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Polybacterial shift in benthic river biofilms attributed to organic pollution – a prospect of a new biosentinel?

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ABSTRACT

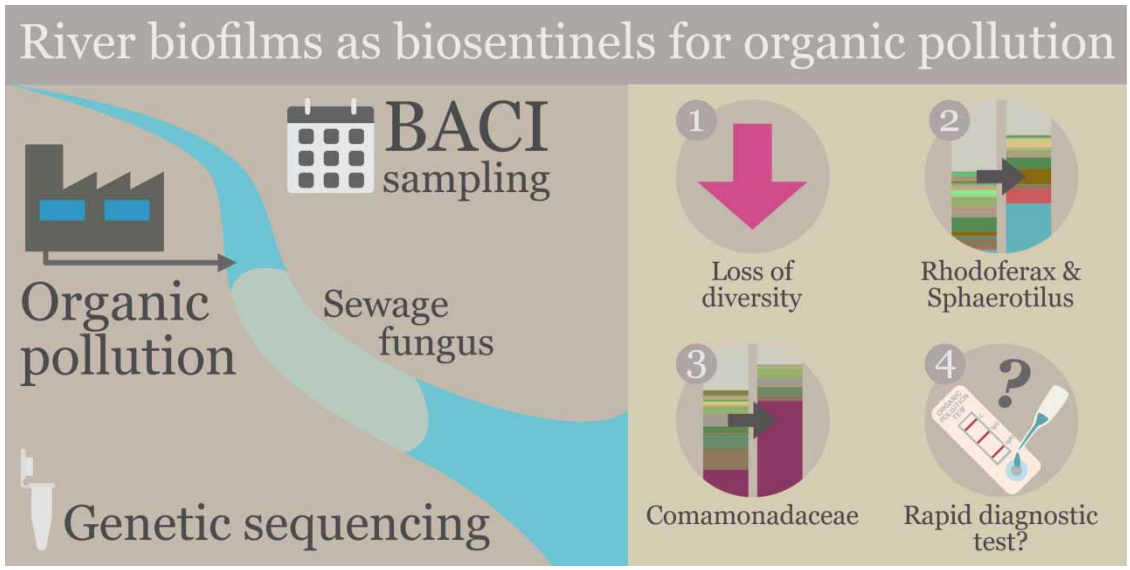
Organic pollution continues to contaminate river water and degrade aquatic ecosystems worldwide. In heavily modified river systems with high organic loading, sewage fungus, a heterotrophic biofilm, can form on the riverbed. The aim of this study was to determine how the polybacterial community of riverbed 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 design. The polybacterial community was characterized using targeted amplicon sequencing (16s rRNA). The results indicate that the community became dominated by two 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.

Key words: 16s rRNA amplicon analysis, bioindicator, *Rhodoferax*, sewage fungus, *Sphaerotilus*, water quality

HIGHLIGHTS

- Riverbed biofilm community was monitored using 16s rDNA in a BACI design.
- Visible sewage fungus outbreak dominated by genera *Rhodoferax* and *Sphaerotilus*.
- A single family, *Comamonadaceae*, accounted for 64.1% of the relative abundance during the outbreak.
- Significant changes in community occurred before the sewage fungus appeared.
- Potential for riverbed biofilm to act as biosentinels for organic river pollution

GRAPHICAL ABSTRACT



INTRODUCTION

Organic river pollution is resurfacing as a major water management problem, even in countries with robust environmental regulation and advanced wastewater treatment (Environmental Audit Committee 2022). 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) (Whitty *et al.* 2022). Currently, the renewed attention has focused on untreated discharges of wastewater (e.g. combined sewage outfalls, CSOs) (Hammond *et al.* 2021), which have increased in frequency due to: (i) a lack of wastewater storage capacity, (ii) increased population density in many areas, (iii) aging infrastructure, and (iv) more intense and unpredictable rainfall attributed to climate change (Semenza & Menne 2009; Hassard *et al.* 2016). 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 (Harrison & Heukelekian 1958; Curtis & Harrington 1971; Global Food Security 2015). A key challenge for monitoring and assessment is the development of sensitive indicators of organic pollutions that can be applied robustly in river systems to identify the location, precise the timing, assess the 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 (BOD_5) 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 of organic pollutants may be not be captured through water quality surveys (Parmar *et al.* 2016; Murray-Bligh & Griffiths 2022). Furthermore, whilst numerous biological indicators (i.e. bioindicators) (Markert *et al.* 2003; Li *et al.* 2010) 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) (Murray-Bligh & Griffiths 2022), 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 (Li *et al.* 2010; Parmar *et al.* 2016).

