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RESEARCH ARTICLE

Impacts of temperature and hydraulic regime on discolouration and biofilm fouling in drinking water distribution systems

Katherine E. Fish[1]*, Rebecca L. Sharpe[2], Catherine A. Biggs[3], Joby B. Boxall[1]

1 Civil and Structural Engineering, Sheffield Water Centre, The University of Sheffield, Sheffield, United Kingdom, **2** Department of the Natural & Built Environment, Sheffield Hallam University, Sheffield, United Kingdom, **3** School of Engineering, Newcastle University, Newcastle, United Kingdom

* K.Fish@sheffield.ac.uk

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Abstract

Discolouration is the greatest cause of customer dissatisfaction with drinking water quality, potentially masking other failures, including microbial issues, which can impact public health and well-being. The theorised association between biofilms (complex microbial communities) and discolouration within drinking water distribution systems (DWDS) was explored, whilst studying the impact and interactions of seasonal temperature variations and hydraulic regime. Transferability of findings to operational DWDS was ensured by using a temperature controlled, full-scale distribution experimental facility. This allowed isolation of the factors of interest, with integration of physical, chemical and microbial analyses. Greater discolouration and biofilm cell accumulation was observed under warmer (summer, 16˚C) temperatures compared to cooler (winter, 8˚C), evidence of microbiology being an important driver in DWDS discolouration behaviour. Temperature was generally more influential upon discolouration and biofilm cell volumes than the shear stress imposed by the hydraulic regimes, which included three steady state and two varied flow patterns. However, the trends were complex, indicating interactions between the two parameters in governing microbial accumulation and discolouration. These results are important in informing sustainable management of our ageing DWDS infrastructure to deliver safe high quality drinking water. By providing new evidence that discolouration is a biofilm/microbiologically-mediated process, we can better understand the importance of targeting interventions to hotter seasons, and manipulating hydraulic conditions (which we can control), to minimise the long-term impacts of impending changing climates on water quality.

1. Introduction

The climate crisis, an increasing population, and increasing urbanisation are all driving an accelerating need to improve the resilience and sustainability of the water sector. It is critical that sustainability conserves water quality not just quantity; this is essential for society, the economy and, arguably, the environment. To protect public health and well-being, water utilities are tasked with maintaining (and improving) drinking water quality, in increasing

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volumes, to a growing number of customers, via an ageing distribution system exposed to new and increasing pressures (e.g. water shortages, temperature changes) [1]. Achieving this in such an uncertain socio-environmental climate requires better understanding of the interactions within the system, and how environmental changes may impact these.

Drinking water is delivered to customers through a pipe network termed the drinking water distribution system (DWDS). Various interactions and reactions occur between the pipe wall and bulk water within DWDS, which degrade the quality of the water as it is transported through the system. The largest number of drinking water quality-related consumer contacts are due to aesthetic degradation, of which discolouration is a principal example worldwide [2, 3]. Discolouration is the result of dissolved colloidal or suspended substances being present in the water column [4], often this material has accumulated within the DWDS pipes and is subsequently mobilised in volumes sufficient to cause visible discolouration [2]. OFWAT (the water services regulation authority in England and Wales) have highlighted the importance of investment in reducing discolouration, introducing penalties for companies if customer contacts regarding discolouration exceed an acceptable level in their Outcome Delivery Incentive reports [5]. Furthermore, customers rated the reduction in discoloured water as high in a "willingness to pay" survey, being prepared to pay more than a third more for this than other service improvements [6]. Even with regulatory and customer pressures, the Drinking Water Inspectorate (DWI) reported that discolouration events affected 1.81 million customers in England in 2020 (61% more than in 2019) [7]. This is a substantial increase from the 1.14 million customers in England and Wales who were estimated to have been affected by "significant, serious and major" discolouration events in 2016 [8]. This figure excludes events that were due to planned work or attributed to treatment work failure and hence captures those that were likely occurring during distribution. Reducing discolouration is an ongoing challenge likely to be intensified by global climate change, particularly temperature changes. In order to mitigate current (and future) water quality failures, water companies are required to shift from reactive to proactive management. Achieving this requires a better understanding of the processes/behaviour of water quality degradation occurring within DWDS and the impact that temperature changes have upon this, especially within the context of discolouration.

Discolouration-causing material consists of fine-sized matter originating from corrosion, chemical reactions and/or biological interactions [9]. Increases in turbidity (the measurement of discolouration) have also been associated with increases in iron and manganese concentrations [10–12], with one study reporting positive correlations of 80.58% and 71.94%, respectively, in UK district management areas [13]. This suggests an association between discolouration and metal concentrations, demonstrating that discolouration events may mask failures of other water quality parameters. Two concepts are generally proposed to describe and understand discolouration: (i) sedimentation, where behaviour is governed by the particles self-weight, as applied for rivers and sewers and with the threshold of motion described by concepts like the Shield criterion; or (ii) the idea of cohesive layers based on the observation that the particles in DWDS are generally too small and low density for their behaviour to be dominated by their self-weight. This cohesive layer concept is captured in the Prediction of Discolouration in Distribution Systems (PODDS) model, which suggests that interactions at the pipe interface lead to particles actively concentrating into attached layers at different adhesive strengths, with the minimum strength determined by the normal hydraulic regime within the pipe [14]. The attached material is then mobilised if the hydraulic forces exceed the layer strength. This PODDS modelled discolouration behaviour has been observed across many DWDS and is conserved between differences in infrastructure, water matrices and countries [12, 15, 16]. PODDS has been validated as an empirical tool by various field and laboratory studies and can accurately predict a discolouration response to changing hydraulics. However,

the model provides limited understanding of the processes causing material accumulation or the effects of temperature on discolouration. Interestingly, the discolouration behaviour that PODDS predicts is analogous to the accumulation and mobilisation of microbial biofilms, which have been shown to be influenced by hydraulic regime, and governed by the cohesive properties of the biofilm [17].

