm sampling would open up new opportunities to identify the location and timing of organic pollution inputs and monitor their impact, regardless of if it is a diffuse or point source or a continuous or intermittent discharge. Given the many pressures exerted on river systems, effective diagnostic tools are urgently needed to support the application of control, mitigation, and restoration measures to improve severely degraded and depleted freshwater ecosystems.

3.4 Conclusions and Future Work

This study collected novel high-resolution taxonomic sequencing of the polybacterial community of riverbed biofilms over time at a control site and a site that experiences infrequent sewage fungus outbreaks. Sewage fungus is a bioindicator of organic pollution which remains an ecological problem for rivers and needs renewed research and regulatory interest. Key findings of this study are the identification of the dominant bacterial taxa in sewage fungus and clear shifts in the polybacterial community composition prior to a visible sewage fungus. The study found that *Rhodoferrax*, a group not previously identified in sewage fungus, was the dominant bacterial genera, followed by *Sphaerotilus*, which is synonymous with sewage fungus. When the genetic data was aggregated at a higher taxonomic level, the dominance of a single family (*Comamonadaceae*) was noted in the polybacterial community during and immediately prior to a visible sewage fungus outbreak. These results provide new genetic information on the shifts in the polybacterial community that generate sewage fungus outbreaks and provide the foundation for a genetics-based biosentinel approach to map and monitor organic pollution pressure on river systems.

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Data access statement

Supporting data for this study can be accessed through the Cranfield University repository (CORD): DOI 10.17862/cranfield.rd.21395136 and 10.17862/cranfield.rd.21395109.

Conflicts of Interest

The authors declare there are no conflicts of interest for this research.

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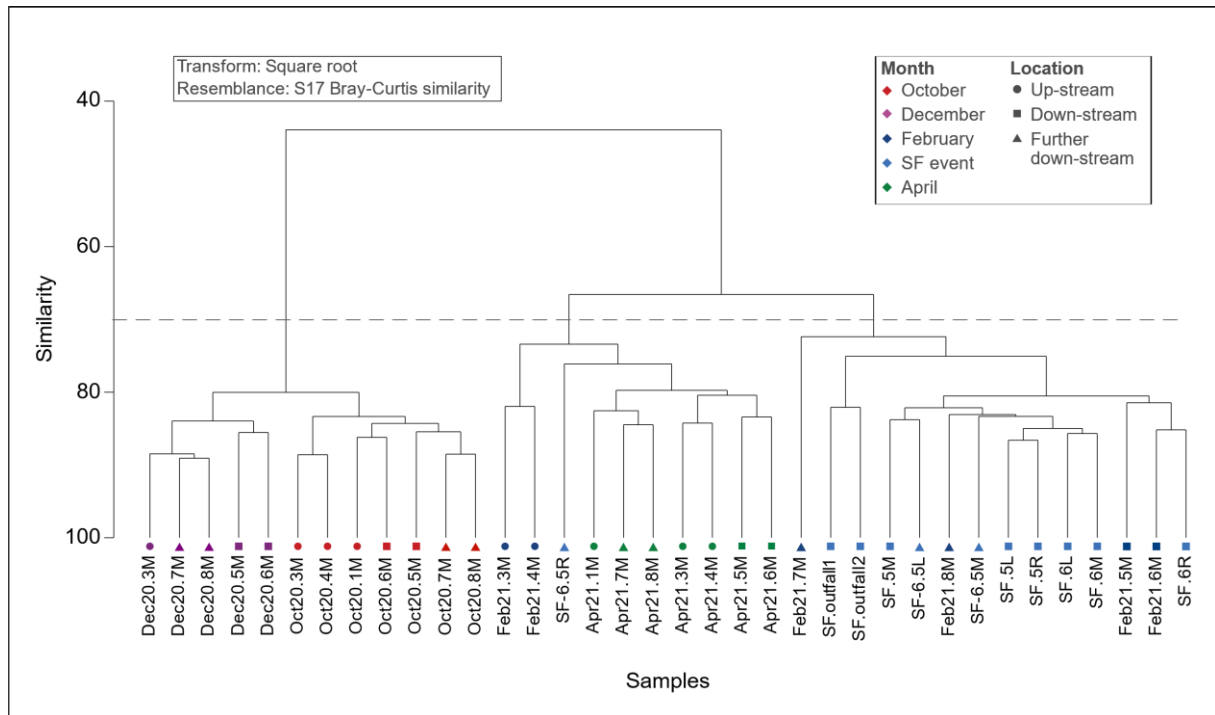
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Supplementary materials

Appendix A – Dendrogram



S3-1: Dendrogram of clustering analysis (group average) with the 70% similarity marked.

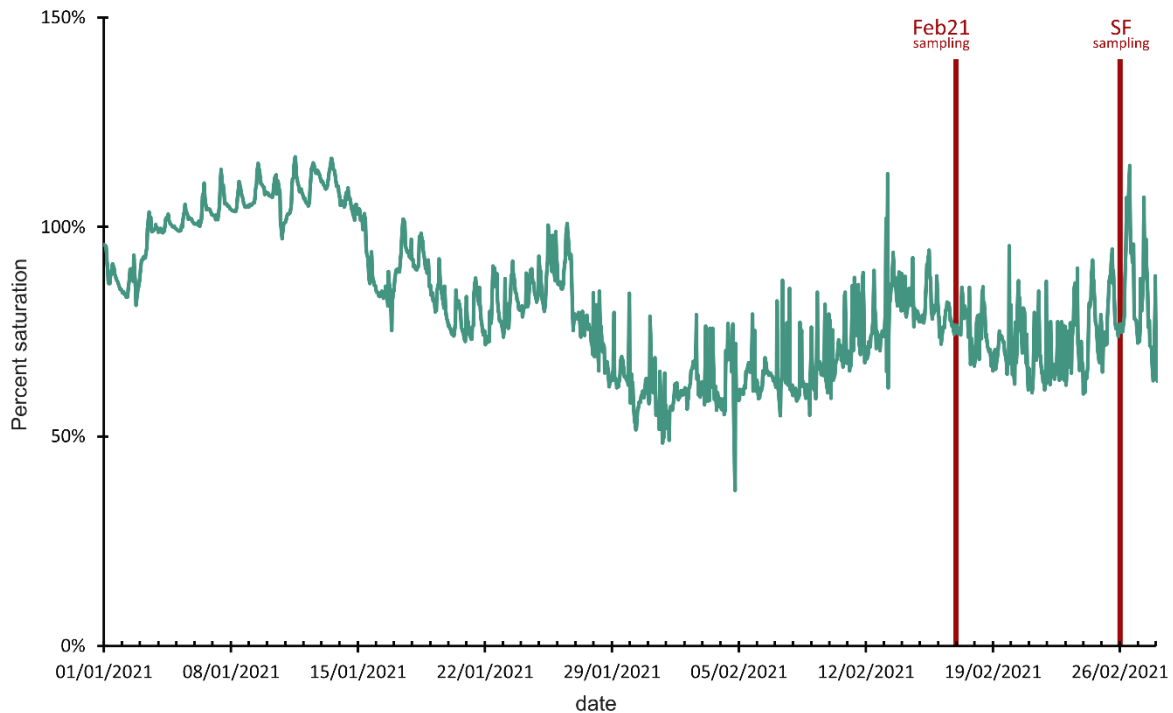
Appendix B – Pearson correlation

S3-2: Pearson correlation of PCOs and the families noted in this study.

	Rhodobacteraceae	Sphingomonadaceae	Moraxellaceae	Comamonadaceae	Burkholderiaceae	Unidentified Alphaproteobacteria	Unidentified Acidimicrobiia	Unidentified Actinobacteria	Reyranellaceae	Unknown Family
PCO1	0.7198	0.7433	0.6496	-0.9004	0.868364	0.9780	0.9691	0.9713	-0.8207	-0.8073
PCO2	0.4217	0.4050	0.0599	-0.4039	-0.38948	-0.1291	-0.1316	-0.1375	0.5282	0.5546
PCO3	0.2652	0.0422	-0.2611	0.0843	-0.08873	-0.0142	0.0536	-0.0161	-0.0944	-0.1361
PCO4	-0.1078	-0.2288	-0.2755	-0.0471	0.165621	0.0592	0.0948	0.0124	0.0302	0.0331
PCO5	-0.2828	0.3721	0.3909	-0.0162	0.176862	-0.0272	-0.0890	0.0197	0.0377	-0.0121
PCO6	-0.2180	-0.0898	-0.1012	0.0888	-0.06148	0.0669	0.0400	0.0567	0.0032	-0.0507
PCO7	0.0681	-0.0843	-0.0705	0.0448	0.01466	0.0137	0.0421	0.0184	0.0558	-0.0088
PCO8	0.0616	0.1233	-0.0960	-0.0071	-0.03387	-0.0201	0.0178	-0.0382	0.0989	0.0131
PCO9	0.0345	0.0728	-0.0667	0.0029	-0.00103	0.0435	0.0083	0.0203	-0.0509	0.0015
PCO10	0.0215	0.0501	-0.1043	-0.0142	-0.01562	-0.0449	-0.0125	-0.0170	0.0108	0.0067
PCO11	0.1031	-0.1202	0.3446	0.0214	-0.03104	-0.0145	-0.0173	-0.0005	0.0654	0.0083
PCO12	-0.0962	-0.0773	0.2113	0.0101	-0.0046	0.0414	0.0198	0.0425	-0.0441	-0.0187
PCO13	-0.1128	-0.0250	-0.0608	-0.0107	-0.02468	0.0122	0.0515	0.0014	0.0214	0.0263
PCO14	-0.0017	0.0363	-0.0277	-0.0044	-0.03362	-0.0020	-0.0123	-0.0132	0.0104	0.0281
PCO15	-0.0444	-0.0211	0.0662	-0.0335	-0.01835	-0.0103	-0.0206	-0.0556	-0.0003	0.0093
PCO16	0.0351	0.0136	-0.0614	0.0281	0.058509	0.0180	-0.0014	-0.0044	-0.0454	-0.0318
PCO17	0.0679	-0.0437	-0.0987	0.0115	0.003255	-0.0096	0.0118	0.0155	0.0079	0.0116
PCO18	-0.0253	0.0129	-0.0309	-0.0097	-0.02252	0.0079	0.0316	0.0187	-0.0574	-0.0388
PCO19	-0.0996	-0.0312	0.0314	0.0024	0.020443	-0.0028	-0.0237	0.0147	-0.0072	0.0110
PCO20	0.0041	0.0578	0.0177	-0.0045	0.021764	-0.0164	-0.0452	0.0450	0.0301	0.0041
PCO21	-0.1086	-0.0278	0.0509	-0.0027	0.059215	0.0299	-0.0060	-0.0214	-0.0019	0.0277
PCO22	0.0491	-0.1056	-0.0694	-0.0067	0.040925	-0.0198	0.0129	-0.0222	0.0050	0.0192
PCO23	0.0276	-0.0022	0.0012	-0.0161	-0.01548	-0.0051	0.0004	0.0736	0.0306	0.0182
PCO24	0.0085	0.0315	-0.0575	-0.0085	0.004285	-0.0191	-0.0315	0.0863	0.0082	0.0387
PCO25	0.0673	-0.0746	-0.0280	0.0153	-0.06114	0.0007	0.0555	0.0101	0.0239	0.0103
PCO26	-0.0130	0.0338	0.0156	-0.0083	-0.00468	-0.0001	-0.0105	0.0156	-0.0397	0.0263
PCO27	-0.0427	0.0298	0.0147	0.0125	-0.01805	0.0167	-0.0337	0.0393	-0.0003	-0.0428
PCO28	0.0435	0.0252	-0.0295	-0.0058	0.007129	-0.0160	0.0288	0.0503	-0.0027	-0.0054
PCO29	0.0383	-0.0025	0.0111	-0.0163	-0.02175	0.0332	-0.0255	0.0345	0.0155	0.0030
PCO30	0.0517	-0.0037	-0.0640	-0.0392	-0.03371	-0.0316	0.0197	-0.0133	0.0440	0.0302
PCO31	0.0298	0.0385	0.0245	0.0024	-0.0256	0.0146	0.0420	-0.0480	0.0119	0.0203
PCO32	-0.0061	-0.0190	0.0780	0.0011	-0.03499	-0.0004	-0.0198	0.0117	0.0038	0.0211
PCO33	-0.0248	0.0176	-0.1264	-0.0086	-0.02561	0.0378	0.0330	-0.0230	0.0294	0.0218
PCO34	-0.0629	-0.0024	0.0205	0.0258	0.000893	0.0619	-0.0263	-0.0108	-0.0103	-0.0739
PCO35	-0.0196	-0.0031	0.0983	0.0142	-0.0257	-0.0258	-0.0275	-0.0243	0.0306	-0.0022

Appendix C – Percent saturation

Percent saturation derived from dissolved oxygen (mg/L), water temperature, and altitude (to derive pressure) from online water quality and meteorological monitoring by the Environment Agency and Met Office.



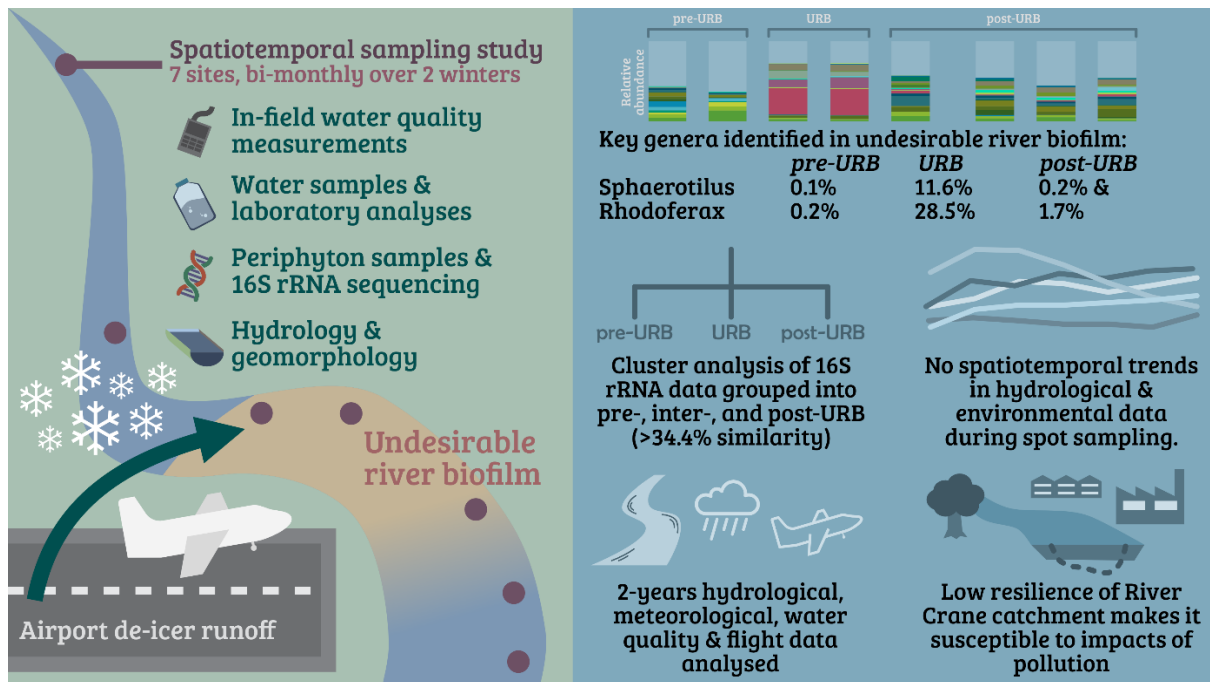
S3-3: Percent saturation (of dissolved oxygen) in the River Crane at the “impact” sampling site calculated from dissolved oxygen (mg/L), water temperature, and altitude (to derive pressure) from online water quality and meteorological monitoring. Weather data was obtained from CEDA archive at Heathrow weather station, and water quality data was obtained from the Environment Agency sonde in the River Crane downstream of outfalls from the neighbouring airport.

4 Persistence of Undesirable River Biofilms: Spatiotemporal Variations of River Periphyton in Response to Poor Water Quality

Abstract

Rivers are vital to our natural environment but are increasingly subjected to water quality or scarcity impacts depleting ecosystems and biodiversity. A persistent concern is the prevalence of undesirable river biofilms (URBs), a response to organic pollution that exacerbates ecological degradation. While most strategies for river health improvement emphasize chemical controls, this research, for the first time, explores the biological response of periphyton to variable water quality. Over a two-year period, seven sites in an urban river with a history of de-icer pollution and URB outbreaks were sampled. The results showed a significant reduction in microbial diversity directly before the URB was visually detected, lasting throughout the URB event during the first winter (Feb-21). Several keystone URB genera were identified including *Sphaerotilus* (3.6 – 25.4 % relative abundance) and *Rhodospirillum* (19.5 – 56.7%) linked to periods of elevated degradable forms of organic material and strongly reducing conditions despite similar dissolved oxygen levels. In contrast, the second winter (Oct-21 to Apr-22) saw limited changes to the periphyton diversity or abundance possibly due to more recalcitrant organics and less reducing conditions. The URB taxa persisted in low relative abundances after the URB event had diminished and throughout the second winter. DistLM analysis highlighted strong correlation between levels of dissolved oxygen, biological oxygen demand (BOD₅), total organic carbon (TOC), conductivity, and nitrate with the shifted periphyton community during the URB event. This study underscores the need for further research into other factors influencing URBs, such as bed substrate, water velocity, and their impacts in a highly modified, urban river. These rivers are less resilient to organic pollution and highly susceptible to further degradation. The enhanced resolution provided by current generation sequencing of URBs offers a dynamic tool for quantifying river processes, enabling more targeted and effective catchment interventions.

Visual abstract



Highlights

1. Bi-monthly winter river sampling for periphyton, water quality and hydrology
2. Periphyton was significantly altered by URB, mainly by *Sphaerotilus* spp., *Rhodoferax* spp.
3. URBs impact on periphyton is persistent, evidencing long-term detriment to rivers
4. Microbiome offers an alternative to chemical monitoring for legacy pollution detection
5. Additional research is required on catchment factors promoting URB proliferation

Keywords

River health; undesirable river biofilms (URBs); organic pollution; ecological degradation; periphyton; water quality

4.1 Introduction

Global freshwater quality remains unsatisfactory, most notable in river reaches (such as in the UK [1], the EU [2], China [3] and the USA [4]) due, for example, to suboptimal nutrient levels, toxic stress, habitat degradation and significant ecological deterioration [5]. The question of improving or deteriorating water quality remains complex. For example in the UK, as an exemplar of higher income countries, levels of biological oxygen demand (BOD_5), ammonia, and metals have improved since pre-industrialisation [6], whereas levels of phosphorus [7], nitrogen [7–9], and dissolved organic carbon (DOC) [7,10,11] have deteriorated [6]. However, in recent years many water quality metrics (including BOD_5 , ammonia, DOC, acid, phosphorus, and nitrogen) [6] have improved, when compared to 1980 – 2020 peaks, primarily driven by infrastructure investment and legislation in high-income countries [12,13]. Concurrently, these advancements are challenged by other contemporary pressures and alterations in land use to environmental health in freshwaters – such as frequent discharges from ageing combined sewer overflows (CSOs) [14]; increasing fine sediments in runoff from road dust (urban) and degraded soil quality (rural) [15–18]; fertilisers [19–21]; microplastics [22–25]; pharmaceutical products [26–28] and other personal care products such as sanitary products and detergents [29,30] – a situation likely to worsen with climate change and growing populations should infrastructure and land management practises not significantly improve. As an exemplar of a global issue, no river in the UK has good chemical status and only 14% have good ecological status (2019) [1,31] according to standards within the water and environmental regulations in the EU [12] and UK [13]. Despite broadly improving chemical water quality, one of the biggest challenges to rivers are the global declines in freshwater biodiversity [5,32–34]. A 2022 report [1] concluded that, after the physical modification of rivers, the three main drivers preventing rivers from achieving good status are: agricultural runoff (40%) [35]; wastewater as both point and diffuse sources of pollution (36%) [14]; and urban runoff and drainage (18%) [29,30]. Emerging concerns include newer sources of pollution, such as microplastics, pharmaceuticals and anti-microbial resistant genes (AMR) [36,37]. These aspects are particularly relevant given the increasing recreational use of rivers and coastal waters [1,38]. The presence of AMR in these environments underscores the importance of the One Health agenda, which

recognizes that human health is interconnected with the health of animals and the environment [39].

A persistent outcome of ongoing river pollution is the proliferation of undesirable river biofilms (URBs, formerly known as “sewage fungus”). URBs are intricate polysaprobic biofilms, long-utilized as bioindicators for heightened levels of organic carbon in rivers [40–44]. These biofilms present an unsightly appearance, influenced by their specific microbial composition within a matrix of extracellular polymeric substances [45–47]. The microbial composition of URBs is intricate but several key taxa are widely recognised across its global distribution [45,46]. These include the genus *Sphaerotilus*, often used synonymously with URBs, as well as *Beggiatoa*, *Zoogloea*, *Leptomitus lacteus*, and more recently identified through genomic techniques, *Rhodoferax* [48] and *Thiothrix* [49]. While labile forms of organic carbon are primary drivers of microbial growth (especially for URBs), other key nutrients also influence its development, including nitrogen sources [50,51], phosphorus sources [52–55], and a broad spectrum of micronutrients [56,57]. Environmental and hydrological factors, including flow (both velocity and discharge) [58,59], substrate type, and sunlight (including shading) [60], also regulate the composition and morphotype of URBs. The proliferation of URBs poses challenges for river ecosystems, as the increased heterotrophic activity depletes large amounts of dissolved oxygen (DO). This depletion places stress on sensitive species, such as invertebrates [61–63] and fish [40,52,64], whereas URBs thrive under these low DO conditions [65–68]. As a result, a substantial microbial biofilm overgrows the riverbed and benthos, leading to further ecological decline as habitats for fish and macroinvertebrates are lost. The striking visual impact associated with URBs further reduce the river’s ecosystem services [40].

Despite over 6025 occurrences of URBs recorded in England between 2000-2020 (**Chapter 2**), research on URBs has been limited since the 1980s, even though well-established drivers such as untreated wastewater discharge are known. One less explored aspect is the impact of winter operations at airports, which require large quantities of anti- and de-icers [69,70]. These substances are usually mobilised by rainfall and transported through airport drainage networks into treatment systems (most commonly aerated wetlands/reed beds, moving bed biofilm reactors (MBBRs), or activated sludge [71]). However, the efficiency of these systems can sometimes be

suboptimal for treating de-icers which have variable shock loads, especially during periods of high rainfall, due to the capacity of the biological systems to degrade excess organic matter [72]. As such, storage reservoirs are often used as part of an airports stormwater treatment system to buffer influxes of contaminated surface runoff into aerated wetlands or MBBRs [71]. The knowledge gap is how the unique properties of de-icers (e.g., high BOD₅ [73]) and airport winter operations influence the drivers and microbial composition of URBs. To address this, a 2-year winter sampling programme was designed focusing on a case study river that has a historic association with URBs, believed to be influenced by de-icing activity at a neighbouring airport.

The aim of this work was to determine the spatial and temporal variations in riverbed periphyton composition in relation to water quality and the context of airport de-icing. We hypothesise that, firstly, there will be substantial temporal changes in river periphyton composition throughout the winter period as inputs into the river change and de-icing operations ramp up. Additionally, spatial variations in microbial composition will develop up- and downstream of airport discharges. Secondly, there will be a decrease in water quality (such as increased BOD₅, total organic carbon (TOC) concentrations and depleted DO) in the river throughout the winter period as a reflection of ephemeral de-icing activity caused by cold weather conditions. Furthermore, these findings will be considered in the greater context of URBs, airport de-icing and persistent and (re)-emerging water quality challenges. The novelty of the research presented herein lies in the integration of cutting-edge genomic sequencing with repeated seasonal sampling, offering a direct insight into the changing composition of URBs. This approach enabled us to highlight the impact airport de-icers can have on river water quality and consequent biological health effects, significantly enhancing our understanding of environmental contamination dynamics.

4.2 Materials and Methods

4.2.1 Study location

A series of sample sites in the River Crane (London, UK) were selected (**Table 4-1**, **Figure 4-1**). These sites were concentrated proximal to outfalls of Heathrow Airport's eastern catchment and were assessed bi-monthly over two successive winter periods.

Table 4-1: Overview of the sample locations along the River Crane (London, UK) utilized for this work. The average river widths and depths were computed based on all sampling dates. The 'Bed Substrate' column represents the predominant bed cover at each site, while the 'Shading' column approximates the degree of bed shading at each location.

Site no.	Grid reference	Site description	Avg. width (m)	Avg. depth (m)	Bed substrate	Shading
1	TQ 10417 78752	Pre-airport control, upstream of the M4 motorway.	7.0	0.17	Gravel, pebbles & cobbles	Moderate
2	TQ 10337 78142	(Used in Oct-20, however, was replaced with Site-1 in subsequent trips due to access issues and practicalities in sample collection.)	-	-	Silt, sand, & gravel	Moderate
3	TQ 10599 75536	Control immediately upstream of airport outfalls.	5.9	0.30	Gravel, pebbles & cobbles	Moderate
4	TQ 10683 75494	Between airport outfalls ('clean' and 'secondary treatment').	5.2	0.39	Silt & sand	Heavy
5	TQ 10958 75229	Immediately downstream of airport outfalls.	5.4	0.20	Pebbles & cobbles	None
6	TQ 11099 75004	Upstream of Duke of Northumberland confluence.	8.4	0.25	Gravel, pebbles & cobbles	Moderate
7	TQ 11160 74456	Downstream of Duke of Northumberland confluence and Baber Bridge. Upstream of a weir.	13.5	0.20	Pebbles & cobbles	Moderate (banks) to none (middle)
8	TQ 11800 73919	Substantially further downstream (ca. 1.9 km from airport outfalls).	8.1	0.35	Gravel, pebbles & few cobbles	Little



Figure 4-1: Annotated map of the study area (River Crane, London, UK) illustrating (a) the locations of sampling sites and (b) the land cover, with indications of permitted discharges into the River Crane. Data provided by the UK Centre for Ecology and Hydrology (land cover) and the Environment Agency (permitted discharges).

The River Crane is an urban river flowing through west London substantially altered over centuries of industrial activities, situated in a primarily urban and sub-urban (65.2% land cover) catchment of 57.1 km² with large areas of grassland and some arable/horticultural land (**Figure 3-1**) [74]. With a mean discharge of 0.506 m³/s (Q10 = 1.27 m³/s) [75], the river receives effluents from various industries, including treated sewage, industrial effluents (e.g., rubber and plastic manufacturing) and treated surface runoff from the neighbouring airport (**Figure 4-1b** and **3.2.1 Study location**). The use of anti- and de-icers at this airport during winter months has been long associated with URBs [76–78]. Consequently, the River Crane has been closely monitored by citizen scientists (Friends of the River Crane (FORCE), Citizen Crane, and the Crane Valley Partnership) [79] and Heathrow Airport Ltd [77,80], providing useful inter-annual datasets and including metadata on the occurrence of URBs. The specific monitoring locations (**Table 4-1**, **Figure 4-1**) were chosen to assess the impacts of the airport's surface water discharges into the River Crane. These locations were informed by the sites regularly sampled monthly by OHES Environmental on behalf of Heathrow Airport Ltd (2016-present). Sites-3 and 5 for Year-1 were used in Exton *et al* (2023) as “control” and “impact” sites respectively [48].

Bi-monthly sampling was conducted during the winters of 2020-21 and 2021-22. For the first year, samples were collected on 19th October 2020, 7th December 2020, 16th February 2021, and 20th April 2021 (Oct-20, Dec-20, Feb-21, and Apr-21). An additional 'event' sample was taken on 24th February 2021, in response to a visible URB event (URB-21). For the second year, samples were collected on 19th October 2021, 7th December 2021, 1st March 2022, and 19th April 2022 (Oct-21, Dec-21, Feb-22, and Apr-22). The Feb-22 sample was initially scheduled for 22nd February 2022, but due to heavy rainfall and high-water levels (peaked at 3.02 m³/s at Cranford Park gauging station on 21/02/2022), sampling was postponed and performed a week later

on 1st March 2022. The elevated river level and flow rate during this period could have influenced the Feb-22 results due to potential scouring and the introduction of pollutants from urban surface runoff [81,82].

4.2.2 Periphyton sampling

Periphyton samples were collected for each site and date and were treated as described by UK and EU guidance (UKTAG River DARLEQ2 [83] and EU COST Action DNAqua-Net [84]). A representative selection of coarse gravel and cobble-sized sediments were collected from the riverbed at each location, stored combined in sealable polythene bags, transported on ice, and refrigerated at 4 °C within 24 hours. Biofilm and periphyton matter were aseptically sampled from the rock samples using a single-use toothbrush. The samples were then rinsed and resuspended in filtered (0.22 µm), sterile de-ionised water, vortex homogenised, and centrifuged (3,800 rpm, 12 min at 20 °C). The supernatant was discarded, and the pelleted microbial biomass was frozen at –20 °C for short-term storage. For DNA extraction, microbial samples were defrosted and processed using the Macherey-Nagel NucleoSpin® Soil kit according to manufacturer instructions. The quality of the extracted DNA was quantified using NanoDrop, and the concentration was determined by a Qubit II fluorometer using the DNA HS kit. The DNA extracts were then frozen at –20 °C and stored until they were shipped for amplicon sequencing.

4.2.3 Genetic sequencing and taxonomic assignment

Sequencing was conducted following the prior method [48]. Briefly, DNA samples were sequenced for the 16S rRNA v3-4 region by Novogene Co., LTD using Illumina MiSeq (250-bp paired-end sequencing) (Illumina Inc., USA). The QIIME (1.7.0 pipeline) [85] was employed for operational taxonomy unit (OTU) assignment for the 16S rRNA gene amplicons. OTUs were clustered against UPARSE (v7.0.1090) [86], and reads that did not match the reference were subsequently clustered de novo at the 97% similarity level using UCLUST [87]. OTU sequences were aligned using PyNAST [88]. The taxonomy of OTUs was determined using the naïve Bayesian Ribosomal Database Project (RDP) classifier for the Mothur method, and the SSUrRNA database from SILVA [89–91] was used as a reference adhering to standard protocols for microbial sequencing. Whilst no replication was conducted in the sequencing of samples, the

periphyton of a representative collection of pebble and cobble-sized sediments from the riverbed were combined prior to DNA extraction and sequencing.

4.2.4 Water quality and hydrological measurements

In-field measurements of conductivity, oxidation-reduction potential (ORP), total dissolved solids (TDS), dissolved oxygen (DO), pH, and water temperature were taken at three intervals (1 m away from each bank and in the middle of the channel), at each site, at approximately 10 cm depth from the water surface. Water velocity measurements (at 20 intervals across the channel) were also taken and used to calculate discharge, determine channel cross-section morphology diagrams and calculate a width average flow velocity [92]. In-field water quality measurements were recorded using HANNA Instruments' HI-9147-04 (DO), HI-98190 (pH, ORP, water temperature), and HI-9813-61 (conductivity, TDS) portable meters (HANNA Instruments Inc., USA). Depth and velocity data were captured using SonTek FlowTracker Handheld-ADV (Xylem Inc., USA). Water samples (1 L) were collected, stored on ice, transported and refrigerated overnight until analysed the following day.

Biochemical oxygen demand 5-day (BOD_5) was determined according to standard methods [93]. Concentrations of ammonium (NH_4-N) [94], nitrate (NO_3-N) [95], phosphate as total phosphorus (PO_4-P) [96], and chemical oxygen demand (COD) [97] were determined by photometry (cell test) and quantified using NOVA 30-A photometer (Spectroquant, Germany). To measure de-icer concentrations, filtered (0.45 μm) water samples were analysed by high-performance liquid chromatography (HPLC) (Agilent Technologies 1200 series, USA) and total organic carbon (TOC) (Shimadzu TOC-V CSH with Shimadzu TNM-1, Japan). For HPLC (100 μL injection volume, 0.400 mL/min flow rate, 19-20 bar, 60 °C. ROA Rezex Phenomenex (150 x 7.8 mm) column, refractive index detector, H_2SO_4 (0.5 mM) mobile phase), mixed standards (0.005, 0.01, 0.05, 0.1, 0.5, and 1.0 %v/v for each of propylene glycol, ethylene glycol, acetic acid, and formic acid) were used to calculate freeze-point depressant (FPD) concentration in river water samples. Calibration curves and quality control for HPLC analysis can be found in the Supplementary Information (**S4-1**).

4.2.5 Other water quality data sources

Water quality metrics were sourced from a multi-parameter sonde, managed by the Environment Agency (Defra), situated downstream of the airport outfall into the River Crane (TQ 1093 7526). River flow information was obtained via Defra's Hydrology Data Explorer, from Cranford Park gauging station (TQ 10312 77840), which is upstream of the airport outfalls and between sampling Sites 1 and 3 (**Figure 3-1**). Weather data, including hourly weather observation [98] and rainfall data [99], were accessed from the Met Office's MIDAS database via the Centre for Environmental Data Analysis (CEDA), at the Heathrow (Greater London) monitoring station (**Figure 3-1**). The location of the Environment Agency sonde is immediately downstream of airport outfalls and therefore provides highly relevant data on water quality, whereas the gauging station in the River Crane used for discharge data is further north of the airfield and so is unable to capture these inputs (**Figure 3-1**). The next nearest gauging station is considerably further downstream than any site sampled and the airport outfalls and therefore encompasses a much larger range of inputs and is also not reflective of the discharge proximal to the airport.

4.2.6 Data analysis

Taxa were consolidated at the lowest taxonomic level (LTL) and family level, using relative OTU abundances (<2 % grouped as “other”). A Principal Coordinate Analysis (PCO) was conducted on relative abundances (without prior transformation) using Bray-Curtis similarity to develop a resemblance matrix in PRIMER v7 / PERMANOVA+ (Primer-e, UK). Apr-22 samples were not included as they were massively skewed to “unknown” taxa. The data was not subjected to transformation prior to the construction of the resemblance matrix to reflect the impact of the URB event more accurately on benthic composition by preserving the original scale of the species abundances, important for capturing the full impact of the event. Distance-based linear modelling (DistLM), visualised as distance-based redundancy analysis (dbRDA), was performed on relative abundances with water quality data as predictor variables (9999 permutations) for multivariate statistics. Environmental data were normalised with missing datapoints given the value 0 for use in DistLM analysis. Shannon's diversity index was calculated to assess taxa richness, diversity, and evenness, using the

combined relative abundance data. Analysis of untransformed water quality and monitoring data was performed using Microsoft Excel and R Studio.

4.3 Results and Discussion

4.3.1 Spatiotemporal variation in periphyton composition

There were substantial temporal changes in periphyton composition between sampling visits at the LTL (**Figure 4-2**), however, considerably less spatial variation between sites. The most notable change was in Feb-21 and URB-21 – i.e., immediately preceding and during an URB event – where there were significant changes to the microbial assembly downstream of airport outfalls when heavy de-icing activity was predicted ($0.001 \leq p \leq 0.035$, PERMANOVA). The significant change in composition is attributable to the increase in the relative abundance of two genera: *Rhodoferrax* and *Sphaerotilus*, as described by the authors in Exton *et al.* (2023) for this catchment [48]. *Sphaerotilus spp.* are well-documented URB taxa and associated with wastewater [40,42,49], whereas *Rhodoferrax spp.* had not previously been associated with URBs [48]. Relative abundances of *Sphaerotilus* and *Rhodoferrax* were $\leq 0.18\%$ and $\leq 0.25\%$ in Oct-20 and Dec-20 (i.e., before significant de-icing activity) which increased slightly at Site-3 upstream of airport outfalls in Feb-21 (0.91% and 2.42%). Immediately downstream of airport discharges (Site-5) saw a significant increase in relative abundances of these two taxa to 9.54% and 32.59%, with a high presence continuing to the final site sampled (Site-8), ca. 1.9 km downstream of airport outfalls. The relative abundance of *Sphaerotilus* remained significantly higher downstream of discharges at 25.36% (Site-6) before reducing to 3.57% (Site-7) and 3.63% (Site-8). *Rhodoferrax*, however, remained highly elevated across all study sites at 19.54% (Site-6), 56.71% (Site-7), and 31.83% (Site-8). *Rhodoferrax* is a genus associated with freshwaters rich in organic matter [100] which could indicate that its larger spatial persistence is a result of it able to utilise a wider range of carbon sources or that it is found in normal river biofilms, unlike *Sphaerotilus* which is heavily linked with URBs [40] and wastewaters [101]. A couple of weeks following Feb-21 sampling there was a visible URB and additional event-based samples were taken (URB-21). Sampling several locations downstream of the airport outfall (including Sites 5 and 6) revealed both *Sphaerotilus* (13.57 – 24.39%) and *Rhodoferrax* (14.71 – 37.61%)

remained dominant. By Apr-21, less than 8-weeks later, microbial data suggests that the water course showed signs of recovery with *Sphaerotilus* and *Rhodoferax* accounting for $\leq 1.39\%$ and $\leq 5.60\%$ relative abundances at all sites sampled.

Figure 4-2: Relative abundances of 16S rRNA (V3-4) sequences at the lowest taxonomic level (LTL). Taxa <2% relative abundance combined as “other” (light grey), taxa >10% maximum relative abundance are labelled in the figure legend with their taxonomic level indicated. (a) Year-1, and (b) Year-2 samples.

In the second winter studied (Oct-21 to Apr-22), there was no discernible shift in community composition (>53.05% similarity, CLUSTER analysis) nor any particularly dominant taxa (no OTU > 16.5% relative abundance). Several OTUs have moderate relative abundances in Year-1 which decrease substantially in Feb-21 and URB-21 and remain at lower proportions throughout Year-2, for example, the family *Rhodobacteraceae* (3.29 – 13.24% pre-URB and 0.10 – 7.86% post-URB) and one of its genus' *Rhodobacter* (4.09 – 9.44% pre-URB and 0.10 – 8.06% post-URB). Whereas *Sphingomonadaceae* and *Luteolibacter* were also prominent pre-URB (i.e., Oct-20 and Dec-20) (0.96 – 5.49%) but their abundance increased again in Feb-22 and Apr-22 (0.10 – 13.15%). Certain taxa are only prominent during certain months in Year-2, such as *Arenimonas* in Feb-22 (3.45 – 9.81%), and *Pseudomonas* at Site-3 in Feb-22 (16.47%, resulting in Feb-22 Site-3 being clustered separately to all other samples, **Figure 4-3**). There were no taxa which accounted for more than 20% relative abundance at any site in Year-2, except in Apr-22 where uncharacterised taxa account for 15.72 – 77.61% relative abundance. Over 2500 uncharacterised OTUs were identified in the sequence data, collectively contributing to minimal relative abundance in April 2022, ($\leq 0.69\%$). This notable presence was not due to a handful of specific taxa, underlining the complexity, variation, and diversity of the periphyton. While their individual abundance might be low, suggesting a minor role in shaping the biofilm structure, their collective contribution underscores their essential role in promoting overall biodiversity. Possibly this is due to the biofilm environment presenting a different ecological niche for bacteria to persist [102].

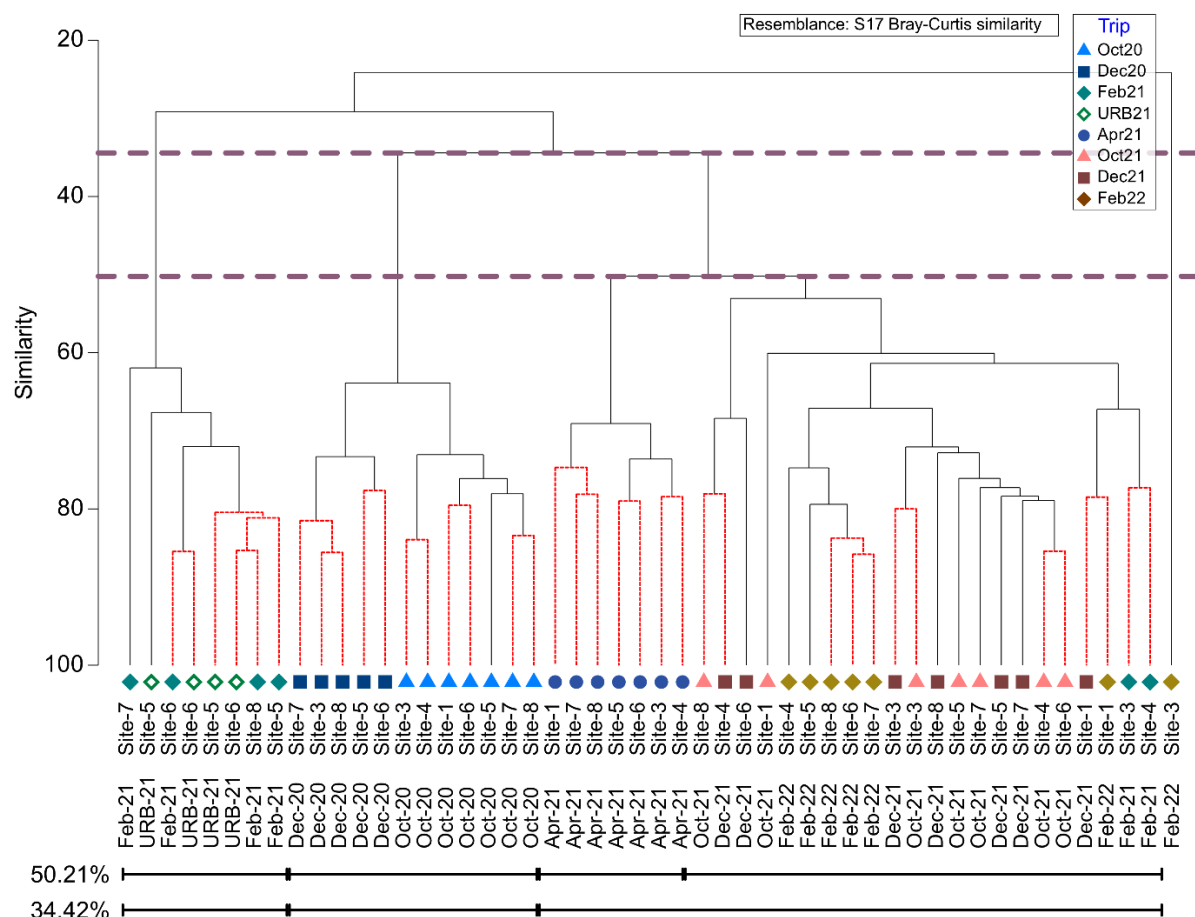
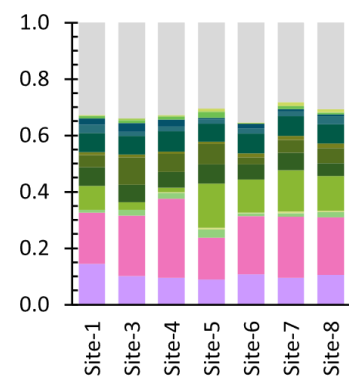


Figure 4-3: Dendrogram constructed from CLUSTER analysis (Group average, 999 permutations, 5% significance) of the resemblance matrix (Bray-Curtis similarity) using 16S rRNA (V3-4) relative abundance data. Purple dashed lines indicate 34.42% and 50.21% similarity.

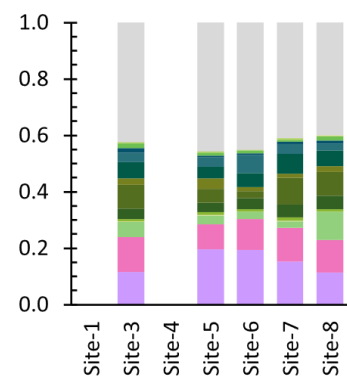
When aggregated at the family taxonomic level (**Figure 4-4**) several taxa have >15% relative abundance and remain prominent in the periphyton community throughout, excluding Feb-21 and URB-21, such as *Sphingomonadaceae* (1.28 – 28.00%), *Rhodobacteraceae* (0.30 – 20.67%), *Rubritaleaceae* (0.20 – 20.16%), *Pseudomonadaceae* (0.10 – 17.06%), *Moraxellaceae* (0.00 – 15.57%). However, during Feb-21 and URB-21, the family *Comamonadaceae* (which contains *Sphaerotilus* and *Rhodoferrax*) shapes the periphyton downstream of airport discharges (17.97 – 81.38%) which then decreases significantly by Apr-21 (8.82 – 13.14%). *Comamonadaceae* had no discernible abundance in the periphyton before

Feb-21, whereas post-URB it maintained a substantial component of the benthos throughout Apr-21 and Year-2 samples (4.54 – 21.28%, excluding Apr-22 possibly due to the presence of unclassified organisms). The persistent presence of *Comamonadaceae* post-URB is also reflected in its genera *Sphaerotilus* and *Rhodoferax* which maintain a presence throughout Apr-21 and Year-2 (0.53 – 1.39% and 0.10 – 5.60%), despite having $\leq 0.18\%$ and $\leq 0.25\%$ relative abundance pre-URB. Thus demonstrating the lasting influence of an URB on the periphyton assemblage explaining their persistent role and rapid appearance.