Sewage fungus is a bioindicator of poor water quality which has been documented extensively (Quinn & Mcfarlane 1985). It is a polymicrobial biofilm that forms on the bed of extremely degraded river channels with high loadings of organic pollution (Hynes 1960; Phaup 1968; Curtis 1969; Curtis & Harrington 1971). 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 (Curtis & Curds 1971; Gray & Hunter 1985; Hickey 1988a; Quinn & Mcfarlane 1989), with wider

ecological impacts (Butcher 1950; Hirsch 1958; Smith & Kramer 1963; Curtis *et al.* 1971; Turnbull & Bevan 1995; United States Environmental Protection Agency 2000; Decho *et al.* 2005; Adeola *et al.* 2009; Flemming & Wingender 2010; Flemming *et al.* 2016). 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 (Ali *et al.* 2013), micropollutants (e.g. pesticides) (Kissane & Shephard 2017), and faecal material in coastal waters (Asahina *et al.* 2009; Winterbourn *et al.* 2016). They are differentiated from bioindicators in that they are the initial organism(s) to respond to changes in ecosystem quality (Asahina *et al.* 2009; Winterbourn *et al.* 2016), rather than simply providing information on 'the quality of the environment' (Li *et al.* 2010). Both bioindicators and biosentinels can be used to track changes in environmental quality over time (i.e. biomonitoring) (Li *et al.* 2010), 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 *Leptomitus lacteus* Curtis 1969; Gray 1985; Geatches *et al.* 2014), 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 (Henry *et al.* 2016) and to assess the environmental microbiome (Thompson *et al.* 2017), but there has been a limited application to sewage fungus (Nott *et al.* 2020) where most of our existing understanding of composition comes from culture-based approaches. 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.

METHODS

Study location

To examine the impacts of organic pollution on the periphyton community, a location on the River Crane (London, UK) was identified that has had historical outbreaks of sewage fungus (Friends of the River Crane Environment 2017; Ricardo 2018; AirportWatch 2019). These outbreaks have occurred in the winter and have been attributed to anti- and de-icing activity in an adjacent airport (ACRP 2014; AirportWatch 2019; BBC News 2019; O'Hare 2022). 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) (Ramakrishna & Viraraghavan 2005; Samuels *et al.* 2006). The riverbed periphyton was sampled at two sites (control vs impact) over the 2020–2021 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 a moving bed biofilm reactor (MMBR) facility, before permitted discharge into the River Crane (Freeman *et al.* 2015, 2018; Pressl *et al.* 2019). 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 October 2020, 7 December 2020, 16 February 2021, and 20 April 2021 (termed Oct20, Dec20, Feb21, and Apr21, respectively) with an additional 'event' sample collected during a putative sewage fungus outbreak which occurred after the Feb21 sampling on 24 February 2021 (referred henceforth as 'SF' in results).

Sampling methodology

Riverbed biofilms were sampled using UK and EU guidance (UKTAG River DARLEQ2, Water Framework Directive – United Kingdom Technical Advisory Group (WFD-UKTAG) 2012 and EU COST Action DNAqua-Net, Bruce *et al.* 2021). 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 h 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 (3,800 rpm, 12 min 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.

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 400–450 bp 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, Caporaso *et al.* 2010a) was used for operational taxonomy unit (OTU) picking for the 16S rRNA gene amplicons. OTUs were clustered against UPARSE (v7.0.1090) (Edgar 2013) and reads failing to hit the reference were subsequently clustered de novo at the 97% similarity level using UCLUST (Edgar 2010). OTU sequences were aligned using PyNAST (Caporaso *et al.* 2010b). OTU taxonomy was determined using the naïve Bayesian Ribosomal Database Project (RDP) classifier for the Mothur method, SSUrRNA database from SILVA (Altschul *et al.* 1990; Wang *et al.* 2007; Quast *et al.* 2013).

Statistical analysis

Taxa richness, diversity, and evenness were calculated at the genus level of taxonomical classification using Microsoft Excel. Shannon's diversity index (Shannon 1948) was used for diversity and evenness as it handles highly diverse samples better than other diversity indices and is commonly used in other studies (Shannon 1948). Relative OTU abundance (≥2%) was plotted in R Studio (RStudio Team 2021) using ggplot2 (Wickham 2016) 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 (PRIMER-e 2015). Pearson correlation coefficients between taxa and each PCO axis were extracted to determine relationships between taxa, sampling locations, and sampling dates.

