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Cyanobacterial Retention and Re-Entrainment in Drinking Water Treatment Systems: From Source Water to Sludge and Backwash Streams

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Abstract

Cyanobacterial blooms present significant operational and water quality challenges for drinking water treatment plants (DWTPs). This study provides a baseline assessment of cyanobacterial removal and microbial community dynamics across three full-scale DWTPs in Victoria, Australia, drawing source water from the Murray River system. Using microscopy, qPCR, 16S rRNA gene amplicon sequencing, and chemical analyses, we characterised the cyanobacterial cell counts and bacterial community composition as well as cyanotoxins and taste-and-odour (T&O) compounds across multiple stages of DWTPs. During autumn sampling, cyanobacteria were detected in clarifier sludge and filter backwash supernatant, highlighting the importance of monitoring residual streams alongside raw water. Subsequent summer sampling revealed a variation in cyanobacterial abundance across the investigated treatment stages, highlighting potential operational challenges during warmer periods. In the autumn observations at K-DWTP and E-DWTP, raw water contained 2217 and 2267 cyanobacterial cells/mL, respectively. Coagulation and clarification reduced the total cyanobacterial cell concentrations in clarified water to below 2000 cells/mL; however, cyanobacteria remained detectable in treatment residuals. All analysed cyanotoxins were below their respective laboratory reporting limits. However, geosmin concentrations reached 110 ng/L in the backwash supernatant at K-DWTP and 470 ng/L at E-DWTP, highlighting the occurrence of T&O compounds in treatment residuals. 16S rRNA gene amplicon sequencing results showed that the relative composition of the cyanobacterial community varied across treatment stages, while clarifier sludge and backwash supernatant samples contained bacterial orders such as Actinomycetales, Rhizobiales, Burkholderiales, and Sphingomonadales. The results also highlight the importance of residual-stream management, particularly at treatment plants where backwash supernatant is recycled to the plant inlet. These findings provide baseline information for future investigations incorporating more frequent sampling, broader seasonal coverage, and measurements of sludge loading to enable more comprehensive statistical analysis and risk assessment.

Keywords: cyanobacteria; drinking water treatment; clarifier sludge; backwash; 16S rRNA gene amplicon sequencing; microbial community

Key Contribution: This study highlights the detection of cyanobacteria in clarifier sludge and filter backwash supernatant, emphasising the importance of monitoring treatment

Check for updates

Received: 24 August 2026
Revised: 19 November 2026
Accepted: 24 November 2026
Published: 27 November 2026

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residuals alongside raw water. Microbial community analysis further revealed differences in bacterial community composition across treatment residual streams.

1. Introduction

Cyanobacteria, also known as blue-green algae (BGA), play a crucial role in ecosystems through oxygenic photosynthesis and their contributions to biogeochemical cycling [1,2]. Beyond these ecological functions, cyanobacteria have potential applications in food production, biotechnology and bioremediation. However, harmful cyanobacterial blooms can negatively affect aquatic biodiversity, fisheries, agriculture, tourism and human health, alongside their impacts on water quality [3]. Cyanobacterial blooms are a growing concern for drinking water utilities worldwide due to their ability to produce harmful cyanotoxins and taste-and-odour (T&O) compounds that compromise both public health and consumer confidence in potable water [4,5]. These events are increasingly linked to climate change [6], nutrient loads [7], and altered hydrology, particularly in warm, slow-flowing river systems such as the Murray River within Australia's Murray–Darling Basin [4]. Consequently, such rivers pose recurring operational challenges for regional water treatment plants in removing cyanobacteria and their metabolites from raw surface water sources [8,9].

The routine monitoring of cyanobacteria in drinking water treatment typically relies on visual inspection, water quality indicators such as turbidity, and microscopic cell counts in raw water to inform operational control measures [10]. However, these monitoring approaches, while simple and practical, provide only a partial overview of bloom dynamics and associated risks throughout the treatment process [11,12]. The main public health risks are associated with cyanotoxins such as microcystins, cylindrospermopsin, saxitoxins and anatoxins, which can cause a range of adverse health effects, including hepatotoxicity and neurotoxicity [13]. Furthermore, T&O compounds including 2-methylisoborneol (2-MIB) and geosmin may aesthetically impair water quality. Conventional treatment processes, including coagulation/flocculation, clarification, filtration and chlorine disinfection, may not consistently remove dissolved cyanotoxins and T&O compounds effectively. The effectiveness of oxidation depends on the target compound and treatment conditions, with geosmin and 2-MIB generally resistant to conventional chlorination [13,14]. However, pretreatment processes such as the addition of granular and powdered activated carbon have been effectively applied to remove T&O compounds [15].

On the other hand, although clarification and filtration can physically remove cyanobacteria from the water column, substantial biomass may be transferred into waste-handling processes such as clarifier sludge and filter backwash waste, where dark, nutrient-rich and oxygen-limited conditions may allow continued viability, gradual decay or cell lysis [12,14,15]. This intracellular breakdown can lead to the release of cyanotoxins and T&O metabolites that may not be detected via microscopic cell counts. Therefore, it is necessary to integrate additional analytical tools such as quantitative polymerase chain reaction (qPCR) and 16S rRNA gene amplicon sequencing to assess the cyanobacterial composition and metabolite risk during treatment [10,12,16]. These techniques provide complementary information on cyanobacterial abundance, selected genetic markers and the bacterial community composition, including the presence of heterotrophic bacterial groups, although their metabolic activities and functional interactions are not directly determined [16,17]. Without these deeper assessments, utilities may fail to detect hidden cyanobacterial hazards that persist beyond conventional removal processes.

The risk may be further amplified when filter backwash supernatant or clarifier supernatant is recycled to the head of the treatment plant, reintroducing dissolved contaminants

into the treatment process and challenging downstream barriers [11,18,19]. Despite the operational importance of these pathways, the microbial dynamics within sludge and wash water streams remain understudied in full-scale facilities. Improved understanding of these overlooked compartments is crucial for informing solids management strategies and preventing the unintended recirculation of harmful biological material within drinking water systems [20].

The aim of this study was to investigate the accumulation and behaviour of cyanobacteria and co-occurring microbial communities in various stages of full-scale drinking water treatment plants. Specifically, the objectives were as follows: (1) integrate microscopic, chemical, and 16S rRNA gene amplicon sequencing analyses to obtain a comprehensive understanding of the cyanobacterial dynamics across two seasonal sampling phases; (2) evaluate the occurrence and treatment-stage distribution of cyanotoxins and T&O compounds throughout the treatment trains; and (3) identify accumulation hotspots, assess treatment effectiveness, and highlight potential operational vulnerabilities associated with solids handling processes that may influence water quality risk.