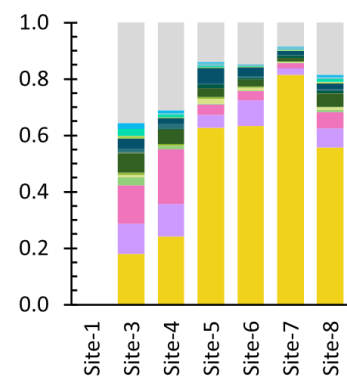
(a-1) Oct-20



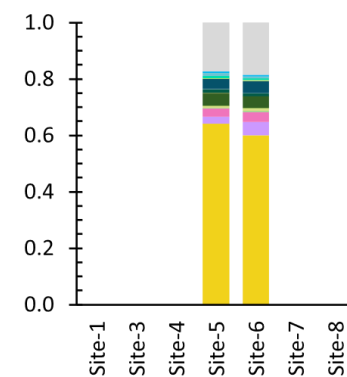
(a-2) Dec-20



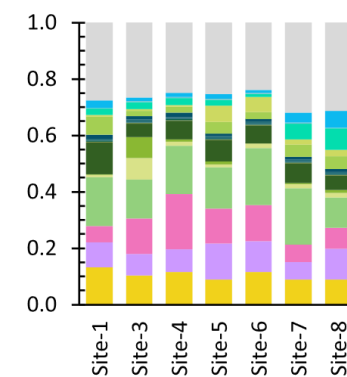
(a-3) Feb-21



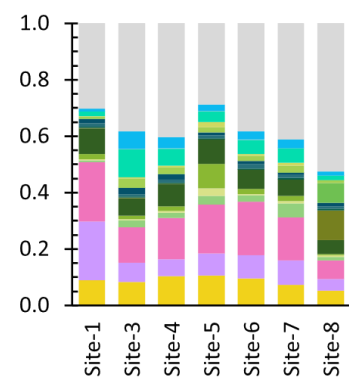
(a-4) URB-21



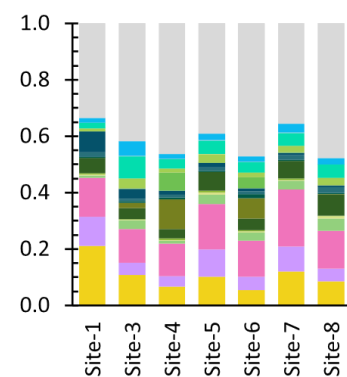
(a-5) Apr-21



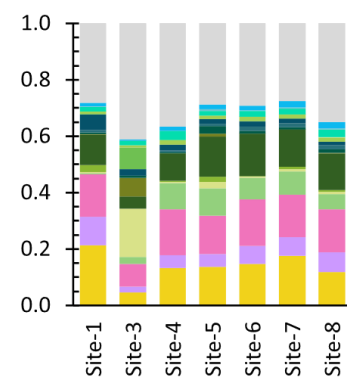
(b-1) Oct-21



(b-2) Dec-21



(b-3) Feb-22



(b-5) Apr-22

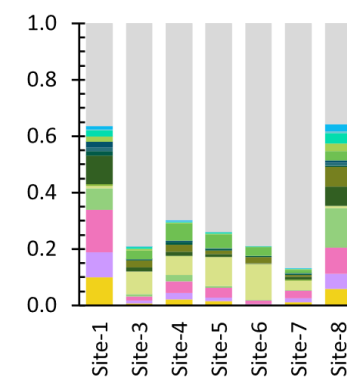
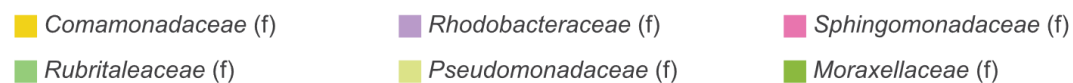


Figure 4-4: Relative abundances of 16S rRNA (V3-4) sequences aggregated at the family taxonomic level. Taxa <2% relative abundance combined as “other” (light grey), taxa >10% maximum relative abundance are labelled in the figure legend. (a) Year-1, and (b) Year-2 samples.

Figure 4-2 suggests there was greater temporal variation in periphyton composition than spatial variation. Statistical analysis (PERMANOVA by date, $P = 0.05$) identified significant differences between most dates sampled with sites grouped (**Table 4-2**). However, Feb-21 and URB-21 are not significantly different from each other ($p = 0.074$) indicating that, although there was no visible URB in Feb-21, the considerable shift to the microbiome had already occurred. The second exceptions are Oct-21 with Dec-21 ($p = 0.421$) and Oct-21 with Feb-22 ($p = 0.087$) showing a more predictable and stable benthos in a winter with no visible URB. Whereas, by performing PERMANOVA analysis by site, only comparison between Site 1 to Sites 4 and 7 reveal significant differences ($p = 0.011$ and 0.028). The greater temporal variation might be due to various factors such as seasonal changes, weather patterns, and fluctuations in environmental conditions that typically exert a profound influence on aquatic microbiomes over time. Low spatial variability might be due to the potential homogeneity of the river ecosystem across different sites, and the mixing action of the river could further reduce spatial differences.

Table 4-2: p values from PERMANOVA analysis by date (P = 0.05, sites grouped) of 16S rRNA (V3-4) relative abundances of river periphyton, comparing dates sampled. Where * indicates a significant difference.

	Oct-20	Dec-20	Feb-21	URB-21	Apr-21	Oct-21	Dec-21	Feb-22	Apr-22
Oct-20									
Dec-20	0.001*								
Feb-21	0.001*	0.002*							
URB-21	0.020*	0.015*	0.074						
Apr-21	0.001*	0.002*	0.005*	0.017*					
Oct-21	0.001*	0.001*	0.002*	0.010*	0.001*				
Dec-21	0.001*	0.001*	0.003*	0.035*	0.001*	0.421			
Feb-22	0.001*	0.001*	0.005*	0.014*	0.001*	0.087	0.035*		
Apr-22	-	-	-	-	-	-	-	-	

Shannon's diversity (**Figure 4-5, S4-2**) remained fairly constant both spatially and temporally (generally between 4.028 and 4.842), except in Feb-21, URB-21, and Apr-22. Diversity in Feb-21 at locations upstream of the airport discharge remained in the range of normal values. However, there was a substantial reduction in Shannon's diversity downstream of the outfall (2.165 – 3.442) which is also observed in URB-21 (3.093 – 3.607), a consequence of the shift in periphyton community to the URB. Expanding the scope to all sites studied, there were considerable impacts to diversity continuing downstream to the final site sampled (diversity remained <3.442 ca. 1.9 km after airport outfall, Sites 5-8). Apr-22 also had a much lower Shannon's diversity for Sites 3-7 (1.408 – 3.122) as a consequence of the high relative abundance of "unknown" taxa which, when aggregated have a substantial effect on the calculated Shannon's diversity. Performing the calculations on relative abundance data without combining "unknown" taxa gave a considerably higher result than other samples (4.493 – 6.356) because some "unknown" taxa may represent identical OTUs that have not been combined.

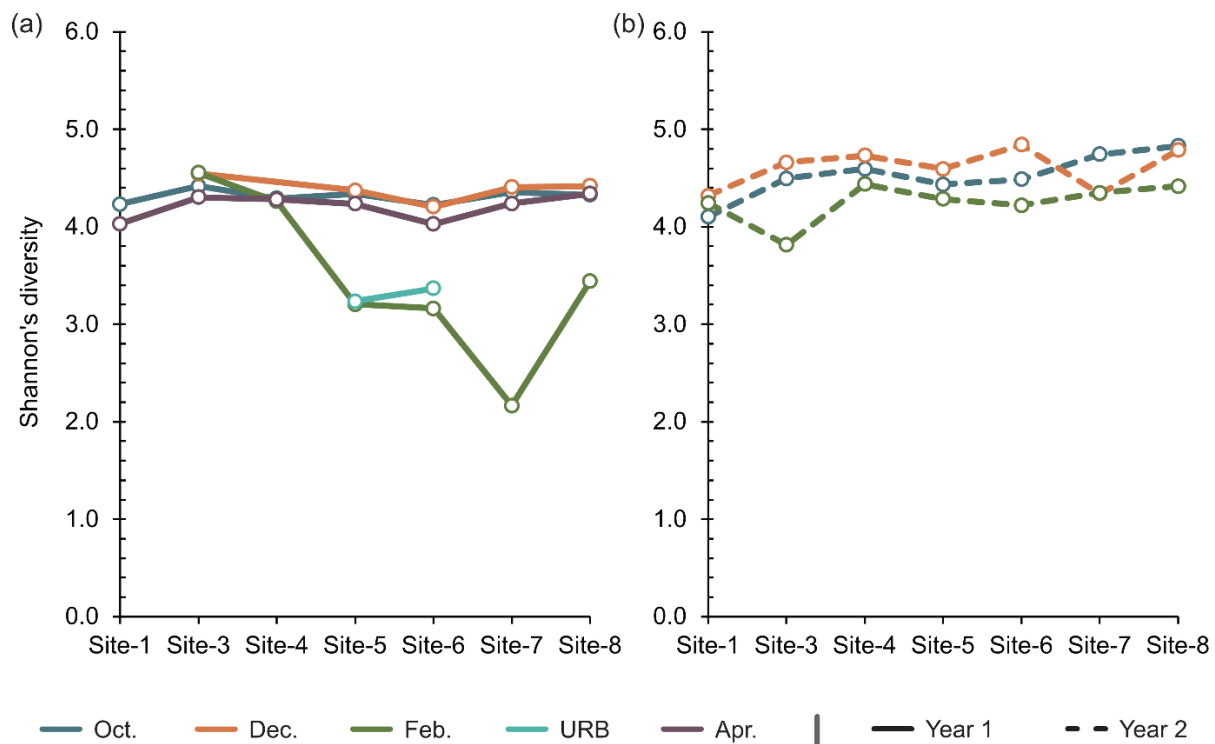


Figure 4-5: Shannon's diversity index calculated for periphyton, employing 16S rRNA (V3-4) relative abundance data, for (a) Year 1 and (b) Year 2 samples. The analysis was performed at the finest taxonomic resolution, often referred to as the lowest taxonomic level (LTL).

4.3.2 Water quality and influences on periphyton

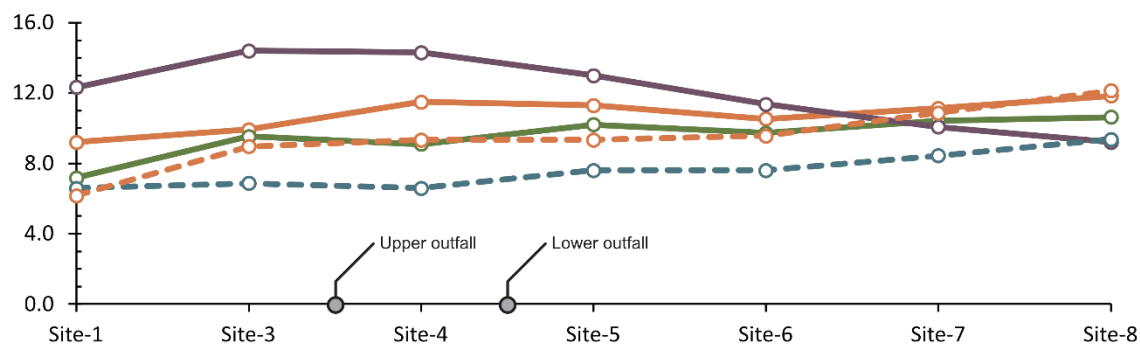
Limited spatial or temporal trends existed in environmental data collected – a summary of select parameters can be seen in **Figure 4-6**. DO was generally 6.17 – 12.3 mg/L and showed small increases progressing downstream (**Figure 4-6a**). Apr-21 was fractionally higher than other sampling dates (9.20 – 14.40 mg/L) and decreased moving downstream. DO was not lower in Feb-21, prior to the URB event, and continued increasing downstream. Water temperature had minimal spatial variation (± 1.92 °C) but changed temporally throughout the winter (**Figure 4-6b**). In both years, two bands emerged (i) October and April (10.9 – 15.2 °C) and (ii) December and February (4.7 – 8.5 °C), with Year-2 samples often fractionally higher than equivalent months in Year-1. BOD₅ was generally higher in February and April for Years 1 and 2 (1.67 – 4.65 mg/L) compared to both Octobers and Decembers (1.01 – 1.55 mg/L,

excluding Dec-21, Site-1) but showed no regular spatial trends (**Figure 4-6c**). BOD₅ did increase downstream of airport outfalls in Feb-21 (from 2.11 to 4.65 mg/L), prior to a visible URB but after the community had already shifted, suggesting heterotrophic activity had already started ramping up. BOD₅ remained high in Apr-21, including above airport outfalls, and decreased progressing downstream suggesting non-URB influences. There were no clear spatial or temporal patterns in TOC (5.06 – 12.25 mg/L), except for an elevated concentration in Dec-20, throughout the entire study area (22.15 – 25.57 mg/L) (**Figure 4-6d**). There were no appreciable trends in NH₄-N (0.03 – 0.47 mg/L), NO₃-N (0.97 – 6.23 mg/L), and PO₄-P (0.16 – 0.32 mg/L), either spatially or temporally (**S4-3**). Chemical oxygen demand (COD) tests were often below the range of the cell test (<25.0 mg/L) and FPDs were mostly below the limit of detection of the HPLC instrument and standards (<0.001 %v/v, which equates to less than 10.4 – 12.2 mg/L of all FPDs). Propylene glycol is not acutely toxic to aquatic organisms, except for at very high concentrations (LC₅₀/EC₅₀ > 18,000 mg/L) [103]. This is also the case for ethylene glycol (LC₅₀/EC₅₀ >10,000 mg/L for fish and aquatic invertebrates) [104], sodium acetate (LC₅₀ >16,000 mg/L) [105], and sodium formate (EC₅₀ > 23,000 mg/L, LC₅₀ >2,300 mg/L) [106]. Therefore, concentrations of FPDs during spot sampling were several orders of magnitude below lethal dose for aquatic organisms such as fish and invertebrates.

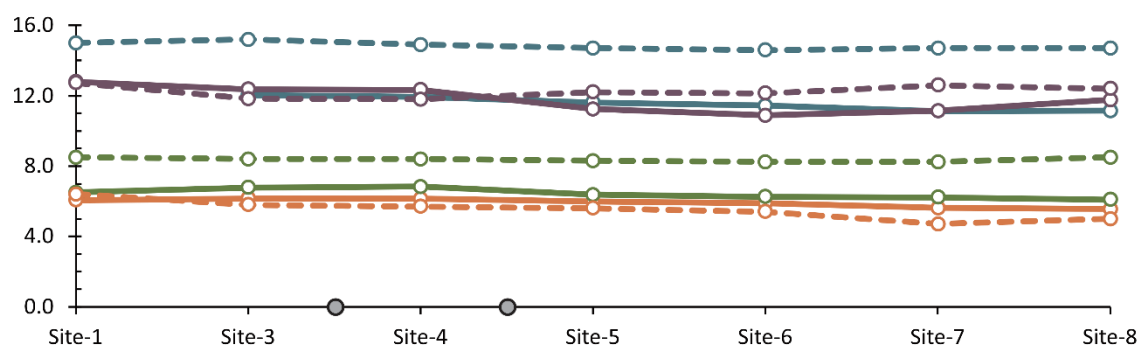
Discharge increased progressing downstream (generally 0.077 – 0.542 m³/s), particularly after the confluence with the Duke of Northumberland River (between Sites 6 and 7), but flows were notably higher in Dec-20 (0.297 – 0.994 m³/s) and especially in Feb-21 (0.872 – 1.746 m³/s). Under normal operation, effluents from airport outfalls can be of better water quality (such as high dissolved oxygen) than the receiving waters which are often in highly urban areas with lots of additional pressures (such as channel modification, CSOs and untreated road runoff). However, during colder periods and the resulting increased de-icer application, elements of effluent quality can decrease, even if still within permitted consents, resulting in URBs. This is further unexpected due to dilution offsets by treated airport surface waters. Despite this, the sudden and harmful manifestation of URBs regularly occurs downstream of airport outfalls [48,49], which is not always expected when examining water quality monitoring – as well-evidenced by local media and campaign groups [76,107–109] (**Table 2-1**).

Calculating pollutant loadings would be a useful addition, however, these data were not available at the time of the study but should be considered in future research.

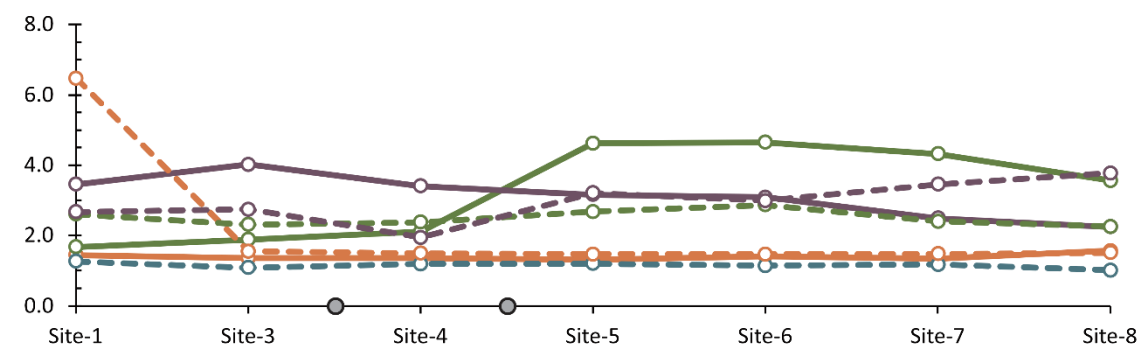
(a) Dissolved oxygen, mg/L



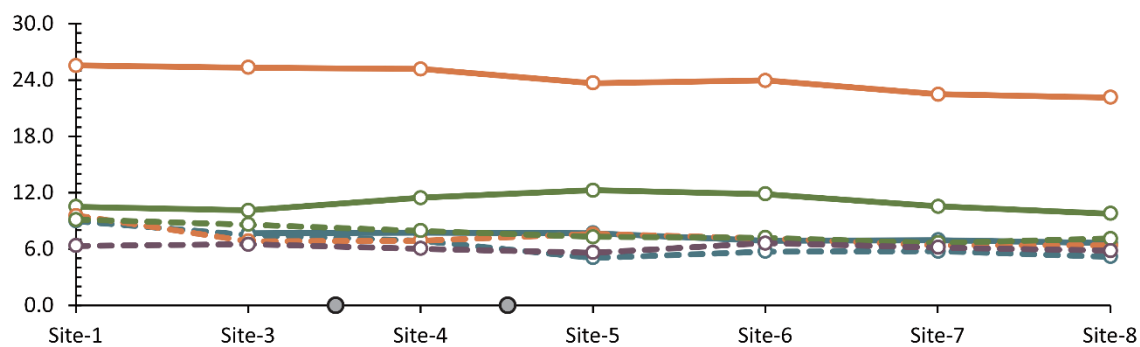
(b) Water temperature, °C



(c) BOD₅, mg/L



(d) Total organic carbon (TOC), mg/L



October December February April | Year 1 - - Year 2

Figure 4-6: Water quality data collected concurrently with periphyton samples from the River Crane in London, UK. The subfigures include (a) in-situ measurements of dissolved oxygen, (b) on-site water temperature recordings, (c) biochemical oxygen demand over 5 days (BOD₅) determined through laboratory analysis, and (d) total organic carbon (TOC) content assessed via laboratory analysis.

The similarity in spatial 16S rRNA sequencing data and differences between temporal samples were further supported by principal coordinate analysis (PCO) of LTL relative abundances (**Figure 4-7a**), which demonstrated three clear groupings of samples at 34.42% similarity (as determined by cluster analysis, **Figure 4-3**): (i) Oct-20 and Dec-20; (ii) Feb-21 (excluding Sites 3 and 4 – i.e., upstream of outfall) and URB-21; and (iii) Sites 3 and 4 in Feb-21, all Apr-21 sites, and Year-2 which can be defined as “pre-URB”, “intra-URB”, and “post-URB”. The latter cluster (post-URB) splits at 50.21% similarity into (i) Apr-21 and (ii) Year-2 plus Feb-21 Sites 3 and 4 – this separation is observable in PCO3. PCO1 explained 36.2% and PCO2 a further 22.1% of the total variation. PCO analysis of the 16S rRNA data further recognises that the date sampled was more important than the location in the river (i.e., temporal is more important than spatial). The Feb-22, Site-3 sample remains separate from all other samples at ≥24.11% similarity further reflecting its difference to all other samples. The high abundance of *Pseudomonas* and unclassified taxon in Feb-22, Site-3 (16.47 and 14.40%) seems to be responsible for the difference to other samples (0.10 – 5.64% *Pseudomonas*). The difference between Feb-22 Site-3 and other samples could be a reflection of the heavy rainfall event in the week prior, however, other sampling sites were not similarly affected. Apr-22 was not included within PCO and cluster analyses because it artificially extended the axes with the incorrect data of “unknown” OTUs.

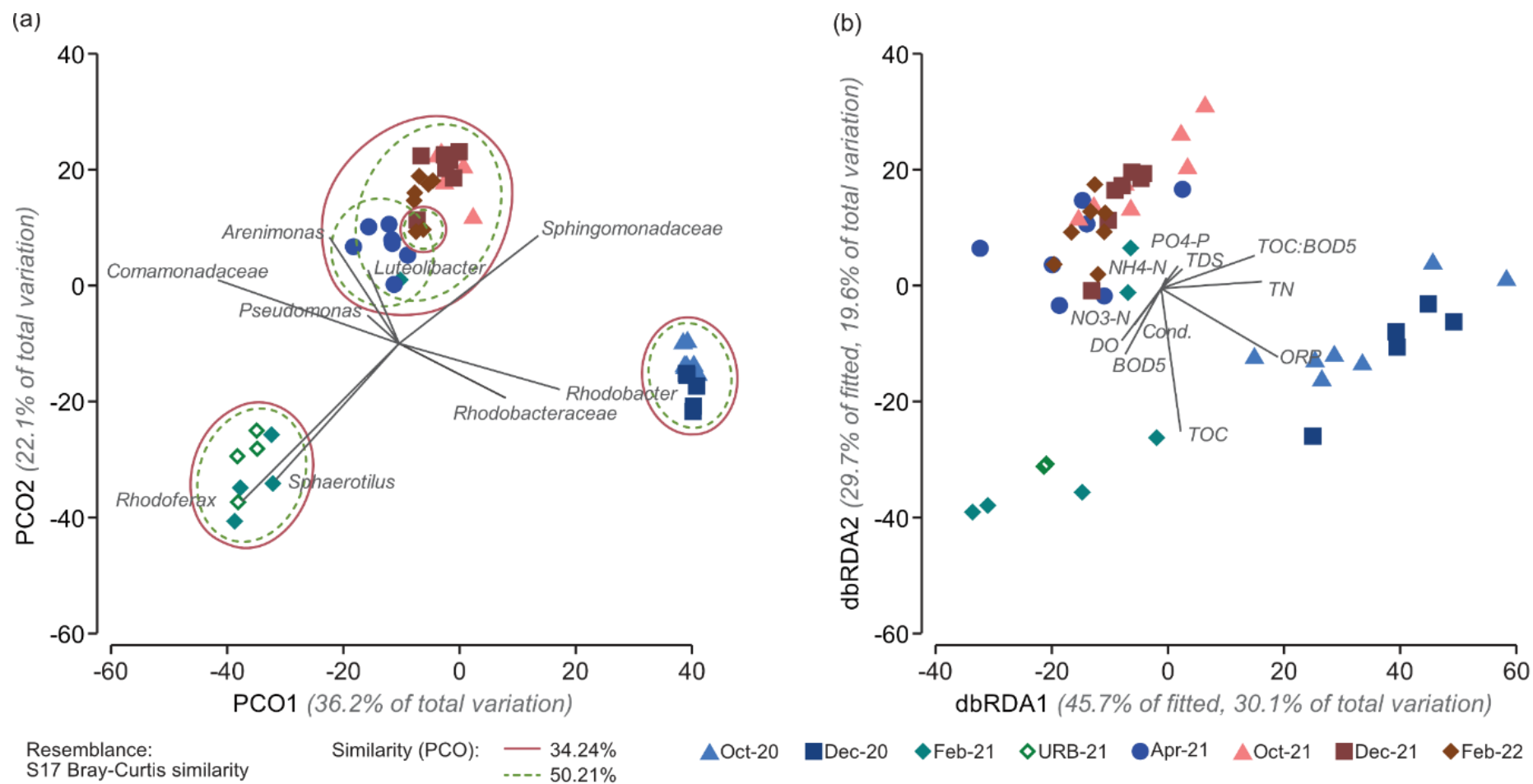


Figure 4-7: Ordination plots using Bray-Curtis similarity of 16S rRNA (V3-4) relative abundance data. (a) Principal coordinate analysis (PCO), labelled taxa are those >10% maximum relative abundance, similarity calculated using CLUSTER analysis (group average, 999 permutations, 5% significance). (b) Distance-based redundancy analysis (dbRDA) with labelled water quality parameters as variables.

In the Feb-21 (URB shifted periphyton) samples the BOD₅ and TOC were 4.29 mg/L and 11.09 mg/L respectively, deriving a TOC:BOD₅ ratio of 2.59. This was different to 2.23 mg/L, 8.43 mg/L and 4.61 for the non-URB samples. The lower TOC:BOD₅ ratios in the Feb-21 water samples are suggestive of the presence of more readily assimilable material, probably encouraging the growth of URB. The URB also appeared to correspond to periods of depleted ORP (**S4-4**). Negative ORP values typically indicate an environment that is reducing, or low in oxygen. In such environments, certain microorganisms, including those that can contribute to URBs, may thrive. These organisms can utilize other electron acceptors, such as nitrate, sulfate, or even organic material, for their metabolic activities under anaerobic or low-oxygen conditions. Additionally, the lack of oxygen can suppress the activities of other microbial communities that usually compete with or predate upon these URB-contributing organisms, providing them with a competitive advantage. However, a slight but discernible increase emerged in DO progressing downstream in each sampling trip, except those collected in April 2021. This augmentation could suggest effective aeration of surface waters by airport treatment systems prior to discharge [71,72]. Conversely, in April 2021, after the URB manifestation, a decline in DO levels was noted downstream of airport outfalls, indicative of sustained impacts from the URB. The sometimes discordant information from ORP and DO measurements underscores the complexities inherent in spot-sampling methods for water quality assessment when contrasted with the insights afforded by continuous water quality monitoring or the longer trends elucidated by riverine bioindicators [110,111]. The shift in microbial community structure can lead to changes in ecosystem function and could potentially result in the development of an URB. Moreover, the reducing conditions signalled by negative ORP values can facilitate the release of certain pollutants from sediments into the water column, potentially providing additional nutrients or resources that encourage the growth of URB-contributing organisms and discourage the higher organisms (e.g., grazers) which thrive in oxic environments [112].

DistLM analysis (presented as a dbRDA plot in **Figure 4-7b**) highlights a strong grouping of Feb-21 (downstream) and URB-21 samples which correlate with TOC, BOD₅, DO, NO₃-N, and conductivity, whereas TOC:BOD₅ demonstrated a strongly negative correlation. DO (9.39%), BOD₅ (8.68%), and the TOC:BOD₅ ratio (8.83%)

accounted for considerable proportions of the variability in bacterial abundance ($0.0001 \leq p \leq 0.0002$) (**Table 4-3**). TOC (5.19%) and conductivity (5.50%) were also influential, albeit explaining slightly less variability ($p = 0.0008$ and 0.0167). Whereas $\text{NO}_3\text{-N}$ (0.87%) had negligible contribution ($p = 0.7659$), despite its positive correlation with URB shifted periphyton. Meanwhile, ORP accounted for the highest proportion of variability (19.54%) in bacterial abundance ($p = 0.0001$), correlating with Oct-20 and Dec-20 sequence data. This suggests that ORP has a strong correlation with periphyton composition. Further investigation is warranted to determine the underlying mechanisms and implications of this. The association of URB shifted periphyton composition with DO, TOC and BOD_5 (**Figure 4-7b**) is consistent with findings by Albini *et al* (2023) [113] and demonstrates the importance of bioavailable organic carbon to URB taxa, which in turn deplete DO, increasing heterotrophic activity and add additional pressures on to the degraded ecosystem.

Table 4-3: Output from distance-based linear modelling (DistLM) analysis of 16S rRNA (V3-4) relative abundance data on river periphyton, with water quality as predictor variables.

(a) Sequential tests							
	R²	SS(trace)	Pseudo-F	p	Prop.	Cumul.	res.df
Conductivity	5.50%	4727.6	2.7951	0.0167*	0.0550	0.0550	48
ORP	19.54%	16787.0	12.2520	0.0001*	0.1954	0.2504	47
TDS	1.59%	1368.4	0.9987	0.3907	0.0159	0.2664	46
DO	9.39%	8063.0	6.6010	0.0002*	0.0939	0.3602	45
NH ₄ -N conc.	0.91%	785.6	0.6380	0.7065	0.0091	0.3694	44
NO ₃ -N conc.	0.87%	743.8	0.5986	0.7659	0.0087	0.3780	43
PO ₄ -P conc.	1.90%	1636.1	1.3265	0.2095	0.0190	0.3971	42
BOD ₅	8.68%	7454.4	6.8918	0.0002*	0.0868	0.4838	41
TOC conc.	5.19%	4459.0	4.4715	0.0008*	0.0519	0.5357	40
TN conc.	3.41%	2927.8	3.0894	0.0078*	0.0341	0.5698	39
TOC:BOD ₅	8.83%	7584.4	9.8110	0.0001*	0.0883	0.6581	38
(b) Specified solution							
R²	0.65808						

4.3.3 Hydrological and catchment influences

Water velocity and the adhered substrate are highly influential in governing URB growth and morphotype. A sufficient minimum velocity is required for nutrient replenishment (0.05 m/s) [43] but if the flow is too fast then growths are scoured away (reported as >0.6 m/s) [41,43]. Average velocity recorded in this study was generally within these limits (0.084 – 0.550 m/s) (**Figure 4-8**). However, the maximum velocity that facilitates URBs is highly dependent upon substrate type. Mechanisms of sediment grain mobilisation are complex [114,115] with several models commonly used [116,117]. However, in general, smaller, unconsolidated sediment grains – such as silt, sand, gravel, and pebbles – are more readily mobilised and so the bed is not stable for long enough for biofilm growths to establish. Whereas larger, more immobile substrates are much better facilitators of URBs, such as large cobbles, boulders, and especially concrete channels or constructs. The majority of sites sampled in the River Crane had similar bed substrates of gravel with some cobbles, whereas Site-4 was entirely unconsolidated fine sediments (primarily sand and silt) (**Table 4-1**). There were more substantial periphyton and URB growths observed on the larger sediment grains. However, lots of silt, sand, and gravel were observed under and between pebbles and cobbles, which clog pores and hinder hyporheic exchange flows (HEFs) [118]. The authors have also noted that submerged objects such as tree branches and large anthropogenic litter (e.g., discarded sinks and bricks) are excellent facilitators of URBs.

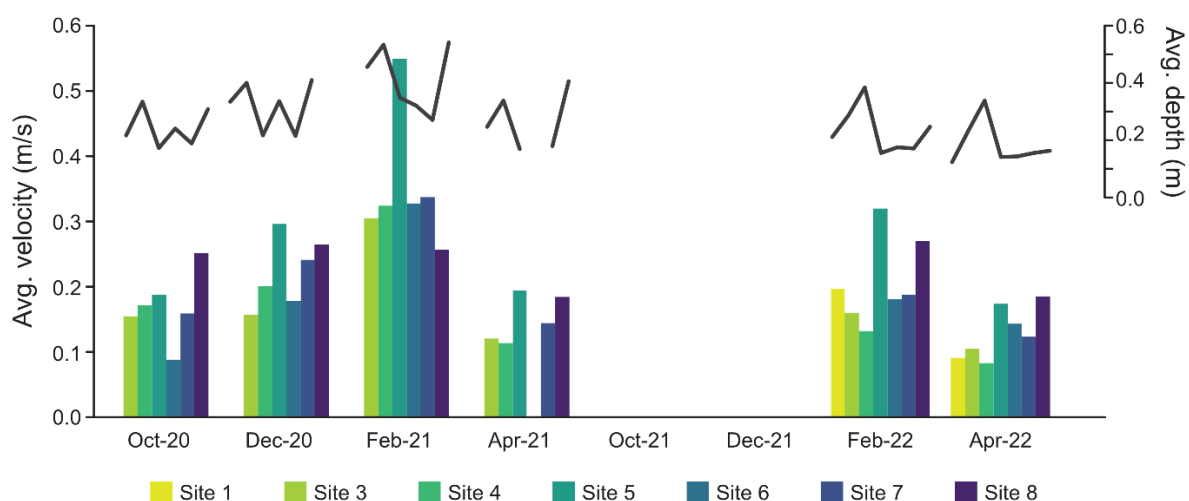
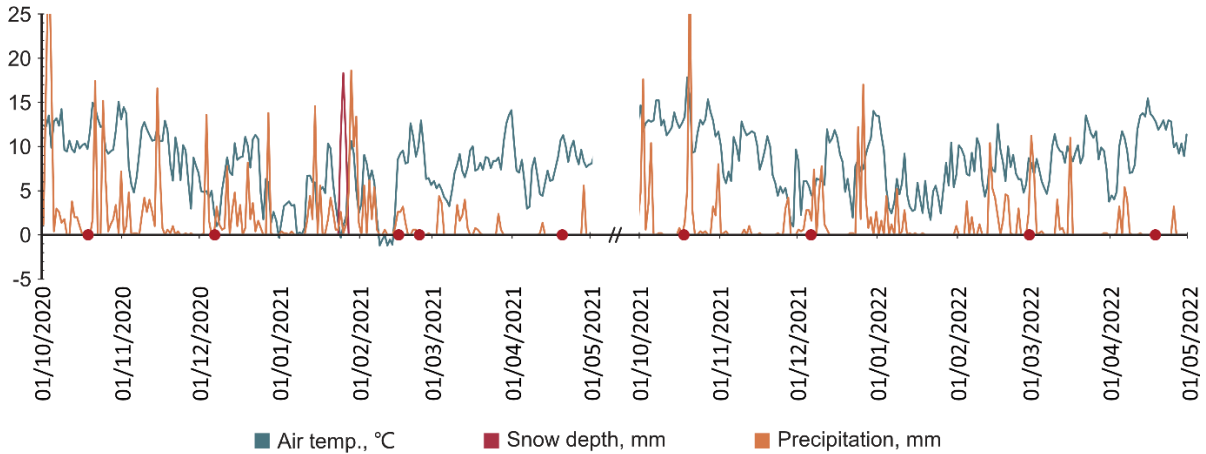


Figure 4-8: Average depth and velocity measured for each sampling location and date, data collected alongside periphyton and water quality samples.

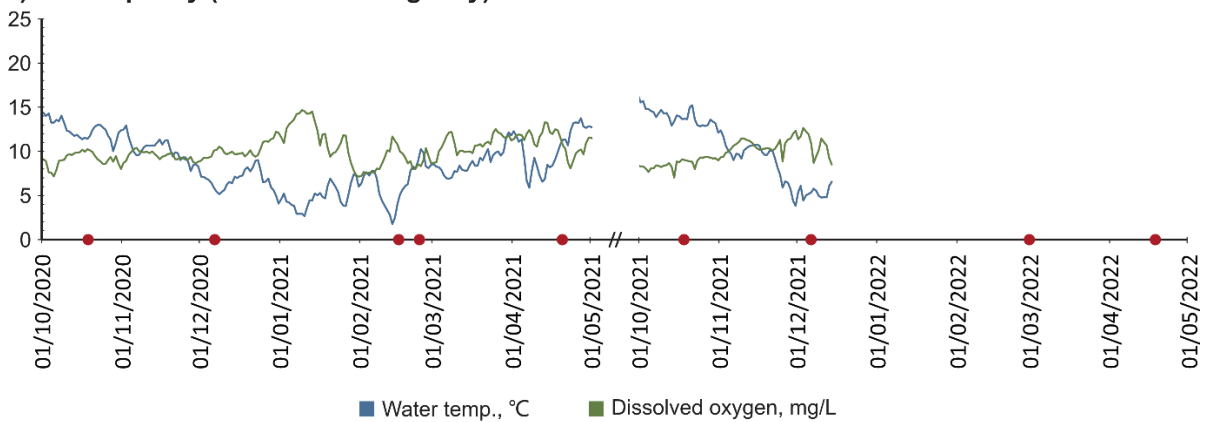
Ongoing monitoring data revealed the Year-1 winter was colder (54.2% more measurements $<5^{\circ}\text{C}$ Year-1 vs 2, October-April) and wetter (45.8% more rainfall Year-1 vs 2, October-April) than Year-2 (**Figure 4-9a**). Additionally, only Year-1 had any recorded snow depth (**Figure 4-9a**). The threshold temperature for automatic de-icing at Heathrow Airport is between 3 and 5°C (Pers. Communication, M. Murphy) and pilots may request aircraft de-icing at any time, therefore substantially more de-icing will likely have taken place in Year-1. The recorded snow in late January 2021 was followed by a substantial surge in discharge in the River Crane over subsequent weeks (up to $12.0\text{ m}^3/\text{s}$), suggesting that de-icers or anti-icers used on the airfield could have been mobilised in surface runoff (**Figure 4-9c**). Discharge measurements at the Cranford Park Environment Agency gauging station upstream of the airport were recorded throughout the study period and baseline flows are similar to spot sampling measurements (**S4-4d**). Significantly higher discharges occurred in Year-1 ($0.859\text{ m}^3/\text{s}$, mean average 01/10/2020 – 30/04/2021) than in Year-2 ($0.464\text{ m}^3/\text{s}$, mean average 01/10/2021 – 30/04/2021), as would be expected with higher rainfall (**Figure 4-9c**). Unfortunately, the Environment Agency sonde, positioned downstream of airport outfalls, malfunctioned, so no water quality data was recorded between 15/12/2021 and 30/04/2022 (**Figure 4-9b**). However, from Year-1 data, DO varies considerably throughout the winter ($4.04 - 20.21\text{ mg/L}$) and is approximately inversely

correlated with water temperature. Ongoing DO monitoring data is similar to spot sampling measurements conducted (**Figure 4-6a, Figure 4-9b**).

a) Meteorological data (Met Office)



b) Water quality (Environment Agency)



c) Discharge, Cranford Park (DEFRA)

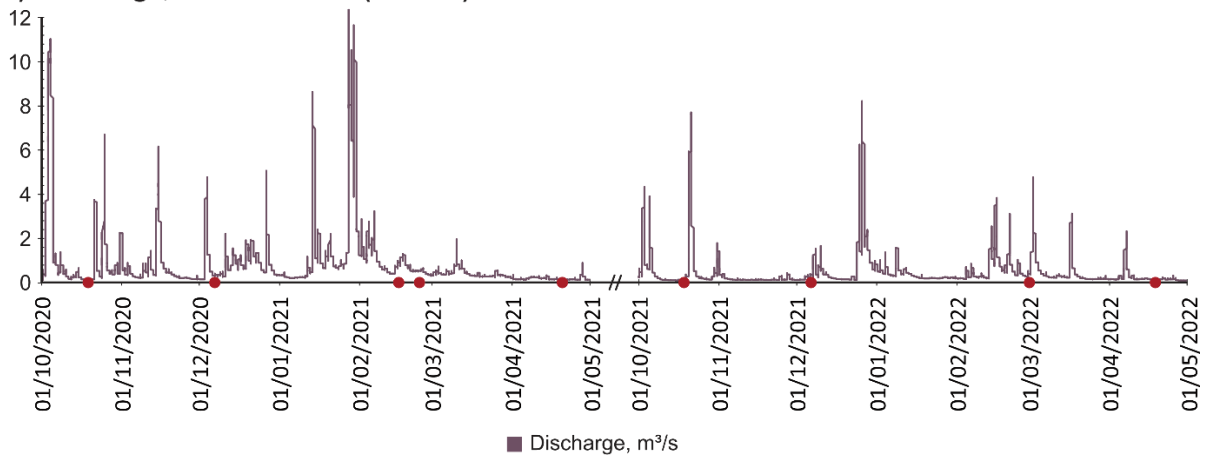


Figure 4-9: Daily averages of monitoring data (precipitation is daily sum) for (a) meteorological data collected by the Met Office (accessed through CEDA) at Heathrow Airport monitoring station; (b) river water quality measurements collected downstream of the airport outfall into the River Crane (London) by the Environment Agency; and (c) river discharge data collected at the Cranford Park gauging station in the River Crane

(London) by DEFRA. Data provided for both winters sampled and dates of sampling are indicated by red dots.

When evaluating influences on poor water quality and the manifestation of URBs, the hydrogeomorphology of the river and its catchment must be considered. The River Crane is a heavily urbanised catchment (residential, commercial, and industrial, with an extensive road network) interspersed with patches of arable and grassland (open grassland, agriculture, and woodland) (**Figure 4-1b**). Therefore, there are many inputs into the river unaccounted for in wastewater treatments. Runoff from urban areas contains a diverse array of contaminants [81,119], for example, road dust, heavy metals, pesticides and fertilisers, organics (including dissolved organic matter (DOM) and volatile organic compounds (VOCs)), polyfluorinated alkyl substances (PFAS), polycyclic hydrocarbons (PHAs), sewage (raw and treated), and various emerging contaminants, in addition to atmospheric deposition – especially from aircraft. Heavy modifications to channels, such as straightening, over-widening, excess loading of fine sediments, and steep artificial banks make rivers much more susceptible to degradation and result in wastewaters discharged into them having a higher impact than the same loading into a more resilient channel [118]. All of these factors are present in the River Crane. For example, sites 1, 3, and 7 all have constructed wooden or constructed vertical banks (**Figure 4-10a**) and Sites 4, 5, 6 and 8 have heavily modified, vertical soil banks (**Figure 4-10b**). Furthermore, there has been substantial amounts of anthropogenic channel engineering in this section of the River Crane, including weirs and the Duke of Northumberland River (a fully artificial watercourse that is a tributary of the River Crane between Sites 6 and 7). The impact of anthropogenic modification on river channels has not previously been studied in relation to the susceptibility of a river to URBs. However, a cross-disciplinary perspective highlights how straight, uniform channels have small hyporheic zones with limited vertical HEFs [118] and the vertical, wooden or concrete banks common in the River Crane prevent lateral HEF [120,121]. HEF helps to filter both river and ground waters, so limited exchange causes the increase of contaminants in rivers [118], such as organic pollutants, and increases their susceptibility to URBs. Furthermore, the excess fine sediments in urban runoff clog pores in the river bed and banks, further

reducing HEFs [122] and limiting the habitat for aquatic insects and fish spawning [123,124] – which is further compounded by the loss of this habitat during an URB event which smothers the riverbed as it proliferates (**Figure 4-11**) [43]. Moreover, the proliferation of URBs further limits HEFs [122,125,126]. The River Crane is also heavily shaded in places by excess vegetation and tree growth on and near riverbanks (Sites 1, 2, 3, 4, 6 and near the banks at Site 7) (**Figure 4-10c**). This affects natural periphyton, such as algal and aquatic plant growth, compounding reduced DO and habitats for aquatic fauna. The River Crane has, in places, naturally improved its overall condition. Excess widening and straightening of the River Crane has resulted in often shallow and low energy flows [79] leading to the development of riffles, longitudinal bars, and vegetated islands (**Figure 4-10d**) which help to increase DO and HEFs [127]. It is therefore important to remember that the geomorphology and river/catchment hydrology substantially affect URB growth, not just chemical water quality. The compounding chemical, morphological, and hydrological influences result in the River Crane being a heavily degraded watercourse that is readily susceptible to URBs and further ecological damage [128].



Figure 4-10: Photos of relevant riverine geomorphology and features in the River Crane (Houndslow, UK) at or near sampling sites. Features include (a) wooden and concrete vertical banks (near Site 3); (b) modified, vertical soil banks (Site 6), and (c) excess shading (Site 4) which contribute to diminished resilience to undesirable river biofilm (URB) manifestation. In addition, (d) where excess widening and straightening of the channel has led to the natural development of longitudinal bars and vegetated islands (Site 7).

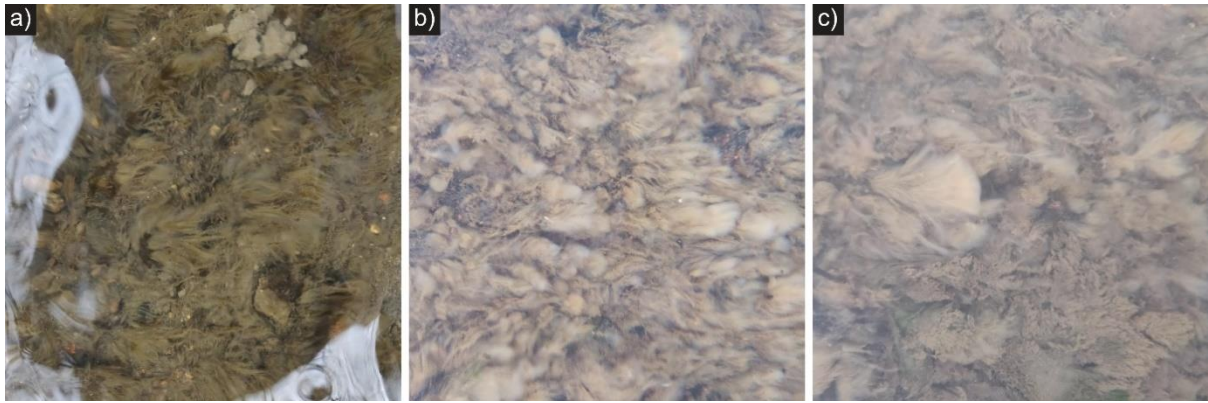


Figure 4-11: Photos of the undesirable river biofilm (URB) during URB-21 sampling event. River flows from left to right in each photo. Photos taken by the author at sampling Site-7 on 24/02/2021.

4.3.4 Influences of COVID-19 on air travel

One of the substantial differences between Years 1 and 2, alongside different temperatures (lower in Year-1) and rainfall (higher in Year-1), was the number of flights from the neighbouring Heathrow Airport and the influence of COVID-19 travel restrictions. In Year-1 (Oct-20 to Apr-21), restrictions on air travel in to and out of the UK remained, whereas, in Year-2 (Oct-21 to Apr-22), travel restrictions had been removed resulting in an approximately fourfold increase in the number of passengers (**Figure 4-12**) [129]. The demonstrable change in microbial composition occurred in Year-1, despite the lower number of flights, suggesting that the weather (most notably temperature), and therefore the more frequent need for de-icing, plays a significantly greater role in periphyton composition, rather than the mere quantity of flights. The lower temperatures in Year-1 also mean that there is likely to have been an increase in the amount of de-icer used domestically (e.g., personal motor vehicles). However, these are not thought to play a significant role, given the sheer volume applied at airports [73]. Additionally, a new moving bed biofilm reactor (MBBR) [71,130,131], for the Eastern catchment within Heathrow Airport has since been constructed with the aim of to improving treatment efficiency and effluent water quality discharged into the River Crane (Pers. Communication, M. Murphy).

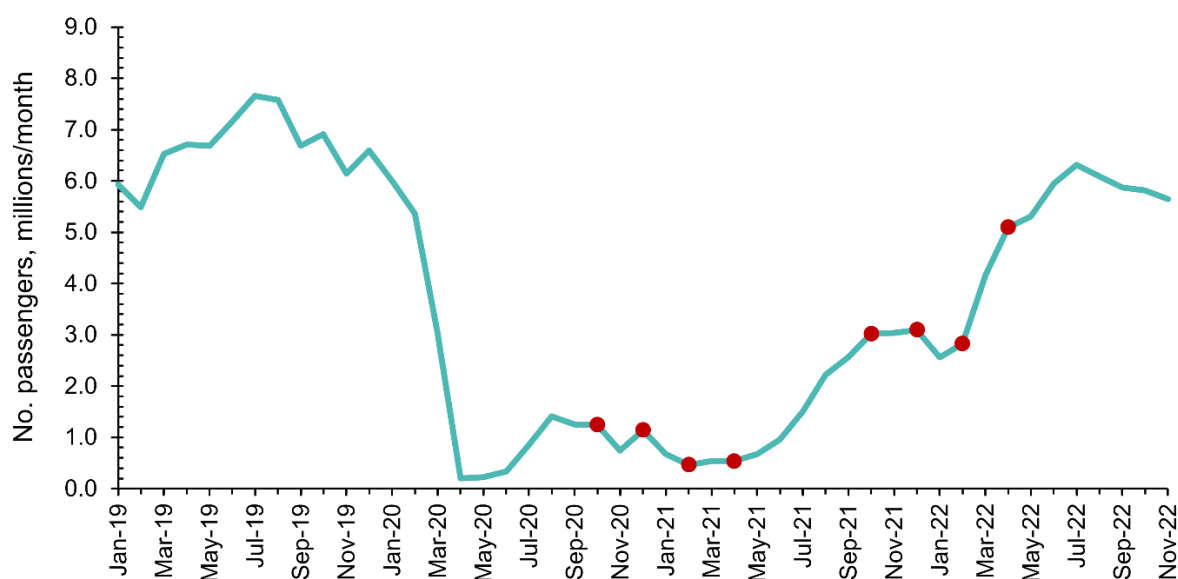


Figure 4-12: Total monthly number of passengers arriving at and departing from Heathrow Airport. Data provided by the Office for National Statistics (UK Government) [129]. Dates of sampling undertaken in this study are highlighted as red dots.