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 1093 7526). 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).

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 1). 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 1). Whilst the relative abundance of the taxa (represented as the lowest taxonomic unit) changed over time, no single taxa was dominant; for example, the greatest relative abundance of OTUs based on the lowest taxonomic unit was 7.1% (Figure 1(a)). The impact site had similar diversity, richness, and evenness to the control during early winter (Oct20 and Dec20). However, from Dec20 to Feb21, there was a reduction in the diversity by 42%, from 4.375 to 3.204, and the evenness also reduced by

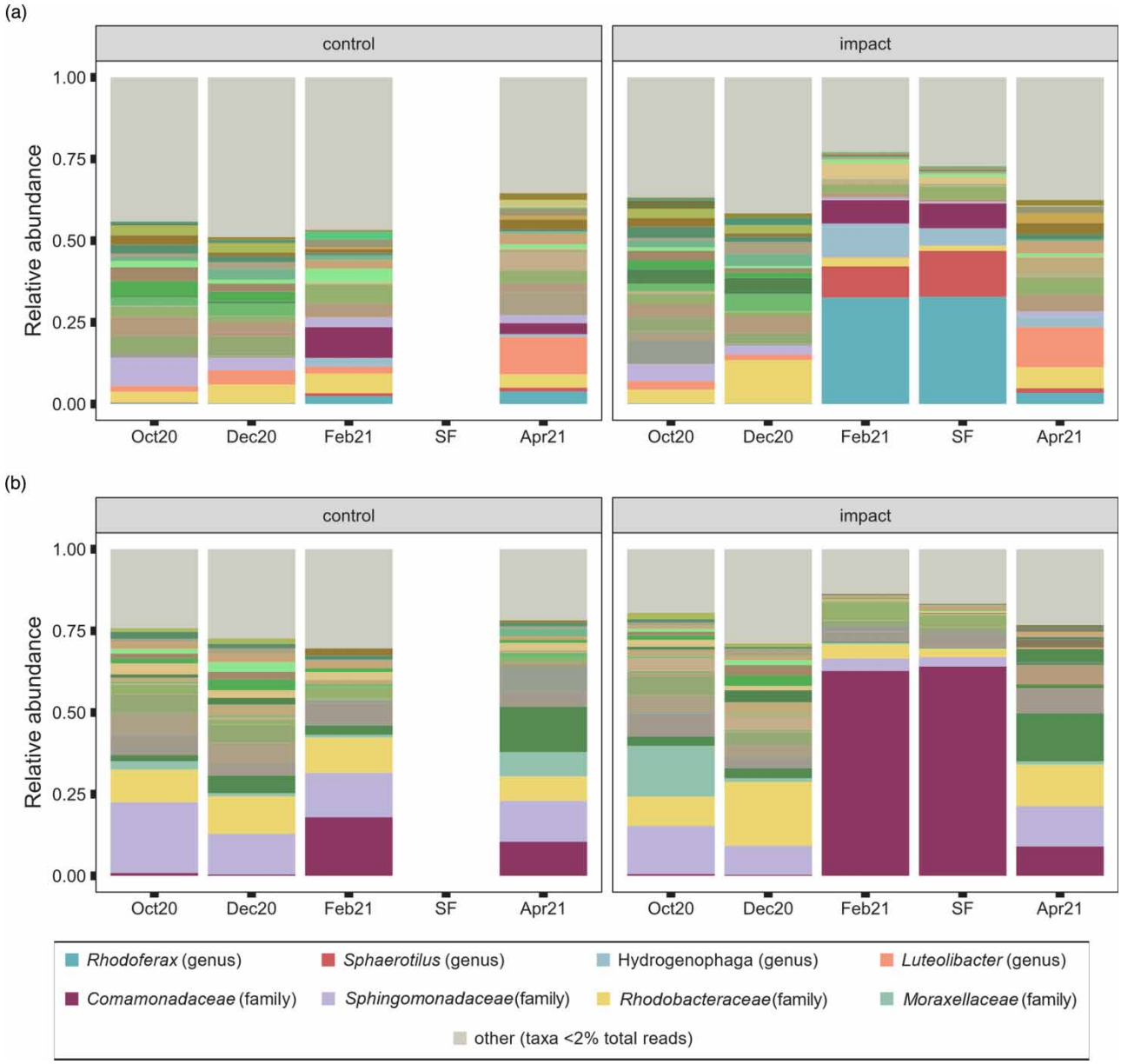


Figure 1 | Relative abundance of periphyton at control and impact site over time at (a) lowest taxonomic level and (b) family taxonomic levels. The minimum threshold for inclusion was set at 2% relative abundance, with taxa below this grouped as 'other'.