Discolouration is reported to be positively correlated with temperature, such that the frequency of reported discolouration increases in the summer months [13, 18]. The accumulation of material within DWDS has also been shown to be elevated at higher temperatures [19]. Water temperature is acknowledged to differ spatially (between networks and along pipes) and temporally (*i.e.* seasonally); 18 countries have a regulation or guideline value for water temperature [20], although an international study on water temperature reported no clear policies are in place should drinking water temperatures exceed these values [21]. Blokker and Pieterse-Quirijns [22] found that water temperature in DWDS in the Netherlands was mostly affected by soil temperature, with annual water temperature ranges of 5–20˚C being reported [19]. A data-driven modelling study of UK and Dutch systems highlighted that temperature, pipe material and bulk-water iron concentration were the three key factors correlated with, and hence influencing, discolouration material accumulation (although temperature correlations were primarily within non-chlorinated DWDS) [23]. Furthermore, in a study of 176 DMAs (supplied with a disinfectant residual) higher water temperatures were correlated with higher iron concentrations (>57% of sites) and higher manganese (>65% of sites) [13].

Despite the recorded temperature variations (and their impact on discolouration), currently only the Netherlands have strict temperature legislation for drinking water (a maximum of 25˚C) [22]. The basis for this legislation is primarily the microbiological quality of bulk-water (i.e. planktonic microorganisms, not biofilm bound microorganisms), rather than discolouration, specifically the awareness that water temperature is influential in the risk of *Legionella pneumophila* (bacteria that causes Legionnaire's disease) colonising DWDS [24–27] and potentially causing an outbreak. Worldwide, microbial drinking water quality is routinely assessed by quantifying coliforms: a group of Gram-negative bacteria used as indicators of the potential presence (or absence) of faecal contamination or pathogens. Changes in temperature have been found to affect the number of coliforms detected within drinking water and microbial growth potential has also been shown to increase at temperatures above a threshold of ~ 15˚C [28–31]. Within DWDS microbial cells are found in both planktonic (*i.e.* in the water column) and biofilm (surface bound) states. DWDS biofilms are mixed-species microbial communities that develop at the pipe-water interface, adhered to the pipe wall by a matrix of extracellular polymeric substances (EPS) and contain the majority of the microbial load of a DWDS. Temperature clearly influences both discolouration and microbial quantity/activity, which suggests that these factors may be linked.

Temperature is an important determinant of drinking water quality as it affects the physical, chemical and biological processes occurring within the DWDS, including microbial growth and the rate of chlorine decay [21]. It has been suggested that the disinfection contact times within DWDS are insufficient to inactivate various microorganisms [32]. The sub-lethal doses can then exert a selective pressure which impacts the planktonic (free-living microorganisms in the bulk-water; [33, 34]) and biofilm microbiomes and cell concentrations [35, 36]. Consequently, temperature could have an indirect impact on the microbial load of chlorinated or chloraminated systems by affecting the disinfection residual concentrations, unless systems are dosed seasonally at treatment works. If biofilm behaviour is analogous to that of discolouration material accumulation/mobilisation within the DWDS, this suggests that the discolouration risk posed by biofilm would be higher in the warmer months. In a one-year study investigating discolouration risk within operational trunk mains, Sunny *et al.* [37] observed a

positive correlation between temperature, material accumulation and total organic carbon (TOC). With respect to organics, assimilable organic carbon (AOC; the microbial available fraction of carbon) is correlated with microbial growth, which can impact water quality with higher AOC concentrations being reported to result in higher discolouration [38]. However, different target AOC concentrations are reported in the literature, with varied impacts [29, 39, 40] and different AOC behaviour being observed in differ assets of the DWDS (pipes vs. service reservoirs; [41]). Clearly, the relationships between nutrients and microbial dynamics in DWDS are complex but crucially the findings by Sunny *et al* [37] indicated that temperature and organic material, and therefore likely associated microbial behaviour, played a critical role in the discolouration risk posed by a DWDS. Further supporting this finding, Cook *et al.* [18] found that a greater number of discolouration complaints were made during higher temperatures in the UK, with fewer in the winter when water temperatures typically fall to $<6^{\circ}C$.

Given that both microbial failures and discolouration events are more likely during warmer periods, there is a hypothesised connection between biofilms and discolouration, with temperature being an important influencing variable. Previous studies have primarily used observational field data to highlight correlations between hydraulics, temperature and discolouration. Where full-scale controlled studies have been conducted, these have considered either just [42, 43] or the impact of future extreme temperatures [44]. Seasonal temperature changes are under-explored, and their impacts have not been considered in combination with different hydraulic regimes, hence causation rather than correlation has thus far not been established at a scale representative of operational DWDS.

This study aimed to determine the impact of seasonal temperature variation (summer to winter in the UK) on the discolouration of drinking water and the quantity of biofilm bound microorganisms within DWDS, when pipes were conditioned to different hydraulic patterns. By investigating only these variables, but in combination, this study set out to determine if one parameter was more influential than another in promoting biofilm development or discolouration whilst also providing further insights into microbiology as a driver for the accumulation and release of material within DWDS.

2. Materials and methods

2.1. Experimental overview

A series of controlled experiments were undertaken using a full-scale, temperature controlled DWDS experimental pipe loop facility (Fig 1) to develop biofilms and condition pipe lengths during an "Accumulation" phase and subsequently assess the discolouration response of the conditioned pipes during a "Mobilisation" phase. Each experiment was conducted under physical, chemical and microbial conditions representative of operational networks to ensure transferability of data to live DWDS.