Despite the significantly reduced number of flights in Year-1, our findings highlight significant temporal shifts in the periphyton community over the Year-1 winter period, particularly during and immediately preceding an URB event – demonstrating the disproportionately large impact de-icing can have on receiving waters. The significant shift in periphyton composition downstream of airport outfalls in Feb-21 and URB-21 was characterised by a significant increase in the relative abundance of the genera *Rhodoferrax* and *Sphaerotilus* (**Figure 4-2, Figure 4-5**). Whilst both have been linked with high organic loading in freshwaters [59,100,132], it is the first time that *Rhodoferrax* has been identified in URBs [48]. When OTUs were aggregated at the family taxonomic level, the overwhelming change was within the family *Comamonadaceae* (17.97 – 81.38% relative abundance) (**Figure 4-4**) and could point to its use as a biosentinel of organic pollution to pre-empt the manifestation of an URB and resulting ecological damage [48]. Notably, the microbial changes preceded a visual manifestation of an URB, suggesting the community had already shifted but had not yet proliferated and caused the ecological deterioration associated with intense heterotrophic activity, such as URBs. While community composition appeared to

recover by Apr-21, the legacy of the URB event persisted, with the presence of these two genera and the family *Comamonadaceae* persisting throughout Year-2 (**Figure 4-2, Figure 4-4**). Despite these changes to the periphyton, there was little direct evidence of a deterioration in water quality in either spot sampling (such as DO, BOD₅, and TOC) (**Figure 4-6**) or ongoing water quality monitoring (air temperature, water temperature, and DO) (**Figure 4-9**).

4.3.5 Future work

Societally, our primary focus lies in the controlling and regulating of inputs into river systems, a crucial approach aimed at preserving and enhancing the health of these aquatic ecosystems. For example, industries in the UK have limits imposed by the regulator (Environment Agency) to control pollution in rivers and maintain good overall health. However, many other influences play key roles. This study looks at the problem differently by exploring what is happening in the river and then how this is affected by inputs. Rivers are complex systems and their overall quality is not solely driven by water chemistry but also, for example, their microbiology, macrobiology, ecology, hydrology, and geomorphology. In the case of the River Crane, it is a heavily modified channel with an often flat, uniform bed and has been repeatedly widened resulting in ordinarily a shallow depth [79], there is a much higher proportion of fine sediments [15–17,133,134] and a total loss of the hyporheic zone [118,135]. These compounding impacts demonstrably affect the river system making it less resilient to stressors like organic pollution. The evidence presented here, especially that of the URB event in late February 2021 (Feb-21 and URB-21), demonstrates the River Crane still receives high organic loading that it is unable to manage, despite strict regulatory consents, resulting in URB growth and subsequent negative impacts. This multidisciplinary approach is needed when considering poor water quality in complex river ecosystems in future research.

URBs are polymicrobial assemblies comprised mostly of bacteria but also fungi and protozoa [43,45,46]. The specific roles of these taxa in URBs remains poorly understood, despite, for example, protozoa being integral components of river periphyton, they are well established indicators of poor water quality [136–138] and should be studied in the context of URBs and poor water quality. Algae has been

reported as only a rare component in URBs and alga are often intolerant to the high levels of organic pollution but may exist below the URB, involved in biofilm adhesion, in the inorganic and eutrophic zone [45]. Therefore, whilst 16S rRNA sequencing is a well-established and cost-effective method, it is restricted in scope, only analysing bacterial components of these complex, mixed microbial ecosystems. Furthermore, there are a lack of reference sequences for taxa associated with wastewaters [139,140] and the regions in which you can observe certain taxa. For example, common URB taxa such as *Rhodospirillum rubrum*, *Sphaerotilus*, and *Leptothrix* are all detected in the V1-3 regions but are hardly observed in V4, in part due to their heavy dependence upon primers [139]. Shotgun metagenomics, however, is less affected by these biases and can identify a much greater range of microorganisms so is a powerful tool to analyse eDNA. However, the cost and increased complexity of shotgun metagenomics (especially in relation to 16S, 23S or ITS rRNA sequencing) is highly prohibitive to many researchers. Therefore a combination of sequencing approaches can be used to obtain an overview of the microbial composition and trends. For example, performing 16S rRNA sequencing to obtain a broad understanding of spatiotemporal microbial composition and then performing targeted metagenomics of selected samples of interest can be a powerful and more cost-effective approach. For future work, we recommend performing shotgun metagenomics on Site-5 samples for all Year-1 dates to gain deeper insights into the composition of and interactions within URBs. Furthermore, including metagenomics in the sampling protocol would enable the investigation of additional areas of interest, such as AMR genes [37,141] which have not yet been studied in the context of URBs (but have been observed in URB taxa, such as *Z. oleivorans* [142]) and could indicate a potentially rich sink of AMR genes in already degraded river ecosystems.

Substantial advancements have been made in this study in developing our understanding of URBs. However, further additional studies should investigate the influence of substrate and flow on URBs (i.e., using flume mesocosms). Further study of the effect of river quality on URBs (e.g., chemical inputs, geomorphology, catchment influences and hyporheic zone) would allow for better prediction and ultimately prevention of URBs in rivers and would contribute significantly towards international and domestic ecological restoration and biodiversity goals for rivers.

4.4 Conclusions

In this research, we have presented a bimonthly monitoring study taken over two consecutive winters examining the spatiotemporal variations of river periphyton composition in relation to river water quality and the context of airport de-icing. There was a considerable change in the microbial composition of periphyton in the first winter, perceived to be in response to high levels of airport de-icing at a neighbouring airport, which included a visible URB following Feb-21 sampling. The dominant genera in the URB were *Sphaerotilus* (3.57 – 25.36 % relative abundance), a common URB taxon, and *Rhodoferax* (19.54 – 56.71%), a genus not previously associated with URBs. There were no substantial spatial or temporal variations in the periphyton community in the second winter which was warmer and drier, despite considerably more air travel following the easing of COVID-19 travel restrictions. However, there remained a minor presence of *Sphaerotilus* and *Rhodoferax* throughout Year-2 as a legacy from the URB. PCO and CLUSTER analysis grouped samples into (i) Oct-20 and Dec-20; (ii) URB samples (Feb-21 impact and URB-21); and (iii) Feb-21 control sites, all Apr-21 sites, and Year-2 samples. Environmental data showed very minor spatial changes and minimal temporal changes demonstrating the importance of examining periphyton as a monitor of water quality and river health. Nevertheless, in DistLM analysis, high biodegradable material (BOD, TOC, DO) and conductivity correlate very strongly with URB taxa. The River Crane has a heavily modified catchment and channel which further exacerbates impacts of chemical inputs. Therefore, whilst regulatory consent limits may be sufficient for an otherwise “healthy” watercourse, the already compounding impacts on the River Crane make it less resilient to organic pollution and highly susceptible to further degradation. The findings of this study have several implications for understanding the influences of airport de-icing on microbial ecology in rivers and the other stressors that exacerbate the ecological impacts of inputs on ecosystems. Findings can be used to inform treatment strategies of de-icer contaminated runoff and river restoration approaches to improve overall river health and water quality.

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Data access statement

Supporting data for this study can be accessed through the Cranfield University repository (CORD): DOI [10.17862/cranfield.rd.23674836](https://doi.org/10.17862/cranfield.rd.23674836).

Conflicts of interest

The authors declare there are no conflicts of interest for this research.

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Supplementary information

Appendix A – HPLC quality control

The calibration and quality control data for HPLC analysis of river water samples. Standards were made up as %v/v but are also shown here in mg/L for each FPD and sampling event. The slope and intercept for the calibration curves are reported, along with R^2 from regression analysis (Data Analysis package, Microsoft Excel), and the limit of quantification (LOQ). LOQ was calculated from the concentration of the lowest standard plus percent accuracy (calculated concentration divided by expected concentration).

Sampling event	FPD	Range, %v/v	Range, mg/L	Slope	Intercept	R^2	LOQ, mg/L
Dec-20	EtG	0.001 – 1.0 %	11.13 – 11,130.0	1.769E+09	1.055E+05	1.0000	48.4
	ProG	0.001 – 1.0 %	10.4 – 10,400.0	1.872E+09	2.960E+04	1.0000	33.4
	Ace-H	0.001 – 1.0 %	10.51 – 10,510.0	1.144E+09	1.449E+05	1.0000	27.7
	Form-H	0.001 – 1.0 %	12.2 – 12,200.0	1.047E+09	1.316E+05	0.9999	59.7
Feb-21	EtG	0.005 – 1.0 %	59.45 – 11,890.0	1.329E+09	4.630E+04	0.9997	83.6
	ProG	0.005 – 1.0 %	61.11 – 12,222.0	1.505E+09	1.388E+04	1.0000	108.3
	Ace-H	0.005 – 1.0 %	62.77 – 12,554.0	7.289E+08	2.407E+04	0.9997	77.1
	Form-H	0.005 – 1.0 %	64.43 – 12,886.0	6.647E+08	1.451E+04	0.9998	97.0
Apr-21	EtG	0.005 – 1.0 %	66.09 – 13,218.0	1.389E+09	5.842E+04	0.9997	76.4
	ProG	0.005 – 1.0 %	67.75 – 13,550.0	1.560E+09	-3.198E+03	1.0000	115.5
	Ace-H	0.005 – 1.0 %	69.41 – 13,882.0	7.130E+08	8.410E+03	0.9999	93.7
	Form-H	0.005 – 1.0 %	71.07 – 14,214.0	7.188E+08	1.218E+04	0.9999	96.5

Dec-21	EtG	0.05 – 1.0 %	727.3 – 14,546.0	1.629E+09	-9.151E+05	0.9948	1242.2
	ProG	0.05 – 1.0 %	743.9 – 14,878.0	1.848E+09	-1.057E+06	0.9946	1162.1
	Ace-H	0.05 – 1.0 %	760.5 – 15,210.0	9.475E+08	-5.685E+05	0.9966	1210.5
	Form-H	0.05 – 1.0 %	777.1 – 15,542.0	9.099E+08	-5.496E+05	0.9969	1415.1

S4-1: Range of standards for each freeze-point depressant (FPD), the calibration curves, regression analysis, and limit of quantification (LOQ, minimum standard + %accuracy) for each sampling event.

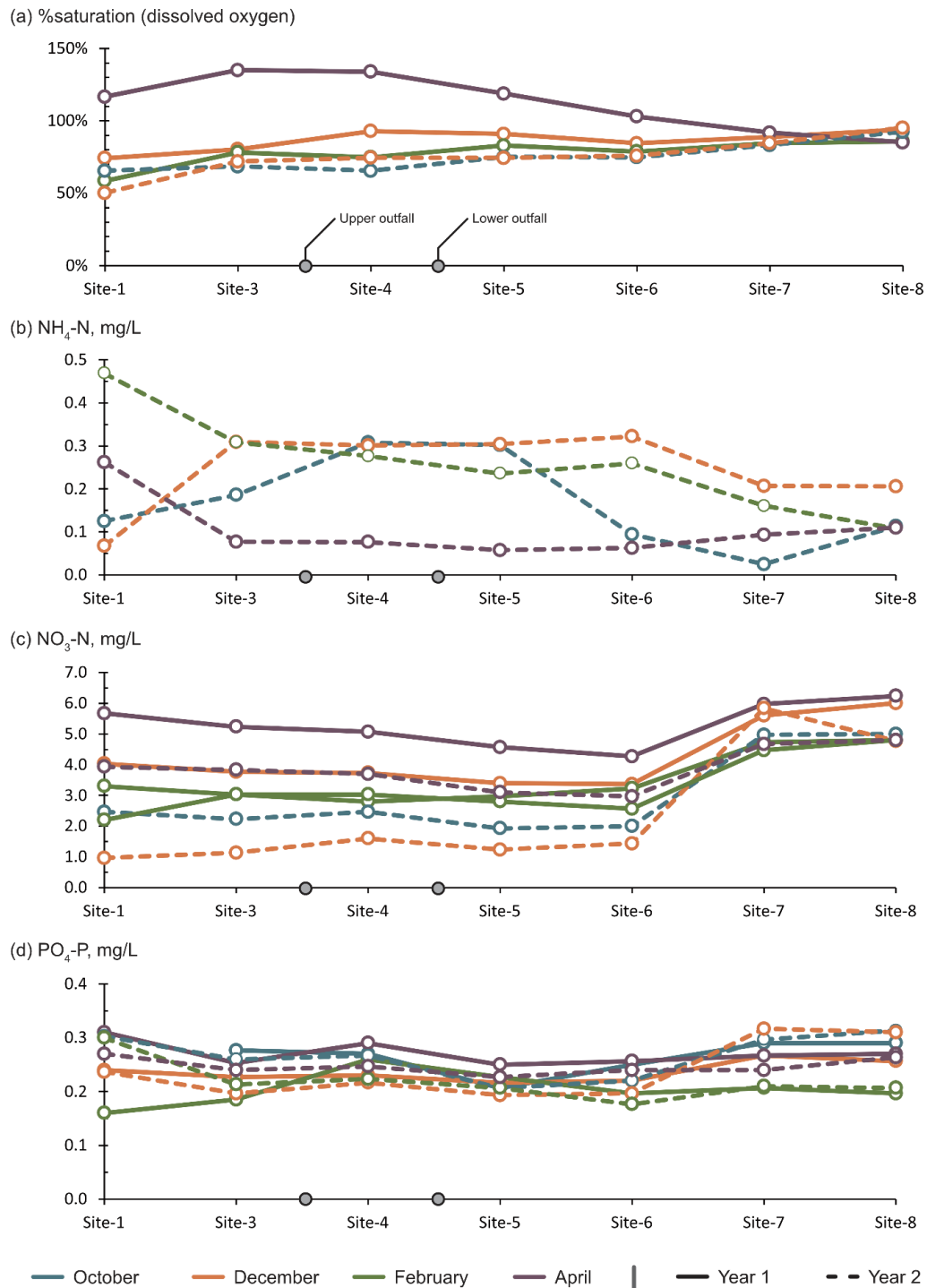
Appendix B – Shannon’s Diversity, Evenness, and Richness

S4-2: Shannon’s diversity, richness, and evenness of periphyton.

	Oct-20							Oct-21						
	Site-1	Site-3	Site-4	Site-5	Site-6	Site-7	Site-8	Site-1	Site-3	Site-4	Site-5	Site-6	Site-7	Site-8
Shannon's diversity	4.231	4.417	4.290	4.336	4.224	4.358	4.328	4.102	4.497	4.593	4.434	4.487	4.744	4.830
Richness	451	492	480	465	486	475	459	544	562	529	536	516	615	687
Evenness	0.692	0.713	0.695	0.706	0.683	0.707	0.706	0.651	0.710	0.732	0.706	0.718	0.739	0.739
	Dec-20							Dec-21						
	Site-1	Site-3	Site-4	Site-5	Site-6	Site-7	Site-8	Site-1	Site-3	Site-4	Site-5	Site-6	Site-7	Site-8
Shannon's diversity	-	4.544	-	4.375	4.206	4.407	4.418	4.318	4.659	4.730	4.593	4.842	4.339	4.786
Richness	-	545	-	550	474	501	527	576	551	610	571	612	523	587
Evenness	-	0.721	-	0.693	0.683	0.709	0.705	0.679	0.738	0.738	0.724	0.755	0.693	0.751
	Feb-21							Feb-22						
	Site-1	Site-3	Site-4	Site-5	Site-6	Site-7	Site-8	Site-1	Site-3	Site-4	Site-5	Site-6	Site-7	Site-8
Shannon's diversity	-	4.555	4.259	3.204	3.164	2.165	3.442	4.243	3.813	4.436	4.285	4.221	4.349	4.415
Richness	-	700	659	597	565	443	554	569	150	486	438	485	559	530
Evenness	-	0.695	0.656	0.501	0.499	0.355	0.545	0.669	0.761	0.717	0.704	0.683	0.687	0.704

URB-21														
	Site-1	Site-3	Site-4	Site-5	Site-6	Site-7	Site-8							
Shannon's diversity	-	-	-	3.237	3.368	-	-							
Richness	-	-	-	549	605	-	-							
Evenness	-	-	-	0.513	0.526	-	-							
Apr-21								Apr-22						
	Site-1	Site-3	Site-4	Site-5	Site-6	Site-7	Site-8	Site-1	Site-3	Site-4	Site-5	Site-6	Site-7	Site-8
Shannon's diversity	4.030	4.303	4.280	4.235	4.028	4.237	4.339	4.554	2.216	3.122	2.404	1.797	1.408	4.393
Richness	575	584	640	581	518	626	614	584	127	159	119	79	90	536
Evenness	0.634	0.675	0.662	0.665	0.644	0.658	0.676	0.715	0.457	0.616	0.503	0.411	0.313	0.699

Appendix C – Additional water quality data (laboratory analyses)

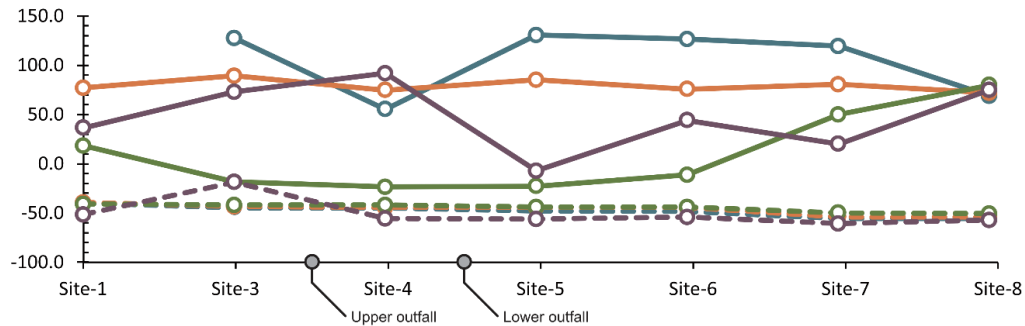


S4-3: Additional water quality data collected concurrently with periphyton samples from the River Crane in London, UK. The subfigures include (a) calculated

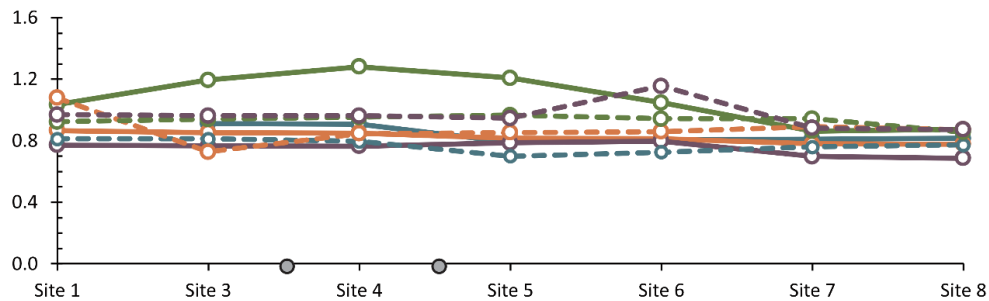
%saturation of dissolved oxygen using in-situ measurements of dissolved oxygen and water temperature, and using photometric laboratory analyses (b) ammonium ($\text{NH}_4\text{-N}$) concentration, (c) nitrate ($\text{NO}_3\text{-N}$) concentration, and (d) phosphate as total phosphorus ($\text{PO}_4\text{-P}$).

Appendix D – Additional water quality data (in-field measurements)

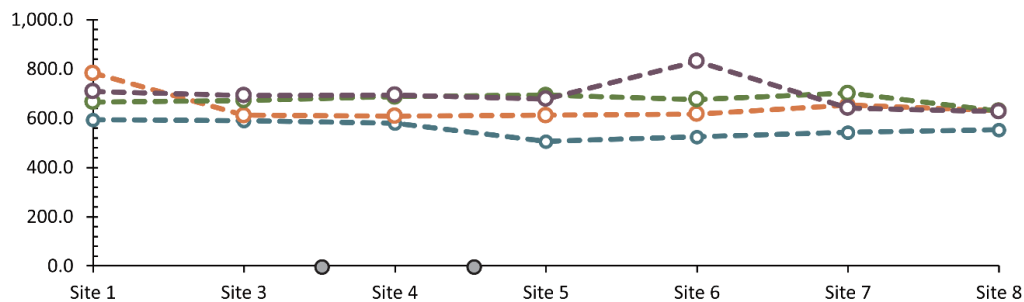
(a) Oxidation-reduction potential (ORP), mV



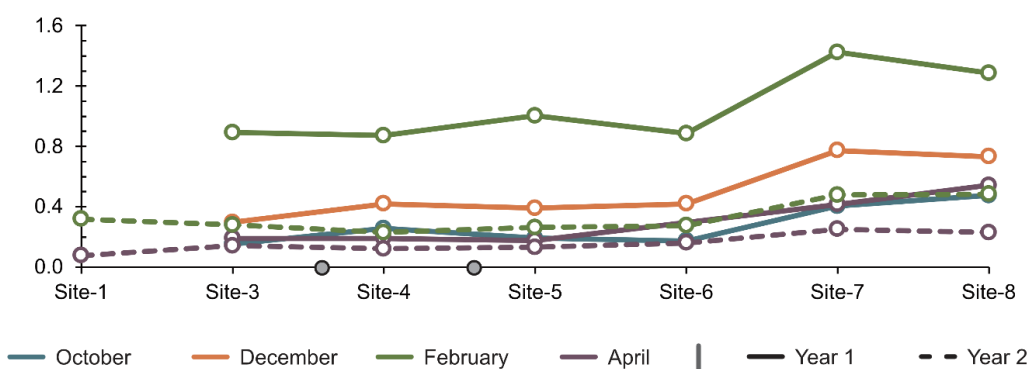
(b) Conductivity, mS/cm



(c) Total dissolved solids (TDS), ppm



(d) Discharge, m³/s



S4-4: Additional water quality data collected concurrently with periphyton samples using in-field probes from the River Crane in London, UK. Subfigures

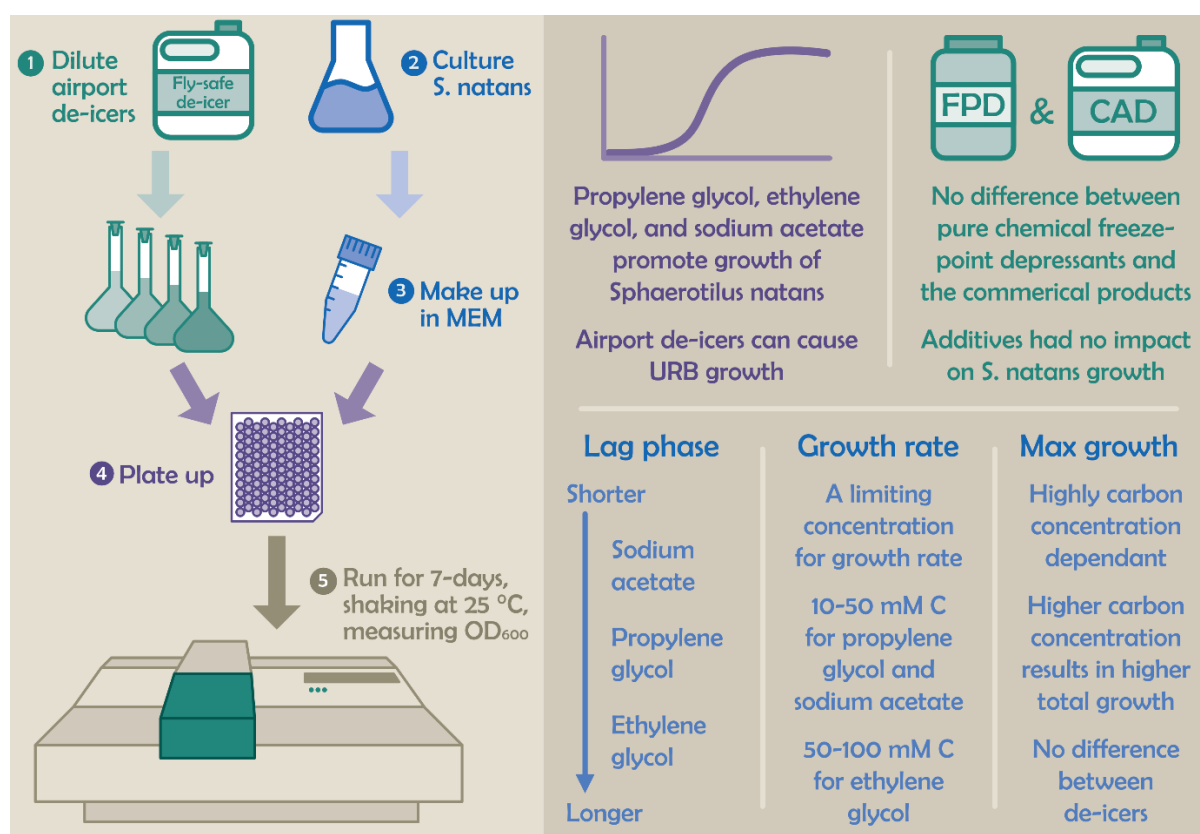
include (a) oxidation-reduction potential (ORP), (b) conductivity, (c) total dissolved solids (TDS); and (d) discharge (calculated from cross-sectional depth and velocity measurements).

5 Impact of Carbon Sources in Airport De-icing Compounds on the Growth of *Sphaerotilus natans*

Abstract

Airport de-icing has been putatively linked with the growth of undesirable river biofilms (URBs, formerly “sewage fungus”), a manifestation of organic pollution causing long-term ecological damage to watercourses. Although surface runoff contaminated with de-icer from airports is usually treated before discharge, URBs are commonly found downstream of airport outfalls. This study used a turbidimetric microcosm design to conduct microbial growth experiments, employing *Sphaerotilus natans* – a keystone URB taxon – and a growth medium supplemented with airport de-icer. Equimolar carbon concentrations were used to compare the effects of common freeze-point depressants (FPDs) and commercial airport de-icers (CADs) – each containing a specific FPD. Growth was assessed via optical density (OD₆₀₀) measurements, from which time-to-detection, maximum rate of change, and maximum optical density were derived and kinetics inferred. *S. natans* was found to grow effectively on all FPDs tested, although the microbial yield was heavily dependent on the carbon concentration for all FPDs and CADs. Under sub-optimal carbon concentrations, both maximum optical density and maximum rate of change were reduced. The mixture of compounds and additives in commercial products did not significantly impact the growth of *S. natans*. This research provides evidence, under controlled conditions, of a link between the growth of URBs and airport de-icers.

Visual abstract



Highlights

- 1 The first definitive link between airport de-icers and URB growth
- 2 *S. natans* grew using propylene glycol, ethylene glycol and sodium acetate freeze-point depressants
- 3 Sodium acetate was consumed earlier and faster, followed by propylene glycol, then ethylene glycol
- 4 Growth was highly carbon concentration dependant
- 5 There were minimal differences between commercial products and pure chemicals indicating additives did not hinder microbial growth.

Keywords

Undesirable river biofilm; airport de-icer; *Sphaerotilus natans*; microcosm; growth kinetics; sewage fungus.

5.1 Introduction

Undesirable river biofilms (URBs), also referred to as “sewage fungus”, are a consequence of organic pollution in inland watercourses [1–4]. These biofilms are widely distributed especially in countries lacking established wastewater treatment infrastructure, and cause significant ecological impact on affected watercourses [5–11]. URBs serve as a visual bioindicator of poor water quality [2] and can provide valuable data regarding overall river health in the face of diffuse and intermittent organic pollution [12–14], for example, spills due to combined sewer overflows. While URBs proliferate in rivers with poor water quality, they also contribute to further degradation in river health. For instance, they consume dissolved oxygen (DO) and nutrients, overgrow riverbeds and lead to significant loss of aquatic fauna and flora [4,15], including macroinvertebrates [16], fish species [8,17] and natural plant life [18]. Moreover, the growth of URBs often results in complaints and disengagement from river users and stakeholders due to their unappealing appearance, olfactory issues, and the subsequent loss of recreational and ecosystem services [19,20]. In the UK, there is substantial evidence to suggest that URBs remain highly prevalent [21–36]. It is also clear that a wide variety of industries and land use changes influence the frequency of URB proliferation (**Table 2-1**). While the specific causes of URBs are variable, catchment specific and usually not directly attributed, one contaminant group implicated in URB growth is runoff from airport anti- and de-icer application. This link has been highlighted by local action groups [37], airport operators [38,39], and local/national news [24,26,28,40], as well as in scientific literature [41–43]. Despite these suggested connections, a direct causal link between the URB growth and airport anti- and de-icers as a nutrient source has yet to be conclusively established or demonstrated empirically.

Sphaerotilus natans is one of the keystone species frequently found in these biofilms [3,8,44–46]. Other notable taxa include the genera *Zoogloea*, *Beggiatoa*, and *Rhodospirillum rubrum* [8,43,46]. A significant amount of research into URBs was conducted between the 1950s and 1980s, often using *S. natans* as a representative taxon for the polymicrobial URB community [47–52]. Recent molecular ecological analysis by Nott *et al.* (2020) [41] applied microarray analysis and shotgun metagenomic sequencing, to URB outbreaks associated with airport de-icer. The study revealed correlations

between elevated antecedent chemical oxygen demand (COD) levels and the abundance of URBs. The taxonomic composition of the URB was predominantly characterised by the presence of *Sphaerotilus*, a characteristic URB taxon, whilst the presence of *Thiothrix*, a genus less commonly associated with URBs, provided new, genomic insights. Further investigation identified the isolated *Sphaerotilus spp.* as a new strain of *S. montanus* KMKE, contrasting with the more frequently reported *S. natans*. Exton et al. (2023) [43] used 16S rRNA amplicon sequencing to discover that *Sphaerotilus* was either absent or very rare prior to airport de-icing activity (0.1% relative abundance) but became dominant (11.6%) in a subsequent URB growth. However, despite these findings and the suspected links between airport de-icing activities and URB growth, no direct assessment of URB taxa growth using freeze-point depressants (FPDs) or commercial airport de-icer (CAD) formulations have been undertaken to date.

Substantial quantities of anti- and de-icers are used to ensure safe airport operation [53–57]. However, heavy rainfall mobilises the highly soluble substances causing them to enter waterways through runoff [56,58]. This runoff travels through airport drainage networks and treatment systems before being discharged into receiving waters and the biosphere. Despite only constituting a small fraction of receiving waters, airport runoff can have significant impacts on water quality [16], including promoting the growth of ecosystem-dominating URBs. Although anti- and de-icers serve slightly different purposes, their chemical compositions are very similar. In this paper, the commercial products used by airports will be collectively referred to as CADs (commercial airport de-icers) for the sake of brevity and euphony. The FPDs are the active ingredients in anti- and de-icers, creating a eutectic solution that lowers the freezing point of water [59–61]. Propylene glycol is often used in aircraft CADs, while CADs for ground application typically use ethylene glycol or formate/acetate salts as their FPDs [62,63] (**Table 5-1**). Aircraft CADs are classified into different types based on their specific use cases. Type I CADs are exclusively for de-icing aircraft; Type II CADs are generally used for de-icing as well but provide more extended anti-icing properties than Type I; and Type IV CADs are primarily anti-icers but can be used for de-icing [64–72]. Past research has suggested that *S. natans* can utilize low molecular weight alcohols, sugars, and acetate salts as carbon sources [10], indicating that most

FPD compounds should foster growth. However, formate salts reportedly cannot support growth when used as a carbon source in wastewater treatment processes including secondary treatment (i.e., the activated sludge process), and where filamentous bacterial growth has been extensively studied [73].

Table 5-1: Overview of commonly used commercial airport de-icers (CADs), the freeze-point depressant (FPD) incorporated in each, their respective applications, and related chemical data. Chemical structures are represented in Simplified Molecular Input Line Entry System (SMILES) format.

	Commercial product	FPD	State	Use	Type	Chemical structure	M _w (g/mol)	log(KoW)
Aircraft	Kilfrost DF Plus [66]	Propylene glycol	Liquid	Aircraft de-icing	Type I			
	Kilfrost ABC-K Plus [67]	Propylene glycol	Liquid	Primarily aircraft de-icing but can provide anti-icing protection	Type II	CC(O)CO	76.1	-0.92 [74]
	Kilfrost ABC-S Plus [64]	Propylene glycol	Liquid	Primarily aircraft anti-icing but can be used for de-icing	Type IV			
Ground	Konsin	Ethylene glycol	Liquid	Runway de-icing	-	OCCO	62.1	-1.36 [74]
	Clearway F1 [65]	Potassium formate	Liquid	Runway, taxiway, and apron de-icing	-	C(=O)[O-].[K+]	84.1	-
	Clearway SF3 [69]	Sodium formate	Solid	Snow and ice melt	-	[Na+].[O-]C=O	68.0	-
	Clearway 6S [68]	Sodium acetate	Solid	Snow and ice melt	-	[Na+].[O-]C(=O)C	82.0	-

In addition to the FPD, CADs contain a variety of proprietary additives designed to enhance their properties, including corrosion inhibitors, non-ionic surfactants, flame retardants, thickeners for adherence, chemicals to reduce surface tension, pH buffers, and anti-precipitation agents [63,75–78]. The concentrations of these additives in CADs are generally low, reportedly ranging from 1-2 %v/v [54] to 10-20 %v/v [62]). However, these additives are often difficult to remove with wastewater treatment processes [79,80], and thus present additional challenges for river ecology. Some additives have been shown to degrade very poorly (e.g., triazole functional groups) [81], persist in the environment (e.g., benzotriazoles) [82] or degrade into more toxic compounds (e.g., alkyphenol ethoxylates) [81] which can act as putative endocrine disruptors [75]. Other additives have been reported as toxic to aquatic organisms [81,83,84] and in isolated cases shown to decrease the biodegradation rates of FPDs such as propylene glycol [85].

Against this background, our study aims to investigate the growth kinetics of *S. natans* in relation to URBs associated with airport anti- and de-icing activities, using controlled laboratory experiments. Specifically, our objectives are to: (i) determine which FPDs can be utilized by *S. natans*; (ii) examine differences in the growth kinetics of different FPDs; and (iii) compare pure FPDs to CADs as carbon sources, investigating any discrepancies.

It was hypothesized that: (i) ethylene glycol, propylene glycol, and sodium acetate can be utilized as carbon sources by *S. natans*, but formate cannot owing to its reportedly poor degradation by similar wastewater bacteria; (ii) lower molecular weight FPDs with higher oxygen content will promote more rapid initiation of growth leading to higher maximum growth rates (acetate > ethylene glycol > propylene glycol); (iii) CADs will have a longer lag period prior to growth but similar bacteria growth levels, due to the reported toxicity of additives in the commercial products stunting initial substrate utilisation but not overall yield at concentrations expected in catchments. The microbial growth data presented herein will help describe how airport de-icers contribute to URB growth. This information can then inform targeted treatment strategies at airports, with the ultimate goals of improving river health and quality, promoting biodiversity, and reducing anthropogenic impacts on the environment.

5.2 Materials and Methods

Microcosm experiments were conducted using a turbidimetric approach to monitor the growth of *S. natans* cultures using various media which incorporated different FPDs and CADs (**Table 5-2**). Optical density at 600 nm (OD₆₀₀) of microcosms housed in 100-well microtiter plates, containing 200µL culture per well, was recorded every 20-minutes over a 7-day period at a constant temperature of 25 °C (high amplitude, low speed shaking ceasing 10 seconds prior to each measurement) [86].

Table 5-2: The carbon sources utilized in this study, along with the corresponding active ingredients in each commercial airport de-icer (CAD), and the abbreviations used to refer to each throughout this study.

Carbon source	Active ingredient	Abbreviation
Propylene glycol	-	ProG
Kilfrost DF Plus	Propylene glycol	DF
Kilfrost ABC-K Plus	Propylene glycol	K+
Kilfrost ABC-S Plus	Propylene glycol	S+
Ethylene glycol	-	EtG
Konsin	Ethylene glycol	Kon
Sodium acetate	-	NaAce

The BioScreen C MBR (Oy Growth Curves Ab Ltd, Finland), a microtiter plate reader with controlled temperature and shaking, was utilised due to its ability to measure absorbance (optical density) at selected wavelengths while allowing high-level control over incubation and measurement conditions and facilitating a large number of samples per run. Given the reported potential challenges of using optical density as a proxy for growth in microorganisms known to clump or form biofilms, like *S. natans* [87]. A high number of replicates were performed to minimize any effects of clumping (10 replicates and 5 blank replicates per condition). The replicates were distributed across the plate to minimize discrepancies due to potential uneven heating of the microtiter plates. A strain of *S. natans* (DSM 6575) was procured from DSMZ (Leibniz

Institute DSMZ GmbH, Germany). It was initially grown via incubation on its prescribed optimal growth medium (DSMZ medium 51) of beef extract (Lab Lemco, Oxoid) (5 g/L), at room temperature on a 100 rpm shaker. The culture was routinely refreshed with new media.

5.2.1 Method development

The River Crane (London, UK) receives treated surface water effluents from airport de-icing in a highly urbanised catchment [43]. During preliminary experiments, a range of peak FPD concentrations from the River Crane were initially used, as reported by the Environment Agency [88]. However, these concentrations, while environmentally relevant, did not stimulate sufficient growth to produce a discernible ΔOD_{600} . Sampling conducted by the authors [43], over two winter seasons (October 2020 to April 2022) in the River Crane (downstream of airport outfalls) reported total organic carbon (TOC) concentrations primarily between 0.2 and 2.0 mM C (**Figure 5-1**). Furthermore, monthly reporting by contractors on behalf of Heathrow Airport Ltd recorded TOC between 0.35 – 0.90 mM C. Despite these findings, initial tests with concentrations of 0.1 and 1.0 mM C of FDP in microcosms did not lead to detectable growth. This may be attributed to the fixed contents in a microcosm, whereas a river's flow provides constant nutrient replenishment – a requirement for URB growth [89]. Additional pilot experiments established that an initial OD_{600} of 0.005 (measured at 0.5 and diluted 1:100) allowed clear and distinct growth profiles to be observed over time within the operational range of the BioScreen C MBR (0.0 – 2.0 abs) [90].

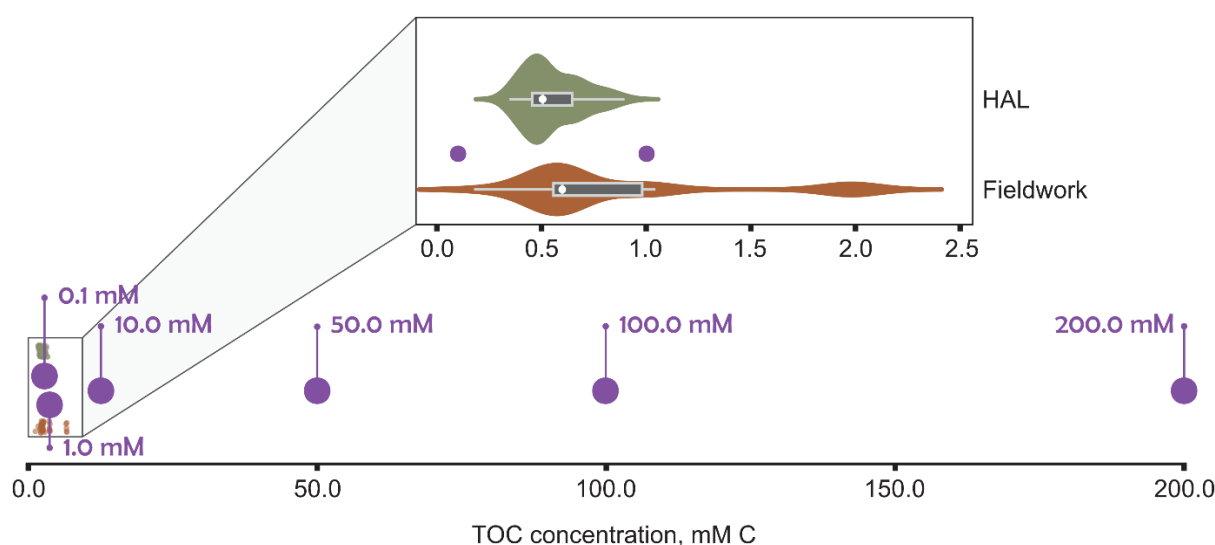


Figure 5-1: Comparison of the carbon concentration used in BioScreen wells (purple) with total organic carbon (TOC) measurements from an urban river (River Crane, London; TQ 10958 75226) receiving airport runoff recorded by the authors (orange) (Chapter 4), in addition to monthly monitoring samples by contractors on behalf of Heathrow Airport Ltd (HAL) (green).

5.2.2 Media selection and preparation

To meet the objectives of this study, various concentrations of several pure-FPDs and CADs (summarised in **Table 5-2**) were tested. Given that assimilable organic carbon is the main driver of URB growth, de-icer concentrations were standardised based on the carbon concentration - in terms of mmol/L of carbon (mM C). Based on the pilot experiments described earlier, it was determined that concentrations of 10, 50, 100, and 200 mM C would provide clear growth profiles. These concentrations do not promote growth which exceeds the maximum OD₆₀₀ absorbance of the instrument (Abs ≤ 2.0) but are sufficient to promote discernible growth. It should be noted that the exact concentrations of FPDs in different CAD products are not universally reported. As such, these concentrations were determined using high-performance liquid chromatography (HPLC, Agilent Technologies 1200 series, USA) with specific settings (10.0 µL injection volume, 0.400 mL/min flow rate, 21-24 bar, 60 °C), column (ROA Rezex Phenomenex 150 x 7.8 mm column, refractive index detector), and mobile phase (H₂SO₄, 0.5 mM). Calibration curves and quality control for HPLC standards

can be found in Supplementary Information (**S5-1**). Serial dilutions of CADs were performed to validate concentrations and to calculate FPD concentration for each CAD. Stock solutions of FPDs/CADs were prepared in a sterile environment. The solutions had double the final concentrations and were prepared in a carbon-free minimum essential media (MEM). This MEM was based on the M9 minimal medium with calcium added to better support *S. natans* growth [91] using: ATCC® MD-VS™ (10 mL/L); ATCC® MD-TMS™ (10 mL/L), Na₂HPO₄ (6.8 g/L), KH₂PO₄ (3.0 g/L), NH₄Cl (1.0 g/L); and CaCl₂ (11.098 mg/L). Once prepared, the FPD/CAD stock solutions were refrigerated (4 °C) until use. Aliquots of FPD and CAD stock solutions were dispensed in 100-well sterile BioScreen C MBR plates (100 µL/well). Replicates of each FPD/CAD at their various concentrations were distributed across the plate to reduce errors attributed to unequal heating. A sample of the *S. natans* culture was then taken and diluted to achieve an effective OD₆₀₀ of 0.005 in MEM. Following the setup, both microtiter plates were placed into the BioScreen C MBR, and the pre-configured settings were engaged.

5.2.3 Data analysis and statistics

Data analysis involved the baseline correction of OD₆₀₀ measurements based on the average OD₆₀₀ of 40-100 minutes (i.e., the average for the first hour after readings were allowed to initially settle) of the sample. Averages and confidence intervals of all replicates were then calculated for each time point. Three indicators of growth kinetics were calculated: time-to-detection (TTD), maximum rate of change (maxROC), and maximum optical density at 600 nm (maxOD). TTD, the time taken for the sample to reach an OD₆₀₀ of 0.1 (after baseline correction), indicates the delay in the utilisation of the carbon source. TTD is a direct assessment of the lag phase duration (“lag-time”) by setting an arbitrary OD₆₀₀ achieved to indicate the start of the exponential phase of microbial growth [86,92,93]. An absorbance of 0.1 was chosen following a visual inspection of growth curves to determine the start of exponential growth in relation to the maxOD achieved in these media for this starting cell concentration of this specific taxon. Secondly, maxROC indicates the highest linear rate of ΔOD_{600} in the exponential growth phase [94] and was calculated by taking the maximum gradient calculated over 3 hours of 1-hour moving average values (“growth rate”) [95]. Moving averages and a 3-hour gradient were used to smooth out sudden fluctuations in the

OD₆₀₀ seen between individual measurements – a limitation with using optical density measurements, especially in taxa that clump or form flocs [87]. Finally, maxOD indicates the maximum turbidity of each sample, calculated simply by finding the maximum corrected OD₆₀₀ of each sample, as a proxy for the highest bacterial load the specific medium can support (“total growth”). These kinetic indicators allowed for direct comparison between different media at varying concentrations and statistical analyses to be performed. The Kruskal-Wallis non-parametric test of difference and pairwise comparisons post-hoc tests were performed in SPSS Statistics v28.0.1.1 (IBM, USA), as the assumptions of ANOVA (normality) were not met.

5.3 Results and discussion

The recorded changes in OD₆₀₀ (Δ OD₆₀₀) show that *S. natans* can utilize propylene glycol, ethylene glycol, and sodium acetate as primary carbon sources (**Figure 5-2**). This substantiates, for the first time and under controlled conditions, a direct correlation between FPD-contaminated discharges and the support or facilitation of URB growth. At concentrations equal to or above 10 mM C, the OD₆₀₀/time growth curves showcase sigmoidal (“S-shaped”) growth, which is characteristic of microbial growth in microcosm experiments without significant carbon inhibition. These patterns are consistently similar across various FPDs, albeit with minor differences observed between them. It is also evident that there is a significant variation in growth kinetics with carbon concentration, which underscores the importance of these assimilable carbon sources as primary drivers of *S. natans* growth and limiters at low carbon concentrations. *S. natans* growth was not stimulated by either sodium or potassium formate, which are commonly used as ground de-icers. To substantiate these findings and validate the viability of the culture-based approach, experiments with formate salts were repeated, including wells with other inoculated FPDs and ideal medium (medium 51, DSMZ). Consistently, *S. natans* showed no detectable increase in OD₆₀₀ in the presence of formate salts, yet robust growth was observed in wells containing all other carbon sources. It should be noted, however, that the inability of *S. natans* to utilize formate salts as a carbon source does not imply that URBs, as a whole, share this limitation. Given the complex assemblage of microorganisms that constitute URBs, formate salts may still be bioavailable to other taxa within these complex polybacterial biofilms, such as certain *Zoogloea spp.* [96,97]. Moreover, the presence of specific

nutrients, like formate, can potentially affect the microbial composition of URBs, thereby optimizing the biofilm's ability to metabolize available nutrients [13]. Decreases in OD₆₀₀ at later stages of growth profiles could indicate clumping of cell [98] or biofilm formation on the walls of microcosms [99,100]. Additional research is required to establish the mechanisms involved in the findings reported in **Figure 5-2**.