2. Results and Discussion

During autumn sampling (Phase 1), cyanobacteria were detected in clarifier sludge and filter backwash supernatant, highlighting the importance of monitoring treatment residuals alongside raw water. Microscopic cell counts (Figure 1) showed that the total cyanobacterial concentrations in raw water were 2217 cells/mL at K-DWTP and 2267 cells/mL at E-DWTP, both exceeding 2000 cells/mL. The 2000 cells/mL reference value corresponds to Alert level 1 in the WHO's earlier alert level framework for cyanobacteria in drinking water sources [21], rather than a universal drinking water safety limit. Following coagulation and clarification, the concentrations decreased to 0 and 443 cells/mL, respectively. Nevertheless, cyanobacteria remained detectable in treatment residual streams. All the analysed cyanotoxins were below their respective laboratory reporting limits, including microcystins (<0.3 µg/L), for which the Australian Drinking Water Guidelines (ADWG) specify a health-based guideline value of 1.3 µg/L expressed as microcystin-LR toxicity equivalents. Specifically, anatoxin-a concentrations were below the laboratory reporting limit of 0.1 µg/L in all analysed samples. At K-DWTP, the backwash supernatant contained 110 ng/L geosmin and 4 ng/L 2-MIB, with the geosmin concentration exceeding the approximate odour detection threshold of 10 ng/L described in the ADWG [22]. These findings indicate that taste-and-odour compounds can be detected in treatment residuals even when analysed cyanotoxins are below their laboratory reporting limits, highlighting potential aesthetic water quality concerns [23]. The elevated geosmin concentration in the backwash supernatant suggests that filter backwashing may mobilise previously retained geosmin or geosmin-associated material, although the mechanisms responsible were not directly investigated [24,25]. This was particularly evident at E-DWTP, where the backwash supernatant recorded 470 ng/L of geosmin, compared with only 19 ng/L in the raw water and 110 ng/L in the clarifier's sludge. These observations are consistent with findings reported by Zamyadi et al. [18], highlighting the potential influence of treatment design, operation, and residuals handling on the distribution of cyanobacteria and associated taste-and-odour compounds. Further investigation of cyanobacterial abundance in sludge and backwash streams is warranted.

Further qPCR analysis of a clarifier sludge sample from K-DWTP reported 3400 copies/mL of the cyanobacterial 16S rRNA gene target (Table 1), whereas microscopy reported a very low total cyanobacterial cell concentration of 1 cell/mL. The laboratory noted that high sludge concentrations may obscure cyanobacterial detection by microscopy. All the targeted toxin-biosynthesis genes (*mcyE*/*ndaF*, *cyrA*, and *sxtA*) were reported at <18 copies/mL,

and toxin production was not directly assessed by qPCR. These findings demonstrate differences in cyanobacterial detection between the two analytical methods. Elevated geosmin concentrations were observed in the backwash supernatant, indicating the presence of T&O compounds in this residual stream. Previous research suggests that sludge may retain cyanobacterial cells and potentially contribute to the release of intracellular metabolites into the surrounding water [11]. Similarly, Pestana et al. [26] demonstrated that cyanobacteria can survive, proliferate and continue producing metabolites in treatment plant sludge lagoons, further supporting the role of sludge as a reservoir of cyanobacterial cells and T&O-producing taxa. At K-DWTP, where a portion of the backwash supernatant is recycled to the treatment inlet, cyanobacterial biomass and associated metabolites may be reintroduced into the treatment process, potentially contributing to taste-and-odour management challenges.

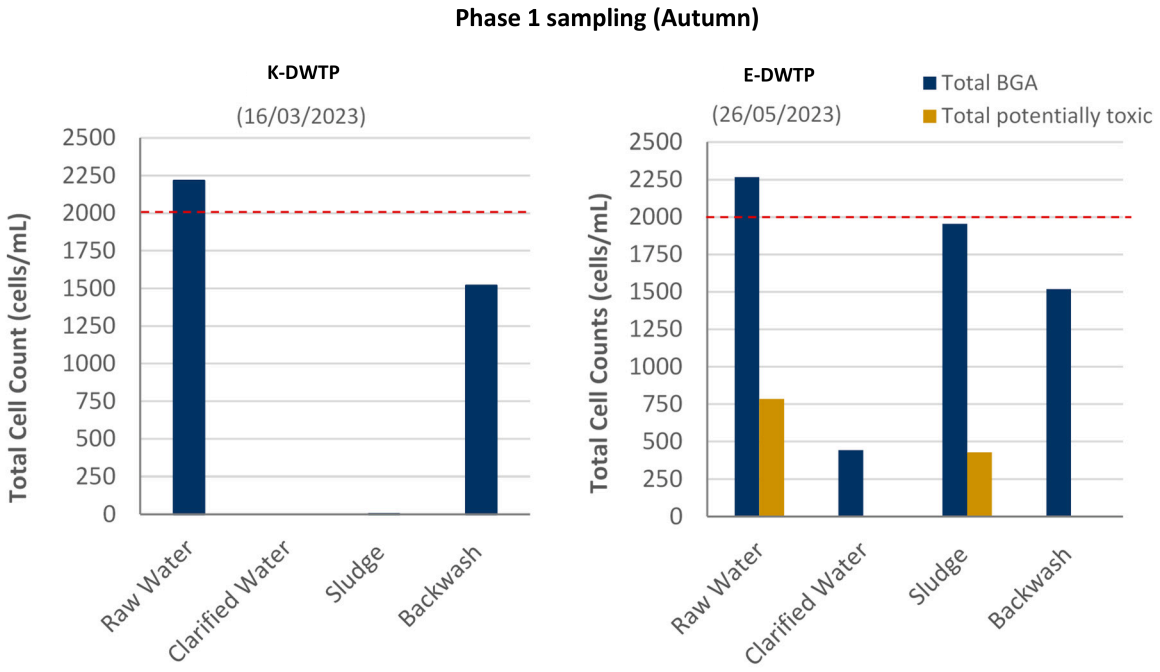


Figure 1. Phase 1: Total cyanobacterial cell counts and counts of potentially toxic cyanobacteria determined by microscopy in raw water, clarified water, clarifier sludge and filter backwash supernatant samples from K-DWTP and E-DWTP. The red dashed line indicates the WHO Alert Level 1 reference value of 2000 cells/mL from the earlier cyanobacterial alert level framework for drinking water sources.

Table 1. qPCR results for cyanobacterial 16S rRNA gene and toxin-biosynthesis gene targets in a K-DWTP clarifier sludge sample during Phase 1 sampling.

qPCR Analysis (Target)	ALS-Reported Concentration (Copies/mL)
Cyanotoxin qPCR—Total cyanobacteria (16S rRNA)	3400
Cyanotoxin qPCR—*Microcystin*/*Nodularin* gene (*mcyE*/*ndaF*)	<18
Cyanotoxin qPCR—*Cylindrospermopsin* gene (*cyrA*)	<18
Cyanotoxin qPCR—*Saxitoxin* gene (*sxtA*)	<18

Microscopic analysis of the E-DWTP clarifier sludge identified a total cyanobacterial concentration of 1955 cells/mL, 428 cells/mL of which were classified as potentially toxin-producing cyanobacteria, including 273 cells/mL of Microcystis and 155 cells/mL of coiled Dolichospermum (≥6 µm). Further 16S rRNA gene amplicon sequencing of the

E-DWTP clarifier sludge sample identified Solibacterales as the most abundant bacterial order (37.2% relative abundance). Other detected orders included Nostocales, Actinomycetales, Burkholderiales, Pseudomonadales, and Rhizobiales. Some of these bacterial groups have been associated with processes such as chemoheterotrophy, fermentation, and nitrate reduction in previous studies [27,28]. However, taxonomic profiles obtained through 16S rRNA gene amplicon sequencing do not establish whether these metabolic processes occurred in the sampled sludge.

Cyanobacterial sequences, including those assigned to Nostocales, were detected in the 16S rRNA gene sequencing results, consistent with the observation of cyanobacterial cells by microscopy. However, the specific contributions of the identified bacterial groups to taste-and-odour compound production or transformation were not directly assessed in this study.

Residual-stream management differed among the three DWTPs. At K-DWTP, a portion of the backwash supernatant was recycled to the treatment inlet, whereas backwash residuals from E-DWTP and C-DWTP were discharged externally. Sludge supernatant was directed to the sewer system at all three DWTPs. The potential ecological effects of these discharges were not assessed in the present study. Characterising microbial community composition across treatment stages may help identify changes in bacterial populations relevant to drinking water treatment operations, although the functional activities of these communities require further investigation.