Accumulation experiments were conducted at two temperatures representative of UK seasonal differences from winter ($8^{\circ}C$) to summer ($16^{\circ}C$) and under one of five hydraulic regimes (three steady state and two varied flow patterns; Fig 2). At the end of each Accumulation phase, incremental increases in flow rate (and hence the shear stress imposed by the near-wall velocity gradient) were applied to each pipe during a Mobilisation phase, for which changes in bulk-water quality were monitored throughout. The ranges of flow rates (and shear stresses) during the Mobilisation phase were representative of those occurring in operational networks during routine maintenance or following bursts. Shear stresses were quantified through evaluation of primary losses due to overcoming friction. The experimental setup was the same as used by Sharpe *et al.* [43] to investigate the impact of hydraulics on discolouration. The current study addresses different research questions, providing an extended dataset with the inclusion

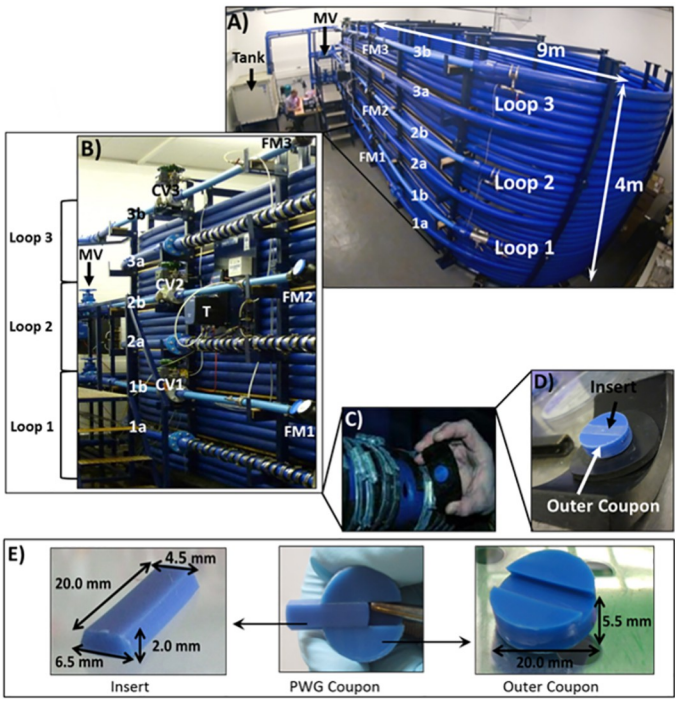

Fig 1. Drinking water distribution system experimental facility. (Reproduced from Fish et al. [45]). A & B) Entire facility comprising high density polyethylene loops and a reservoir tank, total volume 4.5 m^3 (tank 1.53 m^3), split into three loops (1–3) of 200 m via manual valves (MV), "a" = section of loops into which coupons were inserted, "b" = section of loops containing flow meters (FM) and control valves. C & D) Apertures into which Pennine Water Group (PWG) coupons [46] (E) were inserted.

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of summer and winter temperature comparisons across bulk-water and biofilm cell data, but does draw on some data points from the previous study.

2.2. DWDS experimental facility

The DWDS experimental facility (Fig 1) has been described in detail elsewhere [43, 45]. Briefly, the facility consisted of three high-density polyethylene (HDPE PE100) loops, each of 79.3 mm

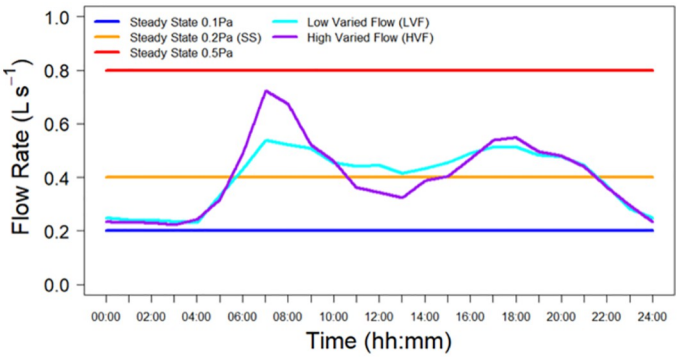

Fig 2. Accumulation-phase diurnal hydraulic regimes tested in this study at 8˚C and 16˚C. Note that the flow rates of the three steady state regimes were 0.2, 0.4 and 0.8 Ls^{-1}, which are named in the key according to the shear stress they imposed upon the pipe wall/biofilm 0.1, 0.2 and 0.5 Pa, respectively. All flows were either just transitional or fully turbulent as per Sharpe et al. [43].

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internal diameter and 203 m in length. The facility combined laboratory level control of environmental parameters and sampling regime, with full-scale replication of DWDS hydraulics (flow and pressure), materials, water supply (local water supply, no seeding or dosing with microbial inoculum), ecology and exchange mechanisms. The entire facility was set within a temperature-controlled room (cooling unit, ICS group) with a range of 4–21˚C (±1˚C). Water sourced directly from the local DWDS (upland peat runoff surface water source, supplied via cast iron trunk main) was pumped around each of the loops from an enclosed reservoir tank. During the accumulation phase, to maintain water quality representative of a live network, a system residence time of 24 hours was set (via drain and trickle feed) for the whole system (tank plus loop volume), such that the average water age within the pipes was 12 hours: note this system residence time is independent of the hydraulic residence time to complete a single pass of the pipe loop. The system was supplied via a single reservoir and pump, with a manifold to split the flow to the three loop, each loop has an independent final control valve before returning to the common reservoir. Thus, the three pipe loops simulate different pipe lengths in an interconnected system. A LabVIEW programme (v8.2, National Instrument Corporation, UK) regulated the hydraulic regime (and flushing flow rates) by adjusting valves and pump speed, care was taken to ensure that all changes were made slowly to eliminate transients. Flow meters (Flownetix ultrasonic, Birmingham, UK) and turbidity meters (Chemtrac TM2200, Chemtrac Inc. USA) provided continuous monitoring of flow rate and discolouration during the accumulation and mobilisation phases.