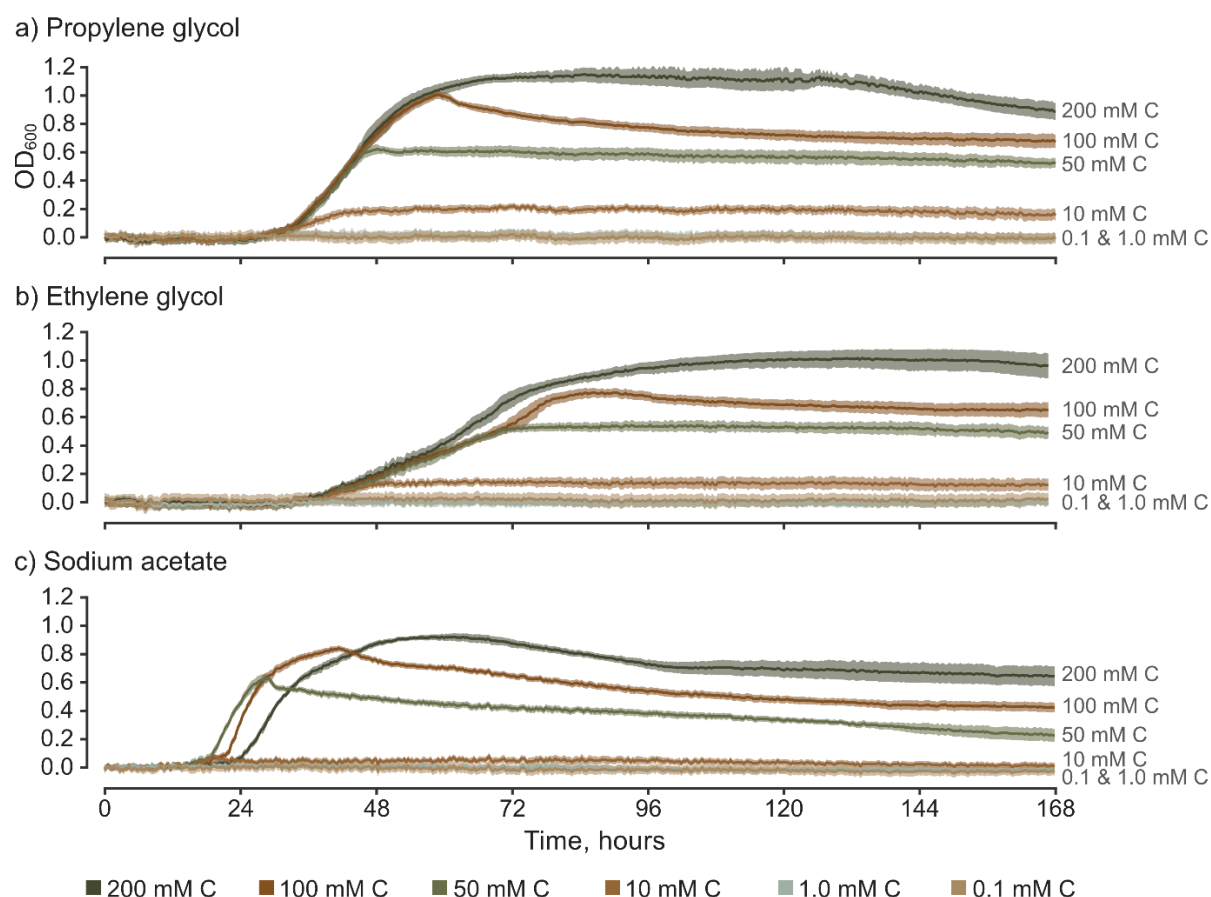
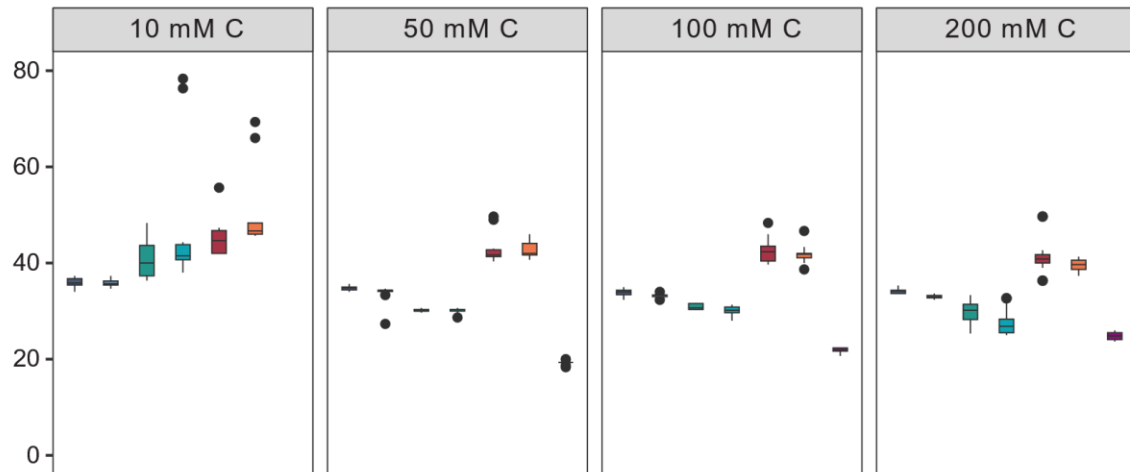


Figure 5-2: Time-series of optical density for *S. natans* cultures grown in media with different concentrations of three freeze-point depressants, presented across a range of carbon concentrations (mM C). Error bars represent 95% confidence intervals ($z = 1.96$) derived from 10 replicates. Graphs show results for (a) propylene glycol, (b) ethylene glycol, and (c) sodium acetate.

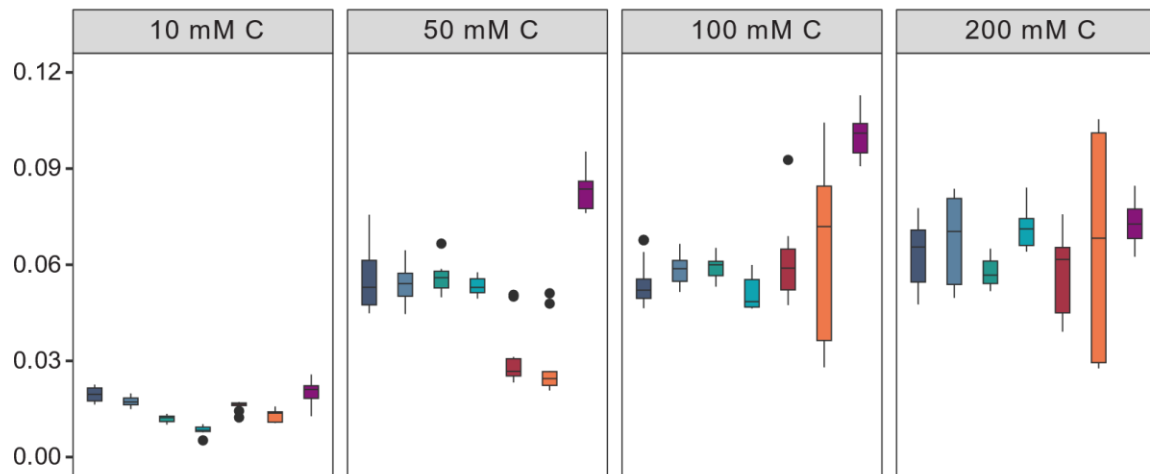
5.3.1 FPD Active Ingredients and Concentrations on *S. natans* Growth

In an effort to delineate the similarities and differences among various growth kinetics, TTD, maxROC, and maxOD were examined (**Figure 5-3**). Initially focused on comparing distinct pure-FPDs (active ingredients), to ascertain the viability of each as a carbon source for *S. natans* at various concentrations. **Figure 5-3** represents the growth kinetics of pure FPDs and CADs (**Table 5-2**) across an array of concentrations.

(a) time-to-detection (TTD), hours



(b) maximum rate of change (maxROC), $\Delta OD/\text{hour}$



(c) maximum optical density (maxOD)

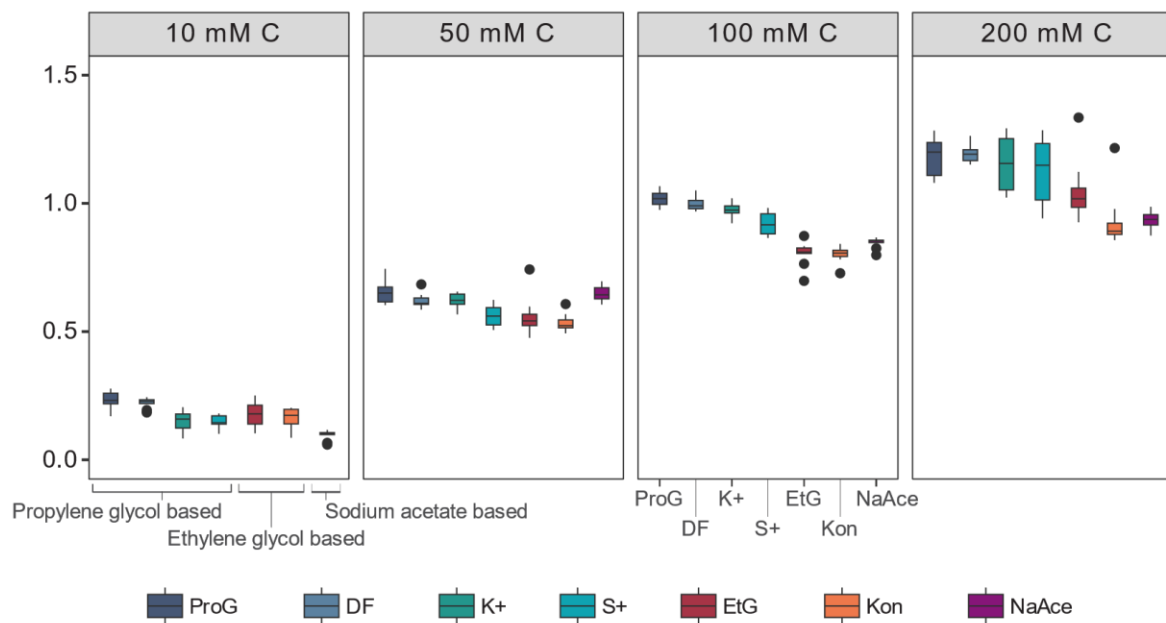


Figure 5-3: Evaluation of growth kinetic parameters of *S. natans* in response to different pure freeze-point depressants (FPDs) and commercial airport de-icers (CADs) across various concentrations. Presented parameters include (a) time-to-detection (TTD), (b) maximum rate of change (maxROC), and (c) maximum optical density (maxOD). The utilized carbon sources include propylene glycol (ProG), Kilfrost DF Plus (DF), Kilfrost ABC-K Plus (K+), Kilfrost ABC-S Plus (S+), ethylene glycol (EtG), Konsin (Kon), and sodium acetate (NaAce). Box plots depict the interquartile ranges, and whiskers represent the ranges, extending up to 1.5 times the interquartile range, and outlying dots signify values outside of this range.

The TTD varied depending on the type of FPD used (**Figure 5-3a**). Ethylene glycol had a significantly longer TTD (averaging 41.2 to 44.2 hours across concentrations tested) compared to propylene glycol (33.9 – 35.9 hours) and sodium acetate (19.2 – 24.8 hours) ($p = 0.000 - 0.028$), which had a notably shorter TTD than either glycol-based FPD ($p = 0.000 - 0.003$) (**Table 5-3**). These variations in TTD could point to differences in the bioavailability of each carbon source for *S. natans*. The longer TTD for ethylene glycol implies that *S. natans* takes more time to initiate growth in this medium, suggesting that it may be less readily utilized by the organism. Conversely, the shorter TTD for sodium acetate indicates that *S. natans* can begin using this compound more quickly, implying it might be more easily assimilated as a carbon source. Therefore, TTD can be seen as a reflection of the lag phase of growth, demonstrating the time taken by the organism to adapt to the medium and initiate exponential growth. The extended TTD for ethylene glycol was unexpected, given its elemental structure and molecular mass closely resemble the acetate anion, and it has a lower molecular weight, more oxygen-rich structure compared to propylene glycol.

Table 5-3: Pairwise comparisons from Kruskal-Wallis test of difference of the time-to-detection (TTD), maximum rate of change (maxROC) and maximum optical density (maxOD) between freeze-point depressants (propylene glycol (ProG), ethylene glycol (EtG), and sodium acetate (NaAce)) at the concentrations (a) 200 mM C, (b) 100 mM C, (c) 50 mM C, and (d) 10 mM C. * indicates significant difference (P = 0.05).

a) 200 mM C									
		TTD			maxROC			maxOD	
	ProG	EtG	NaAce	ProG	EtG	NaAce	ProG	EtG	NaAce
ProG	-			-			-		
EtG	0.028*	-		0.472	-		0.411	-	
NaAce	0.003*	<0.001*	-	0.264	0.066	-	0.060	0.290	-
b) 100 mM C									
		TTD			maxROC			maxOD	
	ProG	EtG	NaAce	ProG	EtG	NaAce	ProG	EtG	NaAce
ProG	-			-			-		
EtG	0.005*	-		0.407	-		0.039*	-	
NaAce	0.002*	<0.001*	-	0.001*	0.009*	-	0.073	0.787	-

c) 50 mM C									
	TTD			maxROC			maxOD		
	ProG	EtG	NaAce	ProG	EtG	NaAce	ProG	EtG	NaAce
ProG	-			-			-		
EtG	0.022*	-		0.074	-		0.364	-	
NaAce	<0.001*	<0.001*	-	0.006*	<0.001*	-	0.982	0.352	-

d) 10 mM C									
	TTD			maxROC			maxOD		
	ProG	EtG	NaAce	ProG	EtG	NaAce	ProG	EtG	NaAce
ProG	-			-			-		
EtG	0.017*	-		0.642	-		0.559	-	
NaAce	-	-	-	0.982	0.626	-	0.164	0.418	-

While the FPD concentration exerts a considerable influence on the overall growth pattern of *S. natans*, it appears to exert minimal impact on the TTD (**Figure 5-3a**, **Table 5-4**). No significant differences were observed in the TTD across different concentrations (≥ 10 mM C) of each FPD ($p \geq 0.306$). However, it is worth noting that at a concentration of 10 mM C, a larger range of TTD values is observed compared to higher concentrations. This suggests that while 10 mM C is the minimum concentration that facilitates growth, it may also serve as a limiting factor for *S. natans*. Furthermore, sodium acetate displayed a smaller standard deviation (0.45 – 0.85 hours) across repetitions, indicating a lesser variance in TTD. This is particularly noteworthy when compared to other carbon sources, such as propylene and ethylene glycol, which had much larger standard deviations (0.50 – 1.06 and 2.80 – 4.23 hours, respectively; **Figure 5-3a**). This data suggests that sodium acetate may provide a more consistent energy source for the growth of *S. natans*. Sodium acetate's suitability as a superior growth substrate for *S. natans* could stem from its water solubility, facilitating easier assimilation, and its chemical structure, favouring metabolic incorporation. Its high solubility allows acetate to be readily available in the environment for bacterial uptake. Additionally, as a small two-carbon compound, acetate might be more easily processed by *S. natans*. Importantly, acetate can directly feed into the Krebs cycle, a central metabolic pathway for energy production. Acetate is converted into acetyl-CoA, a key intermediate in the cycle. This efficient utilization might explain the shorter TTD with sodium acetate, suggesting quicker growth initiation. Propylene and ethylene glycol, despite their similar two and three-carbon structures respectively, do not exhibit the same ease of use by *S. natans*. This might be because their metabolic incorporation involves a more complex process, likely passing through the glycolytic pathway, a less direct route compared to acetate. Additionally, their larger molecular size might also contribute to slower assimilation rates, as observed by the longer TTD.

The data presented in **Table 5-4** implies a concentration threshold for FPDs that significantly impacts microbial growth, most visible in the maxROC (**Figure 5-4**). For treatments utilizing propylene glycol and sodium acetate, a significant difference was observed between 10 mM C and the higher concentrations (50, 100, and 200 mM C) ($p \leq 0.012$, **Table 5-4a,c**). This implies a limiting concentration between 10 and 50 mM C for these carbon sources (**Figure 5-3b**). For ethylene glycol, the notable change in

maxROC occurs between 50 and 100 mM C ($p = 0.015$, **Table 5-4b**), suggesting a higher growth limit concentration. Above these threshold concentrations, the variations in maxROC between concentrations appear consistent for both propylene glycol ($p = 0.230 - 0.857$, **Table 5-4a**), ethylene glycol ($p = 0.729$, **Table 5-4b**), and sodium acetate ($p = 0.266 - 0.603$, **Table 5-4c**). If concentrations of FPDs are exceptionally high, they could have toxic effects on *S. natans* growth.

Table 5-4: Pairwise comparisons using the Kruskal-Wallis test to analyse differences in time-to-detection (TTD), maximum rate of change (maxROC), and maximum optical density (maxOD) between concentrations (10.0, 50.0, 100.0 and 200.0 mM C) of the freeze-point depressants (a) propylene glycol, (b) ethylene glycol, and (c) sodium acetate. Where * indicates significant difference (P = 0.05).

a) Propylene glycol

	TTD				maxROC				maxOD			
	10.0	50.0	100.0	200.0	10.0	50.0	100.0	200.0	10.0	50.0	100.0	200.0
10.0	-				-				-			
50.0	0.671	-			0.007*	-			0.074	-		
100.0	0.306	0.549	-		0.012*	0.857	-		<0.001*	0.004*	-	
200.0	0.403	0.681	0.851	-	<0.001*	0.307	0.230	-	<0.001*	<0.001*	0.372	-

b) Ethylene glycol

	TTD				maxROC				maxOD			
	10.0	50.0	100.0	200.0	10.0	50.0	100.0	200.0	10.0	50.0	100.0	200.0
10.0	-				-				-			
50.0	0.604	-			0.167	-			0.144	-		
100.0	0.555	0.942	-		<0.001*	0.015*	-		0.001*	0.082	-	
200.0	0.307	0.615	0.666	-	0.001*	0.037*	0.729	-	<0.001*	<0.001*	0.033*	-

c) Sodium acetate

	TTD				maxROC				maxOD			
	10	50	100	200	10	50	100	200	10	50	100	200
10	-				-				-			
50	-	-			<0.001*	-			0.001*	-		
100	-	0.773	-		<0.001*	0.603	-		<0.001*	0.281	-	
200	-	0.536	0.741	-	<0.001*	0.554	0.266	-	<0.001*	0.060	0.421	-

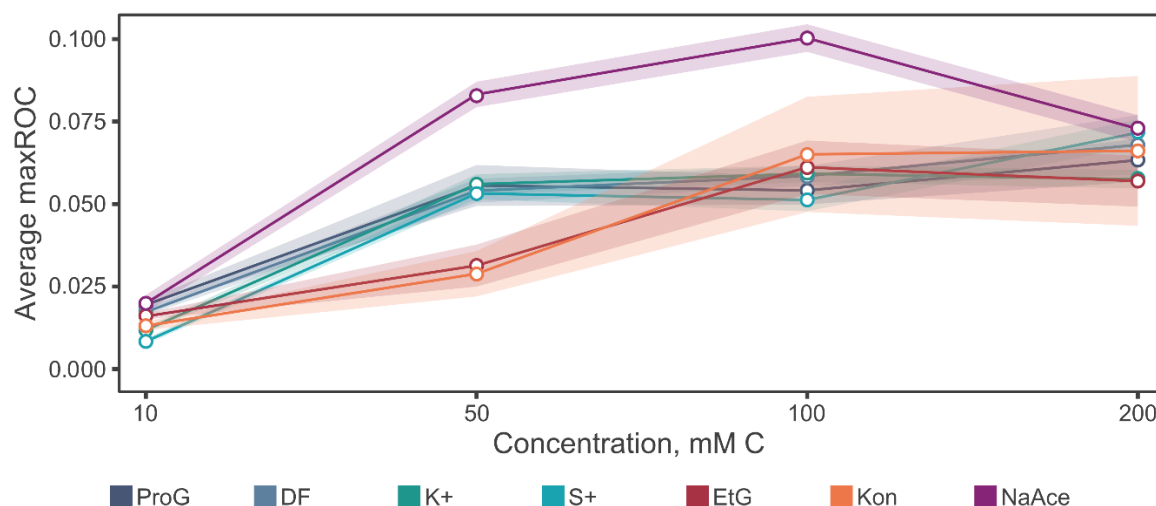


Figure 5-4: The relationship between maximum rate of change (maxROC, $\Delta\text{OD}_{600}/\text{hour}$) and concentration of carbon source. Carbon sources tested include propylene glycol (ProG), Kilfroast DF Plus (DF), Kilfroast ABC-K Plus (K+), Kilfroast ABC-S Plus (S+), ethylene glycol (EtG), Konsin (Kon), and sodium acetate (NaAce). Error is denoted by mean $\pm$ confidence intervals ($z = 1.96$) based on 10 biological replicates for *S. natans* cultures.

Interestingly, sodium acetate often exhibits a significantly higher maxROC compared to propylene and ethylene glycol, except at limiting concentrations of 10 mM C and at 200 mM C where the maxROC drops (**Figure 5-3b, Table 5-3**). The decline in maxROC between 100 and 200 mM C of sodium acetate is not significant ($p = 0.266$), but it may hint towards osmotic stress, toxicity at elevated concentrations, or metabolic burden. Further research is warranted to elucidate this phenomenon.

At elevated substrate concentrations, the suppression of growth (as indicated by the reduction in maxROC) can occur due to several factors. A primary reason could be osmotic stress. High concentrations of solutes in the medium can cause water to move out of the microbial cells to balance the osmotic pressure, leading to dehydration and slower growth. Another factor could be substrate toxicity. Even essential nutrients can become toxic at high concentrations. The FPDs may interfere with cellular mechanisms, enzymatic reactions, or membrane integrity at elevated levels, causing a decrease in cell viability and consequently growth. In addition, metabolic burden or

overflow metabolism might come into play. When substrate availability is high, cells could take up the substrate at a rate exceeding the capacity of the downstream metabolic pathways. This may lead to the accumulation of metabolic intermediates or by-products that are toxic to the cells, thereby inhibiting growth. Among the FPDs, the effect of high substrate concentration might be more pronounced for those that are metabolized more slowly or those that have more potential for toxicity at high levels. For instance, ethylene glycol, which is metabolized in several steps involving conversion to toxic intermediates like glycolic acid and oxalic acid, could show a greater suppressive effect at high concentrations compared to others.

As expected, maxOD escalated with carbon concentration (**Figure 5-3c**), leading to significant differences between each concentration for all FPDs (**Table 5-3**). This indicates *S. natans* and URBs' strong dependency on organic carbon. Propylene glycol consistently induced a slightly higher maxOD than other carbon sources (**Figure 5-3c**), but with no statistically significant difference (**Table 5-3**), except against ethylene glycol at 100 mM C ($p = 0.039$). The comparable maxOD between FPDs was anticipated, as concentration was standardized by moles of carbon to facilitate direct comparison across FPDs.

In conclusion, there were some differences in the growth kinetics of propylene glycol, ethylene glycol, and sodium acetate. The major findings were (i) sodium acetate had a faster TTD and higher maxROC than propylene glycol and finally ethylene glycol; (ii) the influence of a limiting concentration on growth kinetics, specifically on maxROC; and (iii) maxOD was highly carbon dependant.

5.3.2 Analysis of pure freeze-point depressants and corresponding commercial airport de-icers

Additives in CADs appear to exhibit minimal effects on *S. natans* growth as shown in **Figure 5-5**. Minor variations are noticeable in select parameters, for example, the TTD between K⁺ and ProG at 50 mM C ($p=0.016$), and S⁺ and ProG at 50 and 200 mM C ($p=0.019$ and 0.017) (**Figure 5-3a**, **Table 5-5**). These slight differences suggest that the impact of additives on growth, if any, is minor, and does not demonstrably alter the TTD. The lack of disparities in maxROC and maxOD (**Figure 5-3b-c**, **Table 5-5**) further substantiates that commercial additives do not substantially influence *S. natans*

growth kinetics. The marginal impact of additives may be due to their inert nature or low concentration in CADs, which may not sufficiently interact or interfere with *S. natans* to influence growth. Additionally, these additives might not be metabolically usable by *S. natans* or their impact is overshadowed by the more abundant FPDs. Future studies could further investigate the metabolic interactions between these additives and *S. natans* to determine if any specific additive influences the growth kinetics more than others.

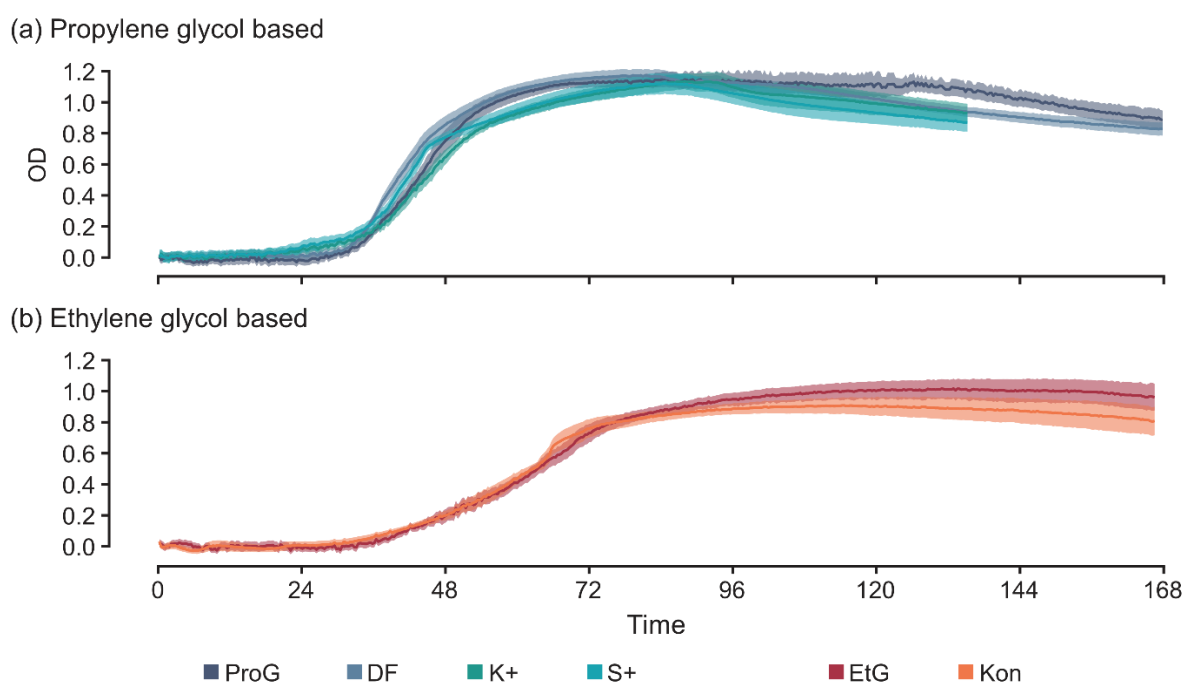


Figure 5-5: Time-series of optical density for *S. natans* culture between pure freeze-point depressants (FPDs) and their commercial products (CADs) at 200 mM C for *S. natans* cultures in (a) propylene glycol based and (b) ethylene glycol based media. Error is denoted by mean $\pm$ confidence intervals ($z = 1.96$) based on 10 biological replicates for *S. natans* cultures. Carbon sources include propylene glycol (ProG), Kilfrost DF Plus (DF), Kilfrost ABC-K Plus (K+), Kilfrost ABC-S Plus (S+), ethylene glycol (EtG), Konsin (Kon), and sodium acetate (NaAce).

Table 5-5: Pairwise comparisons from Kruskal-Wallis test of difference of the time-to-detection (TTD), maximum rate of change (maxROC) and maximum optical density (maxOD) of *S. natans* cultures between freeze-point depressants and their commercial product at concentrations (a) 200 mM C, (b) 100 mM C, (c) 50 mM C, and (d) 10 mM C. Carbon sources include propylene glycol (ProG) and its commercial products (CADs) Kilfrost DF Plus (DF), Kilfrost ABC-K Plus (K+), Kilfrost ABC-S Plus (S+) and ethylene glycol (EtG) and the CAD Konsin (Kon). Where * indicates significant difference (P = 0.05).

a) 200 mM C												
	TTD				maxROC				maxOD			
	DF	K+	S+	Kon	DF	K+	S+	Kon	DF	K+	S+	Kon
ProG	0.417	0.071	0.017*		0.772	0.570	0.306		0.987	0.883	0.701	
EtG				0.615				0.655				0.268
b) 100 mM C												
	TTD				maxROC				maxOD			
	DF	K+	S+	Kon	DF	K+	S+	Kon	DF	K+	S+	Kon
ProG	0.618	0.178	0.081		0.448	0.373	0.660		0.813	0.561	0.274	
EtG				0.904				0.630				0.930

c) 50 mM C

	TTD				maxROC				maxOD			
	DF	K+	S+	Kon	DF	K+	S+	Kon	DF	K+	S+	Kon
ProG	0.525	0.016*	0.019*		0.889	0.861	0.792		0.747	0.787	0.389	
EtG				0.933				0.826				0.845

d) 10 mM C

	TTD				maxROC				maxOD			
	DF	K+	S+	Kon	DF	K+	S+	Kon	DF	K+	S+	Kon
ProG	0.962	0.174	0.080		0.779	0.317	0.169		0.958	0.389	0.376	
EtG				0.682				0.690				0.886

Existing literature provides limited information on the specific chemicals used as additives in CADs [81], although reports frequently note their toxic effects [75,83]. Additives such as benzotriazoles, commonly included in CAD formulas as corrosion inhibitors, are known for their acute and chronic toxicities to various aquatic species [77,82] including the fathead minnow [18]. As a result, manufacturers are reportedly reducing the quantity of benzotriazoles in their CADs [101]. Other additives include alkylphenol ethoxylates, which serve to reduce surface tension but are also reported to be toxic to aquatic organisms, degrading into by-products identified as endocrine disruptors [75]. However, not all additives have harmful effects; for example, phosphate salts are used as benign pH buffers [63]. Previous studies predominantly focus on the toxicity of these compounds to fish and invertebrates. The current research indicates that the additives in CADs exert negligible impact on *S. natans* growth and are, therefore, unlikely to adversely affect URBs. Nonetheless, when organic pollution ceases and URBs decompose, any remaining undegraded compounds are released back into the water system, concentrated by the biofilm [13,102], potentially causing further ecological deterioration. Thus, a holistic understanding of the impact of these additives on various elements of aquatic ecosystems is essential for sustainable management.

5.3.3 New insights in URB growth from high-frequency measurements

In this research, a turbidimetric microcosm design was deployed to evaluate the growth of URB taxa, using airport de-icers as the sole carbon source. The study revealed that FPDs such as propylene glycol, ethylene glycol, and sodium acetate, can stimulate *S. natans* growth. Moreover, negligible differences were observed between the growth induced by pure FPDs and CADs. The Monod equation has been widely utilized in previous studies to model the growth kinetics of *S. natans* [103–105]. Building upon this established framework, this study extends the understanding of *S. natans* growth dynamics, shedding light on its behaviour under varying treatments emulating de-icer contaminated surface water discharges and provides valuable insights into its ecological significance and potential implications for environmental management.

Carbon concentrations below 10.0 mM C failed to stimulate growth, despite observed TOC in a case study river (River Crane, London) receiving treated airport surface runoff not surpassing 2.0 mM C. However, in flowing water bodies, nutrients like organic carbon, nitrogen and phosphorus are continuously replenished [2,8,11,44], necessitating lower background nutrient concentrations. Hence, in fixed microcosms, higher initial nutrient concentrations are required.

It is worth noting that turbidimetric approaches might lead to under- or overestimation of cell numbers in taxa known for cell clumping [87]. To counter this, a high number of replicates were conducted to mitigate the impact of any outlier values. Despite these constraints, turbidimetric microcosm methods offer several key advantages including robustness, wide application in literature enabling comparative studies, high-throughput capacity, and cost-effectiveness. In considering alternative methodologies for evaluating microbial growth dynamics beyond conventional optical density measurements, there are a range of viable alternatives. Among these, flow cytometry stands out, providing the distinct advantage of direct cell enumeration while enabling discrimination between live and dead cells [106]. Fluorescence in-situ hybridisation (FISH) represents another targeted approach even within mixed microbial communities [107,108]. The increasing popularity of quantitative PCR (qPCR) reflects advancements in genomics, offering precise quantification of microbial abundance and gene expression dynamics [107]. Traditional microscopy techniques, such as haemocytometry, remain relevant, alongside newer automated methodologies driven by machine learning algorithms [107]. As we transition into the AI age, many novel machine-learning approaches continue to emerge, such as ScanGrow [109], ColonyLive [110], Growth Profiler (EnzyScreen) [111], and oCelloScope (BioSense Solutions ApS, Denmark) [112], each contributing unique insights into microbial growth kinetics and dynamics. The exponential evolution of machine learning and AI models suggests that these techniques may rapidly develop into reliable alternatives to traditional approaches, providing invaluable tools for studying microbial growth dynamics in diverse environmental contexts. Techniques even extend to monitoring biomass in recirculating channels, as has been applied to sewage fungus with varying concentrations of glucose as the carbon source [10]. Whilst many of these approaches offer advantages over traditional optical density assessments, optical density remains

a robust and well-established approach offering substantial benefits, including a high-throughput of samples allowing researchers to rapidly study a wide range of treatments and conditions and compare the resulting microbial growth and kinetics [86,93].

This work significantly contributes to our understanding of how airport de-icing compounds, both pure and in commercial formulations, influence the growth of ubiquitous river bacteria, *S. natans*. It elucidates key growth parameters such as TTD, maxROC, and maxOD across various concentrations of FPDs, offering valuable insights into the microbial response to these compounds. This research uniquely demonstrates that the additives within commercial de-icers have minimal impact on bacterial growth kinetics, providing a more nuanced understanding of these agents' ecological impacts. Additionally, the findings challenge preconceived notions about the toxicity of these compounds to URBs and highlight the necessity for a more comprehensive understanding of the environmental implications of de-icing chemicals, particularly in light of changing climatic conditions. As such, this study forms a solid basis for future research on microbial responses to pollutants in aquatic environments.

Future research could expand on this study's findings, exploring the growth of other URB taxa and extending the focus to mixed species cultures that more accurately represent polymicrobial biofilms. Methods such as time-lapse microscopy could help understand interactions between taxa and relative growth rates while retaining the benefits of optical density measures [113].

Furthermore, this study looked at a suspended monoculture rather than fixed biofilm communities. Additional studies would be beneficially complemented by larger, mesocosm experiments such as flumes, to investigate the effects of flow rate, background water quality, and mixed cultures on URB formation and growth [114]. Such studies should consider the predicted effects of climate change – such as more extreme weather and temperature fluctuations – and how these could impact FPD usage, runoff and URB growth. More intense rainfall could boost FPD runoff, while warmer winters may lessen FPD usage. URB growth may be enhanced or hindered by these temperature shifts. Altered river flow can also disrupt biofilm formation. Future

research should incorporate these climate-related factors to fully understand the evolving impacts on URB growth and other environmental consequences of FPDs.

5.4 Conclusions

In conclusion, this research investigated the influence of various freeze-point depressants (FPDs) and commercial airport de-icers (CADs) on the growth of *S. natans*, as a representative taxon of URBs. The study demonstrated that carbon sources including propylene glycol, ethylene glycol, and sodium acetate fostered *S. natans* growth, highlighting the metabolic versatility of URBs. Furthermore, a negligible difference was observed between pure-FPDs and CADs, indicating that additives in commercial de-icers exert minimal impact on microbial growth.

The research revealed a clear link between carbon concentration and microbial growth kinetics, identifying a limiting concentration, above which the growth rate did not increase significantly. This underscores the importance of carbon concentration in controlling microbial growth in environments affected by de-icers. Comparing FPDs sodium acetate had a shorter TTD and faster maxROC than propylene glycol, which was likewise shorter and faster than ethylene glycol. Overall, these findings contribute significantly to our understanding of URBs' growth response to carbon sources prevalent in airport de-icers. However, further research is needed to expand these findings to other URB taxa, study the impacts of FPDs on mixed species cultures, and explore the potential ecological consequences of these substances in natural aquatic environments. Such insights would further enhance our knowledge of microbial growth kinetics, aiding the development of sustainable management strategies for ecosystems impacted by de-icers.

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Data access statement

Supporting data for this study can be accessed through the Cranfield University repository (CORD): DOI 10.17862/cranfield.rd.23674914.

Conflict of interest

The authors have no conflict of interest to declare.

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Supplementary information

Appendix A – HPLC quality control

The calibration and quality control data for HPLC analysis of FPD concentration in CADs. Standards were made up as %v/v but have also been converted to mg/L for each FPD. The slope and intercept for the calibration curves are reported, along with R^2 from regression analysis (Data Analysis package, Microsoft Excel), and the limit of quantification (LOQ). LOQ was calculated from the concentration of the lowest standard plus percent accuracy (calculated concentration divided by expected concentration).

FPD	Range, %v/v	Range, mg/L	Slope	Intercept	R^2	LOQ, mg/L
ProG	0.01 – 10%	111.3 – 111,300.0	1.49E+08	-4.54E+03	1.000	298.1
EtG	0.01 – 10%	104 – 104,000.0	1.74E+08	2.42E+04	1.000	184.8
Ace-H	0.01 – 10%	105.1 – 105,100.0	1.18E+08	-5.91E+03	1.000	157.5
Form-H	0.01 – 10%	122 – 122,000.0	1.04E+08	2.52E+04	1.000	289.1

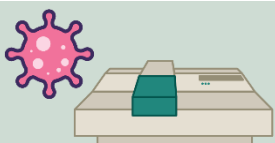
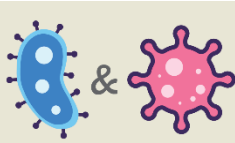
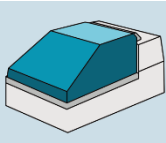
S5-1: Range of standards for each freeze-point depressant (FPD), the calibration curves, regression analysis, and limit of quantification (LOQ, minimum standard + %accuracy).

6 Growth and Interactions in Undesirable River Biofilms on Airport De-Icer Media

Abstract

Anthropogenic pollution in rivers – originating from sources such as wastewater effluents, agricultural runoff and airport de-icing – has been implicated in the proliferation of undesirable river biofilms (URBs). This study delves into the complex interplay between the growth of URBs and freeze-point depressants (FPDs), active ingredients of de-icers, in microcosms of river water contaminated with de-icer, thereby emulating the potential effects of airport surface runoff on the benthic microbiome. A comparative analysis was conducted on the growth of *Sphaerotilus natans*, a characteristic URB taxon, and the impact of the background water quality. No significant growth was observed in urban river water treated with FPDs, suggesting a deficiency of other vital nutrients in the microcosms, confirmed through increased growth in supplement-amended microcosms. A subsequent investigation into another URB taxon, *Zoogloea oleivorans*, revealed limited growth in various FPD-based media. This finding implies that a different *Zoogloea* species might be responsible for degradation, or that *Z. oleivorans* may utilize degradation by-products. Furthermore, the introduction of *S. natans* and *Z. oleivorans* in varying proportions in mixed cultures led to substantially different growth profiles between samples containing *S. natans* (sigmoidal growth) and those with only *Z. oleivorans* (diauxic growth), with significant differences in the initial lag phase (~2.5× greater lag in *Z. oleivorans* only cultures). The application of time-lapse microscopy in growth experiments demonstrated that *S. natans* forms large clusters of cells alongside suspended growth, demonstrating the dual mode of growth of *S. natans*. This research provides further insights into the growth dynamics of two key taxa implicated in URB development when exposed to airport de-icer runoff. It underscores the urgency for the development of enhanced treatment and management strategies to control biofilm growth induced by airport de-icer runoff in aquatic ecosystems.

Visual abstract

			
Objective 1 <i>To investigate the growth of <i>S. natans</i> in river water and airport de-icer media</i> River water is insufficient to promote growth Augmented river water has shorter lag phase than minimal media but lower total growth	Objective 2 <i>To determine the growth of second URB taxon <i>Zoogloea</i> on de-icers</i> Airport de-icers did not facilitate growth Could be misidentified <i>Zoogloea</i> species or could use degradation products from other URB taxa	Objective 3 <i>To examine growth of mixed species cultures at various initial ratios</i> All <i>S. natans</i> containing cultures had no differences in growth <i>Z. oleivorans</i> only cultures had significantly different growth profiles	Objective 4 <i>To explore the use of time-lapse microscopy for URB growth experiments</i> Can see clumping and filamentous growth types Discernible growth is observable and method should be developed for future URB research

Highlights

1. De-icer dosed river water had insufficient nutrients to facilitate growth in microcosms
2. De-icers were unable to promote the growth of *Zoogloea oleivorans*
3. Mixed cultures containing *Sphaerotilus natans* had a shorter lag phase than *Z. oleivorans* only
4. Time-lapse microscopy revealed *S. natans* self-aggregation and suspended growth forms
5. New strategies to manage URBs and organic pollution in saprobic rivers are needed

Keywords

sewage fungus; *Sphaerotilus natans*; *Zoogloea oleivorans*; river water quality; optical density; time-lapse microscopy; microbial synergy

6.1 Introduction

Recent emphasis on poor freshwater quality has increased the need for multidisciplinary research into the anthropogenic impacts on fluvial systems [1–4]. Much of the focus on this issue has been on the influence of raw sewage discharges [5–7] and agricultural runoff [8–10]. However, other point and diffuse sources of river pollution remain relatively understudied, such as airport de-icing. Airport de-icing operations have been linked to the deterioration of water quality in receiving waters, leading to deleterious ecological impacts, most notably the prolific growth of undesirable river biofilms (URBs, formerly “sewage fungus”) [11–13]. The use of large quantities of chemical anti- and de-icers are essential for safe winter operations of airports during cold weather [14,15]. Typical anti- and de-icer formulations consist of either propylene glycol, ethylene glycol, or formate/acetate salts as the freeze-point depressant (FPD) [16,17]. These anti-/de-icers, once mobilised by rainfall [18,19], travel through drainage and treatment systems before being discharged into receiving waters. Conventional treatments, such as aerated wetlands, under certain conditions exhibit limited efficiency in treating anti-/de-icer contaminated runoff [15,20], leading to discharges that significantly degrade water quality [21] and stimulate the growth of URBs [11–13]. Given the renewed focus on the pervasive and persistent nature of URBs, it is imperative to better understand the relationship between pollutants, such as airport anti-/de-icers, and URBs in the complex and dynamic context of river environments. Previous research by the authors (**Chapter 5**) has demonstrated that the URB taxon, *Sphaerotilus natans*, can utilize airport anti-/de-icers as its primary carbon source, underscoring the need for further investigation into these pollution sources.

6.1.1 Taxonomy of undesirable river biofilms

URBs are highly variable polymicrobial assemblies [22,23], despite the emphasis on *S. natans* [24–26]. Their microbial composition is highly variable, influenced by available nutrients [27] and environmental factors such as velocity and substrate [28,29]. Commonly associated URB taxa include the bacterial genera *Zoogloea* [30,31], *Beggiatoa* [22,23,32], and *Thiothrix* [22,32,33]; in addition to fungi, such as *Leptomitopus lacteus* [34–37], and algae, such as *Cladophora glomerata* [22]. Recent,

sequencing based enumeration of URBs associated with airport de-icing, has further validated the genera *Sphaerotilus* [11,13], *Zoogloea* [11], *Thiothrix* [13], and shown the potential involvement of *Rhodoferrax* [11].

Zoogloea spp. have long been associated with wastewaters [38,39] and sewage fungus [22,32,40]. They are reportedly able to use various organic acids, amino acids and alcohols [30]. Moreover, certain *Zoogloea spp.* have been found to utilise simple electron donors including acetate [41,42] and formate [43], which are commonly used as active ingredients in airport anti-/de-icers alongside propylene and ethylene glycol [16,44]. 16S rRNA (V3-4) amplicon analysis data revealed the presence of *Zoogloea* in an airport de-icer implicated URB (4.8% relative abundance) [11] and gene sequence analysis found it most closely matched that of *Zoogloea oleivorans* ($\geq 99.99\%$ identity, accession length 1444). Accordingly, studying *Z. oleivorans* in conjunction with *S. natans* as an URB taxon will enhance knowledge of the growth dynamics and interaction patterns within URBs.

6.1.2 Studying microbial growth of URBs

Two previous studies [11,13] have established a connection between airport de-icing and the proliferation of URBs. Utilizing microcosms, it was demonstrated that *S. natans* utilised many compounds commonly applied as FPDs and in commercial airport de-icers (CADs) (**Chapter 5**). Overall this research highlights the need to improve organic carbon management within airport water treatment, thus reducing overall discharge to limit URB growth. A limitation of this study was in studying a single, even if characteristic, URB taxon, whereas URBs are polymicrobial communities with intricate interactions utilising numerous substrates [45]. There are relatively fewer studies addressing taxa present in mixed-species biofilms, particularly in the context of URBs taxa [46]. Future studies may want to consider exploration of how synergistic effects between the microbial taxa might influence interpretation of optical density measurements, such as generation of artifacts.

When bacteria grow within complex communities such as rivers, the interactions between organisms can be positive (synergistic), neutral, or negative (antagonistic) [45–47]. The current understanding of these interactions between species, particularly

in the context of URBs, remains limited [46]. Synergistic growth, defined as a mixed culture growing more densely than any of the corresponding pure cultures [45], is likely predominant given the ubiquitous nature of biofilms in rivers [47]. However, not all mixed-microbial relationships are synergistic. Competition for nutrients is a common interaction among bacteria, and some microorganisms may produce inhibitory compounds to compete through suppression and therefore outgrowing competitors [48,49]. For example, the marine bacterium *Pseudoalteromonas tunicate* [50] produces compounds such as the antibacterial protein (AlpP) to compete for space and nutrients. The interactions between URB taxa have been poorly studied and it is unclear whether synergistic or antagonistic interactions are dominant.