During Phase 2 (summer sampling), the cyanobacterium concentrations varied across the three DWTPs and their treatment stages (Figure 2). Elevated concentrations were observed in several raw water and residual-stream samples, with some exceeding 2000 cells/mL. However, this pattern was not consistent across all sampling locations or treatment stages.

Two potentially toxin-producing taxa, *Microcystis* cf. *aeruginosa* and *Dolichospermum* cf. *circinale*, were identified in the raw water of E-DWTP but were not observed in the examined samples collected after clarification or in the distributed water, suggesting a reduction in their detectable abundance during treatment. Although potentially toxin-producing cyanobacteria were not detected by microscopy in the examined filtered-water samples, geosmin remained detectable following filtration, consistent with previous reports that conventional treatment processes may not effectively remove dissolved taste-and-odour compounds [29]. To manage geosmin concentrations, E-DWTP uses powdered activated carbon (PAC) dosing [30]. Although PAC can adsorb dissolved taste-and-odour compounds, it is not intended to remove intact cyanobacterial cells, which are primarily managed through coagulation, clarification and filtration [15].

Microscopy examination of raw water samples revealed spherical colonies morphologically identified as *Microcystis* cf. *aeruginosa* and filamentous chains identified as *Dolichospermum* cf. *circinale* (Figure 3a,b). These observations confirm the presence of these cyanobacterial morphotypes in the examined samples but do not establish their toxin-producing activity. Previous studies have reported that cyanobacterial blooms may affect water quality and treatment performance, including potential cyanotoxin production and operational challenges such as filter clogging and reduced coagulation and sedimentation efficiency [14,23,31]. However, these effects were not directly evaluated for the cyanobacterial morphotypes observed in the present study. Microscopy results for E-DWTP showed that the total cyanobacterial concentrations in clarified water were below 2000 cells/mL, with no potentially toxin-producing cyanobacteria observed in the examined samples following coagulation and sedimentation (Figure 2). The microscopic examination of flocculated water samples (Figure 3c,d) showed cyanobacteria and algal cells associated with coagulated flocs, consistent with the aggregation of these organisms during coagulation and

flocculation. The presence of cyanobacterial cells within flocs, together with the lower cyanobacterial concentration observed in clarified water (Figure 2), is consistent with the removal of cyanobacterial biomass during coagulation and sedimentation [14]. Following coagulation and clarification, the total cyanobacterial concentrations in the examined clarified water samples were below 500 cells/mL at C-DWTP and 8 cells/mL at K-DWTP (Figure 2). The microscopic examination of clarifier sludge samples (Figure 3e,f) revealed numerous cyanobacterial and algal cells, including small spherical cells and filamentous forms. These observations indicate the presence of different cyanobacterial morphologies within the examined sludge samples. The presence of cyanobacterial cells in clarifier sludge is consistent with their retention during coagulation and clarification [10,14]. However, the presence of cyanobacterial cells in clarifier sludge suggests that sludge may act as a reservoir of retained biomass, with potential for cell lysis and the release of cyanotoxins or taste-and-odour compounds under certain conditions [11]. These processes were not directly assessed in the present study.

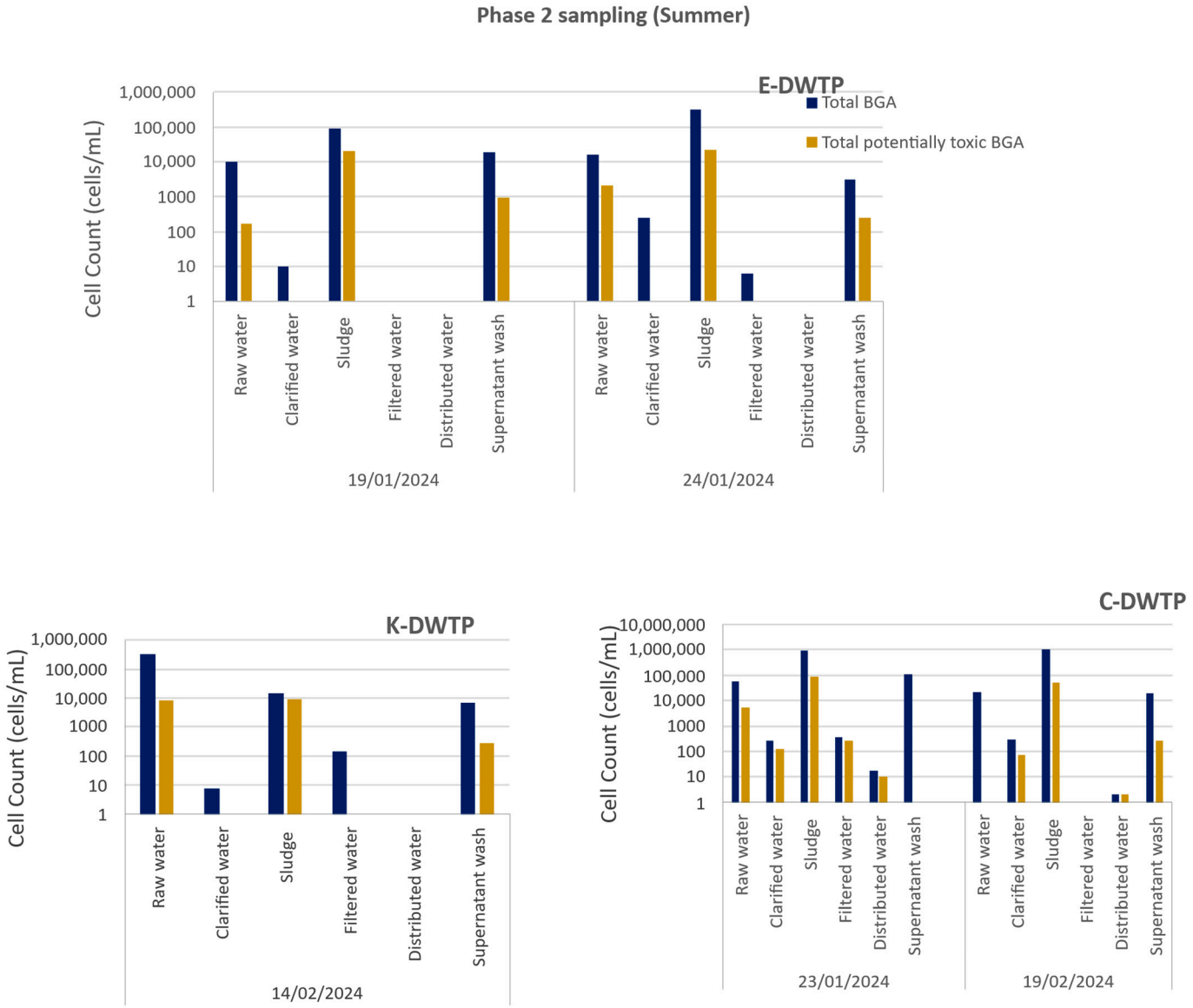


Figure 2. Phase 2: Total cyanobacterial cell concentrations and total potentially toxin-producing cyanobacterial cell concentrations determined by microscopic cell counts in raw water, clarified water, clarifier sludge, filtered water, distributed water, and filter backwash supernatant samples from E-DWTP, K-DWTP, and C-DWTP.