Each of the three loops of the facility had 27 apertures into which Pennine Water Group (PWG) coupons [46] were inserted (Fig 1) allowing the sampling and analysis of material (*i.e.* biofilm) at the pipe wall. PWG coupons were arranged around the entire circumference of the loop following the sequence invert, side and crown. Each PWG coupon comprises an outer and insert components: the outer is curved to fit the inner wall of the pipe and limit distortion of boundary layer hydraulics; the insert (only 4.5 mm wide on the exposed surface) is designed to be flat to facilitate microscopy analysis.

Prior to, and between, experiments, the entire system was disinfected for 24 hours using a 20 mgL^{-1} sodium hypochlorite solution (Rodol Ltd, UK), which was circulated around the system at 4.5 Ls^{-1} and then thoroughly flushed from the system. Prior to use the PWG coupons were sterilised using 2% (w/v) SDS and sonication, followed by being autoclaved, as detailed in Fish *et al.* [45].

2.3. Accumulation phase

2.3.1. Temperature and hydraulic regimes. Material (*i.e.* biofilm) was accumulated for 28 days (indicative of initial colonization of a replaced or relined pipe) at either 8˚C or 16˚C and under one of five hydraulic regimes: Steady State 0.1 Pa, Steady State 0.2 Pa, Steady State 0.5 Pa, Low Varied Flow (LVF) and High Varied Flow (HVF) (Fig 2). These temperatures were chosen with respect to the reported threshold for an increase in biological activity within drinking water [31], while remaining within the average water temperature range expected in the UK during winter and summer, respectively. The hydraulic regimes were based on UK field data for typical diurnal patterns and were previously tested by Sharpe *et al.* [43] but only at one temperature: 8˚C. Where possible the 8˚C experiments were run in the cooler months (Autumn/Winter) and the 16˚C experiments were run in the warmer months (Spring/Summer) to ensure the incoming water temperature was comparable with the experimental conditions.

The five hydraulic regimes were tested by splitting them into two sets—"Steady State" and "Varied Flow"—then running each set of hydraulics at 8˚C and 16˚C. Each set comprised

three hydraulic profiles, which were run simultaneously (*i.e.* one hydraulic regime per loop) with one regime common to both sets as a control (*i.e* 12 experiments were run in total). Initially, the "Steady State" set was run at (8˚C and 16˚C), which applied the 0.1 Pa, 0.2 Pa and 0.5 Pa regimes (Fig 2). The Steady State profiles were applied to ascertain the relationship between increasing shear stress, material accumulation and discolouration, at each temperature. However, steady state flow is rare in operational DWDS, rather there are peaks and troughs in flow rate, and hence shear stress, that are primarily driven by demand. To account for this a "Varied Flow" set of hydraulic regimes were also tested at each temperature. This set included the Low Varied Flow (LVF) and High Varied Flow (HVF) profiles shown in Fig 2, which reproduced typical double-peak diurnal residential patterns of demand based upon UK field data from 75 mm diamater pipes [12]. LVF and HVF were designed to have the same low (0.2 Ls^{-1}, 0.1 Pa) and daily average (0.4 Ls^{-1}, 0.2 Pa) flow rates but different peak flow rates (0.54 Ls^{-1}, 0.34 Pa and 0.75 Ls^{-1}, 0.40 Pa, respectively). Note that the SS0.2 regime was repeated as a control in the Varied Flow set, to facilitate comparison of the impact of average, peak and low flow rates upon accumulation (and subsequent discolouration). For clarity, from herein the hydraulic regimes will be referred to using the notation of SS0.1, SS0.2 and SS0.5 for the Steady State dataset, or codes SS, LVF and HVF, for the Varied Flow dataset.

2.3.2. Water quality. During the accumulation phase of each experiment a range of regulatory water quality parameters were monitored using a combination of water utility supply data and weekly discrete sampling (n = 3) of the reservoir tank in the DWDS experimental facility (S1 Table). Natural variation in water quality parameters was observed but all the measurements were within UK drinking water quality standards and there were no consistent differences between sets of experiments (apart from a spike of iron during the 16˚C Steady State experiments), indicating that the main differences between the experiments were hydraulic pattern or temperature.

2.3.3. Biofilm sampling. Biofilm samples were collected by removing coupons (n = 3) at Day 0 (as a control) and the end of the accumulation phase (Day 28) for each hydraulic experiment, at each temperature. Each set of samples comprised coupons from the three different locations around the circumference of the pipe. The insert from each PWG coupon was removed aseptically, fixed in 5% formaldehyde (48 hours) and rinsed with phosphate buffer solution as described in Fish *et al.* [45]. The inserts were then stored in the dark at 4˚C ready for biofilm cell quantification via confocal laser scanning microscopy (CLSM).