To study growth in mixed assemblages, various methods have been employed, including quantitative polymerase chain reaction (qPCR) [51]. This detects both viable and non-viable cells but can be costly [52]. An alternative, validated approach for monitoring growth is the measurement of optical density at 600nm (OD₆₀₀). This turbidimetric approach measures growth through time (to derive OD/time profiles). To derive aspects of growth kinetics without making assumptions about data fit to existing models, these profiles can be interrogated. Determination of synergistic and antagonistic growth can be achieved by comparing the maximum growth (as maximum optical density at 600 nm, maxOD) between pure and mixed cultures that have the same initial total cell concentration. If the maxOD of the mixed culture surpasses that of both pure cultures, it indicates synergistic growth. If the maxOD falls between the values of the pure cultures, the interaction is considered unresolved [45]. Conversely, if the maxOD is less than that of both pure cultures, it signifies antagonistic growth.

To delve deeper into the polybacterial communities that constitute URBs, recent advancements in time-lapse image analysis instrumentation and algorithms have also been applied [53]. Digital time-lapse microscopy is an optical screening of samples with advanced image processing algorithms to identify and count cells to determine microbial growth. This approach can be applied to monitor culture growth and validate interactions between taxa, such as co-habiting, biofilm formation and potential growth competition [54]. The competitive exclusion principle (also known as Gause's Law) posits that two species vying for the same finite resources cannot coexist indefinitely

or maintain stable population sizes [55]. Therefore, competition among taxa is anticipated within mixed microbial cultures constrained to fixed-volume wells of a multi-well plate. However, it should be noted that this experimental setup may not fully reflect the complexity of a river system, with a complex matrix of nutrients which are less likely to be limiting.

The overall aim of this work is to explore the effect of background water quality, the growth of additional URB taxa, and the relationships within mixed URB species cultures. Microcosm experiments aimed to (i) investigate the growth kinetics of *S. natans* in unaltered and augmented river water using airport FPDs as carbon sources; (ii) determine the growth profiles and kinetics for *Zoogloea spp.* using FPD-based media and comparing to *S. natans*; and (iii) examine the growth of and interactions between mixed-species cultures. It was hypothesised that the growth of *S. natans* in unaltered river water, using airport FPDs as carbon sources, will be greatly reduced due to nutrient limitation in fixed microcosms but there will be increased growth when augmented with additional minerals, compared to those observed in minimum essential media. It was predicted that *Z. oleivorans* would exhibit distinct growth profiles and kinetics when grown in FPD-based media, and that these would be significantly less than those of *S. natans* owing to the smaller role of *Zoogloea spp.* in URBs. Furthermore, in mixed cultures, competition between taxa for growth nutrients and FDP substrates would reduce the overall growth rate of *Z. oleivorans* but not *S. natans*.

6.2 Materials and Methods

6.2.1 Bacterial strains and culture conditions

S. natans (DSM 6575, Germany) was obtained from DSMZ (Leibniz Institute DSMZ GmbH, Germany), cultured (in DSMZ medium 51) to an OD₆₀₀ of ~1.2 and stored with glycerol at -80 °C. Samples were later resuscitated from a frozen stock according to manufacturer instructions and incubated on DSMZ medium 51 with gentle shaking (100 rpm shaker table) at room temperature (25 °C). A second URB taxon, *Z. oleivorans* (DSM 28387, Germany), was likewise sourced from DSMZ and incubated

in its prescribed ideal growth medium (DSMZ medium 1) at 25 °C and 100 rpm shaking.

6.2.2 Microbial growth on de-icers

The overall method for these studies used the FPDs propylene glycol, ethylene glycol, and sodium acetate (Sigma-Aldrich, UK). The FPDs stock solutions (400 mM carbon equivalent) were prepared by dissolving in a minimal media (**Table 6-1**). Essential nutrients for minimal media were: ATCC MD-VS (10 mL/L) (ATCC, USA), ATCC MD-TMS (10 mL/L) (ATCC, USA), Na₂HPO₄ (6.8 g/L), KH₂PO₄ (3.0 g/L), NH₄Cl (1.0 g/L), and CaCl₂ (11.098 mg/L). In total, nitrogen sources made up 20 mmol/L N and phosphorus sources make up 6.7 mmol/L P. FPD stock solutions (400 mM C) were diluted 1:1 (100µL:100µL) with minimal media inoculated with URB culture being tested (diluted to OD₆₀₀ of 0.005) in a 100-well microtiter plate (Honeycomb 2 plate, Growth Curves Ltd, USA) using the BioScreen C MBR plate reader (Oy Growth Curves Ab Ltd, Finland). Comparisons between FPDs or between species were then undertaken. Each condition was tested in ten replicates, with rows of 5 replicates spaced evenly across each plate to minimise localised inter-plate effects. Growth experiments were conducted at 25 °C for 7-days, with 20-minute measurement intervals. The machine was set to continuous, medium amplitude, slow shaking, pausing 10 seconds before each measurement. This procedure was repeated for all samples with 10 replicates per treatment, which appeared to reduce uncertainty in measurement due to cultures forming flocs/clumps [56].

Table 6-1: Names of minimal media used in this study based on river water (RW) or de-ionised water (DI), with added nutrients to formulate a carbon-free minimum essential media (+MEM), and if no *S. natans* was added (_neg).

Name	Base matrix	Added nutrients	<i>S. natans</i> added
RW	River water	×	✓
RW+MEM	River water	✓	✓
RW+MEM_neg	River water	✓	×
DI+MEM	De-ionised water	✓	✓

6.2.3 River water microcosms

River water from an urban, heavily modified river was used to assess the impact of the matrix on the growth of URB taxa. River water was collected from the River Crane (London, UK) (TQ 10958 75226) in December 2021 using new 1L plastic sample bottles (HDPE 2) and refrigerated (4 °C). Concentrations of key nutrients were determined by colourimetry (Spectroquant® NOVA 30-A photometer (Spectroquant, Germany)) and concentration of total organic carbon (TOC), inorganic carbon (IC) and total carbon (TC) were measured on a Shimadzu TOC-V CSH with Shimadzu TNM-1 (Shimadzu Corporation, Japan). These are reported in **Table 6-2**. River water was filtered (0.22 µm syringe filter) to reduce turbidity and suppress the microbiome before use in a minimal media to improve the accuracy of OD measurements. Two different minimal media were prepared: (i) using unaltered river water (RW), and (ii) river water augmented with the essential nutrients (RW+MEM) (**Table 6-1**). *S. natans* negative samples (samples with no added *S. natans* culture) were also prepared in RW+MEM media (RW+MEM_neg) and have been compared to essential nutrients in deionised water (DI+MEM, originally reported in (**Chapter 5**)). FPD stock solutions were prepared using the same minimal media as the culture.

Table 6-2: Concentrations of key nutrients (ammonium (NH₄⁺), nitrate (NO₃⁻), phosphate as total phosphorus (PO₄³⁻), total organic carbon (TOC), inorganic carbon (IC) and total carbon (TC)) in the river water used to create a minimal medium.

Nutrient	Concentration
NH ₄ ⁺	0.30 mg/L N
NO ₃ ⁻	1.5 mg/L N
PO ₄ ³⁻	0.19 mg/L P
TOC	7.04 mg/L C
IC	44.28 mg/L C
TC	51.32 mg/L C

6.2.4 Mixed cultures

To investigate interactions between key URB taxa, *S. natans* and *Z. oleivorans*, mixed species culture microcosm experiments were conducted. The OD₆₀₀ was recorded at 20-minute intervals for seven days for cultures of *S. natans* (S) and *Z. oleivorans* (Z) in the starting ratios of S100:Z0, S75:Z25, S25:Z75, and S0:Z100. FPDs were used as the sole carbon source, prepared in DI+MEM at 400 mM C to 200 mM C final concentration. The mixed species cultures were also added to the wells using the method described above.

6.2.5 Time-lapse microscopy

A digital time-lapse microscopy approach (oCelloScope, BioSense Solutions ApS, Denmark) was used to monitor culture growth that scans through a (semi-)transparent fluid (i.e., media) to generate a series of images to track growth [54]. These images are then processed via one of several algorithms using the UniExplorer companion software (BioSense Solutions ApS, Denmark) to enumerate cell count and differentiate between different organisms. *Sphaerotilus* cells are described as straight rods in a sheath, 1.4 – 1.6 µm width and 4.0 – 6.0 µm in length [57], often found as long, single chains with a bundle of sub-polar flagella that allow them to be actively motile [58]. Whereas *Z. oleivorans* cells are 1.2 – 1.4 µm wide, 2.5 – 3.0 µm in length [59] and cells are described as straight to slightly curved, plump rods which are actively motile owing to the single polar flagellum [30]. Comparing these descriptions and images of *S. natans* [60] and *Z. oleivorans* [59], there appears to be sufficient difference between the cell shapes. Furthermore, the time-lapse microscope has previously been applied for the study of other filamentous bacteria suggesting utility herein [61].

To assess the feasibility of time-lapse microscopy, growth experiments were conducted using *S. natans* in its optimal medium (medium 51, DSMZ) before assessing mixed cultures and specific carbon sources. Different culture volumes (0.2, 0.1, 0.05 mL) and starting cell concentrations (10, 1.0, 0.1, 0.01, and 0.001 %v/v of a starting OD₆₀₀ of 0.5) were tested to optimize image clarity for the instrument's data processing algorithms and to ensure reproducibility of results. The UniExplorer software uses four algorithms: Background Corrected Absorption (BCA), Segmentation Extracted Surface Area (SESA), Segmentation Extracted Average

Length (SEAL), and Total Absorption (TA). BCA and SESA are sensitive and work well at low cell concentrations, while SEAL detects bacterial filamentation but is limited at high concentrations or overlapping filaments. TA quantifies light transmission, similar to a plate reader, and requires high cell concentration. These algorithms offer advantages over traditional methods like turbidimetry (e.g., OD₆₀₀) or haemocytometry, providing real cell counts and differentiating between species in an automated process. These findings could then be used to evaluate the relative proportions and interactions of mixed cultures in FPD-based media.

Other approaches to studying the growth of an interactions within mixed cultures could also be considered, such as mesocosms (e.g., flumes and recirculating channels), respirometers, or metagenomics and gene functions. These methods offer benefits in studying the growth of URBs – such as using fixed biofilms over suspended cultures and deeper insights into the specific interactions between different taxa – however, time-lapse microscopy provides benefits to directly compare the relative growth of different URB taxa in a wide range of treatment conditions.

6.2.6 Data analysis

Baseline correction for optical density at 600nm (OD₆₀₀) was performed by averaging the OD₆₀₀ values between 40-100 minutes, accounting for an initial settling period. Key metrics for growth kinetics, including time-to-detection (TTD), maximum rate of change (maxROC), and maximum OD₆₀₀ (maxOD) were calculated alongside averages and confidence intervals ($z = 1.96$) of replicates for growth profiles. TTD, the time taken for OD₆₀₀ to increase by 0.1 from baseline absorbance, represents the lag phase before exponential growth begins. While TTD may vary across different methods, it allows for direct comparison between studies using the same OD threshold. The maxROC, calculated as the maximum gradient over a 3-hour period of 1-hour moving averages, describes the exponential phase of microbial growth, indicating the peak growth rate. The maxOD, the maximum value of OD₆₀₀ relative to its baseline absorbance, is analogous to the maximum total cell count, although it does not differentiate between viable and dead cells. To determine synergistic and antagonistic growth in cultures with the same initial total cell concentration, maxOD was compared between pure and mixed cultures. Statistical differences were assessed using the non-parametric

Kruskal-Wallis test and pairwise comparisons post hoc test were performed in SPSS Statistics v28.0.1.1 (IBM, USA). Transformations were performed using Microsoft Excel and plotting in R Studio with the ggplot2 package, and statistics using SPSS Statistics v28.0.1.1 (IBM, USA).

6.3 Results and Discussion

6.3.1 Does *S. natans* grow on freeze-point depressants in river water?

The river water-based medium (RW) contains a complex matrix of essential nutrients and natural organic matter, surpassing the minimal thresholds required to preclude growth limitations. However, the growth of *S. natans* was minimal in the RW based media (**Figure 6-1**), a maxOD of 0.19, 0.11, and 0.24 for propylene glycol, ethylene glycol and sodium acetate were obtained. A potential explanation for this limited growth, as indicated by the change in optical density at 600nm (ΔOD_{600}), might stem from the constant flow of nutrients – such as nitrogen and phosphorus – in river water that these fixed microcosms cannot supply. In a fluvial system, flowing water, a requirement for URB growth [32,62], constantly replenishes nutrients for an URB to consume. This limitation could be addressed in future research using microfluidic devices or larger mesocosms, such as flumes or recirculating channels.

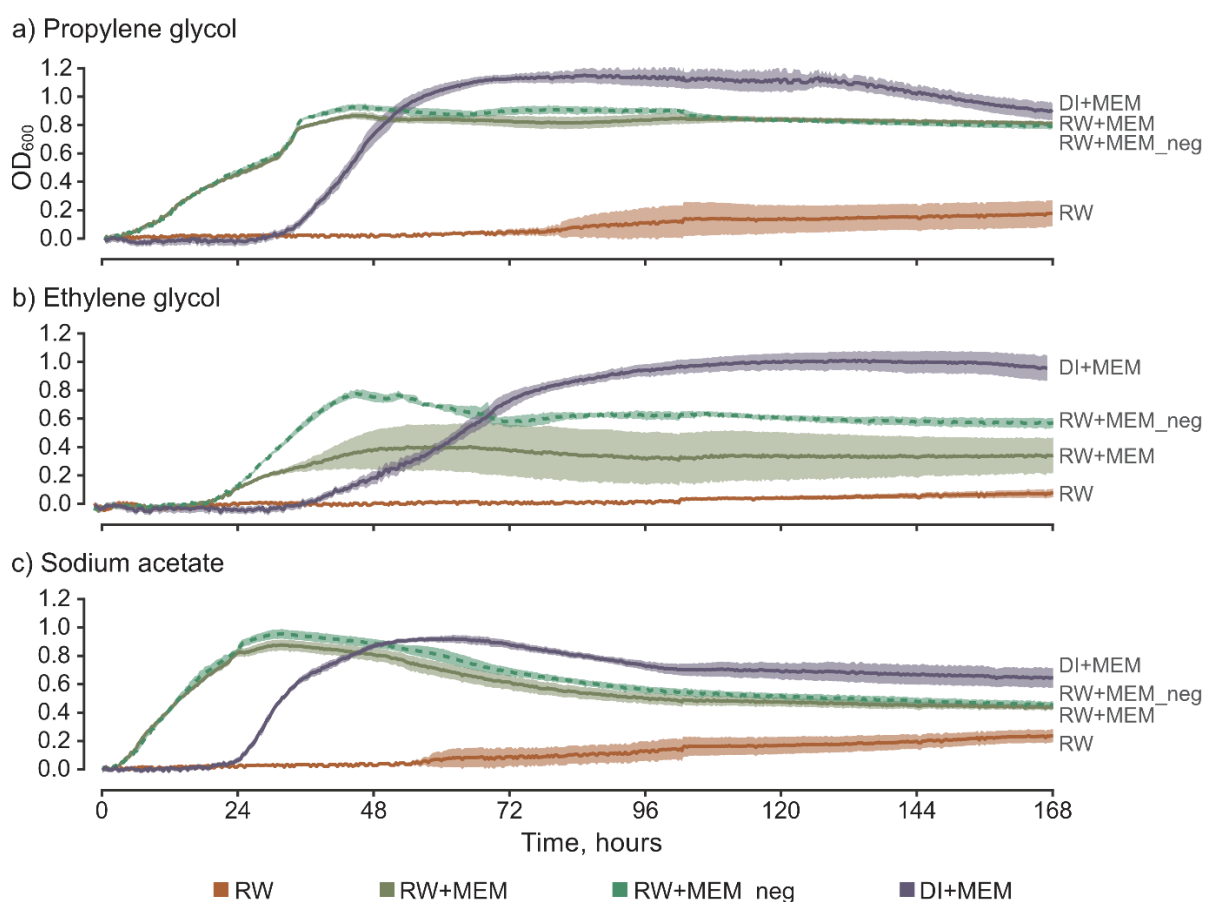


Figure 6-1: Time-series of optical density for *S. natans* grown in media based on various freeze-point depressants at 200 mM C – (a) propylene glycol, (b) ethylene glycol, and (c) sodium acetate. Error is denoted by mean $\pm$ confidence intervals ($z = 1.96$) based on 10 biological replicates for *S. natans* cultures and 5 replicates for negatives. The base media that FPDs were added to are unaltered river water (RW), river water augmented with minimal media (RW+MEM), and de-ionised water with minimal media (DI+MEM). RW+MEM_neg denotes RW+MEM samples with no *S. natans* added.

To examine this, the nutrient content of the minimal media was increased through the addition of carbon-free essential vitamins and minerals for growth (RW+MEM) without any additional carbon source except for a FPD. Under these treatments, the *S. natans* grew immediately (TTD = 4.90 – 23.10 hours) and rapidly (maxROC = 0.032 – 0.062 Δ OD/hr, **Figure 6-2**). Therefore, due to the absence of continual fluvial nutrient replenishment, it would appear there was a deficiency in the concentration of essential nutrients in the RW matrix to support *S. natans*' growth, even with additional carbon

loading. Moreover, there could have been some inhibition of growth in river water due to toxicants in river water. Whilst TTD, maxROC, and maxOD are useful kinetic indicators, kinetic models, such as the Monod equation [103–105], could elucidate additional insights about the growth dynamics of these treatments.

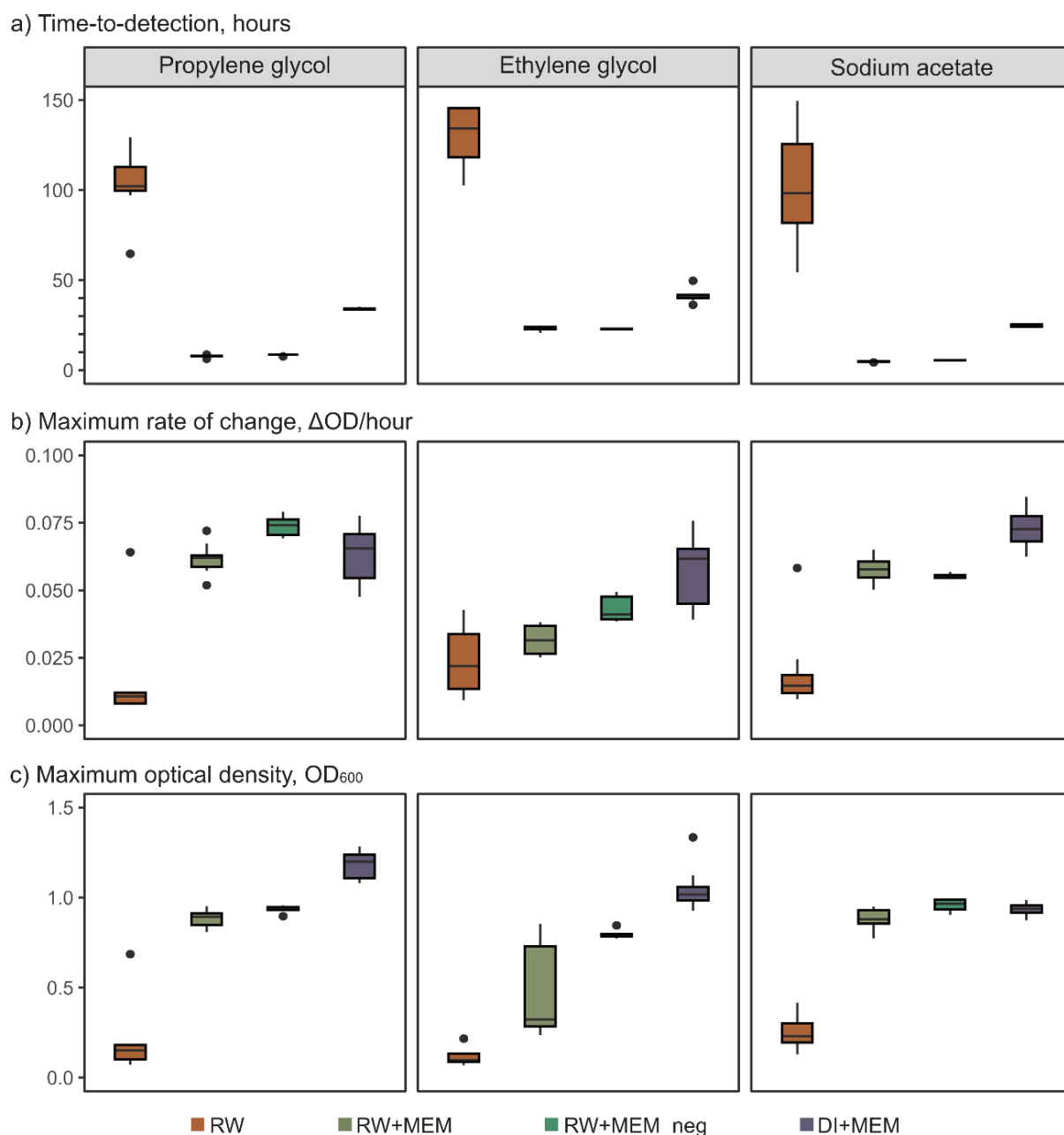


Figure 6-2: Evaluation of growth kinetic parameters of *S. natans* in response to various media containing freeze-point depressants (200 mM C) as the sole carbon source: (i)

propylene glycol, (ii) ethylene glycol, and (iii) sodium acetate, each employed at a 200 mM concentration. Presented parameters include (a) time-to-detection (TTD), (b) maximum rate of change (maxROC), and (c) maximum optical density (maxOD). Base media were river water (RW), river water augmented with additional nutrients (RW+MEM), and de-ionised water with nutrients added. RW+MEM_neg is RW+MEM with no *S. natans* added. Box plots depict the interquartile ranges, and whiskers represent the ranges, extending up to 1.5 times the interquartile range, and outlying dots signify values outside of this range.

The profiles of *S. natans* in RW+MEM are different from that of *S. natans* in DI+MEM for all FPDs (**Figure 6-1**). Most notably there was a significantly shorter TTD (4.90 – 23.10 hours for RW+MEM, compared to 24.77 – 41.24 hours for DI+MEM, **Figure 6-2a**) but significantly lower maxOD in the RW+MEM samples for all FPDs (0.48 – 0.88) compared to DI+MEM based solutions (0.93 – 1.18) (**Figure 6-2c, Table 6-3a,c**). Furthermore, the maxROC (**Figure 6-2b**) was significantly lower for the RW+MEM samples (0.03 – 0.06 Δ OD/hr) in ethylene glycol and sodium acetate compared to DI+MEM (0.06 – 0.07 Δ OD/hr, $p \leq 0.03$, **Table 6-3b**). The increased background TOC from river water (0.59 mM C) in RW+MEM treatments is minimal when compared to the added FPDs (200 mM C), but this extra TOC could offer an initial supply of assimilable organic carbon, despite the majority of TOC being recalcitrant. Another possible explanation for the growth in blank samples is the presence of other microorganisms in the river water. There were no differences in any growth kinetics between RW+MEM and RW+MEM_neg samples ($p \geq 0.1387$) which supports this hypothesis, as the filtration (0.22 μ m) of the river water suppressed, as opposed to inhibited, growth by the native microbiome. The lower maxOD in RW+MEM samples compared to DI+MEM samples indicates that *S. natans* did not readily compete with the background microbiome possibly due to inhibitory microbial interactions which could reduce the observed growth of this taxon. However, the interesting aspect is that these results show that the native microbiome can utilise FDP and that this growth is enhanced in the presence of additional nutrients (**Figure 6-2**). This validates the polymicrobial response associated with organic pollution and the significant role that FDPs can play in enhancing microbial growth.

Table 6-3: Post hoc pairwise comparisons from Kruskal-Wallis test of difference of the (a) time-to-detection (TTD), (b) maximum rate of change (maxROC), and (c) maximum optical density (maxOD) comparing different base media solvents (matrix) and carbon sources. Base media include river water (RW), river water augmented with additional nutrients (RW+MEM), and de-ionised water with nutrients added. RW+MEM_neg is RW+MEM with no *S. natans* added. Carbon sources include propylene glycol, ethylene glycol, and sodium acetate. * indicates a significant difference, $P < 0.05$.

		Propylene glycol				Ethylene glycol				Sodium acetate			
		RW	RW+MEM	RW+MEM_neg	DI+MEM	RW	RW+MEM	RW+MEM_neg	DI+MEM	RW	RW+MEM	RW+MEM_neg	DI+MEM
a) TTD	Propylene glycol	RW											
		RW+MEM	<0.0001*										
		RW+MEM_neg	0.0003*	0.7530									
		DI+MEM	0.0767	0.0017*	0.0245*								
	Ethylene glycol	RW	0.7218										
		RW+MEM	0.0011*	0.1374		0.0019*							
		RW+MEM_neg	0.0032*	0.3126	0.5472	0.0037*	0.8389						
		DI+MEM	0.2978	0.0001*	0.0037*	0.4219	0.2133	0.0140*	0.0271*				
	Sodium acetate	RW	0.9963			0.7089							
		RW+MEM	<0.0001*	0.2147		<0.0001*	0.0064*			<0.0001*			

			Propylene glycol				Ethylene glycol				Sodium acetate							
			RW	RW+MEM	RW+MEM_neg	DI+MEM	RW	RW+MEM	RW+MEM_neg	DI+MEM	RW	RW+MEM	RW+MEM_neg	DI+MEM				
		RW+MEM_neg	<0.0001*	0.5420	0.4233		<0.0001*	0.0683	0.1607		<0.0001*	0.6868						
		DI+MEM	0.0095*	0.0256*	0.1315	0.3641	0.0112*	0.4551	0.4162	0.0872	0.0042*	0.0005*	0.0150*					
b) maxROC	Propylene glycol	RW																
		RW+MEM	<0.0001*															
		RW+MEM_neg	<0.0001*	0.1387														
		DI+MEM	<0.0001*	0.7245	0.2329													
	Ethylene glycol	RW	0.5326															
		RW+MEM	0.2490	0.0027*				0.5971										
		RW+MEM_neg	0.0898	0.0898	0.0059*				0.2352	0.4500								
		DI+MEM	0.0002*	0.6649	0.0666	0.4321	0.0019*	0.0102*	0.1793*									
	Sodium acetate	RW	0.6649				0.8486											
		RW+MEM	0.0003*	0.5718				0.0030*	0.0148*				0.0016*					
		RW+MEM_neg	0.0108*	0.3980	0.0440*				0.0415*	0.1081	0.4610				0.0282*	0.7012		
		DI+MEM	<0.0001*	0.1095	0.8620	0.2120	<0.0001*	<0.0001*	0.0027*	0.0420*	<0.0001*	0.0303*	0.0314*					
c) maxOD	Propylene glycol	RW																
		RW+MEM	0.0013*															
		RW+MEM_neg	0.0006*	0.4288														
		DI+MEM	<0.0001*	0.0027*	0.0980													
	Ethylene glycol	RW	0.7026															
		RW+MEM	0.1420	0.0818				0.0643										

		Propylene glycol				Ethylene glycol				Sodium acetate			
		RW	RW+MEM	RW+MEM_neg	DI+MEM	RW	RW+MEM	RW+MEM_neg	DI+MEM	RW	RW+MEM	RW+MEM_neg	DI+MEM
	RW+MEM_neg	0.0875	0.3622	0.1404		0.0434*	0.6104						
	DI+MEM	<0.0001*	0.0233	0.2886	0.4673	<0.0001*	0.0001*	0.0057*					
	RW	0.4583				0.2613							
	RW+MEM	0.0014*	0.9854			0.0004*	0.0851			0.0143*			
	RW+MEM_neg	0.0002*	0.2448	0.7475		<0.0001*	0.0098*	0.0724		0.0015*	0.2388		
	DI+MEM	<0.0001*	0.3380	0.9928	0.0416*	<0.0001*	0.0070*	0.0903	0.1900	0.0006*	0.3288	0.7034	

Some RW+MEM cultures in ethylene glycol based media exhibited negligible growth ($\text{maxOD} < 0.33$ for 6 of 10 replicates, **S6-1**) which explains the large confidence intervals in **Figure 6-1b** and a large range in maxOD visible in **Figure 6-2c**. Strangely, this variable response between seemingly identical replicates was isolated to the ethylene glycol and could be a facet of possible toxicity or interactions between organisms activating genes linked to growth for available frequently mixed C-sources and those for escaping or adapting to physicochemical stress. Further work beyond the scope of this thesis is required to elucidate these subtle and reproducible differences associated with equivalent molar concentrations of similar substrates.

This study examined the growth dynamics of suspended cultures of a characteristic microorganism within a type of biofilm (i.e., URBs), which is not truly representative of their growth in natural ecosystems. Alternative methodologies, such as microfluidic devices [63,64] or flow cells with microscopy [65], offer opportunities to study microbial growth within biofilms, thus with greater ecological relevance. However, whilst these methods provide valuable insights into biofilm formation and microbial interactions, studying a diverse range of treatments and environmental conditions that affect microbial growth is more challenging than using highly robust optical density methods. Despite not directly studying biofilm-based growth, the nutrient uptake and resulting microbial growth of specific organisms, as determined by OD remains a reliable approach.

6.3.2 Can *Zoogloea* sp. grow on freeze-point depressants?

Z. oleivorans did not exhibit any discernible growth in any of propylene glycol, ethylene glycol, or sodium acetate based media (**S6-3**). The lack of growth, especially using sodium acetate based media, was unexpected as other *Zoogloea* spp. have been shown to use acetate [41,42] and formate [66,67], but not all strains [68]. *Zoogloea*, much like *Sphaerotilus*, is a genus strongly associated with wastewaters and wastewater treatment [30,33], especially *Zoogloea ramigera* [66]. Research conducted by the authors on an airport de-icer implicated URB event identified the genus *Zoogloea* (4.8% relative abundance) [11] with gene sequence analysis and found it most closely matched *Z. oleivorans*. Nonetheless, *Z. oleivorans* did not

demonstrate a change in OD₆₀₀ during the experiment when using FPDs in DI+MEM indicating either (i) its role in the URB was consuming other sources of organic carbon in the river and/or the degradation products of FPDs as metabolised by other taxa within the biofilm.

The growth profile generated of *Z. oleivorans* in an ideal medium (medium 1, DSMZ), did not represent sigmoidal (“S-shaped”) growth observed elsewhere in this study (**S6-2**). Instead, there was sigmoidal growth for approximately 24-hours, followed by a gradual increase after the stationary phase (ca. 40 hours) and finally a decline in OD₆₀₀ (ca. 120 hours) (**S6-2**). The shape of the OD₆₀₀/time profile was different to the sigmoidal growth of *S. natans* in FPD-based media (reported in previous work (**Chapter 5**) and in **Figure 6-1**).

The growth kinetics were different between *S. natans* and *Z. oleivorans*. TTD was much shorter for *Z. oleivorans* (9.20 ± 0.16 hours) in medium 1 (DSMZ) than *S. natans* (24.8 – 41.2 hours) in FPD and DI+MEM based media, likely a result of *Z. oleivorans* not being transferred into a new media with a single carbon source. Furthermore, the maxROC was higher for *Z. oleivorans* ($0.083 \pm 0.003 \Delta OD_{600}/hr$) than *S. natans* in DI+MEM ($0.057 - 0.073 \Delta OD_{600}/hr$) which likewise could be a result of the different media and their intrinsic differences in substrate assimilability between taxa possibly due to enzyme plasticity (the adaptability of enzyme in an organism in response to its environment to maintain the rate of nutrient intake [69–71]), kinetics and specificity for substrates. The maxOD for *Z. oleivorans* cultures was, however, much lower than that of *S. natans* in FPD based media (0.62 ± 0.11 compared to $0.93 - 1.18$). Whilst *Z. oleivorans* is using its ideal medium (medium 51, DSMZ), values for *S. natans* were determined using FPDs in DI+MEM. *S. natans* only had one specific carbon source (compared to the diversity of carbon sources that it could use from rich pollution sources such as raw sewage) and may have had to have overcome potential toxic effects or lower bioavailability.

Sphaerotilus and *Zoogloea* cultures have previously been compared by Unz and Dondero (1967) who examined both of these taxa associated with wastewaters and organic pollution, comparing the depth at which different species grew in agar tubes [67] providing an important foundation to examine the interactions between these taxa.

Results from *Z. oleivorans* using FPD in DI+MEM solutions would have allowed for a more direct comparison (especially of their role in airport de-icer implicated URBs), however, *Z. oleivorans* did not show a change in OD₆₀₀ over time when using any FPD based media and even in an ideal medium, *Z. oleivorans* (maxOD = 0.61) did not grow as well as *S. natans* in FPD based media (maxOD = 1.10).

6.3.3 Interactions between URB taxa

The mixed cultures of *S. natans* and *Z. oleivorans* only demonstrated a discernible Δ OD₆₀₀ in sodium acetate based media (**Figure 6-3**) and not for propylene glycol, ethylene glycol, or sodium formate. *S. natans* has previously demonstrated significant growth using propylene and ethylene glycol (maxOD = 0.93 – 1.33) (**Chapter 5**) so the lack of Δ OD₆₀₀ here was unexpected. Previous experimental cultures had been frozen in glycerol (25 %v/v, -80 °C) between these and previous experiments suggesting that *S. natans* may not tolerate ultracold freezing for long-term storage. Several different frozen stocks were tested.

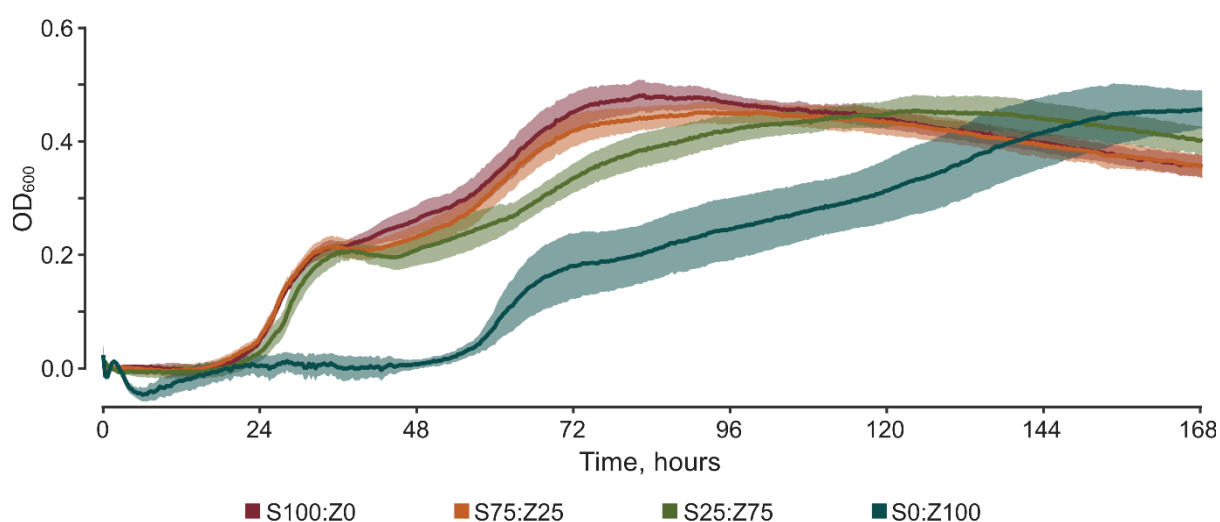


Figure 6-3: Time-series of optical density for *S. natans* (“S”) and *Z. oleivorans* (“Z”) cultures at different starting proportions in a sodium acetate (200 mM C) based medium. Error is denoted by mean $\pm$ confidence intervals ($z = 1.96$) based on 10 biological replicates for each treatment.

In sodium acetate based media, the growth profile for cultures containing *S. natans* (i.e., S100:Z0, S75:Z25, and S25:Z75) can be described as diauxic (**Figure 6-3**) – a sigmoidal (“S-shaped”) curve, followed by a second sigmoidal curve following a stationary phase. Whereas those containing only *Z. oleivorans* (i.e., S0:Z100) had more of a classic sigmoidal profile with OD₆₀₀ continuing to slowly increase after a brief stationary phase.

Analysis of growth kinetics – via TTD, maxROC, and maxOD – (**Figure 6-4**) revealed significant differences only in TTD when comparing different S:Z ratios (**Table 6-4**), despite the vastly different OD₆₀₀/time profiles (**Figure 6-3**). The average TTD in *S. natans* microcosms (S100:Z0, S75:Z25, S25:Z75) was shorter (26.17 – 35.43 hours) than those containing only *Z. oleivorans* (S0:Z100) (69.04 hours) ($p = <0.0001$). The average maxROC was similar between all ratios of S:Z (0.0244 – 0.0271 Δ OD/hr) with no significant differences observed ($p \geq 0.5540$). However, S0:Z100 had a much larger interquartile range (0.0106 – 0.0326 Δ OD/hr). Likewise, maxOD was similar across S:Z ratios (0.4647 – 0.4971, $p > 0.3240$). This suggests that there is unresolved growth – where total growth (maxOD) of mixed cultures is between that of both monocultures under the same conditions – in mixed *S. natans* and *Z. oleivorans* cultures in sodium acetate [45].

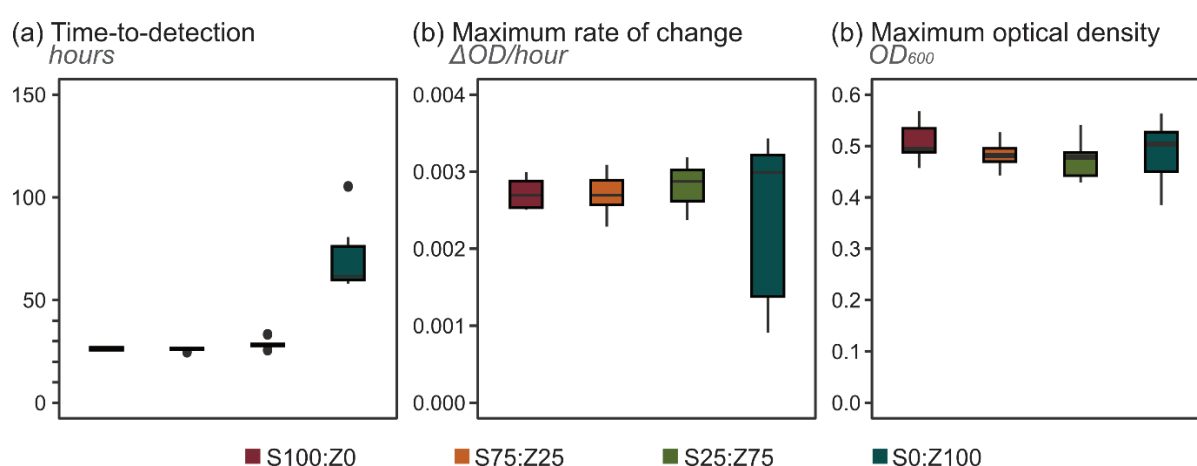


Figure 6-4: Evaluation of growth kinetic parameters of mixed *S. natans* (“S”) and *Z. oleivorans* (“Z”) cultures at different starting proportions in a sodium acetate (200 mM C) based medium. Presented parameters include (a) time-to-detection (TTD), (b)

maximum rate of change (maxROC), and (c) maximum optical density (maxOD). Box plots depict the interquartile ranges, and whiskers represent the ranges, extending up to 1.5 times the interquartile range.

Table 6-4: Pairwise comparisons from Kruskal-Wallis tests of difference for the (a) time-to-detection (TTD), (b) maximum rate of change (maxROC) and (c) maximum optical density (maxOD) between mixed cultures containing different *S. natans* to *Z. oleivorans* (“S:Z”) starting proportions. * indicates a significant difference ($p < 0.05$).

		100:0	75:25	25:75	0:100
(a) TTD	100:0	-	-	-	-
	75:25	0.8890	-	-	-
	25:75	0.2940	0.2345	-	-
	0:100	<0.0001*	<0.0001*	<0.0001*	-
(b) maxROC	100:0	-	-	-	-
	75:25	0.9501	-	-	-
	25:75	0.5540	0.5966	-	-
	0:100	0.5572	0.6000	0.9962	-
(c) maxOD	100:0	-	-	-	-
	75:25	0.4529	-	-	-
	25:75	0.3240	0.8136	-	-
	0:100	0.6826	0.7327	0.5637	-

These results highlight the importance of presenting both growth profiles and kinetics. Whilst parameters like maxROC are difficult to compare by visual inspection of OD/time curves, reporting the kinetics on their own diminishes what can be substantially different growth profiles. In this instance, there was only a significant difference for TTD, despite the growth profiles of cultures containing *S. natans* (S100:Z0, S75:Z25, S25:Z75) and those containing only *Z. oleivorans* (S0:Z100) are noticeably distinct. The interactions between *S. natans* and *Z. oleivorans* in sodium acetate can be categorised as unresolved – i.e., the growth of mixed cultures is not superior or inferior to monocultures (**Figure 6-5**). Statistical tests on maxOD (**Table**

6-4c) further support this categorisation as there are no significant differences between cultures. However, if maxOD were taken at 72 hours (maxOD_{72hr}), differences between cultures emerge. The maxOD_{72hr} for *S. natans* containing cultures (0.34 – 0.45) is substantially higher than that of *Z. oleivorans* only cultures (0.18). Whilst this still indicates unresolved growth, it demonstrates the importance of considering temporal aspects of maxOD determination when assessing species interactions using turbidimetric growth experiments. Future work, therefore, may want to include additional parameters to understand more complex growth, for example, in mixed-species cultures. Taken together, these results suggest there are minimal differences as you alter the ratio of *S. natans* to *Z. oleivorans*. However, eliminating the presence of *S. natans* entirely will affect the growth of the culture. Furthermore, the available nutrients are equally important when determining synergistic versus antagonistic growth [45] and should be a consideration in future work.

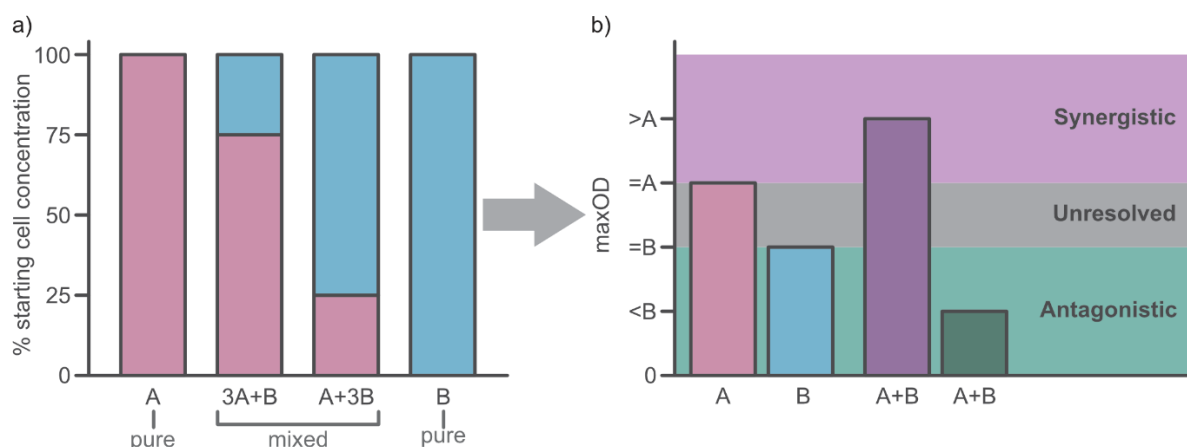


Figure 6-5: Graphical illustration of (a) equal starting total cell concentrations across different mixed culture assemblages; and (b) categorization of interactions in mixed-species cultures, assuming identical initial cell concentrations in all cultures.

Future work should build on the work presented here by using a wider variety of URB taxa, such as a fresh stock of *S. natans* and an alternative *Zoogloea* spp. linked with wastewaters (e.g., *Z. ramigera*) [41], in addition to other key URB taxa, including

Beggiatoa spp. [32], *Rhodospirillum rubrum* spp. [11,72] and fungi (e.g., *Leptomyces lacteus* [22,34]). Many other pollutants also cause URBs, such as raw sewage [5,32] and agricultural runoff [73,74] which will result in different synergistic/antagonistic interactions between taxa in a complex polymicrobial URB. However, optical density can only provide a limited understanding of microbial interactions and relative growth of individual taxa which alternative approaches (such as time-lapse microscopy) are able to examine in finer detail.

6.3.4 Dynamics of URB development using time-lapse microscopy

Whilst robust, turbidimetric determinations of mixed cultures cannot readily yield information about specific proportions of taxa and provide only a surface-level comprehension of their complex interactions. Furthermore, optical density can vary between measurements in cultures that clump together, depending on whether the light beam hits a group of cells (high absorbance) or empty medium (low absorbance) [56]. To offset this, a high number of repeats for each sample were taken and growth kinetics, such as maxROC gradients, were calculated over longer durations of 1-hour moving averages. To build on and compare to results from turbidimetric methods, a time-lapse imaging approach was employed, using the oCelloScope (BioSense Solutions ApS, Denmark). Direct comparisons of the plate reader and time-lapse microscopy tool have been made in antibiotic and phage time-to-kill experiments [75–78] but here, for the first time, mixed cultures of URB taxa were compared. Firstly, different starting concentrations of cells in a media and at different volumes of the inoculated media were tested, employing the different image processing algorithms (BCA, SESA, TA, and SEAL) to determine the optimal test setup.

Several technical challenges were encountered whilst optimising methodological design (summarised in **S6-4**, **S6-5**, **S6-6**). One challenge was the movement of cell aggregates between images. It is unlikely that media advection caused cell movement after the initial few images since the plate and instrument remained stationary. Instead, it is probable the flagella of *S. natans* [58,62,79] and *Z. oleivorans* [30,59] caused cell motility. The active motility of these taxa presents an opportunity within the study of dynamic and time-lapse microscopy which can track not just the optical density properties (such as OD) or gross microbial number as determined by flow cytometry,

but also dynamics in association – i.e., biofilm formation and potential interactions with species. For example, there was distinct clumping within *S. natans* cultures (**Figure 6-6a**) which could explain to the high relative variability in OD for some culture experiments (mitigated by running numerous method replicates alongside biological repeats). This cell aggregation could be indicative of oxygen limitation [80] which may arise as a consequence of no stirring or shaking of the plate, unlike in previous turbidimetric assays (**5.2 Materials and Methods**). Furthermore, cell aggregation can be indicative of biofilm formation [81]. The time-lapse also revealed that not all cultures clump, some remain as ‘free’ cells (**Figure 6-6b**), in a more homogenous solution. Furthermore, in some samples the filamentous growth form characteristic of *S. natans* cultures can be observed (**Figure 6-6c**) [82]. Microscopy-based approaches can be used not only to potentially provide more accurate growth curves for taxa that flocculate, clump, or form biofilms, but are also able to explain unexpected trends in growth curves, such as cell decay and specific proportions of cells in mixed cultures.