38.7%, from 0.693 to 0.501 (Table 1). Diversity and evenness remained consistently low during the sewage fungus incident but increased to values comparable to the control in the Apr21 sampling suggesting a recovery of the periphyton community in terms of evenness and richness.

Table 1 | Ecological diversity, richness, and evenness of microbial biofilm taxa at control and impact sites over time

	Control site					Impact site				
	Oct20	Dec20	Feb21	SF	Apr21	Oct20	Dec20	Feb21	SF	Apr21
Shannon's diversity	4.417	4.544	4.555	–	4.303	4.336	4.375	3.204	3.237	4.235
Richness	492	545	700	–	584	465	550	597	549	581
Evenness	0.713	0.721	0.695	–	0.675	0.706	0.693	0.501	0.513	0.665

The apparent loss of diversity which occurred prior to the Feb21 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 1(a)). 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' (Okafor 2011) that has been isolated from ditch waters and activated sludge (Hochkoeppler *et al.* 1995; GmbH 2022), 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 (Imhoff 2006) 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 (Hickey 1988a, 1988b). *Sphaerotilus* sp. is the genus most commonly associated with sewage fungus (Curtis & Curds 1971; Geatches *et al.* 2014) and accounted for 14.2% of the relative microbial abundance in the sewage fungus event at the impact site (SF, Figure 1(a)). *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 (Butcher 1932; Curtis & Curds 1971; Gray 1982) but were not found in a recent study using sequencing approaches in the USA (Nott *et al.* 2020). Additional studies would be needed to validate whether there is redundancy in terms of key taxon associated with sewage fungus both spatially or temporally. There are strengths and shortcomings of both sequencing and culturing approaches (for example, the detection of genome fragments of environmental bacteria does not always correlate to viable bacteria; whereas culturing underrepresents 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 the community-level assessments of the impact of pollution and sewage fungus have yet to be fully realised.

The shift in the polybacterial community at the impact site became even more pronounced when the taxonomic data were aggregated to the family level. In the Feb21 and SF events, the community was dominated by a single family, *Comamonadaceae*, which increased significantly from <0.9% relative abundance in Dec20 to 64.1% in Feb21 (PERMANOVA, $P < 0.01$; Figure 1(b)). The bacterial community did not differ significantly between Feb21 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 the impact caused by pollution events, prior to ecosystem degradation. These results are supported by a recent study that identified *Comamonadaceae* in river biofilms receiving airport de-icer runoff (Nott *et al.* 2020). 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 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 Feb21, but at a much lower abundance than at the impact site (18.0 vs. 62.7%, respectively; Figure 1(b)), with most of this difference attributed to reduced relative abundances of the genera *Rhodoferax* and *Sphaerotilus* (Figure 1(a)). 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. Additional research is required to determine the 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 1). The relative abundances of these taxa were greatly reduced in Feb21 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 1(b)). The genus *Luteolibacter* was reduced to negligible abundance during the sewage fungus event (Feb21 and SF) but had returned in Apr21 to a much higher abundance (Figure 1(a)). 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 Feb21 and SF).

Interrogation of the chemical water quality monitoring data (Figure 2(b)) 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. 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