2.3.4. Biofilm cell analysis. The cells of biofilm samples were evaluated using the fluorescent microscopy and digital image analysis (DIA) methods described in detail by Fish *et al.* [45]. The insert of each coupon was stained with a 20μM solution of Syto 63 (Molecular Probes, Califronia, USA), which targets DNA. An LSM510 meta up-right CLSM (Zeiss, Germany) within the Kroto Imaging Facility (The University of Sheffield, UK) was used to generate Z-stacks (a series of XY images) for seven (random) Fields of View (FOV; each 420 μm x 420 μm) per sample (*i.e.* n = 3 x FOV = 7). The Z-stack images were taken using the lambda imaging capability of the LSM510 meta upright CLSM (with a x20 EC Plan Neofluar objective, 0.5 NA). Lambda-imaging facilitated the subsequent unmixing of the scaffold/biofilm autofluorescence and Syto 63 signals via the CLSM associated software. DIA was applied as described in Fish *et al.* [45], to remove noise from the unmixed Z-stack via median filtering and threshold the images (at 2401). Subsequently, the area covered by the stained cells was calculated for each XY image in a Z-stack by dividing the number of stain-associated pixels by the total pixels in the image (832 × 832). This data was visualised as area distribution plots (S1 and S2 Figs), for which normalised slice number refers to the labelling of the XY image (or slice) with the maximum cell coverage set as slice "0" and numbering slices above and below consecutively. This was necessary to enable visual comparison of the area distribution plots

because the size of each Z-stack differed between FOV, hence slice "1" was not a comparable point of reference. Area coverage (expressed as a fraction or proportion of the area) was the principal quantified parameter and was used to calculate the volume of stained cells (μm^3 per 420 μm^2), as explained in Fish *et al.* [45]. This DIA analysis used a combination of the open access software Python (www.python.org) and R v.4.0.2 [47].

2.4. Mobilisation phase

2.4.1. Flushing sequence. The Mobilisation phase protocol was detailed in Sharpe *et al.* [43]. In brief, a series of six increasing shear stresses (Table 2) were applied by flushing the loop at different flow rates (the system was run for three turnovers at each step to ensure the complete removal and mixing of material prior to transitioning to the next step). Each loop of the system was flushed independently, using a fresh volume of water in the reservoir tank for each loop and isolating the loops not being flushed (sealing water within them). An initial flushing step (Step 0 in Table 2) was used to mix the reservoir tank water with the loop water at the previous Accumulation phase conditioning flow rate and shear stress (for LVF and HVF the daily average of 0.4 Ls^{-1}, 0.2 Pa was used). This provided a baseline for the water quality parameters prior to the Mobilisation phase experiments, as described in Sharpe *et al.* [43].

2.4.2. Discolouration measurements. Bulk-water discolouration was monitored during the Mobilisation phase(s) via the collection of continuous turbidity data (online Chemtrac meters; Fig 1) and discrete water samples (100 mL) for iron and manganese analysis (Table 1). The discrete water samples (n = 3) were collected prior to the start of the Mobilisation phase and then at the end of each flushing step (0–6 in Table 2).

2.5. Data analysis

All the statistical tests detailed below and associated plots were generated using R v.4.0.2 [47] unless otherwise specified.

2.5.1. Water quality changes during Mobilisation. For the Mobilisation-phase(s), turbidity was averaged across the three turnovers for each flushing step. Then standardised by subtracting the mean value during the last 10 minutes of the initial mixing phase (Step 0 in Table 2) from each of the subsequent flushing steps (steps1-6). If the standardised average was ≤ 0 this indicated that no change from the background water quality was detected. Similarly, the average iron and manganese concentrations were calculated for each flushing step and normalised by subtracting the average concentration in the water sampled from the reservoir tank at the end of the mixing phase. Note that, due to this standardisation process, some of the measurements taken in the early flushing steps of the Mobilisation phases were similar to that of the mixing phase, in a few instances causing the average to drop below "0". For clarity, we have plotted all the standardised data as it was calculated, rather than further processing the data to adjust negative measurements to "0".

Table 1. Details of the flow rates and shear stresses used during the Mobilisation phase, each step was run for three turn-overs.

Step Number	0[A]	1	2	3	4	5	6
Flow Rate (Ls^{-1})	0.2–0.8	0.80	1.20	1.75	3.20	4.00	4.50
Velocity (m/s)	0.04–0.16	0.16	0.24	0.36	0.65	0.81	0.91
Shear Stress (Pa)	0.1–0.5	0.50	0.73	1.07	2.00	2.50	3.00

[A] The initial mixing phase which was carried out at the previous conditioning flow during the Accumulation phase, for the varied flow regimes the daily average of 0.4 Ls^{-1} / 0.2 Pa was used.

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Table 2. Total discolouration and metal concentration increases in bulk-water during the Mobilisation phase of each hydraulic experiment at 8˚C and 16˚C. Values presented were calculated using area under the curve analysis, based on averages of normalised data.

Hydraulic Dataset	Hydraulic Regime [A]	Turbidity (NTU)		Iron (μg l^{-1})		Manganese (μg l^{-1})	
		8˚C	16˚C	8˚C	16˚C	8˚C	16˚C
Steady State	SS0.1	0.62	1.64	124.5	174.3	14.4	24.8
	SS0.2	0.20	0.84	56.7	144.6	7.7	15.2
	SS0.5	0.21	0.55	19.9	36.0	2.3	3.6
Varied Flow	SS[B]	0.33	0.78	11.5	20.8	2.6	14.2
	LVF	0.29	0.69	3.8	24.5	-1.0	8.6
	HVF	0.12	0.69	5.9	22.7	3.9	9.9

[A] Codes refer to the conditioning hydraulic regime during the growth phase, as set out in Methods 2.1: Steady State 0.1Pa / 0.2Pa /0.5Pa, Low Varied Flow and High Varied Flow.

[B] SS regime was a repeat of SS0.2.