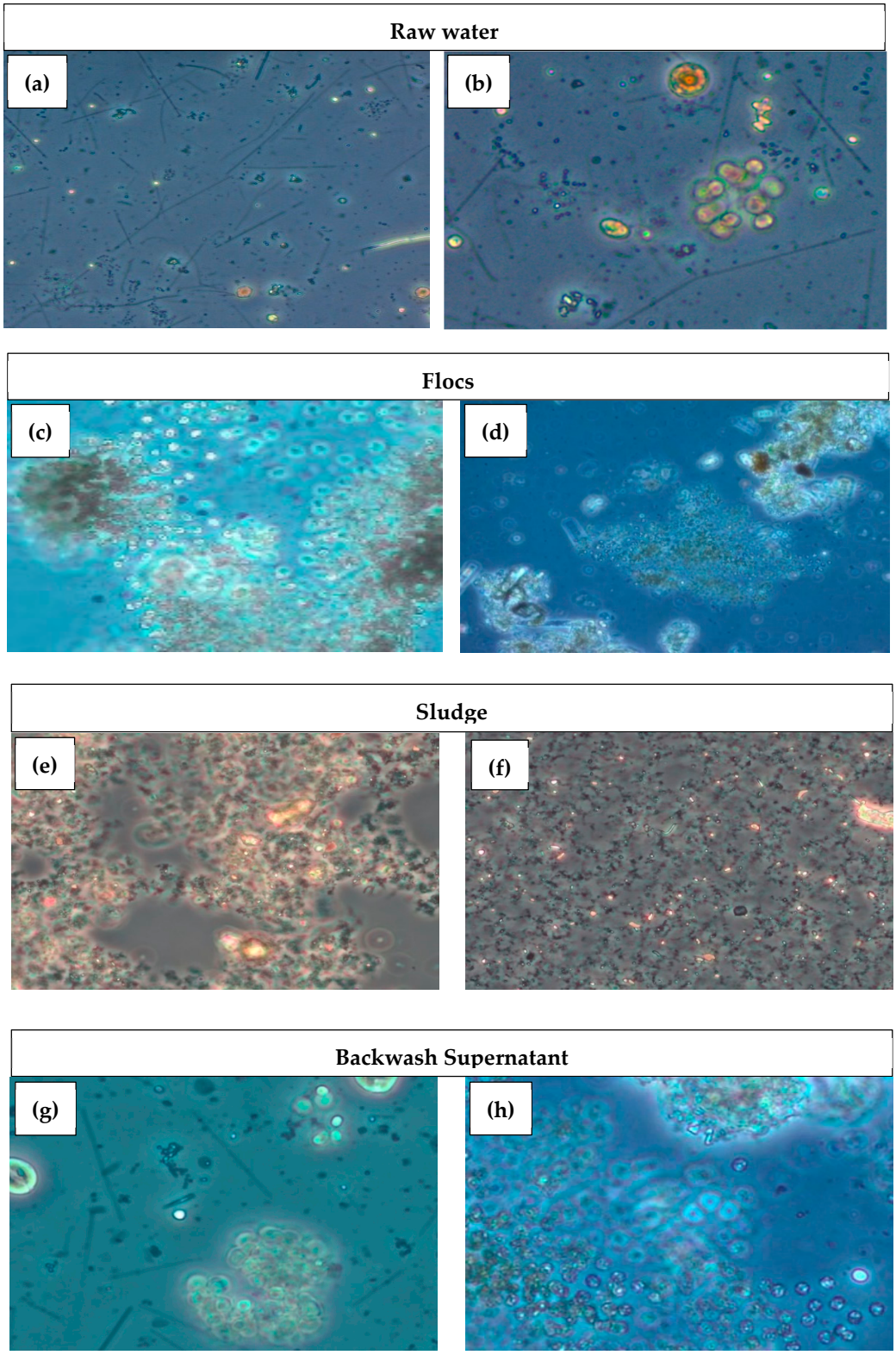


Figure 3. Light micrographs of samples collected from E-DWTP during the summer sampling phase: (**a**,**b**) raw water; (**c**,**d**) flocs formed during coagulation–flocculation; (**e**,**f**) clarifier sludge; and (**g**,**h**) backwash supernatant. Microscopic analyses were performed at magnifications of 100× and 200× according to the laboratory protocol. The images were provided by ALS without individual image calibration information; therefore, scale bars could not be included, and the images are presented for qualitative illustration only.

The microscopic examination of backwash supernatant samples (Figure 3g,h) revealed numerous small cyanobacterial cells, with morphological similarities to those observed in the flocculated water samples (Figure 3c). The presence of cyanobacterial cells in backwash supernatant is consistent with the mobilisation of biomass retained within filter media during backwashing, although the origin of individual cells could not be directly established from microscopy alone. At K-DWTP, where a portion of the backwash supernatant is recycled to the treatment inlet, this practice may reintroduce retained cyanobacterial biomass into the treatment process, potentially increasing cyanobacterial loading and associated operational challenges [11,12].

Bacterial orders previously associated with heterotrophic metabolism were prominent in sludge and backwash supernatant samples, reflecting differences in microbial community composition across treatment stages (Figures 4–6). Raw water samples from the three DWTPs contained a range of bacterial orders, including Actinomycetales, Rhizobiales, Burkholderiales, Flavobacteriales, Cytophagales and Pseudomonadales (Figures 4–6). Some of these bacterial groups have been associated with nutrient cycling and organic matter turnover in previous studies [32], although these processes were not directly assessed in the present study. The variation in bacterial community composition observed in raw water highlights the importance of treatment processes capable of managing changes in source water microbial communities [14,33]. Compared with raw water, clarified water samples showed differences in bacterial community composition and the relative abundance of several bacterial orders (Figures 5 and 6). Actinomycetales, Rhizobiales, and Burkholderiales were detected at lower relative proportions, whereas Flavobacteriales and Sphingobacteriales showed higher relative proportions in some samples. These relative abundance patterns indicate shifts in taxonomic composition but do not independently demonstrate absolute biomass removal or changes in microbial activity.

Clarifier sludge samples from the three DWTPs contained several bacterial orders in common, particularly Actinomycetales, Rhizobiales, and Burkholderiales, alongside Flavobacteriales and Cytophagales, although their relative abundances varied among treatment plants (Figures 4–6). These bacterial groups have been associated with organic matter degradation in previous studies, although their metabolic activity was not directly assessed in the present study [34]. While Actinomycetales and Rhizobiales were prominent across the three DWTPs, the relative abundances of Sphingomonadales and Bacillales varied among sludge samples and treatment plants (Figures 4–6). These variations reflect differences in taxonomic composition rather than direct evidence of changes in microbial metabolic activity [34]. Cyanobacterial orders, including Nostocales and Pseudanabaenales, were detected in the clarifier sludge sequencing profiles (Figures 4–6). Sequences assigned to Prochlorales were also reported by ALS; however, these assignments should be interpreted cautiously because the corresponding species-level identification of *Prochlorococcus marinus* could not be independently verified. Relative abundance data alone cannot establish absolute cyanobacterial retention or the metabolic activity of other bacterial groups. The presence of diverse bacterial orders in clarifier sludge suggests a microbial community with potentially varied ecological functions, although these functions were not directly assessed. Previous studies indicate that retained cyanobacterial biomass may undergo cell lysis and release intracellular metabolites, including geosmin, 2-MIB, and cyanotoxins, under certain conditions [11,12].