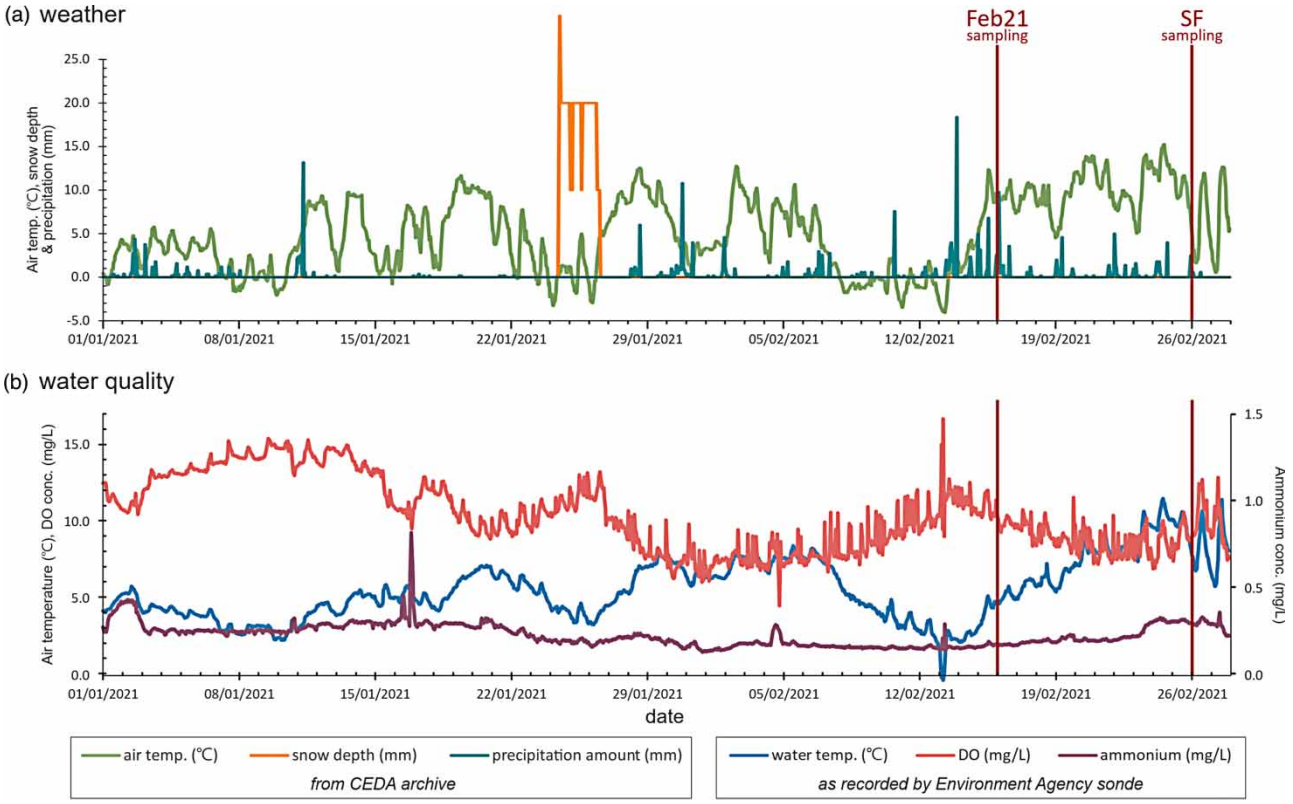


Figure 2 | Weather and water quality preceding Feb21 and SF sampling. Weather data were obtained from CEDA archive at Heathrow weather station, and water quality data were obtained from the Environment Agency sonde in the River Crane downstream of the EBR outfall.

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 Met Office 2022), suggesting that de-icers were likely used and mobilized through the drainage system (Figure 2(a)). Though, the airport's surface water discharge, is treated and is monitored for compliance with 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 (Harrison & Heukelekian 1958; Lock *et al.* 1984; Hall-Stoodley & Stoodley 2002). 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.

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 1(a)). 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 1(b)). 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) Oct20 and Dec20, (ii) Feb21 (impact) and SF, and (iii) Feb21 (control) and Apr21 (Figure 3, S1). There are several important observations from these results that support the potential development of a biosentinel approach. First, the Feb21 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). Whereas Oct20 and Dec20 samples have a strong positive correlation with PCO1 and little correlation along PCO2, with Feb21 control and Apr21 samples correlate weakly in a negative direction to PCO1 but positively with PCO2. Thus, the biofilm community sampled immediately prior to and during the visible sewage fungus outbreak have a significantly different bacterial composition from samples collected earlier in the winter. With a >92.5% correlation threshold,