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The average (mean) and standard deviation of the turbidity, iron and manganese concentrations were plotted against shear stress. The response of these water quality parameters was not linear. Therefore, in order to compare to the total increase in NTU, iron or manganese throughout the mobilisation phases between experiments, area under the curve (AUC) values were calculated. These enabled comparison of total amounts of material mobilised regardless of when the most mobilisation (*i.e.* peak) occurred. AUC values were calculated for each experiment of the temperature and hydraulic data sets using trapezoidal integration (the "trapz" method from the R package "pracma"). AUC provides a quantitative way to summarise the different water quality responses / profiles in a single, computed value—rather than only qualitatively describing different shapes.

2.5.2. Biofilm cell analysis. The volume of biofilm cells were plotted using box and whisker plots (showing the entire range of the data) because the data were not normally distributed. Non-parametric statistical tests were used to compare between experiments—specifically Kruskal Wallis and Wilcoxon tests, for which χ^2 or W values are presented with the p values (significance set at ≤ 0.05). Samples were compared for any positional effects (*i.e.* coupons take from the crown, side or invert of the pipe) upon biofilm cell volumes. None were found so it was unnecessary to distinguish samples based on coupon location in downstream analysis.

3. Results

3.1. Water quality response to Mobilisation phase. Regardless of conditioning hydraulics (SS0.1, SS0.2/SS, SS0.5, LVF or HVF), at both temperatures (8˚C or 16˚C) turbidity, iron and manganese concentrations increased over the course of the Mobilisation phase as the shear stress at the pipe wall increased (Figs 3 and 4). The water quality responses were not linear with respect to increasing shear stress. Rather each parameter (turbidity or metal concentration) exhibited a unique and varying profile, with peaks at different stages of the Mobilisation phase.

3.1.1. Mobilisation response across Steady State hydraulics at different temperatures. Discolouration responses consistently increased with temperature for each of the Steady State hydraulic regimes (*i.e.* conditioning shear stress) with greater increases in turbidity and metal concentrations at 16˚C than to 8˚C (Fig 3; Table 2). This increase in discolouration was particularly evident for turbidity at 16˚C, which has at least double the AUC value of that at 8˚C, for each Steady State regime (Table 2). Additionally, regardless of conditioning hydraulics,

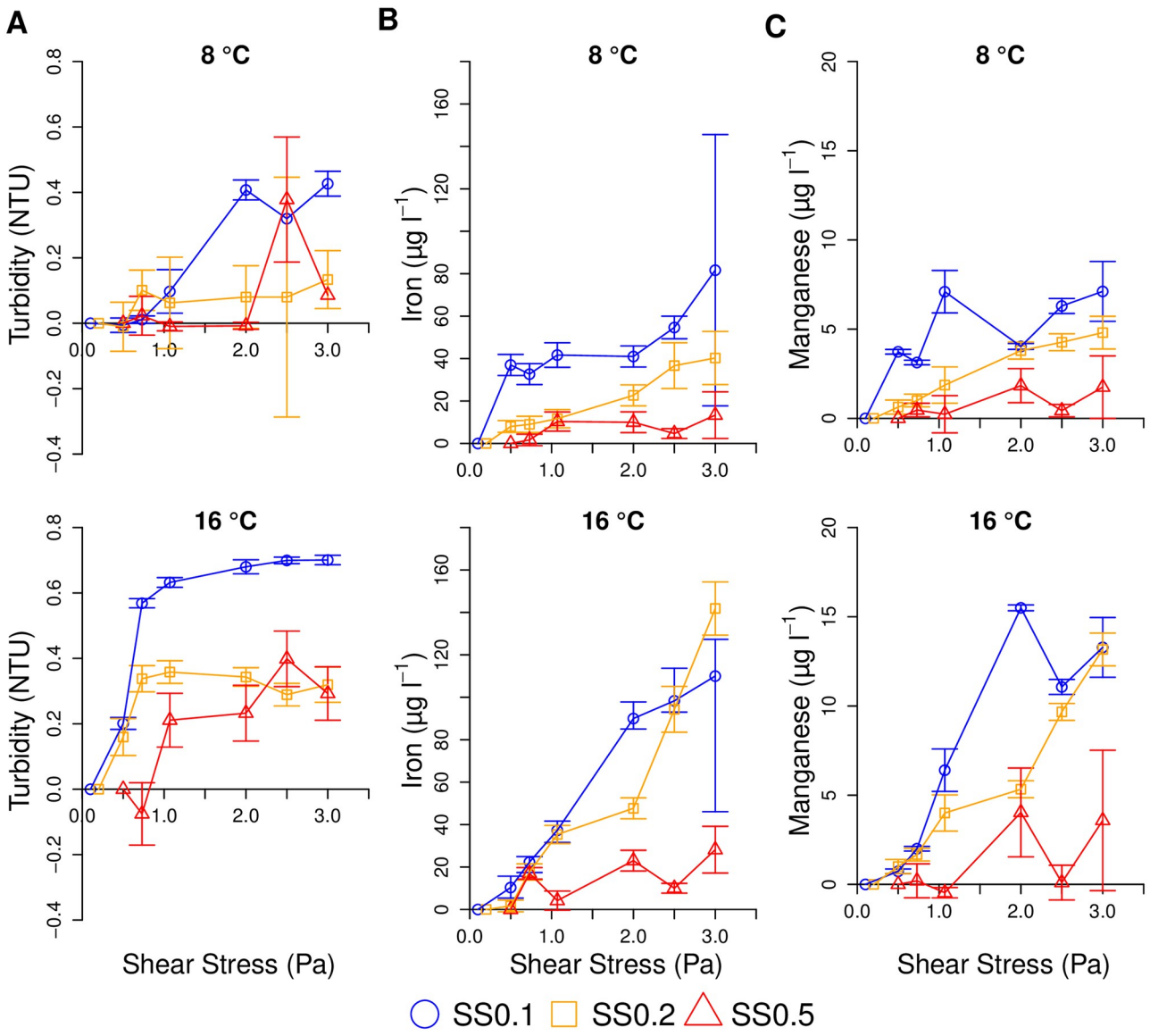

Fig 3. Water quality responses of Steady State conditioned pipes to elevated shear stress during the Mobilisation phase. Pipes previously conditioned to three Steady State hydraulic regimes (SS0.1, SS0.2 and SS0.5). Experiments run at 8˚C and 16˚C, as indicated. Discolouration parameters monitored were A) Turbidity, B) Iron and C) Manganese concentration. Normalised data is presented as averages ± standard deviation. A) Averages of time series data from 0–3 turnovers, replication was a function of flow rate, turbidity was measured every second so n≥1199 (duration for three turnovers at the highest flowrate); B/C) averages based on discrete samples (n = 3) at the end of 3 turnovers.