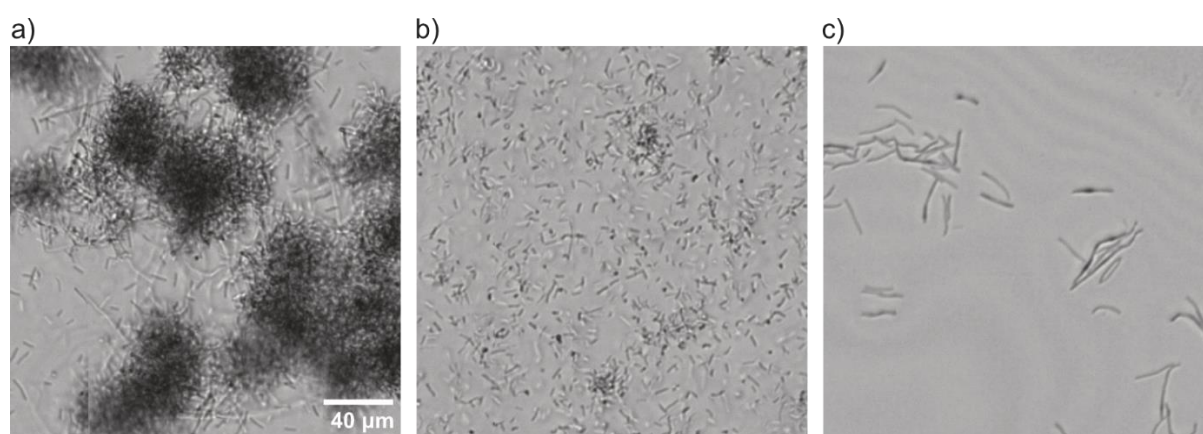


Figure 6-6: Images demonstrating growth patterns of *S. natans* using the oCelloScope. (a) reveals the clumping of *S. natans* in wells; (b) demonstrates some *S. natans* cultures exist as ‘free’ cells; and (c) the filamentous growth form of *S. natans*.

Examination of select images from certain wells clearly shows cell growth over time (**Figure 6-7**), demonstrating the viability of time-lapse microscopy for this application.

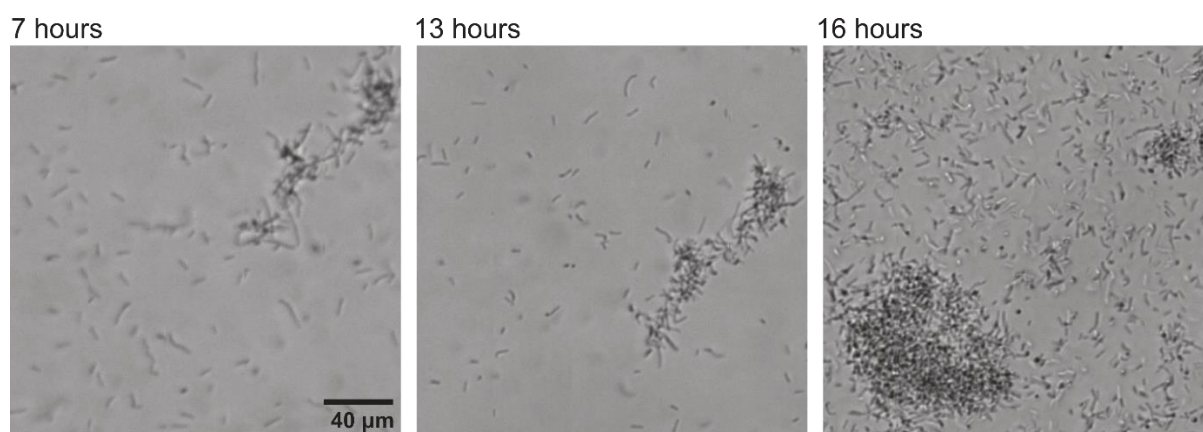


Figure 6-7: Selected images showing cell growth over time, even if insufficient to calculate growth curves using the oCelloScope.

These findings provide an important potential contribution to method development for monitoring the kinetics and growth of URB taxa. Time-lapse microscopy can provide additional detail that complements robust OD assessments [53] and has the potential to address long-held gaps in knowledge. Firstly, time-lapse microscopy can differentiate between different cell morphologies to monitor the growth of individual taxa, cell interactions, and has been used in studies alongside investigations into biofilms [83,84]. Furthermore, recent trends in wastewater epidemiology and the river microbiome have demonstrated the importance of anti-microbial resistance (AMR) genes. AMR has been detected in URB taxa, such as *Zoogloea* [85], and has been studied using time-lapse microscopy [54]. AMR genes have dynamic bearing on understanding the river microbiome for the One Health agenda [86,87] and their role in URBs has not yet been studied. Recent advancements in mathematical modelling have enabled the algorithms behind time-lapse microscopy instruments to provide advantages over existing techniques such as optical density. For the first time, we demonstrate how optical-based time-lapse microscopy could enhance future research on URB growth.

6.3.5 Future work

This paper has sought to provide additional insights into how URBs proliferate in polluted rivers. It builds on research by examining a single URB taxon using urban

river water rather than de-ionised water as the base matrix for minimal media; studying the growth of additional URB taxa; and multi-species cultures. URBs are complex polymicrobial communities [13,23], implying that there is broadly synergistic interaction but has not been tested. In this study we sought to examine these interactions. However, several challenges were encountered centring around microbial growth. For future work, we recommend further development of the methods for studying filamentous wastewater bacteria using imaging approaches [53] and using fresh stocks of a wider range of URB taxa, such as *Sphaerotilus*, *Rhodoferax*, *Zoogloea*, *Beggiatoa*, *Hydrogenophaga*, *Leptothrix*, and *Thiothrix* [11,13,22,32]. This would allow for a much richer discussion about the interactions between taxa and to explore complex interactions like predation, competition, mutualism, neutralism, catabolism, anabolism, commensalism, and amensalism [88]. Studying the specific growth of URBs is challenging because they are diverse and variable polymicrobial biofilms that when distilled down into individual taxa and individual pollutants only give a tiny snapshot of these complex systems.

Moreover, URBs are fixed biofilms, rather than suspended colonies. Therefore, future studies should consider employing methodologies designed to study fixed biofilms, such as microfluidic devices [63,64] or flow cells with microscopy [65]. Larger scale growth studies using mesocosm experiments should also be considered, such as flumes [24], to measure biomass over time. This would also enable periodic sampling for genomic sequencing and evaluate how microbial composition or gene expression may change with different treatments. In addition, further exploration of nutrient requirements and utilisation over time, for example requirements to initiate compared to sustain URB growths, could be studied which is of much greater challenge in microcosm experiments. However, this approach also has limitations, such as scale and the volume of water and nutrients required.

6.4 Conclusions

In this study, we set out to build upon existing work examining the growth of URB taxa using airport freeze-point depressants (FPDs) and commercial airport de-icers (CADs) as the carbon source by: (i) using nutritionally diverse water samples from an urban river; (ii) measuring the growth of a second URB taxon, *Z. oleivorans*; to (iii) to evaluate

the synergistic or antagonistic effects of each bacteria in mixed-species cultures; and (iv) to explore the use of imaging technology (i.e., oCelloScope) instead of optical density to monitor the growth of the mixed-species cultures in.

In the first part of this study, using river water as the matrix for media, we found that although the concentration of nutrients in the RW matrix were sufficient for growth in natural fluvial systems, there were insufficient nutrients in each fixed microcosm to facilitate *S. natans* growth; so river water had to be augmented with essential vitamins and minerals (RW+MEM) containing minimal carbon sources so as to isolate different FPDs and CADs as carbon sources. There were only significant differences between RW+MEM samples inoculated with *S. natans* and “negative” controls (RE+MEM_neg) for ethylene glycol based media. This suggested that the native microbiome was able to utilise common airport FPDs for growth as well as *S. natans*. Furthermore, negative controls had a shorter time-to-detection (TTD) than *S. natans* cultures in a de-ionised water matrix.

In the second experiment, *Z. oleivorans* unexpectedly did not demonstrate a discernible ΔOD_{600} when using FPDs as the sole carbon source. *Zoogloea* spp. are heavily associated with wastewaters and have been shown to utilise acetate and formate as carbon sources and whilst 16S rRNA (V3-4) sequences from an airport de-icer implicated URB most closely matched *Z. oleivorans*, another *Zoogloea* species, such as *Z. ramigera*, could be responsible. Further exploration of the growth of different *Zoogloea* species in FPD-based media is recommended for future work.

In the third phase of this research, mixed cultures of *S. natans* and *Z. oleivorans* in varying starting proportions in sodium acetate based media were compared. Samples containing *S. natans* had very similar growth profiles (sigmoidal growth) and no statistical differences in their growth kinetics. However, samples containing only *Z. oleivorans* produced vastly different growth profiles with a diauxic profile and had significantly different TTD compared to those containing *S. natans*. Furthermore, it highlights the value of reporting both growth profiles and the calculated kinetics.

In the final experiments, a novel time-lapse microscopy approach was tested to assess its potential application in studying mixed species URB cultures. Whilst several

challenges were identified, the approach demonstrated that it is applicable to URB research and presents a powerful tool to improve understanding of the complex interactions between species within URBs.

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Data access statement

Supporting data for this study can be accessed through the Cranfield University repository (CORD): [10.17862/cranfield.rd.23675316](https://doi.org/10.17862/cranfield.rd.23675316).

Conflict of interest

The authors have no conflict of interest to declare.

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DOI:<https://doi.org/10.1002/psc.3080>

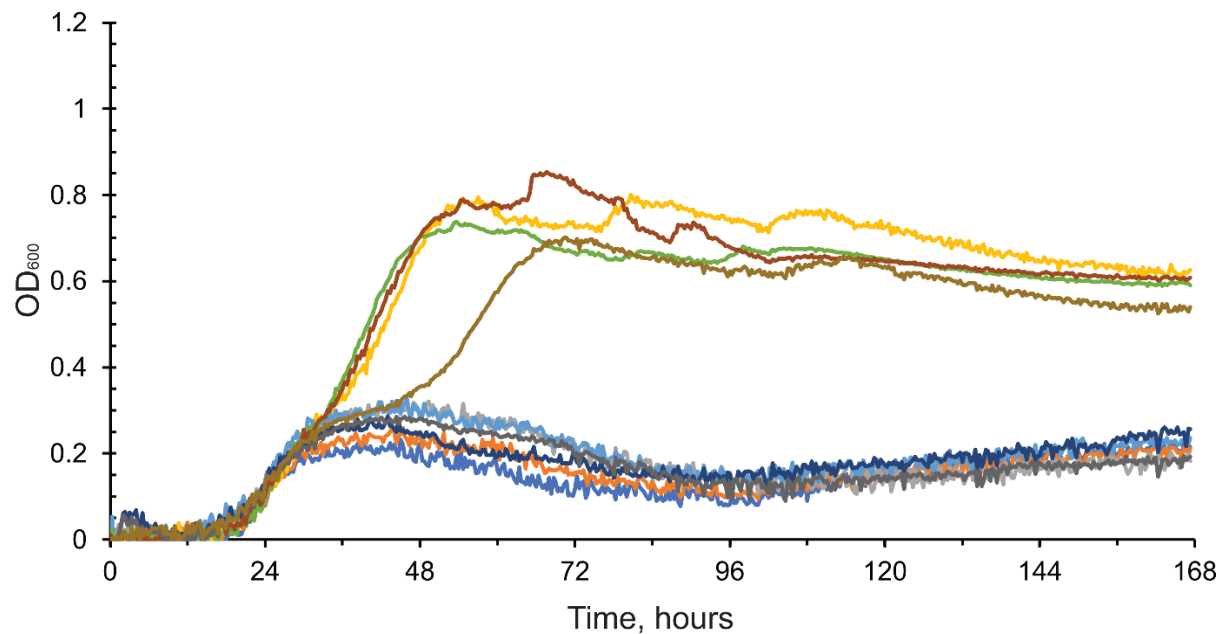
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Supplementary information

Appendix A – Ethylene glycol growth curves

A.1 Ethylene glycol growth curves in RW+MEM based samples

In some microcosms, the growth of *Sphaerotilus natans* in ethylene glycol based RW+MEM media was significantly diminished and is presented below.

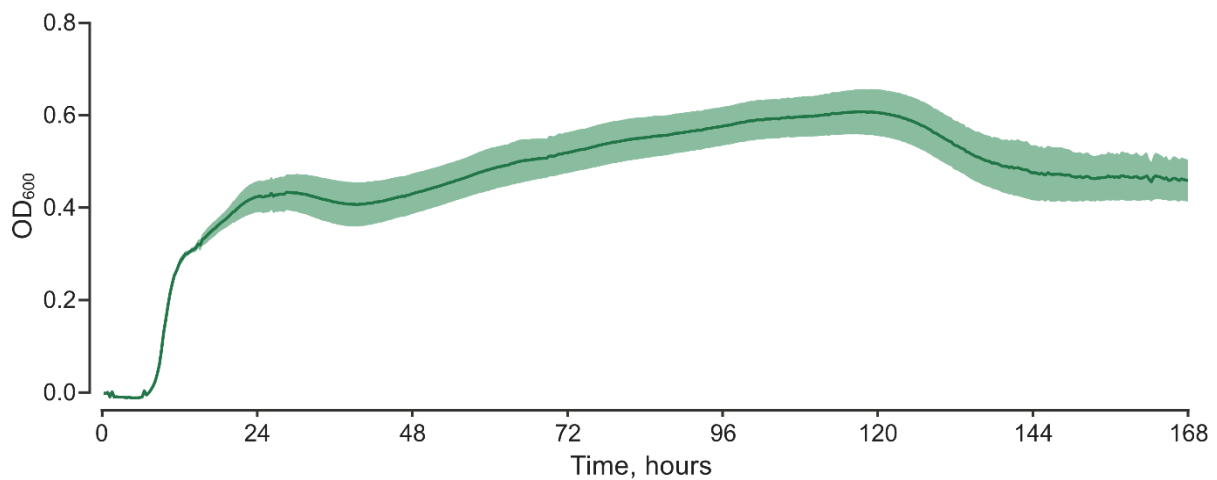


S6-1: Time-series of optical density for *S. natans* culture in a matrix comprised of a river water augmented with essential vitamins and minerals to support microbial growth (RW+MEM) matrix supplemented with ethylene glycol (200 mM of carbon). Individual, base-line corrected replicates are presented.

Appendix B – *Zoogloea oleivorans* growth

B.1 Growth profile of *Z. oleivorans* in an ideal media

Below is the growth profile for *Zoogloea oleivorans* in its prescribed media (medium 1, DSMZ, Germany). The culture had a starting optical density of 0.005 and was baseline corrected based on OD₆₀₀ readings between 40 – 100 minutes.

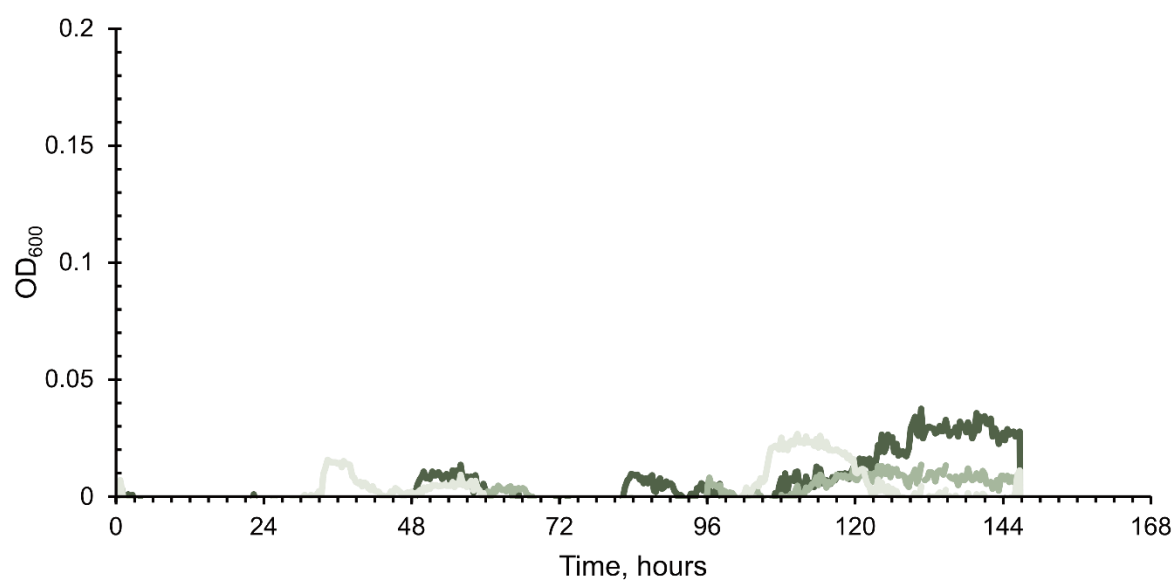


S6-2: Time-series of optical density for *Z. oleivorans* culture grown in an ideal medium (medium 1, DSMZ). Error is denoted by mean $\pm$ confidence intervals ($z = 1.96$) based on 10 biological replicates for *Z. oleivorans* cultures.

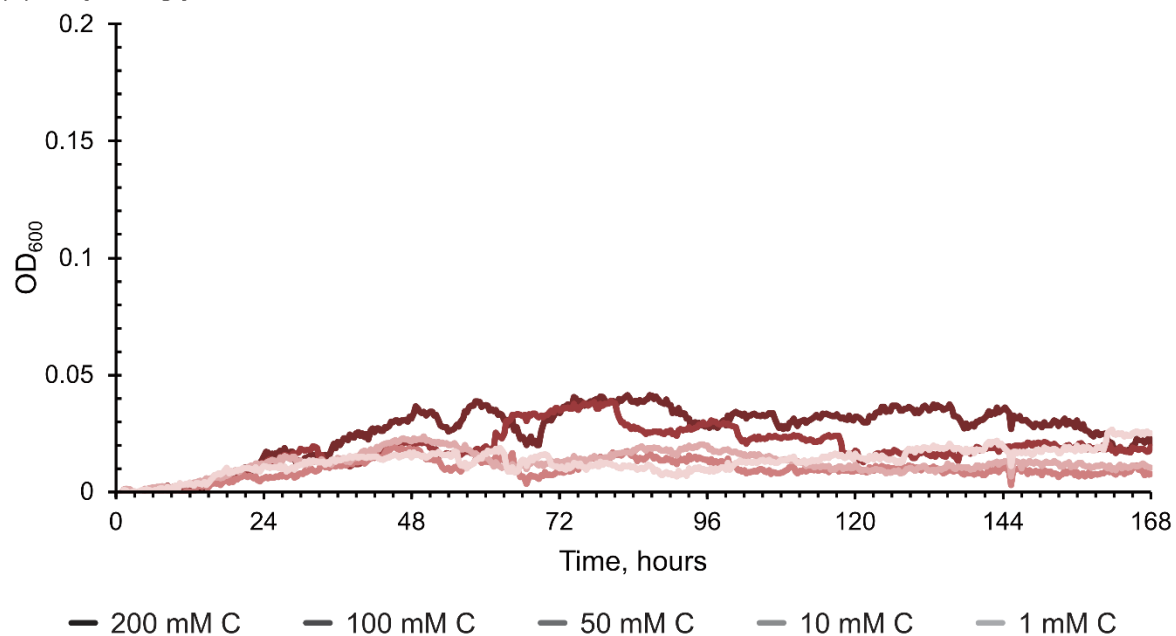
B.2 Growth profile of *Z. oleivorans* in freeze-point depressant based media

Z. oleivorans did not exhibit any discernible growth in propylene glycol or ethylene glycol based media.

(a) Propylene glycol



(b) Ethylene glycol

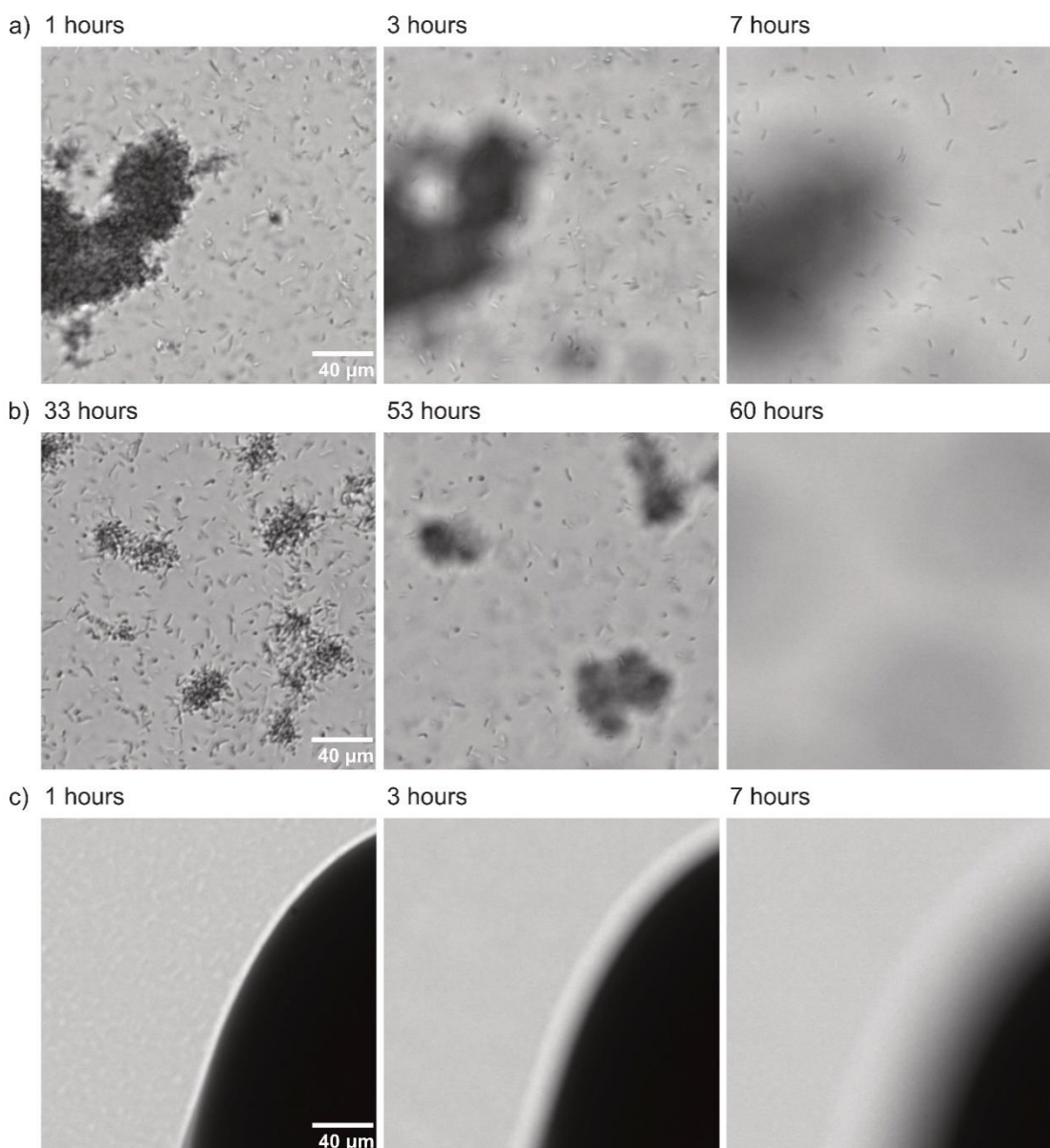


S6-3: Time-series of average optical density for *Z. oleivorans* (based on 10 replicates) grown in propylene glycol (green) and ethylene glycol (red) based media over a range of carbon concentrations (more faded colour denotes lower carbon concentration).

Appendix C Issues encountered using oCelloScope

C.1 Focal issues in images

There were several issues with the autofocus option for the instrument (**S6-4a**). Therefore, focal length was manually set for each well on the multiplate but focus on cells was again lost over time (**S6-4b**). Possible reasons for this could be bacteria settling or media evaporation, therefore, a black dot was drawn in an empty well as a reference point. However, even with the dot, the images still went out of focus (**S6-4c**), suggesting that the camera and its sensors were likely the underlying technological issue.

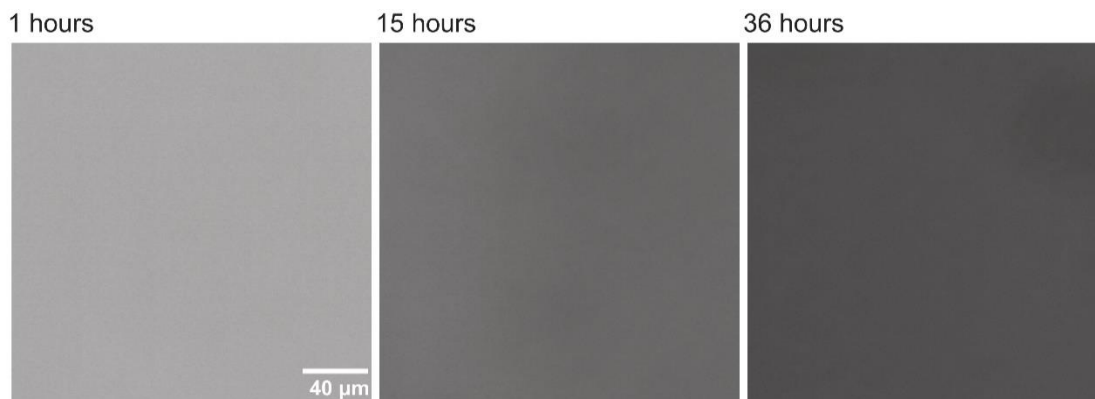


S6-4: Focus issues experienced using the oCelloScope over time including using (a) autofocus, (b) manual focus, and (c) an ink dot on the bottom of a well to manually set the focus.

C.2 Lighting issues in images

Furthermore, lighting of images also became a problem in some methodological tests. Over time images became darker (**S6-5**), even when manually setting illumination levels. A possible cause of this could be condensation on the plate lid, which may also

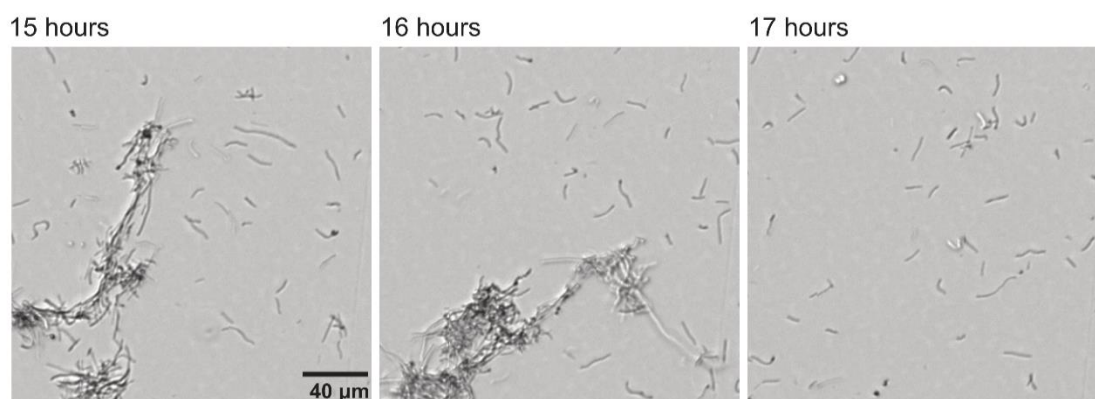
relate to focus issues. Dark images affect the image analysis algorithms (especially BCA algorithm which may detect “false objects”).



S6-5: Illumination issues experienced over time when testing method design with the oCelloScope.

C.3 Motility of cells in images

The rapid movement of cells between images (1-hour intervals) caused issues in generating microbial growth curves as there were regular, substantial decreases in total cell count (**S6-6**).



S6-6: Issues with cells moving between measurements when testing method design using the oCelloScope.

7 Influence of Temperature, Sunlight, and Wind on the Mobilisation by Rainfall of Propylene Glycol from Airport De-Icers

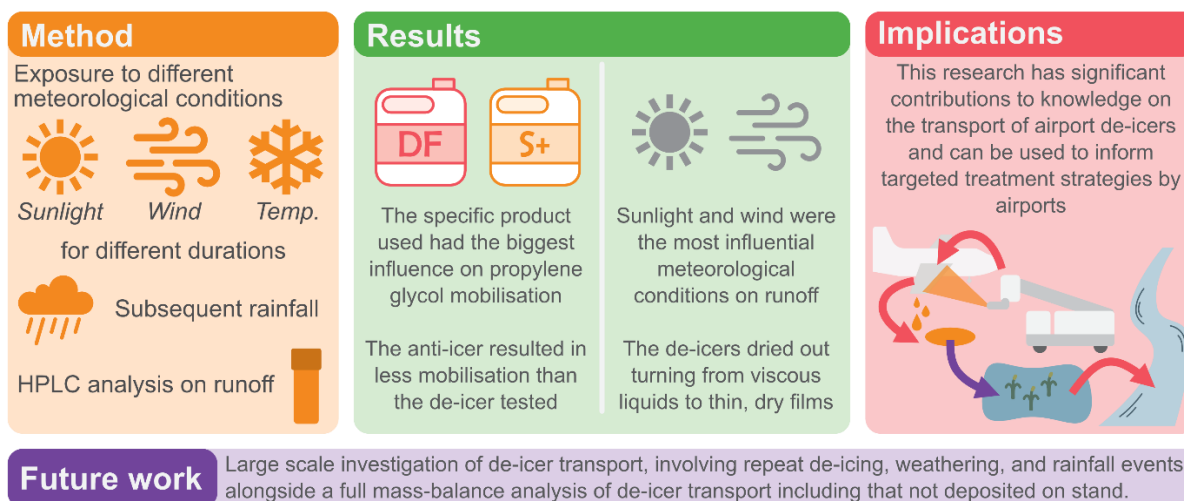
Abstract

The use of chemical anti- and de-icing compounds at airports has profound effects on the environment. It is therefore imperative to better understand the fate and transport of these commercial airport de-icers (CADs, includes anti-icers) to improve the effectiveness of treatment and minimise environmental and ecosystem damage. Observations from airport de-icing operators note physical changes to CADs after exposure to weathering, following application and deposition on airport surfaces. This study aimed to expose CADs to different weathering conditions (different temperatures, sunlight, and wind) for 1- and 2-week durations before subjecting them to artificial rainfall where runoff was sampled every minute. Loss of the freeze point depressant, propylene glycol, was calculated for each CAD, weather type, weather duration, and rainfall duration. All weathering conditions affected the mobilisation of propylene glycol. Different winter temperatures (+5 and −5 °C) had minimal influence and were not statistically different from one another, whereas sun and wind had a drying effect on the CAD that caused the viscous liquids to become thin, dry films and resulted in lower amounts of propylene glycol in runoff. Differences in mobilisation rates were found between the anti-icer and de-icer tested. Nevertheless, the influence of each weathering condition was similar between CADs, except for sunlight which had a significantly higher impact on propylene glycol mobilised from the anti-icer. These results form a key part of understanding de-icer contaminated runoff at airports and can be used to help inform treatment strategies and reduce the impact on the ecosystems of receiving waters.

Visual abstract

Influence of temperature, sunlight, and wind on the mobilisation of de-icers in rainfall

Aim: to determine how key meteorological conditions affect the mobilisation of airport anti-/de-icers from a surface by rainfall



Highlights

1. Novel dataset of airport de-icer mobilization and transport
2. Weathering had an impact on the mobilisation of de-icers by rainfall
3. Sunlight and wind had significant influences on propylene glycol mobilisation
4. The specific product was the biggest influence on propylene glycol mobilisation
5. Duration of weathering had minimal effect on propylene glycol mobilisation

Keywords

Airport de-icer; airport anti-icer; runoff; mobilisation; transport mechanisms; artificial rainfall;

7.1 Introduction

The application of anti- and de-icing chemicals is a critical requirement for ensuring safe winter operations and air travel at airports [1–3]. In a given de-icing season, in excess of one million litres of commercial airport de-icers (CADs) can be used for aircraft with another million litres on ground surfaces at an international airport [4–6]. The active ingredients within CADs are a freeze-point depressant (FPD), commonly propylene glycol, ethylene glycol or acetate/formate salts [7–11], which create a eutectic solution when mixed with water/snow/ice to lower the freezing point and prevent snow/ice build-up [12–14]. Anti- and de-icers have distinct but closely related functions, as do commercial products designed for either ground surfaces or aircraft. Anti-icers are designed to prevent ice build-up, whereas de-icers remove frozen material from critical surfaces. Aircraft CADs are classified into different types depending on their intended use (**Table 5-1**). Type I CADs are exclusively utilized for de-icing aircraft [15], while Type II CADs offer extended anti-icing properties compared to Type I but are primarily used for de-icing [16]. Type IV CADs, on the other hand, are primarily anti-icers but can also be used for de-icing as required [16].

The application of CADs on aircraft is performed by contractors using specialised vehicles with a highly manoeuvrable aerial boom [17] to directly target critical aircraft surfaces. Following application, approximately 75-80% of CADs are deposited on ground surfaces from overspray, deflection, and drip [4,18]. The remaining material can be deposited large distances away from their application by, for example, wind or jet blasts [19]. Additives are used to ensure remaining CAD shears during take-off [6,20,21]. CADs deposited on airport surfaces are readily mobilised in rainfall and overland flow and are transported through the airport surface water drainage network [21,22] (**Figure 7-1**), as FPDs are highly water soluble [21]. The transport pathways of CADs are affected considerably by the volume, intensity, and duration of rainfall, in addition to the specific layout of the airports' surface water drainage networks [21,23]. In general, the drainage systems are designed to transport water away from critical areas (such as aircraft manoeuvring) to minimise flooding [23], however, this does mean large volumes of runoff may be transported rapidly to treatment systems – exacerbated by the large surface area of impermeable surfaces, such as asphalt. Treatment systems for airport surface runoff (such as moving bed biofilm reactors

(MBBR) [24] and aerated wetlands [25]) are designed for continuous steady flows, therefore storage reservoirs are needed to help buffer influxes in runoff volume [26,27].

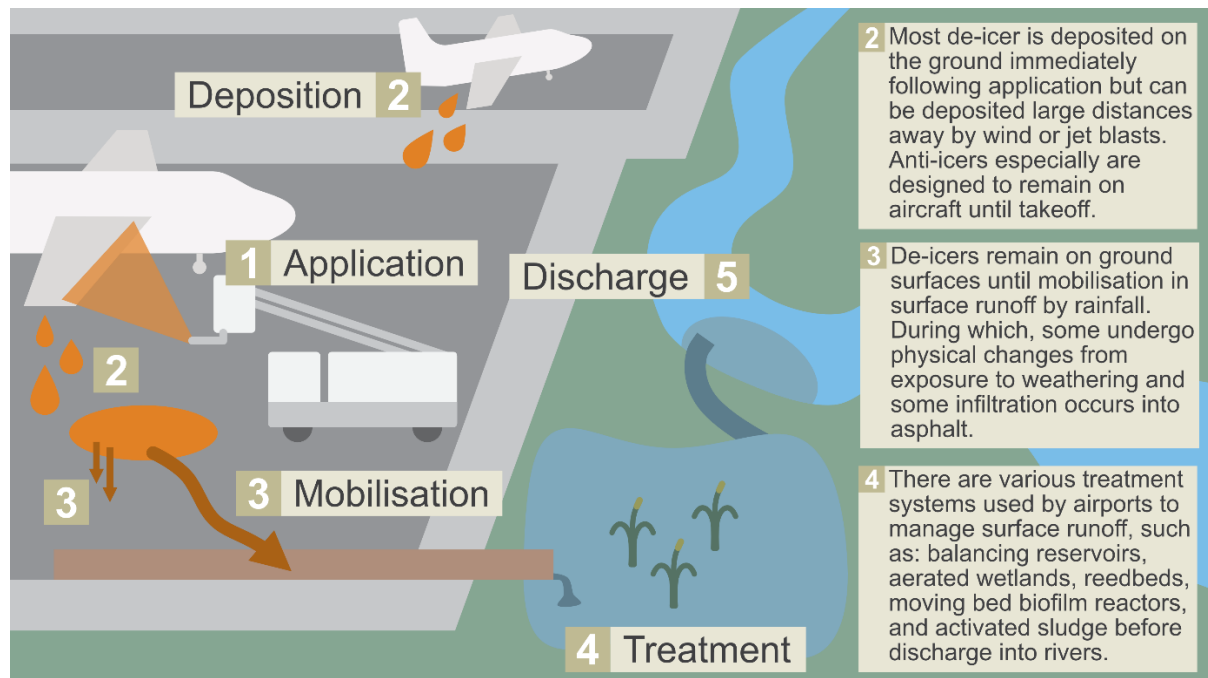


Figure 7-1: Overview of the main transport pathway of airport de-icers: (1) Application of de-icers; (2) deposition on ground surfaces; (3) mobilised in surface runoff; (4) de-icer containing surface runoff is treated; (5) treated effluents are discharged into receiving waters.

Although essential for airport winter operations, anti- and de-icers are major sources of organic pollution in airport surface runoff with severe ecological impacts in rivers [28], including the growth of undesirable river biofilms (URBs, formerly “sewage fungus”) [29–31] which causes dissolved oxygen (DO) depletion [32–37] and impacts to macroinvertebrates and fish [28,36]. Additionally, additives in CADs may have toxic effects on aquatic flora [38] and fauna [39], such as from benzotriazoles used as corrosion inhibitors [21]. Commercial products contain approximately 1-2% proprietary additives [6,21,40] but can be as high as 10-20% [7], to act as non-ionic surfactants, flame retardants, corrosion inhibitors, chemicals to reduce surface tension or thickeners for adherence, pH buffers, and/or anti-precipitation agents [8,41–44].

Therefore, an improved understanding of the transport of CADs following their application is needed to better inform airports' winter operations and reduce their environmental impact.

The scope of this study was an initial investigation into the mobilisation of CADs in a rainfall event following exposure to different meteorological treatments, using observations from collaborators at a major UK airport (Pers. Communication, M. Murphy). It has been noted that, following application, CADs can remain on airfield surfaces for long periods of time until mobilisation by rainfall mobilised [5]. It has also been observed that, in the time between CAD application and rainfall, physical changes occurred in the deposited CADs; they had become more gelatinous and sometimes developed a texture described as “like a boiled sweet” (Pers. Communication, M. Murphy). Several meteorological conditions could alter the chemical and physical structure of CADs, such as sunlight, wind and temperature. Sunlight can lead to evaporation of volatile or water fractions of mixtures [45,46], whereas wind increases evaporation by increasing turbulent eddy exchanges with the atmosphere [47]. Whilst the drying of CADs has not been directly studied, propylene and ethylene glycol, common FPDs in CADs, are expected to be non-volatile from water and dry surfaces [48]. Whereas lower temperatures often increase the viscosity and lower the solubility of liquids suggesting that lower temperatures could reduce mobilisation in runoff. Therefore, these physical, and perhaps, chemical changes form an important part of understanding the fate and transport of CADs following application which in turn has important implications for treatment strategies and reducing ecological stressors on receiving waters. As a result, the methodology of this study was highly controlled to reduce the influence of external complexities (such as substrate effects) and directly examine the role of different weathering treatments on the mobilisation of CADs. The knowledge gap here is the influence of weathering prior to a rainfall event on the mobilisation of airport de-icers by rainfall, a key part of the transport pathway.

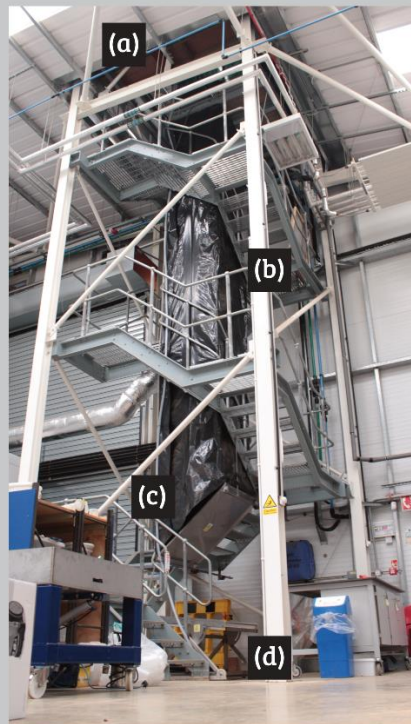
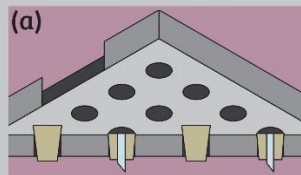
The overall aim of this study is, therefore, to determine how key meteorological conditions affect the mobilisation of airport anti-/de-icers from a surface by rainfall. We hypothesize that, (i) sun and wind weathering treatments will cause higher evaporation of the water fraction, drying out the sample and making it less soluble; (ii) longer

exposure to meteorological treatments will have a greater impact on the mobilisation of propylene glycol as samples become more dehydrated (sun and wind) or more viscous (low temperature); (iii) small temperature changes will have negligible impact on the solubility and therefore mobilisation of propylene glycol; and (iv) Type IV aircraft anti-icers will be less readily mobilised by rainfall than Type I aircraft de-icers (Type I) because they are more viscous.

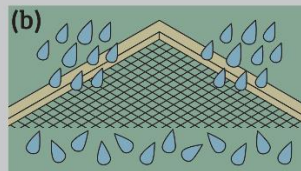
7.2 Methods

To establish the impact of weathering on CAD transport from airfields, samples were exposed to different environmental treatments (temperature, sunlight and wind) over two durations (1 and 2 weeks) before using an artificial rainfall tower to simulate rainfall, where runoff was collected every minute for high-performance liquid chromatography (HPLC) analysis to quantify de-icer concentrations. The gravity-fed rainfall tower (CHAP Soil Science Unit, Cranfield University, UK) is approximately 9 m tall with a head tank that fills a needle bed with de-ionised water (**Figure 7-2**). Rainfall characteristics (such as droplet size, kinetic energy and intensity) can be controlled through the weir height, number of needles used, and size of needles. Droplets then fall from hypodermic needles and hit a suspended mesh splitting and randomising before hitting the ground as artificial rainfall. The height of the rainfall tower is to allow droplets to reach terminal velocity under gravity when they impact with the sample.

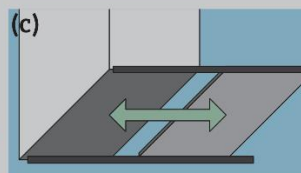
The CHAP rainfall tower is 9m tall and can simulate a range of rainfall conditions with adjustable needle bed, weir height, scattering mesh to control rainfall characteristics. It is used here to simulate the mobilisation of airport de-icers in stormwater runoff.



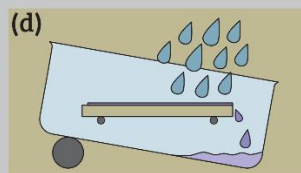
Bungs in the needle bed can be replaced with needles and the weir height changed to control rainfall intensity, droplet size, and kinetic energy.



Mesh suspended halfway down the tower splits and randomises droplets. The mesh size can be changed to further control droplet characteristics.



A sliding hatch at the bottom of the shaft allows rainfall to be diverted whilst setting up samples and to control specific rainfall duration.



Tiles were placed on level rods in plastic tubs, tilted to collect runoff in the bottom. Runoff (100 μ L) was sampled every minute, for 5-minutes.

Figure 7-2: The labelled key features of the CHAP rainfall tower and diagrams of each key feature, including (a) the needle bed and weir, (b) the mesh layer, (c) cover hatch, and (d) runoff trays.

7.2.1.1 Sample preparation

For each sample, 5 mL CAD was applied to a tile using a precision pipette and spread evenly across the tile, leaving an approximately 1 cm border along each edge as a buffer to minimise the likelihood of spillage. The most commonly used CADs used at Heathrow Airport include Kilfrost DF Plus [49] and Kilfrost ABC S Plus [50] (herein referred to as “DF” and “S+”) so were selected for this study (**Table 5-1**). Both CADs are propylene glycol based with different proportions of the FPD and additives. Each CAD uses a different dyes for quick identification. DF is a type I de-icing fluid with an orange dye, whereas S+ is primarily a type IV anti-icer but can also be used for de-icing purposes and is coloured green.

Several types of tile were tested (**7.2.3 Method development**) with glazed, matt ceramic tiles selected (Matrix® Antique White Matt Tile (14.8 x 14.8 cm), Topps Tiles (696128)). Domestic bathroom tiles were used, despite not being fully representative of materials used at airport stands, such as asphalt, tarmac, or concrete. This deliberate choice was made to isolate weather conditions as variables to better address the aim of this research as an initial investigation into CAD mobilisation. More realistic surfaces will have additional factors what would need to be accounted for, such as absorption into the material, or protection in surface cavities, and exceeded the scope of these novel pilot experiments.

Tiles were then placed into clear, plastic containers (18 x 25 x 9 cm, Beaufort (104601)) which were either left open or closed for exposure to different environmental conditions.

7.2.1.2 Artificial meteorological conditions

Three aspects of weather were evaluated: temperature, sunlight, and airflow (**Table 7-1**). First, the effect of temperature was determined by positioning tiles inside sealed, clear plastic containers and placing them within a temperature-controlled fridge. The threshold for automatic de-icing at airports is at temperatures below 5 °C to maintain safe winter operations (Pers. Communication, M. Murphy), therefore, temperatures of +5 °C (“cool”) and –5 °C (“cold”) were chosen to represent the approximate threshold temperature and colder winter temperatures in the UK. Tiles were sealed inside plastic containers to minimise the impact of the damp atmosphere inside a fridge. Second, the effect of sunlight (“sun”) was determined using a simple presence-absence design. Samples (CADs applied to tiles) were placed inside sealed clear plastic containers in a glasshouse (+18 °C) with no surrounding obstacles to minimise shading (**Figure 7-3a**). Containers were sealed to minimise the effect of any airflow within the glasshouse. Adding a lid to “sun” treatments could impact penetration of solar radiation and result in increased temperature or reduced volatile losses so a control for these samples (“warm”) was employed by placing a cardboard box over samples in sealed containers in the glasshouse so as to match the environment of “sun” samples, excluding light to reduce these potential unintended influences (**Figure 7-3c**). And third, the effect of airflow (“wind”) was assessed as a combination of sunlight and wind,

due to practical difficulties in isolating wind as the sole variable (i.e., sufficient temperature-controlled space with no light). Wind samples were placed in the glasshouse, alongside sunlight samples, except with containers open and a desk fan positioned to provide additional convection of air (**Figure 7-3a,b**). The control for these samples also adopted a presence-absence approach using “sun” as the control for wind conditions, due to the above-mentioned practical constraints in isolating airflow as a variable. Samples within the glasshouse (“sun”, “wind”, and “warm” treatments) were organised by treatment type and chemical agent rather than being randomised. Although a randomised layout is typical to ensure unbiased results, the expansive and open structure of the glasshouse should facilitate a uniform treatment of replicates. For all samples, weathering was tested over 2 durations: 1- and 2-weeks. Measuring identical samples over two durations allowed for the effect of time between CAD application and rainfall for each weathering condition to be evaluated. In addition, freshly applied samples (“untreated”) were also tested as an overall control for no weathering and were exposed to rainfall immediately after CAD application.