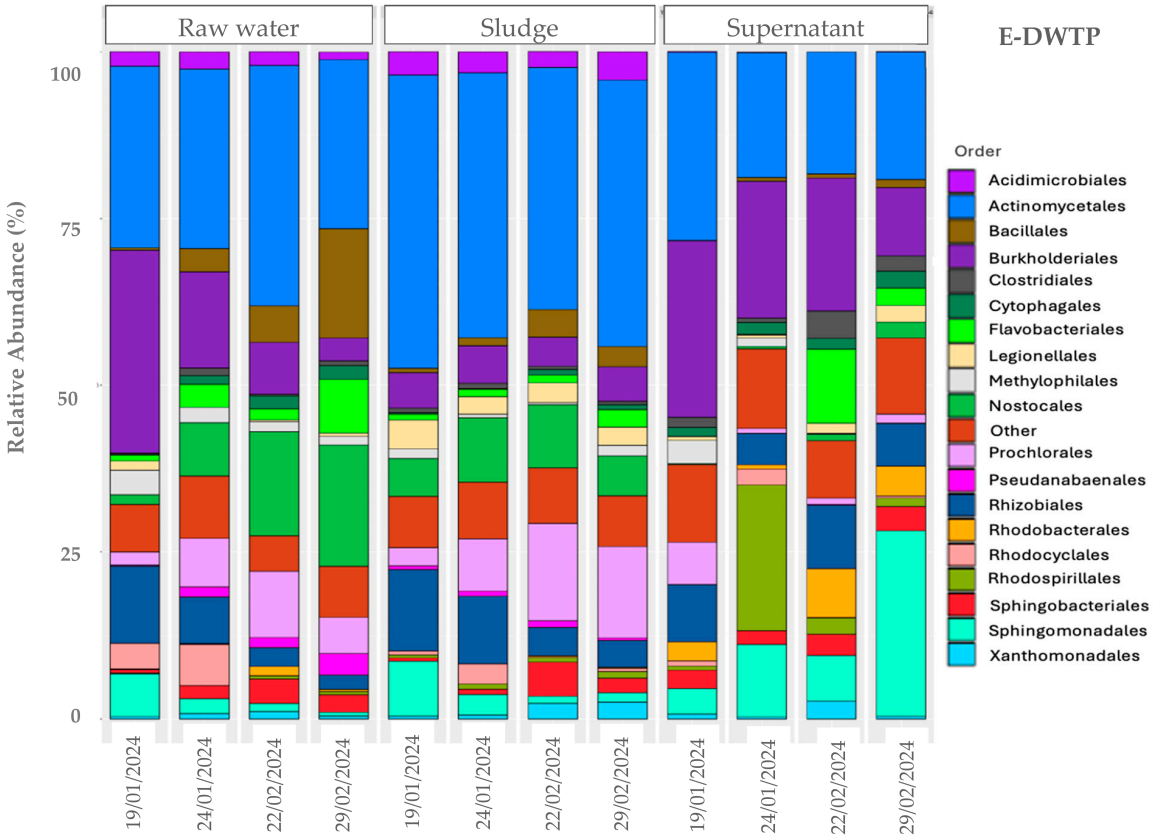


Figure 4. Relative abundance of bacterial orders across sampling compartments at E-DWTP during the second phase of sampling. The bar plots show the relative abundance of bacterial orders in raw water, clarifier sludge and backwash supernatant samples, based on 16S rRNA gene amplicon sequences.

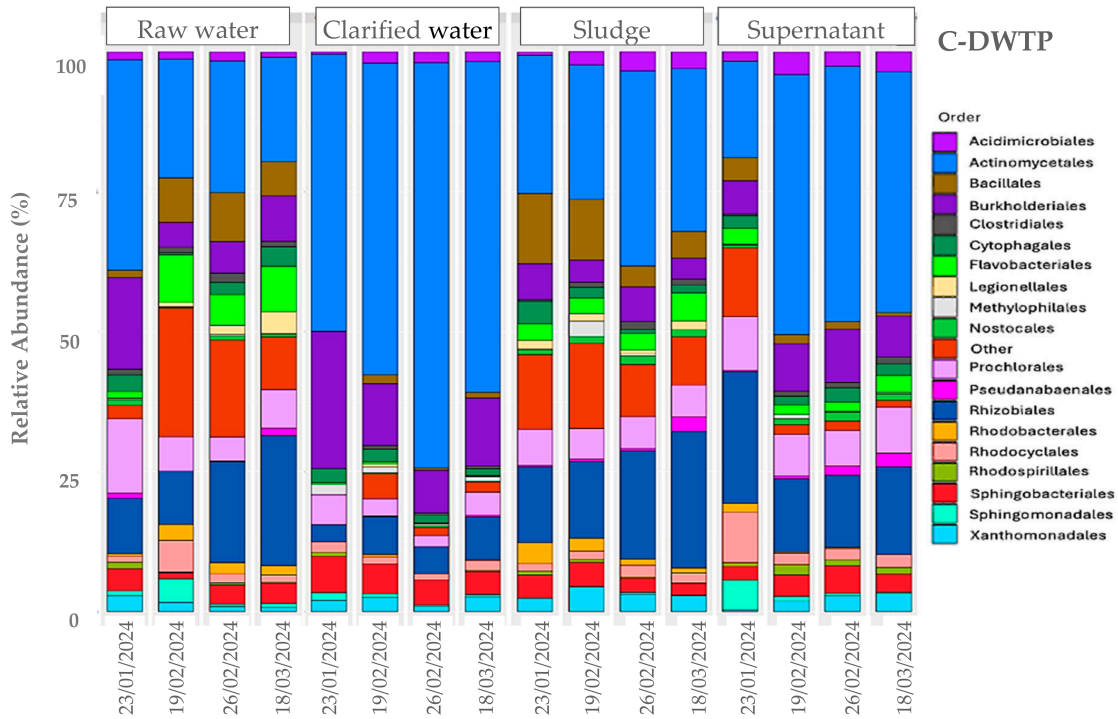


Figure 5. Relative abundance of bacterial orders across water treatment stages at C-DWTP. Bar plot indicating microbial taxa at the order level across samples from raw water, clarified water, clarifier sludge, and backwash supernatant in the second phase of sampling.

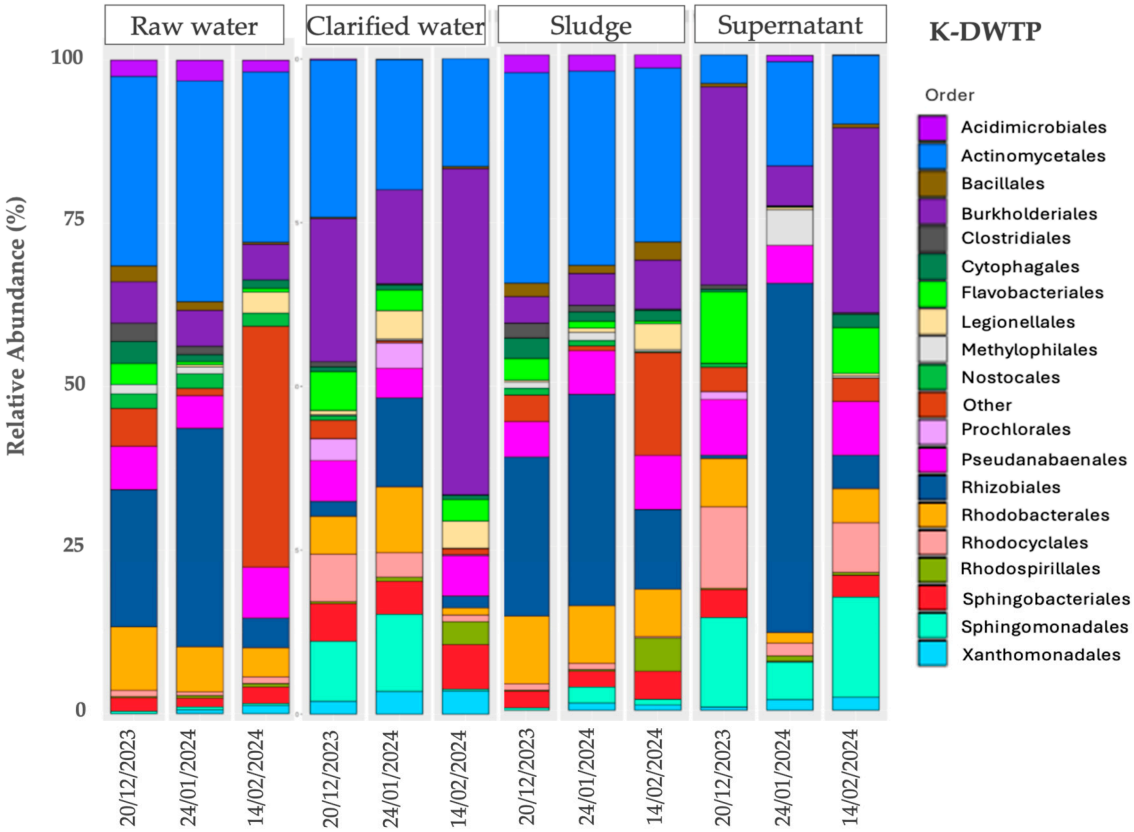


Figure 6. Relative abundance of bacterial orders across water treatment stages at K-DWTP. Bar plot indicating microbial taxa at the order level across samples from raw water, clarified water, clarifier sludge, and backwash supernatant in the second phase of sampling.