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turbidity increases observed at 16˚C predominantly occurred at lower shear stresses (*i.e.* earlier in the mobilisation phase) and then stayed fairly stable, whereas at 8˚C the mobilisation profiles were more varied with peaks at higher shear stresses.

At both temperatures, the lowest conditioning shear stress regime imposed during the accumulation phase (SS0.1) always subsequently exhibited the greatest increases in turbidity, iron and manganese during the Mobilisation phase (Fig 3; Table 2). However, the turbidity and metal mobilisation profiles did differ between temperatures. At 8˚C, during Mobilisation, the peaks in turbidity, iron and manganese generally occurred at a lower flushing shear stress (*i.e.* earlier in the flushing process) for the system that experienced the lowest shear stress during

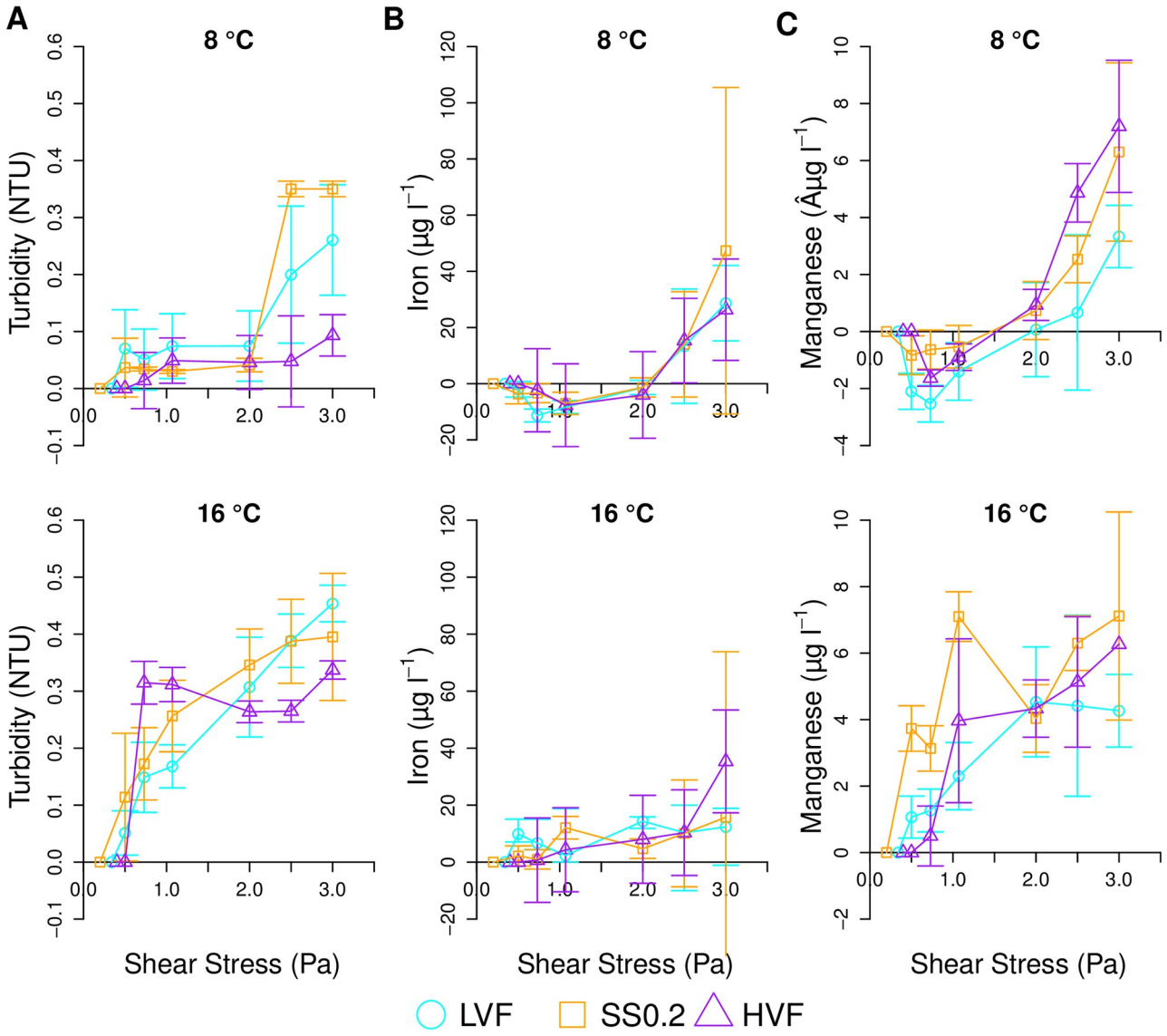

Fig 4. Water quality responses of pipes conditioned to Varied Flow hydraulics to elevated shear stress during the Mobilisation phase. Conditioning hydraulics were Low Varied Flow = LVF, Steady State = SS and High Varied Flow = HVF. Temperatures were 8˚C and 16˚C, as indicated. Discolouration parameters monitored were A) Turbidity, B) Iron and C) Manganese concentration. Normalised data is presented as averages ± standard deviation. A) Averages of time series data from 0–3 turnovers, replication was a function of flow rate, turbidity was measured every second so n≥1199 (duration for three turn overs at the highest flowrate); B/C) averages based on discrete samples at the end of 3 turnovers.