Table 7-1: A summary of the meteorological conditions in each weathering condition tested.

Name	Cold	Cool	Warm	Sun	Wind
Temperature	–5 °C	+5 °C	+18 °C	+18 °C	+18 °C
Sunlight	✗	✗	✗	✓	✓
Airflow	✗	✗	✗	✗	✓

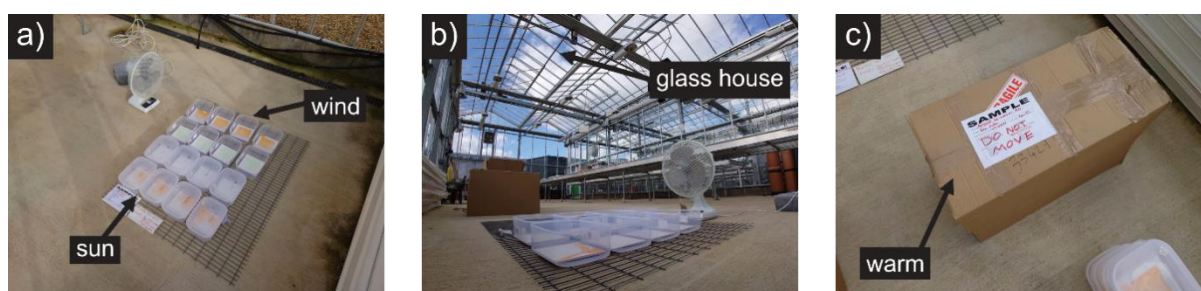


Figure 7-3: Photos of samples during different weathering conditions. (a) “sun” samples in sealed plastic containers and “wind” samples in uncovered plastic containers with a desk fan to provide additional airflow; (b) experimental setup in the glass house; (c) “warm” samples inside sealed plastic containers covered by a cardboard box.

7.2.1.3 Sampling runoff

After exposure of CADs to different treatments, tiles were placed in a runoff tray (a modified 18 x 25 x 9 cm plastic container, Beaufort (104601)) (**Figure 7-4a,b**) under the rainfall tower (**Figure 7-2**) and exposed to artificial rainfall (12.26 ± 0.90 mm/hr, 5 min). After 5 minutes, 38.68 mL rain fell within the runoff tray, 22.38 mL (58%) of which was on the tile. Future calculations account for the dilution observed by the greater surface area of the runoff tray, compared to the sample tile. (**Figure 7-4c,d**). Samples of runoff (100 μ L) were collected every minute and diluted to 1 mL (i.e., 1:10 dilution) in ultra-pure deionised water and refrigerated (4 °C) until analysed the following day.

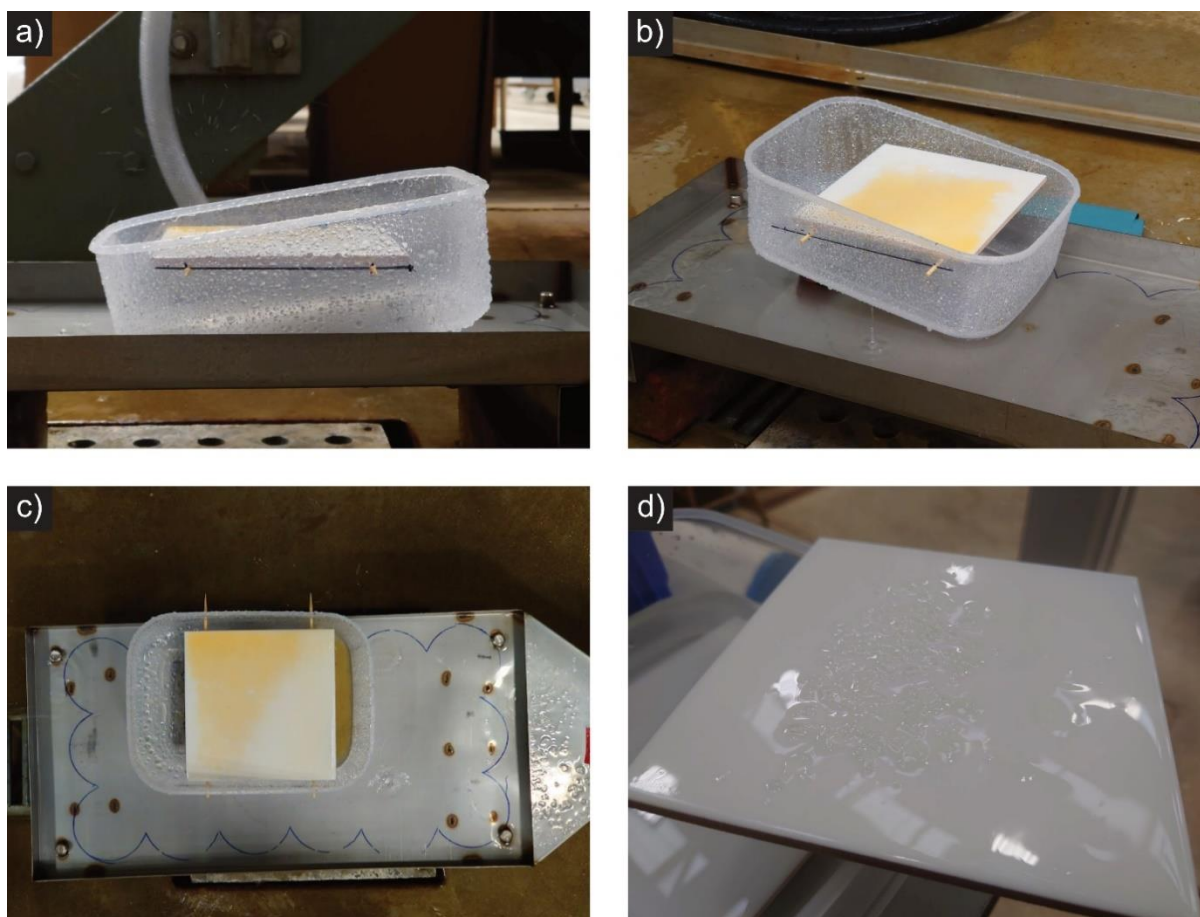


Figure 7-4: Photos of samples and runoff during rainfall.

The rainfall tower was calibrated to ensure evenly distributed rainfall of the desired intensity [51]. A 28 mm weir was chosen for the needle bed and the configuration of needles was optimised for even rainfall across the target area. The rainfall tower was calibrated by arranging pre-weighed catchment cups in a grid in the target area. After running for several minutes to equilibrate, the sliding hatch was opened to collect rainfall for 15-minutes before closing the hatch, drying the outside of collection cups and re-weighing to accurately determine the volume of water, from which intensity (mm/hr) was calculated (**Equation (7-1)**). Before every use, a quick calibration check was performed (5-minutes) to ensure similar rainfall intensity for each set of experiments.

$$intensity\ (mm/hr) = \frac{rain\ collected\ (mL)}{surface\ area\ (cm^2) \times time\ (hr)} \times 10 \quad (7-1)$$

7.2.2 Replication and analyses

To mitigate the potential for variability between samples, three replicates for each condition were prepared and tested (direct replication) – a fourth tile was also prepared in case of any problems during the treatments, such as the tile not quite being level leading to unintended spillage of the CAD from the tile. Sample tiles were chosen at random for rainfall treatment and analysis. Additional replicates of runoff were also taken for a single treatment to ensure mixing was sufficient to homogenise runoff (sample replication) – findings of this are presented in **7.2.3 Method development**.

7.2.2.1 Laboratory analysis

Diluted runoff samples were analysed by HPLC (Agilent Technologies 1200 series, USA) (10.0 μ L injection volume, 0.400 mL/min flow rate, 21-24 bar, 60 °C) using a ROA Rezex Phenomenex (150 x 7.8 mm) column with refractive index detector and H₂SO₄ (0.5 mM) mobile phase. Propylene glycol (extra pure, analytical specification, Fisher Chemical™) was diluted in ultra-pure deionised water to make standards of 3.0, 2.5, 2.0, 1.5, 1.0 and 0.5 %v/v (52.0 – 312.0 g/L propylene glycol) to create the calibration curve. Calibration curve and quality control information can be found in Supplementary Information (**S7-1**).

7.2.2.2 Data analysis and statistics

Peaks for each sample were integrated and the amount of propylene glycol was calculated from the calibration curve in the ChemStation companion software (Agilent Technologies Inc., USA). Data were transformed (to account for dilution and volume of propylene glycol) and plotted in Microsoft Excel (**Equation (7-2)**) (i.e., volume mobilised). The density of propylene glycol is 1.04 g/mL [52], used for mass calculations throughout. Mobilisation per minute was calculated using the change in mass of propylene glycol in runoff between sequential measurements (i.e., Δ ProG/min). Percentage mobilisation of propylene glycol relative to starting concentration (“percentage mobilisation”) was calculated using the percentage of

propylene glycol in each CAD (96.99 %v/v for DF and 44.28 %v/v for S+). Statistical analyses were performed in IBM SPSS Statistics [53]. Data did not fulfil the assumptions of ANOVA (data was not normally distributed) so the Kruskal-Wallis test of difference was used to compare the amount of propylene glycol in runoff after 5-minutes of rainfall, with pairwise comparison post hoc test.

$$vol.ProG (mL) = undilute\ conc. (mL/L) \times vol.rain (L/min) \times time(min) \quad (7-2)$$

7.2.3 Method development

These experiments were designed to test how the erodibility of each de-icer changed with exposure to weather conditions. The choice of substrate for the tests was important, in terms of surface texture and porosity. The sponsor wanted a substrate that was similar to material found at an airport (concrete and bitumen), however, we had concerns that their porous nature could reduce runoff, which would influence the erodibility results. Initial testing was conducted to find a suitable material. Unglazed domestic house tiles were proposed (Quarry Autumn Blend Tile (15.0cm x 15.0cm), Topps Tiles (709218)), as an approximation for airfield ground surfaces whilst maintaining regular uniformity between samples (**Figure 7-5a**). However, during initial testing, a large proportion of the CADs appeared to infiltrate into the porous tiles, which would substantially affect runoff and variability in erodibility measurements. Consequently, glazed, matt tiles were tested so as to provide the non-porous surface of glazing but maintain some texture to the surface. Tiles were sourced (Matrix® Antique White Matt Tile (14.8cm x 14.8cm), Topps Tiles (696128)) and trial experiments found them to be superior to the unglazed quarry tiles, with no detectable infiltration (**Figure 7-5b**). Aluminium plates and glass dishes were also tested; however, the CADs quickly clumped together, and substrates were highly slippery increasing the likelihood of spilling material from them during transport, thus invalidating those samples (**Figure 7-5c**).

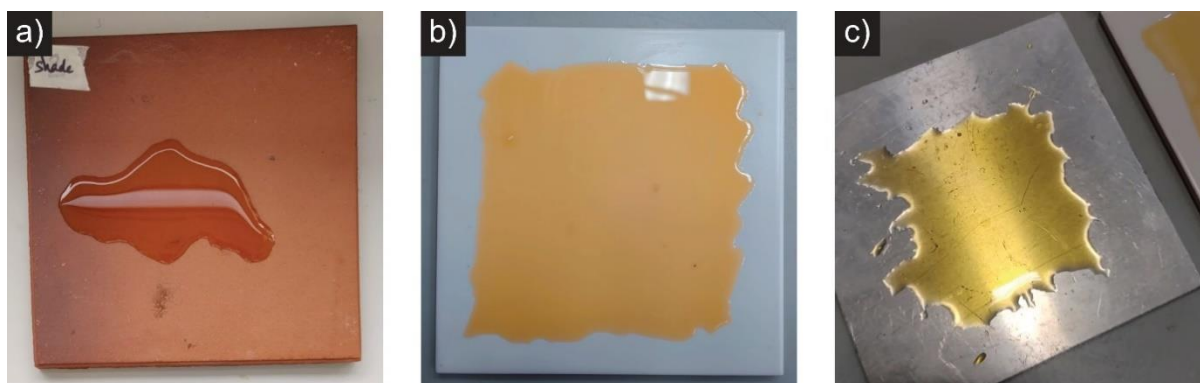


Figure 7-5: Photos of airport de-icers on different types of tile substrates including (a) an unglazed quarry tile; (b) a matte, glazed tile; and (c) an aluminium plate.

It was important that the tile surface had minimal effect on the spread of CADs as the aim of this research was to evaluate the effect of weather conditions on the erodibility of the CADs. Different amounts of CADs were therefore trialled: 2, 5, and 10 mL. We found that using only 2 mL often resulted in the CAD mostly being removed from the tile by various kinds of weathering, whereas 10 mL often spilt off the tile during weathering or when transporting between weathering and rainfall tower. The loss of material for both 2 and 10 mL applications would lead to inaccuracies in results, so 5 mL aliquots were deemed optimal. Runoff rates of 5 mL CADs were then visually assessed to determine sampling duration and frequency which found substantial removal of CAD within the first couple of minutes and often all CAD was visibly gone within 5-minutes. Therefore it was decided that runoff should be sampled every minute for 5-minutes.

Prior to sampling for High Performance Liquid Chromatography (HPLC) analysis, runoff was mixed using a pipette tip. To verify this was effective homogenisation, triplicate samples (sample replicates) were collected at each time point for the cold-2wk treatments (**Figure 7-6**), alongside the three direct replicate tiles for each treatment condition (direct replicates). Analysis of runoff concentrations revealed minimal variability among replicates (standard deviation = 0.15 – 1.33 g/L for DF, 0.05 – 1.80 g/L for S+), where standard deviations were <3.1% of the mean of sample replicates for all but one tile with S+ CAD (red columns for S+ in **Figure 7-6**). Therefore, the pre-sampling mixing procedure was deemed effective, allowing for a

single, well-mixed sample to be considered representative for each sample tile and time point in subsequent treatments.

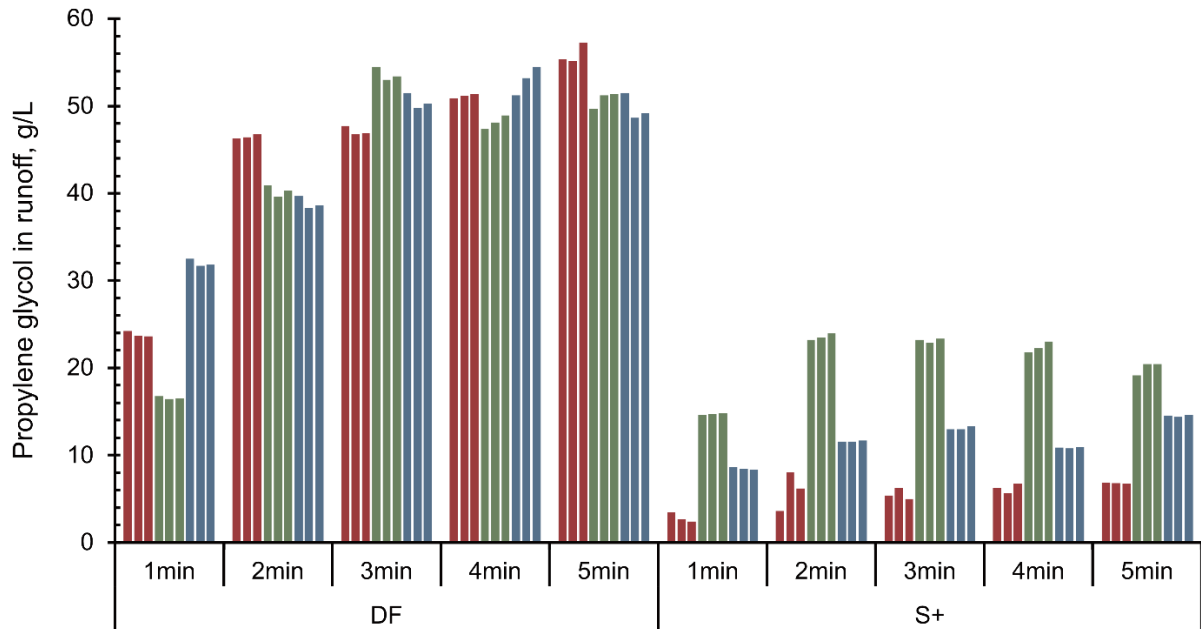


Figure 7-6: Comparing replicates of propylene glycol concentration in runoff to test homogenisation of runoff for cold-2wk samples of the airport de-icer Kilfrost DF Plus (DF) and anti-icer Kilfrost ABC-S Plus (S+). Colour denotes each tile (direct replicates) and clustered columns of each colour are runoff replicates for that tile (sample replicates).

7.3 Results

Simulated meteorological treatments had a significant impact on the mobilisation of CADs in runoff, with those experiencing drying effects (i.e., sun and wind) having the largest impact. There are also substantial differences between the two CADs tested. Differences between treatments were initially discernible in visual observations of tiles (e.g., **Figure 7-7**). Both DF and S+ became visibly more viscous in “cold” and “cool” treatments, likely a result of the lower temperature, and was most pronounced in “cold” conditions (-5°C) where there was also some visible CAD loss (**Figure 7-7c**). There was minimal change in appearance for “warm” samples with some changes for DF

after 2-weeks. However, in “sun” and “wind” treatments, there were profound changes to both CADs (**Figure 7-7b**). There was a substantial drying out of the CADs and samples had the appearance of a thin, dry film than a viscous liquid and was much more noticeable in “wind” which had both sun and wind influences. For many “wind” samples, especially in S+, there was very little CAD visible on tiles after 2 weeks which may suggest a loss of propylene glycol through evaporation. However, propylene glycol is described as non-volatile in water [47] and has a Henry’s law constant of $1.3 \times 10^{-8} \text{ atm} \cdot \text{m}^3/\text{mol}$ [53] implying that it would not readily evaporate under these treatments.

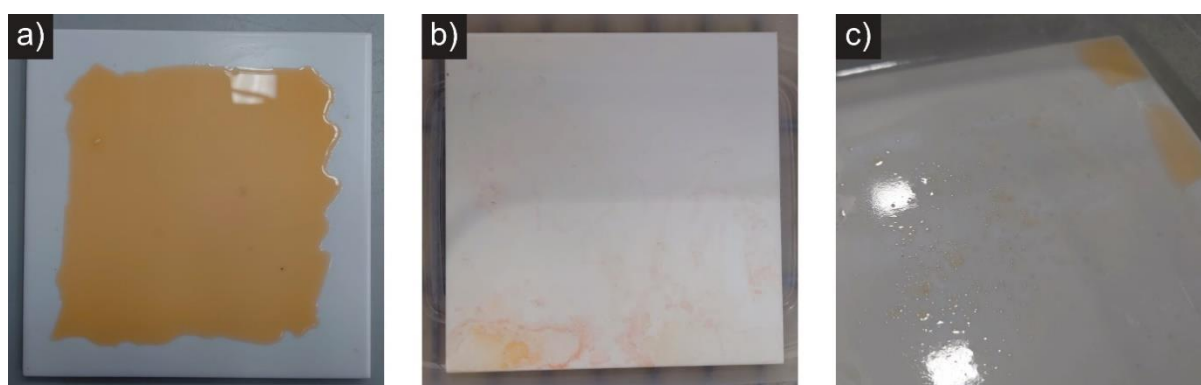


Figure 7-7: Photos of Kilfrost DF Plus aircraft de-icer on tiles following weathering to indicate a change in physical properties of the de-icer. (a) untreated-0wk; (b) wind-1wk; and (c) cold-2wk.

7.3.1 Influence of specific meteorological conditions on CAD mobilisation

The treatment type, the duration of weathering, the choice of CAD, and rainfall duration all had impacts on the mobilisation of propylene glycol in runoff (**Figure 7-8**). After 5-minutes of rainfall, there was often no longer CAD visible on tiles, however, HPLC results indicate that, after 5-minutes of rainfall, <60 % of propylene glycol was mobilised in runoff.

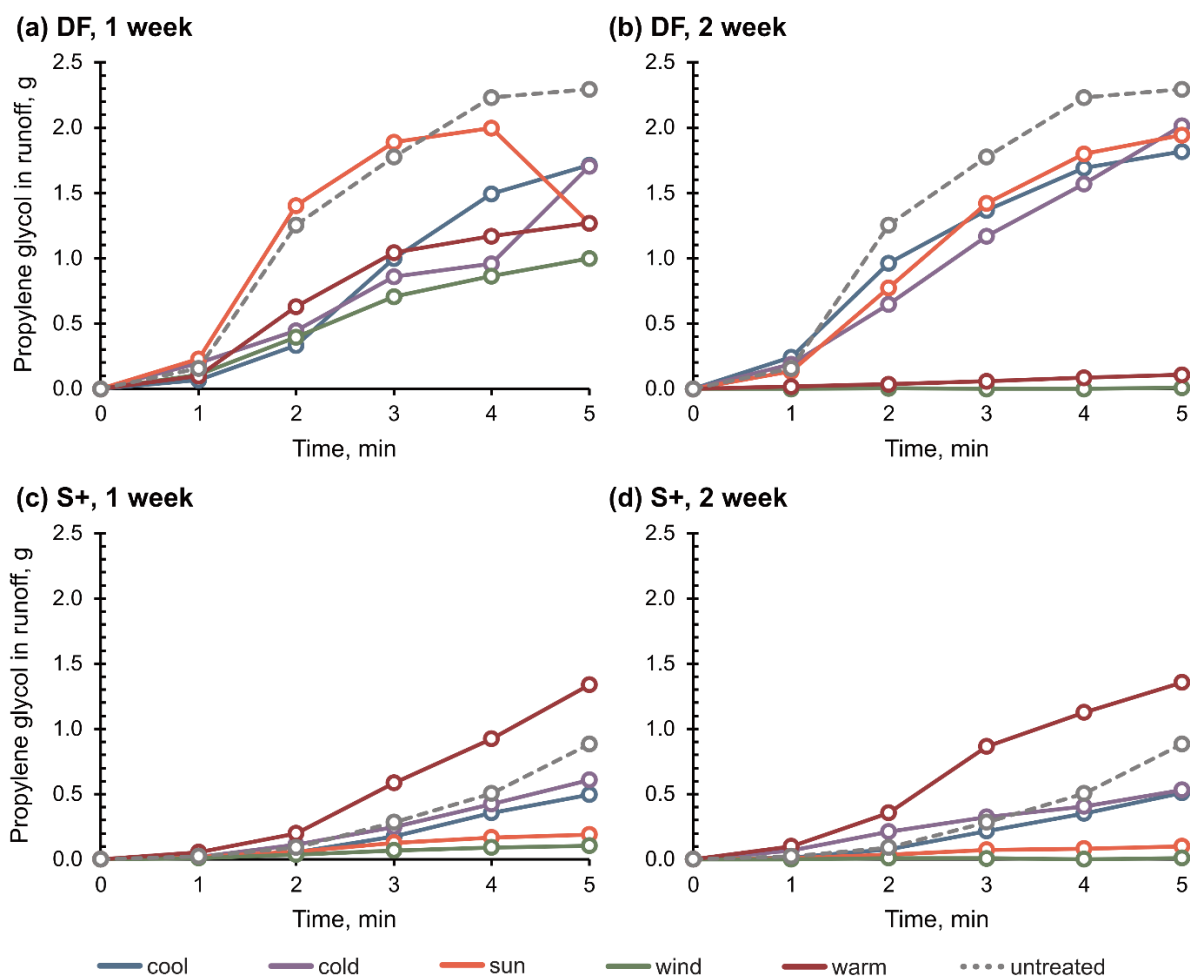


Figure 7-8: Mean cumulative amount of propylene glycol in runoff from the aircraft de-icer Kilfrost DF Plus (DF) and anti-icer Kilfrost ABC-S Plus (S+) following exposure to meteorological conditions for 1- and 2-weeks. (a) DF, 1wk; (b) DF, 2wk; (c) S+, 1wk; (d) S+, 2wk.

Firstly, for DF there was substantial mobilisation of propylene glycol in all treatments, however, after 2-week durations (**Figure 7-8b**) there was negligible mobilisation in both “warm” and “wind” treatments. Visual observations of tiles suggest this could be a consequence of drying effects and loss of CAD to evaporation. There was much less impact on sun samples which, as clear but sealed containers, may have been less susceptible to evaporation. There were also limited differences between different winter temperatures (-5°C and $+5^{\circ}\text{C}$) indicating that a colder temperature does not play a substantial role in influencing the mobilisation of CADs ($p \geq 0.551$, **Table 7-2a**).

Furthermore, after one week treatment duration, there were no significant differences in runoff amount after 5-minutes of rainfall between any treatment in DF samples ($p \geq 0.395$, **Table 7-2a**). After two week treatment duration, there were significant differences between wind-2wk, warm-2wk and all other weathering after 5-minutes rainfall ($p \leq 0.022$, **Table 7-2a**). Untreated-0wk samples generally had more propylene glycol in runoff, as would be expected, demonstrating that weathering treatments affected CAD mobilisation. However, only comparisons with wind-2wk and warm-2wk yielded any significant difference ($p = 0.000$ and 0.002).

Table 7-2: Pairwise comparisons from Kruskal-Wallis test of difference in the amount of propylene glycol in runoff after 5-minutes rainfall (mL) comparing different weathers. For (a) Kilfrost DF Plus for 1- and 2-weeks, and (b) Kilfrost ABC-S Plus for 1- and 2-weeks. Where * indicates a significant difference (P = 0.05).

a) Kilfrost DF Plus											
	1wk						2wk				
	cool	cold	sun	wind	warm		cool	cold	sun	wind	warm
cool	-	1.000	0.444	0.395	0.395		-	0.551	0.718	0.004*	0.022*
cold		-	0.444	0.395	0.395			-	0.815	0.001*	0.004*
sun			-	0.932	0.126				-	0.001*	0.008*
wind				-	1.000					-	0.580
warm					-						-

b) Kilfrost ABC-S Plus											
	1wk						2wk				
	cool	cold	sun	wind	warm		cool	cold	sun	wind	warm
cool	-	0.882	0.625	0.407	0.297		-	0.898	0.349	0.160	0.269
cold		-	0.523	0.328	0.372			-	0.288	0.126	0.328
sun			-	0.734	0.126				-	0.640	0.041*
wind				-	0.061					-	0.012*
warm					-						-

Secondly, for S+ samples, there is substantially less propylene glycol in runoff (**Figure 7-8c,d**) compared to DF. The anti-icer S+ has a much lower FPD concentration (ca. 442.8 g/L propylene glycol in S+, compared to 969.9 g/L in DF) and S+ is much more viscous. Therefore, comparing the percentage of propylene glycol mobilised from the tile relative to its starting concentration, the disparity between DF and S+ shrinks but nonetheless, some significant differences remain (**Figure 7-9**). There are significant differences between CADs for warm-1wk, warm-2wk, and sun-2wk treatments (**Table 7-3**). Using percent propylene glycol mobilised is useful to directly compare the specific runoff between CADs, however, the two products are used for different purposes (anti- vs de-icing) and thus in different quantities. As such, the more relevant metric for environmental management and airport operators is the amount of FPD in runoff (e.g., g/L) and biological oxygen demand (BOD₅).

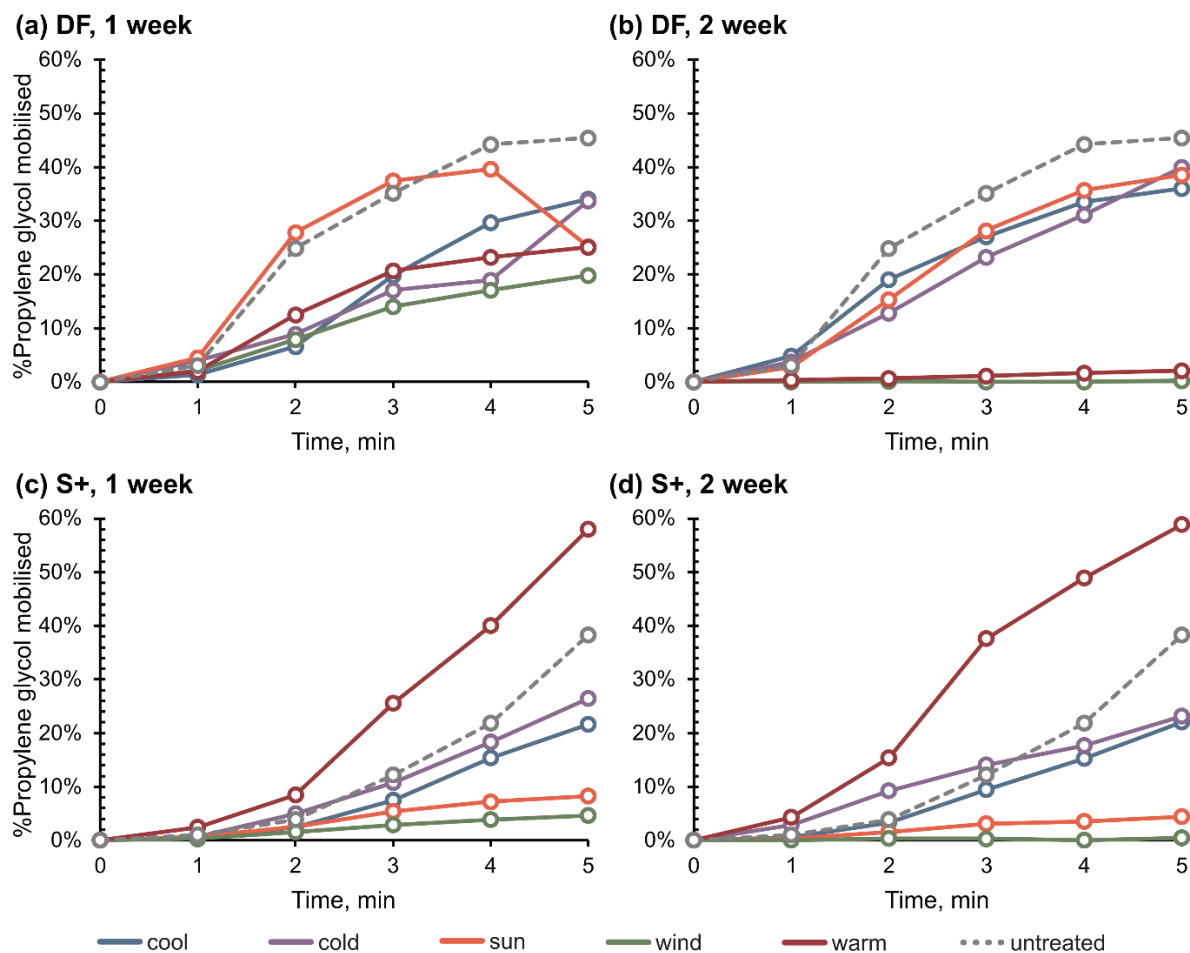


Figure 7-9: Percentage propylene glycol mobilised from tiles relative to starting concentration in the Kilfroast DF Plus (DF) aircraft de-icer (969.9 g/L) and anti-icer Kilfroast ABC-S Plus (S+) (442.8 g/L) following exposure to different meteorological conditions for 1- and 2-weeks.

Table 7-3: Pairwise comparisons from Kruskal-Wallis test of difference on the percentage of propylene glycol mobilised in runoff, relative to starting concentration in the CADs comparing Kilfrost DF Plus to Kilfrost ABC-S Plus, after 5-minutes rainfall. Where * indicates a significant difference (P = 0.05).

Percentage propylene glycol mobilised (relative to starting concentration in each CAD)									
(a) 1wk					(b) 2wk				
cool	cold	sun	wind	warm	cool	cold	sun	wind	warm
0.4047	0.6295	0.0843	0.3924	0.0436*	0.2315	0.2363	0.0372*	0.9650	0.0008*

Within S+ samples, treatments had an impact on runoff. Exposure to “cool” and “cold” treatments results in approximately 21.7 – 26.5 %v/v of propylene glycol mobilisation after 5-minutes compared to untreated-0wk (38.5 %v/v) and “warm” (58.4 – 59.2 %v/v) treatments (**Figure 7-9**). In contrast, “sun” and “wind” treatments had substantially lower mobilisation amounts (0.5 – 8.3 %v/v), resulting in a significant difference between sun-2wk and wind-2wk when compared to warm-2wk samples ($p \leq 0.0381$, **Table 7-3**).

7.3.2 Effect of weathering duration on CAD mobilisation

Duration of each treatment had little influence on the amount of propylene glycol mobilised, except for “wind” for DF (**Figure 7-8a-b**). Whilst there were visible differences in the samples for some treatments (e.g., “sun”, “wind” and “warm” became thin, dry films), the only condition where duration of weathering had a significant influence in mobilised amounts after 5-minutes rainfall is for “wind” for DF ($p = 0.033$, **Table 7-4**).

Table 7-4: Pairwise comparisons from Kruskal-Wallis test of difference in the amount of propylene glycol (mL) in runoff comparing 1-week to 2-week weathering durations at 5-minutes rainfall. For (a) Kilfrost DF Plus, (b) Kilfrost ABC-S Plus. Where * indicates a significant difference (P = 0.05).

a)					b)				
DF					S+				
cool	cold	sun	wind	warm	cool	cold	sun	wind	warm
0.898	0.640	0.317	0.033*	0.115	1.000	0.983	0.655	0.566	0.949

7.3.3 The impact of rainfall duration on CAD mobilisation

Longer durations of rainfall resulted in increased mobilisation of propylene glycol from tiles (**Figure 7-8**). Whilst there was generally a consistent increase in the amount mobilised per minute for S+ ($\bar{x} = 0.107 \pm 0.132$ g/min, mean $\pm$ standard deviation), there was greater variation for DF ($\bar{x} = 0.266 \pm 0.298$ g/min, **Figure 7-10**). High variability was observed in the runoff values for some treatments (e.g., cold-1wk minute-5, warm-1wk minute-2, and cool-2wk minute-2) but these are not consistent for most conditions and their direct replicates. A possible explanation for this could be poor homogenisation of a few samples as there is no discernible trend in meteorological treatment, CAD, date sampled, or minutes rainfall.

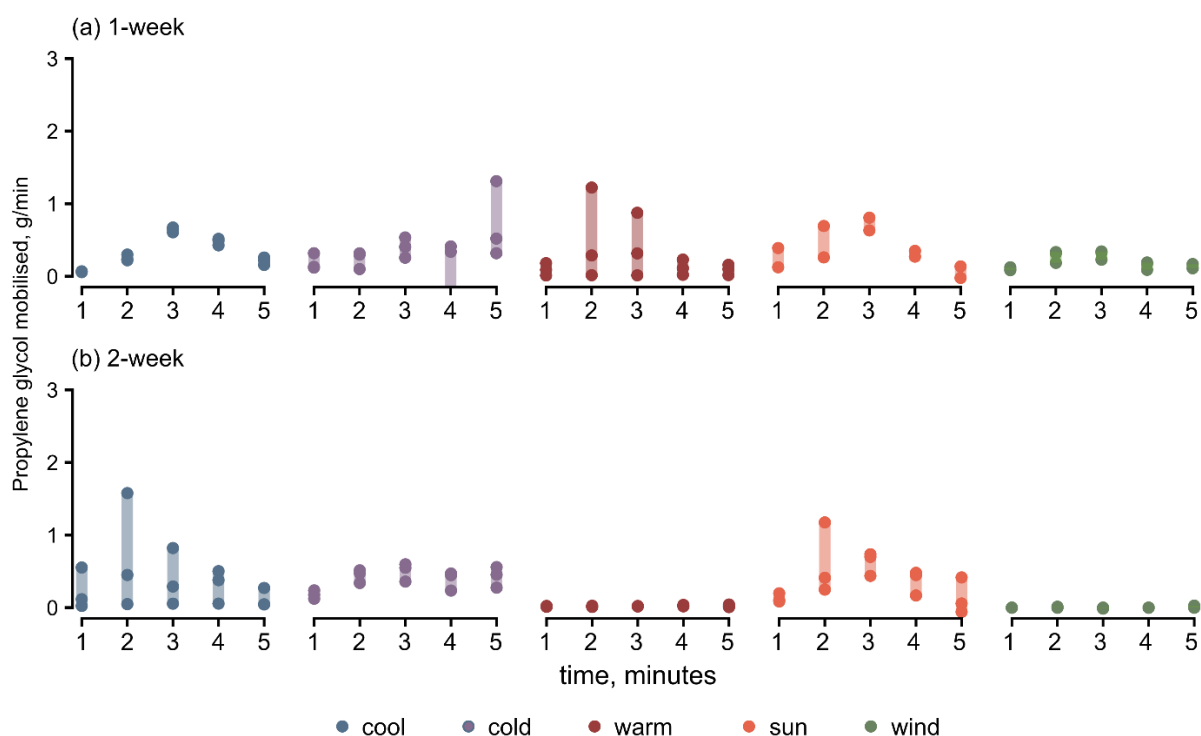


Figure 7-10: Amount of propylene glycol mobilised during each minute from Kilfrost DF Plus ("DF") airport de-icer in rainfall following exposure to different meteorological conditions for (a) 1-week and (b) 2-weeks duration of weathering for all replicates.

7.4 Discussion

The most influential factors on propylene glycol mobilisation were the specific CAD tested and the treatments with attributed drying effects (“sun” and “wind”), whereas the duration of weathering was much less influential. Treatments “wind” and “sun” were combinations of meteorological factors and further evaluation is required to extrapolate individual influences. “Warm” was treated similarly to “cool” and “cold” (in a dark space but at a higher temperature); “sun” had the additional influence of sunlight whilst at the same temperature; and “wind” then further developed this with airflow present in addition to sunlight and higher temperatures (**Table 7-1**). These treatment Effectively acting as controls for one another. First, comparing treatments at lower (“cool” and “cold”) to higher temperatures (“warm”), there are several differences. For DF, there was slightly less propylene glycol mobilised after 1 week of “warm” than “cool” or “cold” treatments, however, after 2-weeks exposure, there are significant differences, with only trace amounts of propylene glycol mobilised in warm-2wk (**Figure 7-8a,b**). Conversely, S+ samples, consistently had the highest amount of propylene glycol in runoff when exposed to “warm” treatment. The amount of propylene glycol mobilised was even higher than untreated-0wk samples which were subjected to rainfall immediately after CAD application (**Figure 7-8c,d**). It is therefore difficult to conclude what the impact of these different temperatures were given the vastly different behaviour between CADs in “warm” treatments.

The second comparison, between “warm” and “sun” treatments, used “cool” samples to assess the influence of sunlight on propylene glycol mobilisation. For DF samples, “sun” resulted in a similar amount of propylene glycol in runoff, however, sun-1wk had higher than expected amounts after 2-4 minutes of rainfall. Whereas, in S+ samples, “sun” treatments resulted in approximately a quarter of the amount of propylene glycol mobilised in runoff compared to “cool” and “cold” treatments, suggesting that each CAD could be affected differently by sunlight.

“Sun” (and to a lesser extent “warm”) were the only treatments to significantly differ between CADs. This difference between DF and S+ could be a consequence of the higher viscosity of the S+ anti-icer.

Comparing “sun” and “wind” treatments, there was consistently slightly less propylene glycol mobilised with the presence of airflow. In wind-2wk treatment there was negligible propylene glycol mobilisation, resulting in a significant difference between it and sun-2wk samples for DF de-icer.

These findings, along with observations of thin, dry films forming on “sun” and “wind” tiles, indicate that the combined influence of sunlight and air convection substantially altered the mobilisation of CADs in runoff, which has significant implications for the treatment approaches employed by airport operators. Understanding the timing of higher organic loads in effluents is crucial for the effective treatment of surface runoff at airports, especially during periods of intensive de-icing activities. Consequently, these findings have direct implications for airport treatment strategies, as they demonstrate that exposure to meteorological conditions affects the quantity of CADs released in runoff. For instance, not all CADs may be mobilised during the initial rainfall event, so even in the absence of de-icing activity between rainfall events treatment systems should anticipate the presence of CADs in runoff. Future research should expand on this and examine repeat rainfall-drying effects.

In all samples tested, <60% of propylene glycol was mobilised in runoff after 5-minutes rainfall. Visual inspection of tiles indicated that some CAD often remained, however, other factors may affect the amount of mobilised propylene glycol on glazed ceramic tiles, such as evaporation of the water fraction and either volatility or photodegradation of the FPD. The evaporation of water from the CAD in sunlight and airflow would increase the FPD concentration and thus viscosity of the CAD [54], thus more rainfall would be required, and this could explain the decrease in the mobilisation of samples exposed to “sun” and “wind” treatments – especially for the more viscous S+ anti-icer. Propylene glycol is reportedly non-volatile in water and dry substrates [48] and has a Henry’s law constant of $1.3 \times 10^{-8} \text{ atm} \cdot \text{m}^3/\text{mol}$ [55], indicating that it does not readily evaporate when exposed to weathering, has environmental persistence in liquid form and evaporation is therefore unlikely to be a significant transport vector. Furthermore, propylene glycol is not thought to undergo photodegradation in water or soil matrices [56] but has been reported as undergoing photolysis in the presence of a TiO_2 catalyst [57]. Furthermore, in all HPLC chromatograms of runoff samples there were no additional peaks observable, an initial indication that there was no, or very limited,

chemical degradation of propylene glycol. However, further work, such as untargeted mass spectrometry (e.g., QTOF), could ascertain any chemical degradation and would also allow for a much-improved understanding of the additives used in the commercial products and how these might affect the physical transport, chemical fate, and environmental impacts of CAD use.

The scope of this study was an initial investigation into the mobilisation of CADs in a rainfall event focussing on exposure to different meteorological treatments, based on observations from collaborators at a major UK airport (Pers. Communication, M. Murphy). The methodology of this study was highly controlled – exploring individual meteorological conditions by reducing external complexities (such as using regular, uniform sample tiles over more realistic substrates with more complex loss pathways) – but is, therefore, not truly representative of the environment in which these challenges exist. For example, the ground surfaces of airports are primarily asphalt, tarmac and concrete which have a rough texture and are semi-permeable, unlike the glazed tiles used here. The justification for this decision has been outlined in **Sample preparation**, but future research should consider using materials from airfields and measure additional loss pathways arising, such as CAD absorption or protection in surface cavities. The HardSPEC model was developed to consider the sorption and degradation of herbicides on hard surfaces (including concrete and asphalt) and estimate herbicide exposure in different surface and ground waters [58,59]. A modified version of this model could have substantial implications in follow on studies incorporating concrete and/or asphalt substrates and could provide substantial benefits to key stakeholders, such as airport operators, and assist in their winter operation plans. Furthermore, the scale of the experiments (5 mL sample on 219.0 cm² tiles) is vastly smaller than the ca. 500 L of CAD used on an Airbus A380 aircraft and ca. 30,000 L CAD used on a runway [60]. A factor also meriting additional scrutiny involves the design of the treatments, particularly how the configuration of the lid impacts "sun" treatments. The lid, used to remove airflow as a variable, could inhibit the full penetration of solar radiation, increase temperature or reduce volatile losses. While "warm" treatments were implemented as a control mechanism to counterbalance the effects observed in "sun" treatments, these potential limitations should be carefully addressed and considered in further research. Future studies

should also aim to build on the results presented here and, using larger-scale designs, assess combinations of weathering conditions with a greater range of CADs.

A factor also meriting additional scrutiny involves the design of the treatments, particularly how the configuration of the lid impacts "sun" treatments. The lid, used to remove airflow as a variable, could inhibit the full penetration of solar radiation, increase temperature or reduce volatile losses. While "warm" treatments were implemented as a control mechanism to counterbalance the effects observed in "sun" treatments, these potential limitations should be carefully addressed and considered in further research. This is compounded by not measuring factors such as solar intensity and wind speed of treatments in this study. These factors are important to quantify to fully understand the drivers influencing CAD mobilisation and future research should ensure they are accurately recorded. Future studies should also measure further physical and chemical parameters, such as viscosity and photodegradation. Additional research should investigate the mass-balance analysis of CAD transport, including the approximately 30% of CADs that are not immediately deposited on the stand [60]. Further research should evaluate the transport of CADs from application to mobilisation throughout drainage networks and treatment systems to more fully understand the physical transport and chemical fate of CADs before runoff is ultimately discharged into receiving waters to reduce stressors on the environment and river ecosystems.

7.5 Conclusions

This study has demonstrated that exposure to meteorological treatments had an influence on the mobilisation of propylene glycol from airport anti- and de-icers deposited on ground surfaces following application. The largest contributor to propylene glycol mobilisation was the choice of CAD. The more viscous Kilfrost ABC-S Plus (S+) anti-icer resulted in substantially less propylene glycol mobilised from the sample tile. However, S+ has a much lower starting concentration of propylene glycol than the de-icer Kilfrost DF Plus (DF). Nonetheless, there is generally proportionally less propylene glycol mobilised from S+ sample tiles in relation to starting amounts than in DF. Different winter temperatures (+5 and -5 °C) had little effect on

mobilisation, whereas exposure to sunlight and airflow ("sun" and "wind" treatments) had a more considerable effect on mobilisation. When exposed to "sun" and "wind" treatments, CADs were observed drying and forming thin, dry films, as opposed to the viscous liquid of freshly applied CADs. Duration of each treatment also had a limited effect on the mobilisation of propylene glycol, with some exceptions (e.g., "wind" for DF samples which has substantially less propylene glycol in runoff after 2-weeks than 1-week exposure). These results provide a significant step towards understanding the transport mechanisms of CADs following application and thus inform treatment strategies to better manage winter surface runoff and prevent ecosystem degradation in receiving waters.

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Data access statement

Supporting data for this study can be accessed through the Cranfield University repository (CORD): DOI 10.17862/cranfield.rd.23676339.

Conflicts of interest

The authors declare there are no conflicts of interest for this research.

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Supplementary information

Appendix A – HPLC calibration curves and quality control

The calibration and quality control data for HPLC analysis of runoff. Standards were made up as %v/v but are also shown here in mg/L for each FPD and sampling event. The slope and intercept for the calibration curves are reported, along with R^2 from regression analysis (Data Analysis package, Microsoft Excel), and the limit of quantification (LOQ). LOQ was calculated from the concentration of the lowest standard plus percent accuracy (calculated concentration divided by expected concentration).