The bacterial community composition of backwash supernatant differed from that of clarifier sludge, with variation in the relative abundances of Actinomycetales, Rhizobiales, Flavobacteriales and Sphingobacteriales across the three DWTPs and sampling dates (Figures 4–6). These groups have been associated with organic matter degradation in previous studies; however, their metabolic activity was not directly assessed here. Backwashing may mobilise previously retained microbial biomass from filter media. Differences in relative abundance between sludge and backwash supernatant indicate changes in microbial community composition but do not independently establish selective resuspension or microbial adaptation. These findings suggest that filter backwashing can redistribute microbial communities within treatment residual streams, although microbial activity and metabolite release were not directly established by the sequencing results.

The original ALS sequencing reports assigned cyanobacterial sequences to six orders: Chroococcales, Gloeobacterales, Nostocales, Oscillatoriales, Prochlorales and Pseudanabaenales (Supplementary Table S3). However, the Prochlorales assignments require cautious interpretation because the associated species-level identification of *Prochlorococcus marinus* could not be independently verified. The table presents all detected cyanobacterial taxa and their relative abundances across the three DWTPs, sampling dates and treatment stages. Nostocales therefore represented only one component of the cyanobacterial community detected across the treatment streams.

Taken together, the 16S rRNA gene amplicon sequencing results show changes in microbial community composition across treatment stages, including differences in the relative abundance of cyanobacterial taxa. However, these relative abundance patterns do not independently establish absolute microbial removal or accumulation. Several bacterial orders commonly associated with heterotrophic metabolism accounted for substantial

proportions of the sequencing profiles in sludge and backwash supernatant. Some of these groups have been associated with organic matter degradation in previous studies, although their metabolic activity was not directly assessed in the present study. These findings highlight the importance of considering microbial communities in the sludge and backwash streams alongside cyanobacterial concentrations in the main treatment flow when assessing potential treatment-related risks. The handling of residual streams may also warrant consideration, particularly at DWTPs where backwash supernatant is recycled to the treatment inlet, such as K-DWTP, as retained cyanobacterial biomass could contribute to metabolite-related risks under certain conditions.

3. Conclusions

This investigation represents an initial baseline assessment of cyanobacterial behaviour and microbial community dynamics across three full-scale DWTPs with source water from the Murray River. As the study relied on a small number of sampling events, formal statistical analyses were not applied. Although triplicate analyses were reported, the limited number of independent sampling events and differences in analytical coverage among treatment plants constrained the scope for formal statistical comparisons. The use of 0.45 µm membrane filtration for DNA extraction may have reduced the recovery of smaller free-living bacterial cells, potentially influencing the microbial community profiles. Therefore, qualitative comparisons and relative abundance patterns across treatment stages were used to interpret microbial community shifts in DWTPs. These results provide a foundational framework for future work incorporating higher temporal resolutions, seasonal variability, and sample replication to support robust statistical evaluation and quantification of treatment performance.

The 16S rRNA gene sequencing results also presented limitations in taxonomic resolution. In particular, the original ALS reports included assignments to *Prochlorococcus marinus*, a species associated with marine environments. Given the freshwater origin of the samples, these species-level assignments are considered uncertain and could not be independently verified. Consequently, the corresponding Prochlorales assignments in the order-level community profiles should also be interpreted cautiously, as they originated from the same taxonomic classification procedure. Future studies should prioritise independent validation of uncertain cyanobacterial taxonomic assignments using curated reference databases and phylogenetic analyses to improve identification at finer taxonomic resolutions.

Despite these limitations, this study provides insights into microbial community composition within drinking water treatment processes. By identifying the bacterial taxa present across treatment stages, operators can better understand microbial community shifts within key treatment units and identify areas requiring further investigation. Clarifier sludge was identified as a retention compartment for cyanobacterial biomass; however, retained cells may undergo lysis and release metabolites, including cyanotoxins and taste-and-odour compounds, under certain conditions. Chemical analyses showed variable geosmin and 2-MIB concentrations across the investigated treatment stages, including elevated geosmin concentrations in clarifier sludge/float samples (Table S4). However, all analysed cyanotoxins were below their respective laboratory reporting limits, and the chemical results do not establish metabolite release or production within these treatment stages.

16S rRNA gene amplicon sequencing results further revealed differences in relative bacterial community composition across treatment stages, including the presence of bacterial orders previously associated with heterotrophic metabolism in clarifier sludge and backwash supernatant. Backwashing may mobilise microbial biomass retained within filter media. At K-DWTP, where a portion of the backwash supernatant is recycled to the plant

inlet, this pathway may reintroduce cyanobacterial biomass or associated metabolites into the treatment process.

Overall, the observed reductions in cyanobacterial cell concentrations following treatment, together with the detection of cyanobacteria in treatment residuals, suggest that cyanobacterial biomass may be retained within residual streams rather than completely eliminated during treatment. Such retained biomass could pose metabolite-related risks under certain conditions. The findings underscore the need for strengthened sludge and backwash management practices, deeper molecular monitoring, and a statistically rigorous follow-up study to evaluate treatment performance across seasonal, hydrological, and operational variability.

4. Materials and Methods

4.1. Sampling Sites and Sample Collection

Location: Samples were collected across different treatment stages at three full-scale drinking water treatment plants (DWTPs) in Victoria, Australia (Figure 7), to investigate cyanobacterial accumulation and microbial community dynamics. Three treatment plants operated by Lower Murray Water (K-DWTP; Mildura, Victoria, Australia), Coliban Water (E-DWTP; Bendigo, Victoria, Australia) and Goulburn Valley Water (C-DWTP; Shepparton, Victoria, Australia) were selected based on prior reports of in-plant algal growth, their shared Murray River water source, geographic distribution and operational readiness to trial new monitoring or treatment strategies. To choose the key sampling locations within each site (Figure 7) and sampling frequency, historical operational records and routine water quality data were reviewed (Lower Murray Water, 2025; Coliban Water, 2025; Goulburn Valley Water, 2025). All treatment plants (Figure 7) included coagulation and flocculation using aluminium sulphate (alum) as a coagulant, sedimentation at K-DWTP and E-DWTP or dissolved air flotation (DAF) at C-DWTP, filtration, and disinfection using chlorine or ultraviolet (UV) treatment, depending on the DWTP. At K-DWTP, backwash supernatant (0–5% of the design flow) is returned to the head of the plant, whereas backwash from E-DWTP and C-DWTP is discharged externally and does not re-enter the treatment train. At all three DWTPs, sludge supernatant is discharged to the sewer system and is not returned to the treatment process. Key operational characteristics and treatment processes along with source water quality at each DWTP are summarised in Table S1.