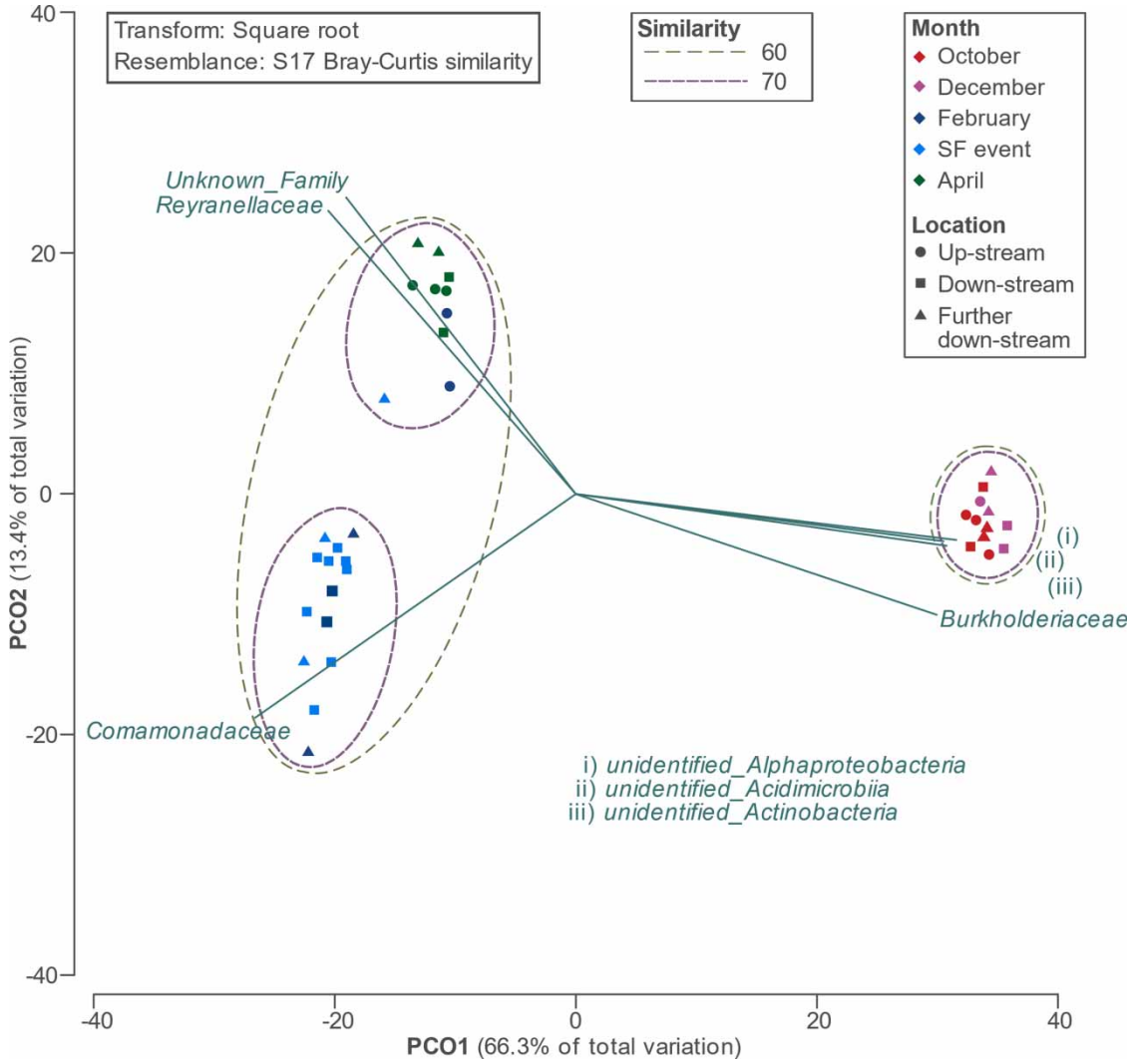


Figure 3 | PCO plot constructed of all winter 2020–2021 samples using a Bray–Curtis-based resemblance matrix of square root-transformed relative abundance at the family taxonomic level.

the only family to correlate to the impact samples (Feb21 impact and SF) is *Comamonadaceae*, suggesting the utility of the *Comamonadaceae* as a biosentinel. At genera level, Rhodoferax, 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.

Second, the Feb21 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 Feb21 sampling are assigned to different clusters (70% similarity) (Figure 3). 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 Feb21 and SF was significantly different from the Apr21 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 (S2). 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 1 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 (S2).

The statistically significant differences in the polybacterial community of the riverbed biofilm prior to and during a sewage fungus outbreak appear to the 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 multiple pressures being exerted on river systems, effective diagnostic tools are urgently needed to support the application of control, mitigation, and restoration measures to halt and reverse the decline of our freshwater ecosystems.

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 *Rhodoferax*, 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 were 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 AVAILABILITY STATEMENT

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

CONFLICT OF INTEREST

The authors declare there is no conflict.

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