Treatment	Duration	Range, %v/v	Range, g/L	Slope	Intercept	R^2	LOQ, g/L
cool	1wk	0.5 - 3.0%	5.20 - 31.20	1.565E+08	-1.830E+04	0.9993	10.38
	2wk	0.5 - 3.0%	5.20 - 31.20	1.568E+08	-2.070E+04	0.9994	10.38
cold	1wk	0.5 - 3.0%	5.20 - 31.20	1.563E+08	-1.814E+04	0.9993	10.38
	2wk	0.5 - 3.0%	5.20 - 31.20	1.522E+08	-1.557E+04	0.9994	10.39
sun	1wk	0.5 - 3.0%	5.20 - 31.20	1.575E+08	-2.643E+04	0.9990	10.31
	2wk	0.5 - 3.0%	5.20 - 31.20	1.565E+08	-1.830E+04	0.9993	10.38
wind	1wk	0.5 - 3.0%	5.20 - 31.20	1.565E+08	-1.830E+04	0.9993	10.38
	2wk	0.5 - 3.0%	5.20 - 31.20	1.568E+08	-2.070E+04	0.9994	10.38
warm	1wk	0.5 - 3.0%	5.20 - 31.20	1.568E+08	-2.460E+04	0.9993	10.40
	2wk	0.5 - 3.0%	5.20 - 31.20	1.568E+08	-2.460E+04	0.9993	10.40
untreated	0wk	0.5 - 3.0%	5.20 - 31.20	1.575E+08	-2.643E+04	0.9990	10.31

S7-1: Range of standards for propylene glycol standard, the calibration curves, regression analysis, and limit of quantification (LOQ, minimum standard + %accuracy) for each treatment and treatment duration.

8 Overall Discussion and Conclusions

8.1 Introduction

Previous chapters have highlighted that undesirable river biofilms (URBs, formerly “sewage fungus”) remain a persistent issue globally, even in higher-income countries with advanced wastewater treatment, such as England (**Figure 2-4**). Despite their continued prevalence, there has been limited scientific investigation of URBs since the mid-1980s [1–3]. As such there is limited current data in literature on the industries implicated in URB proliferation (**Table 2-1**). However, airport de-icing has been well-documented as sources of URBs [4–10]. Airports provide an opportunity through which to continue URB research and their role in reflecting and further impacting water quality in rivers, owing to their point source discharges and the relative simplicity of the organic chemicals emitted. The overall aim of this thesis was to investigate how the delivery of airport de-icer contaminated runoff to receiving waters impacts the growth and composition of sewage fungus. To address this aim, four themes were developed each addressing an objective (**Figure 8-1**).

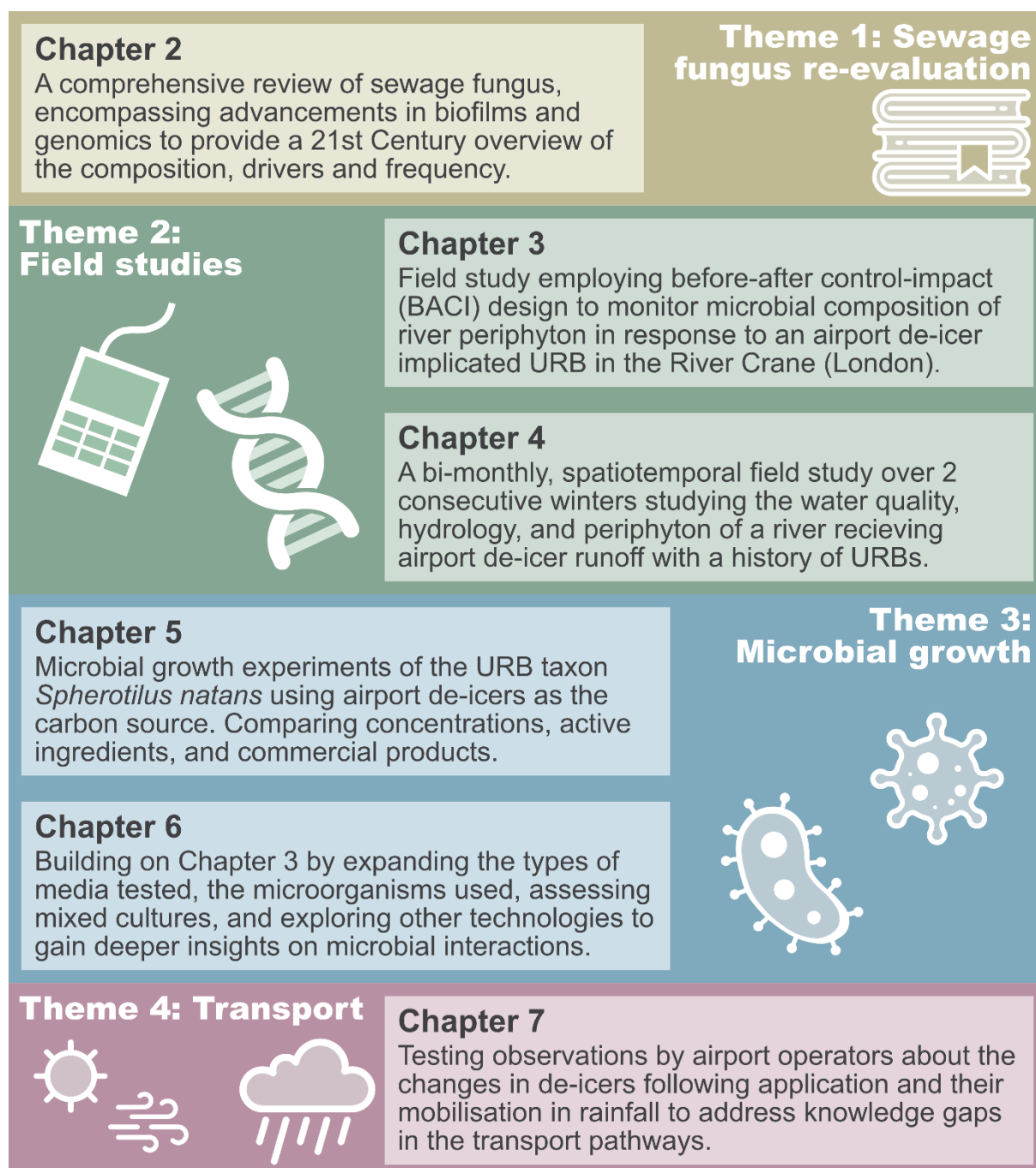


Figure 8-1: An overview of the thesis, with chapters categorised under four themes, each addressing an overall objective. Reproduced from Figure 1-2.

The first objective was to reassess our scientific understanding of sewage fungus in the 21st Century. A comprehensive review of the available literature was undertaken which highlighted the continued extent of URB prevalence. Although bacterial

communities and biofilms are integral self-purification components of rivers [11,12], URBs cause significant ecological damage to rivers [1,13]. The rapid growth of URBs severely depletes dissolved oxygen (DO) [14] (stressing more sensitive aquatic organisms [15–18]) and blankets the riverbed (destroying habitats for invertebrates [19–21] and for fish spawning [22,23]). Impacts of poor water quality are further exacerbated by reduced hyporheic exchange flows (HEFs, the interchange of river and ground waters) which play important roles in the biogeochemical filtering of macro- and micro-pollutants and provide aquatic habitats [24,25]. The growth of URBs is fundamentally caused by high organic loading (such as from airport de-icing) and has long been used as a bioindicator of organic pollution [1].

The second objective sought to investigate the changes in water quality and microbial composition of river periphyton in a river receiving airport surface runoff during winter through an extensive BACI (before-after control-impact) field study. Immediately before and during a visible URB outbreak, the genera *Sphaerotilus* and *Rhodospirillum* were highly dominant in periphyton resulting in a significant reduction in diversity. The scale of this taxonomic shift was most prominent at the family taxonomic level where *Comamonadaceae* was responsible with a relative abundance of up to 81.4%. These taxa were not present in samples before the URB manifestation. However, they remained part of the periphyton throughout the second sampling year, highlighting the long-term influences of an URB on periphyton.

Using findings from the above field study, the third objective of this thesis (to evaluate the growth of sewage fungus taxa in airport de-icer based media) was addressed by turbidimetric assessment of microcosm growth using airport de-icers as the sole carbon source. *Sphaerotilus natans* showed strong growth on common freeze-point depressants (FPDs) (propylene glycol, ethylene glycol, and sodium acetate), where: (i) sodium acetate had a significantly shorter time-to-detection (TTD, “lag phase”) and (ii) a higher maximum rate of change (maxROC, “growth rate”), followed by propylene glycol and finally ethylene glycol. Furthermore, there was (iii) a limiting maxROC (10 – 50 mM C for sodium acetate and propylene glycol, and 50 – 100 mM C for ethylene glycol) and (iv) maximum optical density (maxOD, “total growth”) was highly concentration dependant but with no significant difference between FPDs. Moreover,

there was (v) no significant difference between pure-FPDs and their commercial airport de-icers (CADs), indicating their additives have limited impact on URB growth in rivers – but additives nonetheless impact other aquatic organisms [26–28].

Finally, the fourth objective (to examine the impact of meteorological conditions on the mobilisation of airport de-icers in surface water runoff) addressed the transport of CADs in airport runoff. Key findings were that exposure to meteorological conditions following CAD application decreased the mobilisation of CADs in runoff. Sunlight and wind were the conditions found to have the greatest effect, resulting in less propylene glycol being mobilised from CADs. However, the biggest impact on propylene glycol mobilisation was the specific CAD used.

8.2 Whole system assessment of the influences on airport de-icer use and their environmental impacts

The results presented in each theme of this thesis achieve the aim of this project by improving our understanding of the transport of airport de-icers, the influence they have on periphyton composition, the ability of URBs to utilise FPDs, and why a river may be particularly susceptible to URBs. It is important to examine the factors that contribute to URB development as a consequence of airport de-icing. **Figure 8-2a** outlines the transport pathways of airport de-icers and the riverine influences on URB growth. Different elements of anthropogenic modification and construction, along with natural processes, can either exacerbate or mitigate the impact of airport de-icing and of URBs which feed into a holistic understanding of minimising ecosystem deterioration (**Figure 8-2b**). For example, excess widening and straightening of river channels and modified uniform, flat riverbeds result in shallower and less energetic flows. The River Crane has been modified over centuries of industrial activity and has been used throughout this thesis as a case study and as a study site. Whilst width varies across study sites (5.3 – 14.3 m), the river reaches its maximum width at Site 7 (14.3 m) where it had a consistently shallow measured depth during fieldwork sampling ($\bar{x} = 18.4 \pm 2.1$ cm) and vertical, constructed banks (**Chapter 3** and **Chapter 4**). Consequently, such rivers experience less bed scour and fine particles more easily clog pores, reducing invertebrate habitats and HEFs – a natural self-cleaning process of rivers – further exacerbating impacts of high pollutant loading [24]. In addition,

excess shading from overhanging riparian vegetation (such as trees) reduces available sunlight for freshwater plants, restricting their growth and resulting dissolved oxygen (DO) production – further reducing resilience of the watercourse to the impacts of pollution. These factors, especially in combination, make rivers more susceptible to unconstrained URB proliferation and the subsequent ecological impacts.

This thesis has primarily used Heathrow Airport and the River Crane (London, UK) as a lens through which to investigate URBs. However, the findings can be extrapolated to other rivers and pollution sources. The following assessment of the influences on URB development, particularly concerning those arising from airport de-icing, again uses the River Crane to illustrate the influences but applies to many rivers in both urban and rural environments.

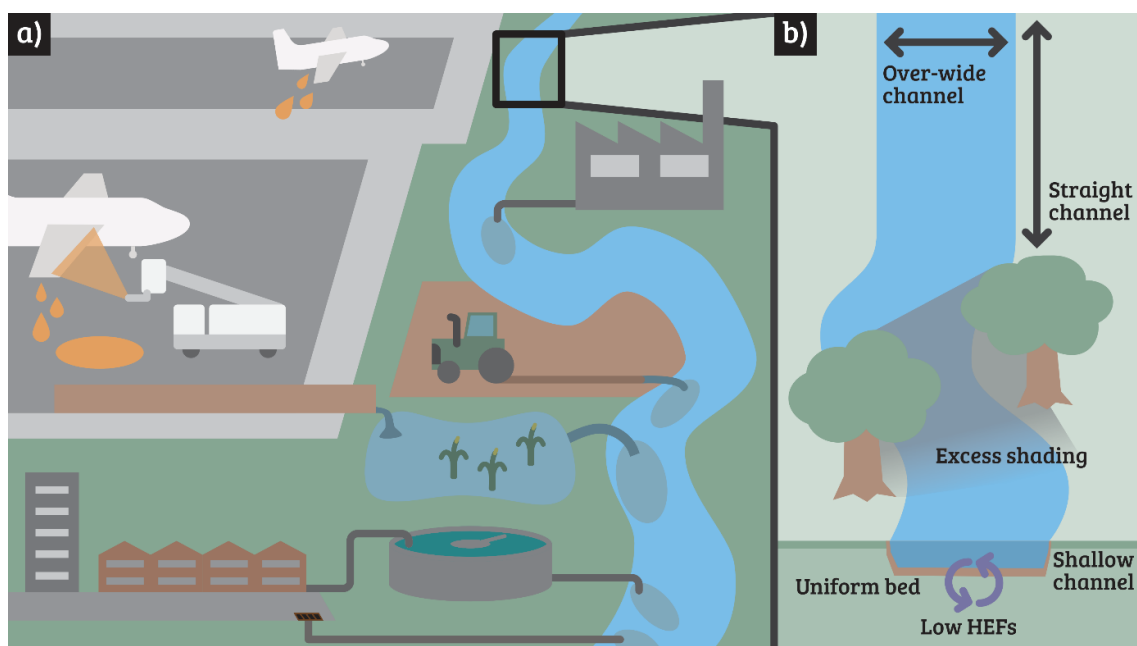


Figure 8-2: An overview of pressures on riverine environments leading to undesirable river biofilms (URBs), including (a) the inputs and (b) the factors in the riverine environment that influence the growth of URBs. HEFs = hyporheic exchange flows.

8.2.1 Meteorological influences on airport de-icing

Firstly, we examine the factors that govern the application of CADs. Primarily, weather conditions drive the use of CADs. When temperatures fall below a specific threshold, airports initiate de-icing operations [29]. The substantial size of aircraft and airfields necessitates the use of considerable quantities of CADs, resulting in vast quantities of carbon and biochemical oxygen demand (BOD₅) that necessitate proper management (approximately 500 L de-icer is used for an Airbus A380 with a BOD₅ of 177.0 kg and 30,000 L de-icer on a runway with a BOD₅ of 8,100.0 kg) [30]. Each specific CAD formulation has different BOD₅ and are a consideration for airport operators, whilst balancing the performance of products (**Table 5-1**).

Furthermore, weather conditions further influence the mobilisation of CADs following their application, as demonstrated in **Chapter 7**. Different meteorological conditions impact the extent of CAD mobilization in runoff, with sunlight and wind exerting the most notable influence, drying CADs into more viscous liquids or even into thin, dry films. Subsequently, rainfall facilitates CAD mobilisation into airport surface runoff and drainage networks. However, after exposure to wind and sun, less CAD might be mobilised in a precipitation event. Thus, the first rainfall event may not be sufficient to mobilise all CAD from ground surfaces and some may be mobilised in subsequent rainfall. Treatment systems (such as aerated wetlands [31–35] and moving-bed biofilm reactors (MBBRs) [36–38]) must, therefore, be prepared for additional loading, even if there has been no CAD use since. . Increased rainfall will increase the discharge of receiving rivers, and therefore dilute CADs, but can also affect background water quality through increased surface runoff (e.g., from urban centres [39,40] and agriculture [41,42]). The influences that weather has on the transport of CADs are complex but cold weather necessitates their use which later contributes to potential ecological damage in rivers [10,43,44].

In this thesis, the effects of weather conditions on the mobilisation of CADs were examined using a laboratory mesocosm approach, chosen because it was better able to remove external complexities of environmental systems so that the effects of temperature, wind and sunlight could be quantified. For this purpose, small, glazed tiles were used to minimise the impacts of surface characteristics as to not influence

the storage or mobilisation pathways (e.g., absorption into the material, or protection in surface cavities). Domestic bathroom tiles were chosen because of their uniform shape, size, texture and have edges conducive to collecting representative runoff (**7.2 Methods**). However, they are not fully representative of the system they seek to emulate. To align the study better with applications at airfields, larger scale studies should be adopted in future research using substrates employed at airfields and larger quantities of CAD. However, this would require a different approach to quantifying mobilisation (e.g., multiple events, of different magnitude, duration and frequency). Furthermore, future studies should use a full factorial cross and a greater number of treatments.

This would be complemented by a large-scale full mass-balance study across an entire airfield – including sampling and monitoring of de-icing areas, drainage network, treatment systems, and discharges into surface waters. Nevertheless the study conducted in (**Chapter 7**) presents novel research which, for the first time, scientifically investigates the influence of weather exposure on the mobilisation of CADs in rainfall, as observed by airport operators (Pers. Communication M. Murphy).

8.2.2 Treatment and discharge of CAD contaminated runoff at airports

Following rainfall, CADs applied at airports are mobilised rapidly into drainage networks to help prevent flooding of critical operational areas [45]. However, within drainage networks, some CAD deposition occurs, forming “gelatinous blobs” that require mechanical removal (Pers. Communication, M. Murphy). These will also gradually dissolve in runoff from subsequent rain events. Treatment systems must, therefore, be prepared for additional CAD loading, long after de-icing activities may have ceased.

Treating de-icer runoff is challenging, in part because of the requirements to carefully dose runoff with appropriate amounts of nutrients such as nitrogen and phosphorus to facilitate biological degradation, but also because of the high BOD₅ of FPDs [30,35]. Commonly used aircraft CADs have a BOD₅ of approximately 354 g/L with an average of 500 L CAD used for an Airbus A380. The resulting BOD₅ is approximately 177,000 g per aircraft, the equivalent of the typical BOD₅ of wastewater generated by 2,950

people in a day [30,31]. In addition, de-icing a single runway results in 8,100,000 g BOD₅ (30,000 L CAD at 270 g/L BOD₅), the equivalent of 135,000 peoples wastewater [30]. With an average of 650 aircraft taking off from Heathrow Airport each day in 2016 [46,47], the surface runoff from airfields has an extremely high BOD₅ during cold periods and presents considerable challenges in effective treatment. At Manchester Airport (Manchester, UK), the mean aircraft de- and anti-icer use was 623,507 L/year between 2010 and 2013, in addition to 545,078 L/year of pavement de-icing fluids [48]. Therefore, with an average BOD₅ of 354,000 mg/L for aircraft de-icers and 250,000 mg/L for pavement de-icers [48], there could be an annual BOD₅ of 357 tonnes over a single winter period. As reported in **Chapter 7**, not all CAD may be mobilised in the first rainfall event. Nevertheless, even after dilution in rainfall or snow melt, a BOD₅ of >20,000 mg/L is not uncommon in runoff waters [17,49]. The high BOD presents a significant challenge for airport operators and the treatment systems.

Various treatment systems can be employed by airports [33,50,51] which commonly use either mechanical systems such as moving bed biofilm reactors (MBBRs) [36–38] and/or nature-based solutions like aerated wetlands [31–35]. The optimal functioning of these treatment systems relies on low, continuous loads, so balancing reservoirs are often used in conjunction with active treatment to distribute influxes more evenly. Large balancing ponds can serve as effective buffers for treatment systems; lowering the water level prior to anticipated heavy rainfall events, creates more holding capacity, and alleviates the strain on the active treatment systems. This strategic approach enables the treatment systems to manage stormwater influx at a lower rate over an extended duration, preventing system overload. There are significant variations in the performance of these systems which need optimising for each specific airfield [15,48,52,53]. For example, aerating wetlands and reedbeds increases biological activity thus enhancing FPD degradation [32,54] and leading to BOD₅ reductions of 74 – 98 % [31,53]. The effectiveness of CAD treatment is further constrained by insufficient availability of crucial nutrients [34,35,55], such as nitrogen and phosphorus, which are essential for bacterial activity but are not present in modern de-icing formulations [56,57]. Thus, careful dosing of additional nutrients is required in many CAD treatment systems. Furthermore, biological activity, and therefore the effectiveness of treatment, may also be affected by colder temperatures [58,59].

Treated effluents are then typically discharged into freshwaters, most frequently rivers adjacent to the airport, where they can have considerable impacts [43,44], including the manifestation of URBs [10,60]. When treated well, effluents from aerated wetlands have the potential to improve river quality due to the high levels of DO in treated effluents following aeration. However, DO is often found to decrease downstream of airport outfalls [43].

Whilst treatment systems at airports are carefully managed, airport runoff during colder periods has extremely high BOD₅ which must be effectively treated in order to discharge effluents within permitted consents [33,52]. This is undeniably challenging and is reflected in numerous reports of environmental issues downstream of airport outfalls, including fish death and most notably URBs [4–8,10,60–62].

8.2.3 River conditions influencing impacts on ecosystem resilience to poor water quality

High BOD₅ inputs result in considerable depletion of DO in rivers, with resulting negative ecological impacts [15,49,56]. However, background water quality in urban areas is often very poor [63,64], with no rivers in the UK that achieve good chemical status [65,66]. The largest single sectors responsible for this are the water industry (36%) and agriculture (40%), with urban diffuse pollution following (18%) [66,67]. For example, the River Crane catchment, used as a case study throughout this thesis, is mostly urban and sub-urban with patches of grassland and agriculture (**Figure 4-1**). It is therefore likely already under significant strain from a range of inputs [68]. Consequently, the River Crane has had several severe ecological incidents in recent years [7,60,69,70] and has also been studied by citizen scientists, local action groups and academia [68,71–76].

In addition to water quality, urban rivers often suffer from hydromorphological alterations (i.e., changes to channel form, river discharge, and sediment loads) which can damage habitat quality and can affect the ability of a river to process nutrients and contaminants. A river possesses several mechanisms for contaminant processing, uptake and removal, such as aquatic plants, the deposition/storage of sediments, and sediment-bound contaminants. The hyporheic zone of a river, a region of hydrological connection between rivers and groundwaters that exists within the riverbed and banks,

is an important part of a river's self-purification [24,25,77]. HEFs are substantially reduced by excess fine sediments, which often increase in surface runoff and in rivers as a result of urbanisation and intensive agriculture [76,78]. Excess fine sediments clog pores in the bed which causes habitat loss for fish and invertebrates in addition to preventing HEFs, further degrading water quality. Moreover, heavy modification of the channel geomorphology, such as a straightened and widened channel, the subsequent shallow flows, and a uniform, flat bed, results in limited mobilisation of fine sediments which causes an additional decrease in HEFs [24,79] – all of which are features of the River Crane. A decline in HEFs leads to a further reduction in the self-purification of rivers, exacerbating poor water quality, negative ecological impacts, and resulting in an increased susceptibility to extensive URBs which further worsen the ecological condition of the river and surrounding environments.

An additional consequence of low energy, shallow flows and flat, uniform beds is the river's limited ability to increase DO levels, which is important for a whole range of aquatic species. In rivers with less modified channel hydrogeomorphology, flows have more energy facilitating greater DO concentration from riffle beds, standing waves and increased water circulation patterns [80,81]. In turn, this improves HEFs which further elevate DO concentrations [82] and facilitate nutrient cycling to self-clean watercourses [79,83]. Furthermore, the high level of shading along the River Crane [74] decreases the amount of in-channel aquatic plant growth which limits the biological generation of DO, as well as reduced biological uptake of nutrients in rivers. Low DO in rivers stresses aquatic fauna and causes further ecological degradation. In addition to impacting DO levels, the limited aquatic plant growth results in limited food for aquatic fauna, reduced nutrient cycling, and habitat loss (in the river and riparian zone). Each of these has severely negative impacts on river ecology and overall river health.

The River Crane is an exemplar of these compounding factors to diminish ecosystem resilience to poor water quality. A field study was conducted to monitor the benthic microbiome over 2 winter seasons in the River Crane along with water quality and channel characteristics (**Chapter 3** and **Chapter 4**). The main focus of this was 16S rRNA sequencing of periphyton taken from bed substrates which supported previous

identification of “sewage fungus” taxa (*Sphaerotilus* and *Zoogloea*) [10,84–86] during an URB shifted microbiome but many taxa were not present in any samples that have been well documented in literature, including *Beggiatoa*, *Leptomitius*, and *Thiothrix* [10,84–86].

A novel component of this study was the temporal data collection which covered not only the URB event itself but covered months leading up to it and afterwards. The spatiotemporal design was an important and deliberate choice in constructing the methodology and is a substantial strength of this research. It allowed not only for spatial monitoring at two different control sites – pre-airport influence (Site 1) and immediately upstream of airport outfalls (Site 3) – but also to monitor throughout the winter period. Bi-monthly sampling was designed to capture prior to any especially cold weather (October), at the start of colder weather (December), towards the end of winter but still colder weather (February) and into spring when temperatures had begun to rise and after de-icing had likely ceased (April).

Periodic periphyton sampling is a good indicator of longer-term river quality [87,88] and complements other types of water quality monitoring data. Monitoring water quality in catchments is challenging and complex [89]. No single approach gives a holistic and comprehensive understanding of relevant, precise, and reliable water quality in space and time [89]. Many handbooks, guidance and directives exist for water quality monitoring programs [90–95] but each watershed has unique constraints and pressures and there is no ‘one-size fits-all’ solution [89]. High-frequency or continuous monitoring of water quality throughout a catchment is able to capture representative spatiotemporal data but is more limited in the parameters it monitors [96,97]. Conversely, spot-sampling (routine or reactive) enables a much wider array of water quality parameters to be analysed (both in-field and samples taken for laboratory analyses). However, spot-sampling requires a physically visiting the site costing human, logistic, and financial resources. Furthermore, it is unable to capture a full temporal perspective of water quality in a dynamic river system. A hybrid approach includes automated monitoring where water samples can be collected at user-defined time intervals, flow values, or triggered by specific water quality thresholds exceeded by sondes, and retrieved for additional analyses [98,99].

Alternatively, biomonitoring can be used to determine short and long-term water quality with a wide array of techniques applied in river systems [90,100–103], including macroinvertebrates [104–107], benthic diatoms [108–111] and microorganisms [60,112–117]. Periphyton, as a biomonitor of water quality, has been heavily used as a key indicator of water quality throughout this thesis (**Chapter 3** and **Chapter 4**) as it integrates all stressors (including short term, intermittent discharges) which can easily be missed with periodic point sampling and may not be captured by the more limited parameters measured by multi-parameter continuous sondes [118]. This research has attempted to integrate several of these approaches (high-frequency multi-parameter sondes alongside spot sampling of chemical water quality, hydrogeomorphology, and periphyton biomonitoring), each highlighting some of the limitations of that approach. A multi-parameter sonde operated by the regulator had a substantial change in conductivity values when replaced, and it stopped working part way through the second year of sampling (**Figure 4-9b**). Furthermore, in recent years the spatial density of Environment Agency water quality sondes has decreased, including in the River Crane, due in part to financial constraints, making it more challenging to identify water quality issues. In addition, handheld probes used in spot sampling developed faults and resulted in missed dates for measurements creating challenges in subsequent multivariate analyses (such as PCO and DistLM). Nevertheless, when brought together, the chemical and physical water quality data presented in this thesis and their relationship to changing periphyton and URBs is consistent with other studies looking at multivariate analysis water quality and URB taxonomic data [119]. Although future research would benefit from near-continuous, long-term monitoring complemented by discrete chemical water quality, hydrogeomorphological and periphyton (for DNA sequencing) synoptic sampling, where measurements and samples are taken spatially within a few hours to be representative of the river.

The most striking finding presented here was the shifted periphyton composition 1 week prior to a visible URB event. Feb-21 sampling (16/02/2021) revealed a significant change in periphyton composition compared to previous months with characteristic URB taxa dominant, including genera with historic strong association with URBs (e.g., *Sphaerotilus*) and those not previously linked with URBs (e.g., *Rhodoferrax*) but were also dominant in the visible URB 1 week later (“URB-21” sampling on 24/02/2021).

This shift prior to a visible URB manifestation suggests that the microbial community had already changed in response to organic pollution and additional loading enabled it to rapidly grow, covering the majority of the riverbed for a long stretch downstream. The shifted periphyton was present in all sites in Feb-21, stretching 1.9km downstream showing the large spatial influence of URBs.

URBs are believed to be largely comprised of bacteria, but other taxa – such as fungi, algae, archaea, and protozoa – are believed to play an important role. This study did not examine the role of these non-bacterial microorganisms as 16S rRNA sequencing on the V3-4 region was performed. Moreover, the role of phototrophs in URBs has not been fully understood and the relationship between chemical composition and light governing the composition of URBs is, therefore, unclear. As a result, future research should be undertaken to examine the role of these organisms using next-generation sequencing, such as 16S V4-5 region (archaea) and 18S or ITS (fungal). Furthermore, whole genome sequencing (WGS) offers valuable and deeper insights into the microbiome. Future studies employing WGS should consider URBs in the context of wastewater epidemiology [113,115,120] and One Health [121–124]. Additional research could further support the development of a rapid diagnostic test of benthic biosentinels of organic pollution and enable quick response of regulators to early identification of pollution events and locate sources allowing for mitigations to be put in place to limit ecosystem degradation, as proposed in **Chapter 3**.

8.2.4 Microbial insights into the influences of de-icers on URB growth

URBs are a natural response to organic pollution in rivers more broadly. The growth of URB taxa has been studied but not in the context of airport de-icing [23,125]. In **Chapter 5** and **Chapter 6** the growth of URB microorganisms were monitored in microcosms with the sole carbon source of different freeze-point depressants (FPDs) and commercial airport de-icers (CADs) to address this knowledge gap. These experiments found *S. natans* was readily able to use common FPDs propylene glycol, ethylene glycol and sodium acetate for growth, with total growth (maxOD) having a strong dependency on carbon concentration. There were few differences in growth kinetics (TTD, maxROC, and maxOD) between pure-FPDs and their commercial products suggesting the additives in commercial formulations do not substantially

impact *S. natans* growth, despite being toxic to aquatic organisms. However, *S. natans* was not able to utilise sodium formate and *Zoogloea oleivorans* was seemingly unable to use any FPD for growth.

These studies primarily used optical density to assess microbial growth as a robust and well-established method. However, several shortcomings in the methodology are noteworthy. Firstly, 200 μ L microcosms were used with a fixed volume of media and culture. This is not truly representative of the flowing rivers in which URBs grow where nutrients are continually replenished. And secondly, the suspended culture in microcosms isn't reflective of the fixed biofilm of URBs in the environment. These limitations underscore the need for further refinement in experimental design to better mimic the dynamic conditions of natural river environments. Future studies could explore alternative methodologies that account for the continuous replenishment of nutrients in flowing rivers, such as microfluidic devices [126,127] or flow cells with microscopy [128]. Alternatively, experiments could be scaled up to more realistic set-ups at mesocosm scale, such as flumes or recirculating channels [23]. Such approaches would ultimately contribute to a more comprehensive understanding of URB dynamics and their ecological implications in aquatic ecosystems. Notwithstanding these limitations, the results obtained from these high-throughput mesocosms offer valuable insights into the relative growth of individual URB taxa in a vast range of media. In a single experimental run, two 100-well plates could be monitored with 10 replicates and 5 blanks (no added bacteria) for 6 different treatments per plate. This setup enabled the testing of a wide array of treatments within a short timeframe. Therefore, insights gained from these new contributions to knowledge could be leveraged for selecting optimal treatments to test in future experiments using microfluidic devices, or larger scale flumes.

8.3 Undesirable river biofilms: Good or bad?

Throughout this thesis, URBs have been consistently portrayed in a negative light. However, delving deeper into their ecological dynamics has a much more nuanced perspective. URBs are able to degrade excess organic matter within river systems, contributing to the self-regulation and cleaning of the ecosystem [1,13]. The presence of even large URBs indicates that a river is still able to manage the organic loading

and has not reached a state of ecological collapse. Therefore, URBs play an important role in maintaining a good chemical status of rivers.

Nevertheless, the impacts of rapid and prolific growth of URBs results in several detrimental consequences and have been fully detailed in **2.1 Introduction**. In summary, the rapid heterotrophic activity of URBs results in the substantial depletion of dissolved oxygen levels stressing many other, more sensitive, aquatic species [14]. Their extensive growth can smother the riverbed, disrupting habitats crucial for various aquatic organisms (such as macroinvertebrates) [19,20]. Furthermore, URBs can cause longer-term impacts on ecosystem health. For example, during critical periods like fish spawning, prolific URBs growth can destroy habitats and significantly diminish fish populations for the entire year [13,16]. Moreover, the ‘smothering’ of the riverbed can lead to the death of aquatic plants, exacerbating ecological damage [129,130]. Additionally, once high organic loading ceases and the URB begins to breakdown and decay, vast quantities of nutrients will be released back into the watercourse, which could include toxic or harmful chemicals previously sequestered within the now decaying biofilm and can be released back into the watercourse [131–134]. URBs are also able to proliferate over large distances stretching downstream, as their negative effects can extend over a significant stretch of the river if left unchecked [2,11,135]. Conversely, the negative impacts of excess organic loading, if not degraded by heterotrophic URB activity, could span much longer river reaches and result in poorer ecological health.

In summary, are URBs a net positive or negative? Whilst this is not something that can readily be objectively answered, it remains my belief that URBs are a net negative for ecosystem health. URBs improve chemical water quality, and this is an important benefit, however, due to the magnitude of their growth, the negatives, such as DO depletion and habitat destruction, become overwhelming to ecological health. Language such as the “blanketing” and “smothering” of riverbeds has long been used to describe URBs which highlights the scale of its domination of the river reach. If the growth of URB microorganisms were not so prolific the benefits may readily outweigh impacts. However, in many rivers, altered by centuries of human and industrial activity, there is poor resilience to pollution events, exacerbating their impact.

Moving forward, enhancing the resilience of rivers to poor water quality emerges as a critical strategy for improving chemical and ecological status and mitigating the severity of URBs. This may involve interventions such as narrowing channels, re-adding meanders, reducing fine sediments, and opening the riverine canopy [136–138]. These measures not only promote ecological restoration but also offer potential benefits for water quality improvement [139,140]. For instance, by increasing the presence of aquatic plants by opening up the riverine canopy, there is the potential to boost DO production through photosynthesis. Aquatic plants have also been shown to decrease FPD content in waters [141]. Additionally, improved river channels may enhance HEFs, facilitating the purification and cleaning of waters through natural filtration processes [24,25,79,83]. Furthermore, such interventions could create more diverse habitats and increase overall ecosystem resilience to pollution, ultimately contributing to the mitigation of URB proliferation and its associated impacts on aquatic ecosystems. These proposed strategies merit further investigation and represent promising avenues for future research in the field of river restoration and water quality management.

8.4 The River Crane: A Petri dish for URBs

The River Crane is a highly modified and degraded ecosystem within an urbanised catchment of various land uses (65.2% urban and sub-urban, also with woodland, arable/horticultural, and grassland areas) [142]. The compounding influences within the riverine environment, such as a modified channel with a stable riverbed and excess shading, in addition to poor chemical quality caused by point source and diffuse inputs, results in a low resilience to any additional pollutant loading (**Figure 8-3**). Consequently, the River Crane is highly susceptible to URBs.

A holistic approach to river restoration is needed within the River Crane and can be applied more broadly to rivers in urban and rural settings with a history of URBs. Numerous approaches could be used based on an appreciation of the hydrological, geomorphic and biochemical processes that contribute to a reduced resistance to organic pollution [151]. For example, a simple and easily achievable element of this can be to open up the canopy above and directly alongside the channel. Decreasing direct shading of the river would increase aquatic plant growth which not only increases DO and increases nutrient cycling but provides a habitat for other aquatic fauna. In addition, the River Crane would benefit from a reduction in channel width and re-introduce meanders to increase flow velocities during high flows (which often occur in winter) to (i) scour URBs (ii) mobilise the riverbed sediment to flush out fine sediment and support HEFs, and (iii) add variation to the otherwise uniform bed. These physical changes to the river should make it less susceptible to URBs whilst also addressing other ecological problems (e.g., limited physical habitat). However, dedicated, thorough study would be needed to determine where physical mediation would be best targeted, ensuring that infrastructure (e.g., bridges) and local and downstream flood risk are not negatively impacted. Nonetheless, it remains clear that the River Crane needs restorative action to improve its chemical water quality, ecology, and biodiversity and reduce the rivers susceptibility to URBs.

8.4.1 Implications for airport operators and river management

The initial scope of this research included a stronger focus on the transport, fate and impacts of airport de-icing. However, as the research naturally evolved, with additional challenges to the aviation industry during the COVID-19 pandemic, the dearth of recent research into the continued prevalence of URBs became evident as an important avenue for investigation. The resulting findings presented throughout this thesis have several implications for airport operators and have been outlined in this discussion. A summary of the key implications for industry and regulators is presented below.

Firstly, the literature review provided an up-to-date summary of URBs – including the taxonomic composition, water quality and environmental drivers of growth and the ecosystem impacts of sewage fungus – whilst highlighting their continued prevalence

using regulator monitoring data in England as a case study. These insights have key implications for environmental regulators, providing a much-needed update on URBs as a bioindicator of poor water quality. The environmental drivers of URBs also provide important insights for improving river resilience to poor water quality and URBs through holistic river restoration. Furthermore, this review presents data highlighting the continued prevalence of URBs, even in a country with advanced wastewater treatment.

Secondly, fieldwork investigations elucidated the interplay between airport de-icing and riverine ecosystem dynamics. The dominance of the Comamonadaceae family and most notably genera *Rhodoferrax* and *Sphaerotilus*, has implications as a potential biosentinel for organic pollution and could lead to the development of rapid diagnostic tests indicating organic pollution and possible URB development, which leads to significant ecological impacts in the river. The development of an affordable and rapid test would have benefits for river users (such as recreational bathing, fishing, and dog walking), local action groups invested in the ecological health and social wellbeing benefits of their local rivers, and environmental regulators to ensure improving the chemical and ecological status of rivers. Moreover, the correlation between the shifted periphyton composition and water quality parameters (conductivity, TOC, BOD₅, DO, and nitrate concentrations) also has implications for environmental regulators and airport operators to monitor treated effluents for these parameters to reduce the likelihood and/or severity of URB growth.

Third, microbial growth experiments demonstrate that URB taxa are able to utilise common FPDs for growth. These findings could have implications in selecting commercial products with a lower risk of URB growth. However, few significant differences between formulations were observed and a limitation of this work – that suspended monocultures were studied, not complex and diverse cultures in a fixed biofilm – further limits the impact for stakeholders. Nevertheless, findings presented provide a useful foundation for further research and testing of future commercial formulations which has direct implications for airport operators.

And fourth, evaluating the influence of weathering treatments on the subsequent mobilisation of CADs in runoff has implications for airport operators. Whilst

methodological limitations (e.g., size and material of substrate) curb the magnitude of implications, these deliberate considerations reduced external complexities of environmental systems and provide useful insights for airport operators about the specific influences of different weather conditions. These findings can then be extrapolated and used to inform treatment strategies (such as when to expect and the amount of CAD).

8.5 Future research

The research conducted in this thesis has significantly improved our understanding of URBs and how they relate to greater water quality but is constrained by time and methodological limitations. Therefore, recommendations for future research include:

1. The microbial composition of URBs. There have been some recent advancements in the microbial composition of URBs. However, these have related solely to airport de-icer implicated occurrences and have only identified a few key taxa [10,60]. Therefore, future work should examine URBs from a range of implicated pollutants and examine additional domains, including 23S (archaea), ITS (fungi) and 18S (algae) genes in addition to targeted shotgun metagenomics on specific samples of interest.

2. The growth of URBs. URBs are polymicrobial assemblies of organisms. Research in this thesis has begun to explore mixed cultures of URB taxa. However, future research should expand on this and examine complex multi-species cultures. Moreover, URBs can grow using a wide number of organic substances, not just airport de-icers. Therefore, future work should also examine URB growth on other substrates and at different scales, such as mesocosms with flowing water (flumes). This research should evaluate the amount of organic loading required to initiate growth versus sustain an existing URB.

3. The transport of CADs. The transport of CADs is complex and highly dependent upon the specific drainage networks of an airfield. The research outlined in this thesis has shown that weather plays a significant role in the initial mobilisation of CADs. Future research should build on these findings to investigate repeat de-icing/weathering events. Additional aspects of the transport of CADs should be examined, such as the transport and deposition within drainage networks and CADs

deposited not on stands (accounts for approximately 30% of applied volume), to further optimise treatment processes that better degrade CADs and design more informed strategies manage surface runoff.

8.6 Contributions to the understanding of water quality in rivers

The scale of poor water quality and conditions of rivers in the UK has seen increasing public pressure during the span of this research. Raw sewage discharges are now frequently and widely reported in the media across the UK. The impacts of intensive agriculture are well documented, and public concern for river health is the highest it has been for decades [66,67]. Whilst URBs remain severely under-reported and under-investigated, they remain highly pervasive globally and have profound and lasting effects on the affected ecosystems. This research has highlighted the continued prevalence and impacts of URBs, improved understanding of their microbial composition, demonstrated a clear link between airport de-icing and the occurrence of URBs, begun to explore the complex transport pathways of CADs, and how background water quality, geomorphology and hydrology of rivers can exacerbate the likelihood of URBs.

The implications of URBs are far-reaching. The negative impacts that URBs have on rivers hinder their use for recreation and livelihoods but, more fundamentally, URBs indicate poor water quality. The EU Water Framework Directive [152] and Water Environment (Water Framework Directive) (England and Wales) Regulations 2017 aim to improve inland and coastal waters but the continued prevalence of URBs is another example to indicate that water quality remains unacceptable [64,66]. URBs have global societal relevance, for example to the One Health agenda [123,124], Sustainable Development Goals 6, 11, and 14 [153], and the World Health Organisation's guidelines on recreational water quality [154]. For several decades the prevalence and impacts of URBs have been much under-reported and understudied but are an important indicator of water quality in rivers. As we strive to improve inland water quality the drivers and impacts of URBs need further investigation and monitoring. The significant contributions to knowledge outlined in this research vastly advance our understanding of the microbial composition and growth of URBs, largely examined through the example of airport de-icing.

8.7 Overall Conclusions

This research used a combination of field surveys and laboratory experiments to investigate how the delivery of runoff contaminated with airport de-icer impacts the growth and composition of sewage fungus in surface waters. This aim was achieved by (i) a critical review of available “sewage fungus” literature, drawing on advancements in biofilm research to reassess them in the 21st Century; (ii) an extensive field survey of the periphyton, water quality and hydrology in a river receiving treated winter airport surface runoff over two consecutive winters; (iii) laboratory experiments measuring the growth of sewage fungus taxa in de-icer based media; and (iv) pilot-scale mobilisation experiments evaluating the mobilisation of airport de-icers in surface runoff.

First, Objective 1 was addressed by a comprehensive review of undesirable river biofilms (URBs, formerly called “sewage fungus”) (**Chapter 2**). URBs are a natural response of rivers to excess organic loading but quickly proliferate and have significant impacts on the ecology and water quality of affected rivers. Key taxa include *Sphaerotilus*, *Zoogloea*, *Beggiatoa*, *Leptothrix*, and *Thiothrix*. Influences on URB growth include water velocity, river substrate(s), shading, and available nutrients, especially organic carbon. However, the chemical and environmental drivers of URBs remain complex and the unique conditions of each URB affect its specific composition and morphology. Furthermore, the continued prevalence of URBs was highlighted in data from England’s environmental regulator (Environment Agency), despite a lack of recent research on URBs.

Second, to achieve Objective 2, a field sampling campaign was conducted to evaluate the spatiotemporal effects of an URB and the drivers of growth (**Chapters 3 and 4**). This work revealed the extent of the shift in the microbial composition of river periphyton as a response to suspected organic pollution: a single family comprised over 80% relative abundance in periphyton. This significant shift occurred even before a visible URB appeared and had a prolonged influence on periphyton composition. New 16S rRNA sequencing (V3-4) data on the riverbed biofilm community collected from the bimonthly sampling over two years showed little spatiotemporal variation, except preceding and during an URB proliferation event. Two genera were responsible

for the demonstrable change in community composition: *Sphaerotilus*, a genus strongly associated with URBs since Kützing (1833); and *Rhodoferrax*, a genus not previously associated with URBs but common in freshwaters receiving excess organic loading. When aggregated at the family taxonomic level, the significant change in microbial composition was within a single family, *Comamonadaceae*, suggesting this may be a biosentinel of organic pollution and could lead to the development of a rapid diagnostic test for organic pollution to pre-empt an URB proliferation and their resulting ecological impacts. Despite this considerable change to the periphyton, neither spot sampling nor ongoing monitoring identified any substantial change in water quality, demonstrating the challenge of interpreting traditional water quality datasets and further demonstrating the potential benefits of a biosentinel approach.

Third, URBs have, for some time, been putatively linked with airport de-icing. To address this, Objective 3 was fulfilled through microbial growth experiments which demonstrated, for the first time, that freeze-point depressants (FPDs) and the commercial products used at airports (commercial airport de-icers, CADs) facilitate the growth of URBs (**Chapters 5 and 6**). New datasets of *Sphaerotilus natans* growth profiles and kinetics using de-icer based media were generated and revealed a heavy dependence on carbon concentration and some differences in growth parameters between different FPDs. For example, there were significant differences between FPDs in the time-to-detection (lag phase) of *S. natans* growth. Furthermore, there were minimal differences between FPDs and their corresponding CADs, indicating that the additives in CADs have no impacts on *S. natans* growth. URBs are polymicrobial assemblies and so the findings from growth experiments and field studies were used to inform subsequent studies that explored the growth kinetics of other URB taxa and mixed cultures. This research trialled time-lapse microscopy, novel in URB research, to improve understanding of the complex interactions between URB taxa competing for available nutrients. Future research should further expand on this to evaluate the interactions between species and the fundamental drivers of URB growth.

Finally, Objective 4 was addressed via contaminant transport on CAD mobilisation, building on observations by airport operators about the influence of exposure to weathering on the physical properties of spent CADs and the subsequent impacts on

mobilisation in runoff (**Chapter 7**). New datasets documented the rates of CAD runoff following prolonged exposure to different weather conditions. Exposure to sunlight and wind caused substantially less CAD mobilisation in runoff during rainfall, whereas lower temperatures had negligible impact. The findings of this study have direct implications for airport operators to inform the optimisation of treatment strategies, in turn reducing the organic loading in receiving waters and protecting ecosystems. Future research is recommended using larger-scale experiments evaluating the transport pathways of CADs in surface runoff from concrete and asphalt prior to discharge.

In conclusion, this research has examined the influence of airport de-icer contaminated runoff on the growth and composition of URBs and the implications of these findings in the greater context of URBs. FPD contaminated runoff significantly affects the microbial composition of river periphyton and can cause the growth of URBs which have demonstrable impacts on river ecosystems. These findings have direct implications for airport operators, informing treatment strategies (including when to expect CAD contaminated runoff and the amount of CAD in runoff), and highlighting the need for meaningful river restoration to improve their resilience to organic pollution incidents and prevent the ecologically damaging growth of URBs.

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