Sample collection: Two phases of sampling were conducted in this study, and due to operational, logistical, and financial constraints, the scope of sampling and analysis varied among sites, providing complementary perspectives on cyanobacterial accumulation and microbial communities across treatment stages (Figure 7). All samples and analyses were conducted in triplicate and averaged accordingly. The first phase of sampling was undertaken during March and May 2023 at K-DWTP and E-DWTP. Although cyanobacterial activity is typically lower during autumn than during summer, the Murray River commonly shows its highest BGA biovolume during the summer and early autumn period. Therefore, the first sampling phase commenced in autumn due to the timing of resource availability and project mobilisation. This phase established a baseline to evaluate microbial conditions during autumn sampling and to identify the presence of cyanobacteria and preliminary accumulation points within the treatment process. Grab samples were collected from different treatment stages, including raw water, clarified water, clarifier sludge, filtered water, distributed water, and backwash supernatant, with the sampling and analytical coverage varying between DWTPs, as detailed in Supplementary Table S2. Cell counts, chemical analyses and molecular analyses of sludge samples were used to investigate cyanobacterial occurrence and potential accumulation within solids-handling streams during Phase 1.

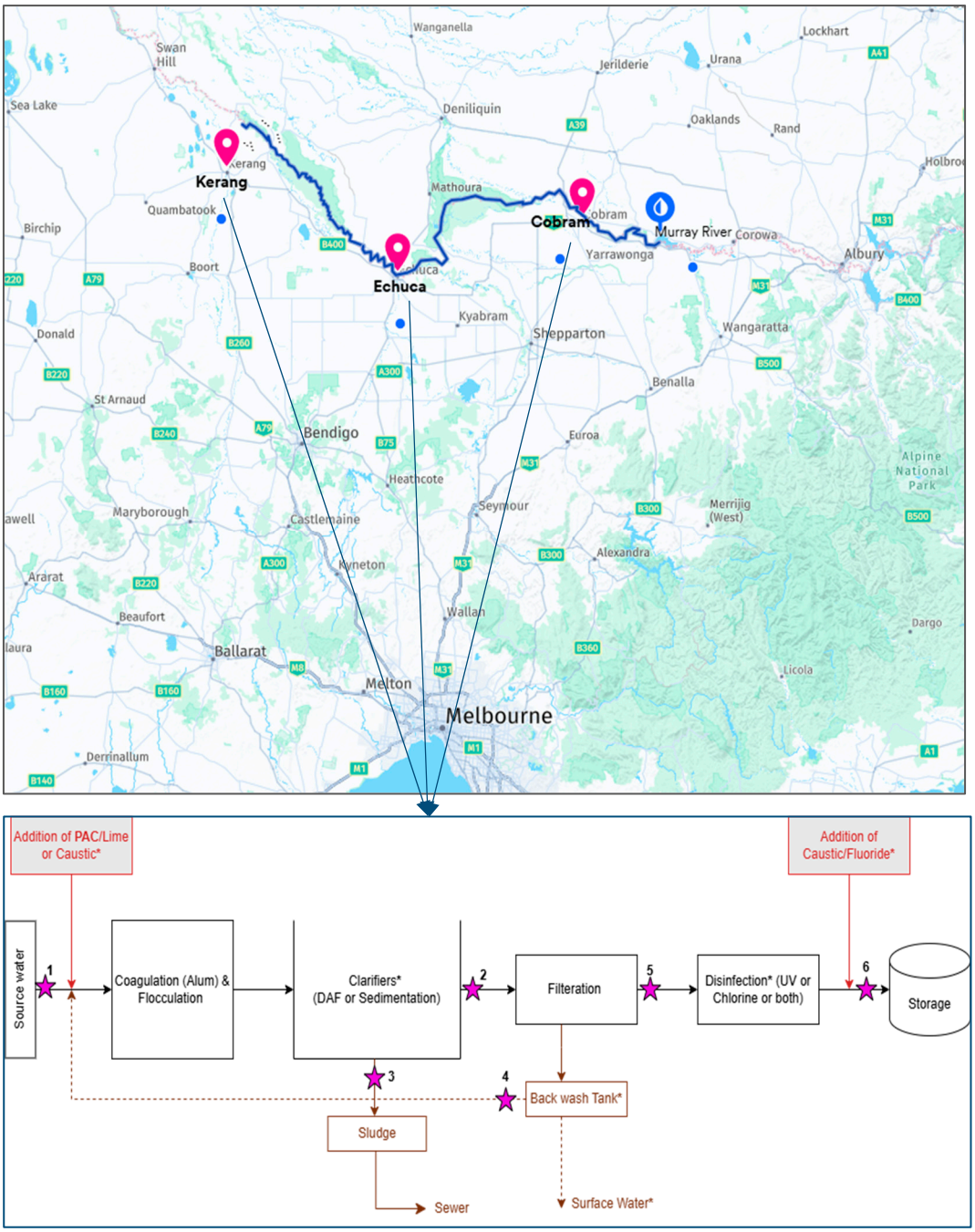


Figure 7. Top: Sampling locations of the three drinking water treatment plants (DWTPs) included in this study, i.e., K-DWTP (Kerang), E-DWTP (Echuca), and C-DWTP (Cobram), located along the Murray River system in northern Victoria, Australia. These facilities source raw water from the Murray River and associated channels. The arrows connecting the three locations to the schematic below indicate that the general treatment process represents all three DWTPs. **Bottom:** General schematic of the DWTPs showing sampling points at various treatment stages, i.e., (1) raw water; (2) clarified water; (3) clarifier sludge; (4) backwash supernatant; (5) filtered water; and (6) distributed water. The numbered purple stars indicate sampling points, while arrows in the schematic indicate the direction of water and residual-stream flow. The small asterisks (*) indicate operational features that differ among the three DWTPs.

Phase 2 focused on expanded sampling during summer (December 2023–March 2024), incorporating 16S rRNA gene amplicon sequencing across multiple treatment stages to assess shifts in microbial community structure and the relative abundance of cyanobacterial

orders under conditions associated with an increased likelihood of cyanobacterial blooms during warmer summer months [6]. During Phase 2, which coincided with active algal bloom events, grab samples were collected from various treatment stages at the three DWTPs (Figure 7).

4.2. Laboratory Analysis

The analytical techniques, sampling points, sampling phases, and analytical coverage are summarised in Table S2.

Sample processing: Upon collection, all samples were transferred to ALS Limited (Scoresby, Australia), which is a National Association of Testing Authorities (NATA)-accredited commercial laboratory contracted to perform routine water quality testing for the participating water authorities. All samples were stored at 4 °C upon delivery to the laboratory and processed within 48 h to maintain sample integrity before concentration. Samples were maintained under these controlled conditions in accordance with the laboratory protocol to minimise degradation prior to analysis. For DNA extraction, between 50 and 500 mL of each water sample was passed through a 0.45 µm membrane. A 1000 mL sterile water blank was processed in parallel as a negative control. For 16S rRNA gene amplicon sequencing, where filtration was not feasible due to high particulate content (clarifier sludge), 0.6 g of concentrated solid material was placed directly into bead tubes for DNA extraction.

Microscopic cell counts and images, cyanotoxins, and taste-and-odour compounds: The cyanobacterial cell counts and biovolume were determined following ALS internal methods MB010.BGA/MW024 VCA, based on which subsamples were preserved with Lugol's iodine and analysed using an inverted light microscope with a Sedgwick–Rafter counting chamber (~1 mL and various dilution factors, i.e., 1:1, 0.1:1, and 0.33:1, according to type of samples). Samples were analysed at magnifications of 100× and 200×, with 20–500 fields of view assessed depending on cell density. For each sample, the cell counts (cells/mL) and biovolume (mm^3/L) were calculated based on the chamber volume, dilution factor, and field count. Cyanotoxins (anatoxin-a, deoxy-cylindrospermopsin, cylindrospermopsin, microcystins and nodularin) were analysed using the NATA-accredited LC-MS/MS methods (WP248 and WP249). Microcystins were reported collectively by the analytical laboratory, and the specific microcystin congeners included in the analysis were not identified in the available laboratory documentation. Taste-and-odour compounds (geosmin and 2-methylisoborneol (2-MIB)) were measured using the GC-MS method (EP115). The laboratory reporting limits (LOR) were 0.1 µg/L for anatoxin-a, 0.05 µg/L for cylindrospermopsin and deoxy-cylindrospermopsin, and 0.3 µg/L for microcystins and nodularin. Reporting limits for geosmin and 2-MIB varied between samples. Separate limits of detection (LOD) and quantification (LOQ) were not specified in the available ALS laboratory documentation. The available chemical analysis results across the three DWTPs and treatment stages, including laboratory reporting limits, are presented in Supplementary Table S4.

DNA extraction: After filtration, the membranes were transferred into bead tubes for DNA extraction. DNA was extracted from samples using either the MagMAX™ Microbiome or MagMAX™ CORE Kit (Life Technologies, ThermoFisher Scientific, Austin, TX, USA), following the manufacturer's recommended protocols. All extractions were automated using KingFisher™ DUO or KingFisher™ Flex platforms (Thermo Fisher Scientific, Vantaa, Finland).

qPCR, library preparation, and sequencing: For a Phase 1 clarifier sludge sample in which microscopy reported a very low cyanobacterial cell concentration, further evaluation was conducted using an in-house qPCR assay targeting the cyanobacterial 16S rRNA gene and cyanotoxin biosynthesis genes, including *mcyE*/*ndaF* (microcystin/nodularin), *cyrA*

(cylindrospermopsin) and *sxtA* (saxitoxin), to assess the presence of cyanobacterial DNA and genes associated with toxin biosynthesis.

The clarifier sludge sample was submitted to ALS Environmental Ltd (Scoresby, Victoria, Australia) as wet sludge (W-SLUDGE) for qPCR analysis, with cyanobacterial gene concentrations reported by the laboratory in copies/mL. Volume-based normalisation of qPCR measurements from wet sludge has been described by Dionisi et al. [35]. However, the original sample volume and laboratory-specific normalisation factors were unavailable for independent verification.

For one sludge sample in Phase 1 and selected samples from Phase 2, bacterial community profiling was performed using the in-house molecular method MM327, which involves 16S rRNA gene amplicon sequencing and high-throughput sequencing to characterise the bacterial community composition in water samples. For clarifier sludge and clarifier float samples, which were unsuitable for membrane filtration due to their high solids content, 0.6 g wet weight was directly transferred to sterile bead tubes for DNA extraction.

For 16S rRNA gene library construction, 8 µL of each sample (or diluted sample) was used, along with a positive *E. coli* control. Libraries were prepared using the Ion 16S™ Metagenomics Kit and the Ion Xpress™ Plus Fragment Library Kit (Thermo Fisher Scientific, Warrington, UK, and Carlsbad, CA, USA). Barcoding was carried out using the Ion Xpress™ Barcode Adapters Kit. Template amplification and enrichment were performed on the Ion Chef™ Instrument using the Ion 510™, 520™, and 530™ Kit-Chef, according to the manufacturer's instructions. Sequencing was conducted on an Ion 530™ chip using the Ion GeneStudio™ S5 System (Thermo Fisher Scientific, Carlsbad, CA, USA).

Base calling and demultiplexing of the sequencing data were carried out using Torrent Suite v5.10.1 (Thermo Fisher Scientific, Carlsbad, CA, USA), with the 400 bp protocol and 850 flows; all other settings were kept at default. Trimmomatic v0.36 (Forschungszentrum Jülich, Jülich, Germany) was used to trim and quality-filter the raw UBAM files, applying a minimum PHRED quality score of 20 and a minimum read length of 180 bp. Sequence data were normalised by rarefaction to 250,000 reads per sample for Phase 1 and 200,000 reads per sample for Phase 2, as documented in the ALS sequencing reports. Taxonomic assignment was completed using Ion Reporter™ Software v5.10 (Thermo Fisher Scientific, Carlsbad, CA, USA), applying the Metagenomics workflow with the MicroSEQ v2013.1 and Greengenes v13.5 16S reference libraries. Assignments used default primers, with a read abundance filter set to 30; all other parameters were left at default.

Quality assurance and quality control (QA/QC) procedures for chemical analyses included method blanks, duplicate analyses and spike recovery tests, as documented in the ALS laboratory reports. The reported QC results met the laboratory's acceptance criteria, although holding-time exceptions were identified for some analyses.

4.3. Data Analysis

The analytical results were first compiled and quality-checked; then, preliminary summaries of cyanobacterial cell count, biovolume, and taste-and-odour (T&O) measurements were generated using Microsoft Excel (v2021; Microsoft Corporation, Redmond, WA, USA). These datasets were then combined with molecular results for integrated interpretation. Following sequencing, the data were reviewed for potential quality control issues and possible contamination using the information available in the ALS laboratory reports. For each sample, relative taxonomic abundances were obtained from the sequencing analysis performed by ALS Water (Scoresby, Victoria, Australia). The bacterial order-level relative abundance data were subsequently visualised using R software (version 4.4.1; R Foundation for Statistical Computing, Vienna, Austria) to generate stacked bar charts illustrating the bacterial community composition across treatment stages, sampling locations and sampling dates.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/toxins18120511/s1, Table S1: Summary of key operational characteristics and treatment processes at three drinking water treatment plants (DWTPs) included in this study, i.e., K-DWTP, E-DWTP, and C-DWTP; Table S2: Analytical techniques and sampling coverage across treatment stages at Kerang (K-DWTP), Echuca (E-DWTP) and Cobram (C-DWTP) during Phases 1 and 2; Table S3: Summary of Cyanobacterial taxa detected across Phase 2 treatment streams (2023–2024); Table S4: Cyanotoxin, geosmin and 2-methylisoborneol (2-MIB) concentrations across treatment stages at three drinking water treatment plants during Phase 1 (March–May 2023) and Phase 2 (December 2023–March 2024).

Author Contributions: Conceptualization, S.I.A. and A.Z.; Methodology, S.I.A. and A.Z.; Investigation, S.I.A.; Formal analysis, S.I.A.; Data curation, S.I.A., S.N., V.I., D.S. and S.B.; Resources, S.N., V.I., D.S. and S.B.; Writing, original draft preparation, S.I.A.; Writing, review and editing, S.I.A. and A.Z.; Visualization, S.I.A.; Supervision, A.Z.; Project administration, A.Z. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the Australian Government's Future Drought Fund and Water Research Australia (WaterRA), project number 4554, and by the Ministry of Education, Saudi Arabia, through a Ph.D. scholarship awarded to S.A.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data supporting the findings of this study are available from the corresponding author upon reasonable request. The data are not publicly available due to partner utility requirements.

Acknowledgments: During the preparation of this manuscript, the authors used ChatGPT (OpenAI, GPT-6) to assist with English language editing, improve clarity and readability, and refine the wording and layout of the graphical abstract. The authors reviewed and edited the output and take full responsibility for the content of the publication.

Conflicts of Interest: Author Steven Newham was employed by the Goulburn Valley Water; Author Vijay Ignatius was employed by the Lower Murray Water; Authors David Sheehan and Sallyanne Bartlett were employed by the Coliban Water. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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