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Accumulation (SS0.1) compared to those experiencing a greater shear stress during the Accumulation phase (SS0.2 or SS0.5). For regime SS0.1, the peak turbidity, iron and manganese responses during the Mobilisation phase occurred at a higher flushing shear stress at 8˚C than was observed at 16˚C. Consequently, at 16˚C, the systems that had previously experienced low (SS0.1) and medium (SS0.2) shear stress during accumulation had similar turbidity profiles in response to the flushing: an initial mobilisation of material and then little variation over the rest of the mobilisation phase. The SS0.1 and SS0.2 regimes also had similar metal mobilisation at 16˚C, which generally increased as flushing shear stress increased throughout the Mobilisation phase. Whereas the system that experienced the highest shear stress during accumulation (SS0.5) had substantially lower mobilisation of turbidity and metals at 16˚C than either SS0.1 or SS0.2,

with the SS0.5 peak in turbidity occurring towards the end of the mobilisation phase. Overall, the SS0.5 regime, which was the highest shear stress during the accumulation phase, consistently had the lowest increase in metal concentrations during mobilisation at both temperatures, and the lowest turbidity response at 16˚C (at 8˚C the turbidity response was similar to SS0.2).

3.1.2. Mobilisation response across Varied Flow hydraulics at different temperatures. Greater mobilisation of material was consistently detected at 16˚C compared to 8˚C, for all discoloration parameters (NTU, Fe, Mn) and across all three of the Varied Flow conditioning hydraulic regimes (Table 2). The greatest increase in turbidity between the two temperatures was in the HVF conditioned pipes, for which the total turbidity mobilised (based on AUC analysis) increased six-fold between 8˚C and 16˚C, for the LVF and SS regimes there was a two-fold increase in turbidity between 8˚C and 16˚C.

At 8˚C, all three Varied Flow hydraulic conditioned pipes had similar turbidity responses to elevated shear stress until step 4 of the Mobilisation process (2.00 Pa), at which point the NTU measurements diverged depending on the conditioning hydraulics. The SS regime experienced a greater peak in turbidity than the LVF or HVF regimes, supported by the AUC analysis (Table 2) in which, at 8˚C, SS had the greatest turbidity response and LVF the lowest metals response. Manganese and iron concentrations only increased beyond the background concentration (from the mixing phase) when shear stress was $\geq$2.0 Pa. The iron responses remained similar for all three regimes throughout the mobilisation phase, whereas the HVF and SS regimes had a greater peak in manganese than the LVF condition.

Generally, at 16˚C, the mobilisation of turbidity and manganese profiles showed an initial increase mainly between 0.5–1 Pa, which then either stabilised or continued to increase but at a slower rate. The HVF conditioned pipe had the greatest initial peak in turbidity, although this then stabilised and over the entire Mobilisation phase the total turbidity mobilised from a HVF pipe was the same as that from the LVF conditioned pipe (based on AUC analysis). The SS regime had the greatest total discolouration response throughout the Mobilisation phase, although the difference between the hydraulic regimes was less pronounced than at 8˚C (Table 2). Manganese concentrations were also greatest when flushing the SS conditioned pipe, with the highest and earliest peak and greatest AUC. Iron responses were similar in profile (Fig 4) and magnitude as demonstrated by similar AUC values (Table 2).

Overall, the SS regime consistently had the greatest discolouration at both temperatures, HVF had the lowest at 8˚C and the distinction between the two varied profiles (LVF and HVF) was less clear at 16˚C than at 8˚C.

3.1.3. Comparing the influence of Steady State and Varied Flow hydraulics. As conditioning Steady State shear stress increased (SS0.1, SS0.2, SS0.5), discolouration response decreased, whether measured as turbidity, iron or manganese concentration. Although at 8˚C the SS0.2 and SS0.5 were similar in their turbidity response. Similarly, introducing variation into a conditioning flow profile decreased discolouration. This was most pronounced at 8˚C where the accumulation regime with the greatest variation (HVF) had half the total turbidity response of the accumulation regime with the greatest shear stress (SS0.5), despite the HVF profile having a lower average (0.4 Ls^{-1}, 0.2Pa) and peak (0.75 Ls^{-1}, 0.40 Pa) flow rate and shear stress than the SS0.5 regime. However, this relationship was not observed at 16˚C, where LVF and HVF had similar turbidity responses to each other and to the SS0.5 conditioning profile, though less than the SS0.2 profile.

3.2. Biofilm analysis

Biofilm cell volumes were calculated for coupon samples from Steady State and Varied Flow experiments at each temperature, across all the samples there were no consistent significant

differences driven by sampling location (crown, invert or side) hence all replicates are presented in the following without the need to define position.

3.2.1. Biofilm cell accumulation across Steady State hydraulics at different temperatures. Comparison of biofilm samples from each Steady State hydraulic regime showed an increase in cell volume at 16˚C compared to 8˚C (Fig 5; Table 3 and area distribution plots in S1 Fig). At 8˚C there were no statistically significant differences in biofilm cell volumes between the hydraulic regimes (χ^2 = 0.02, df = 2, p = 0.99). However, at 16˚C biofilm cell volume did differ between hydraulic regimes (χ^2 = 11.89, df = 2, p = 0.003), which was driven by the SS0.5 biofilms having the lowest cell volume (little difference between SS0.1 and SS0.2).

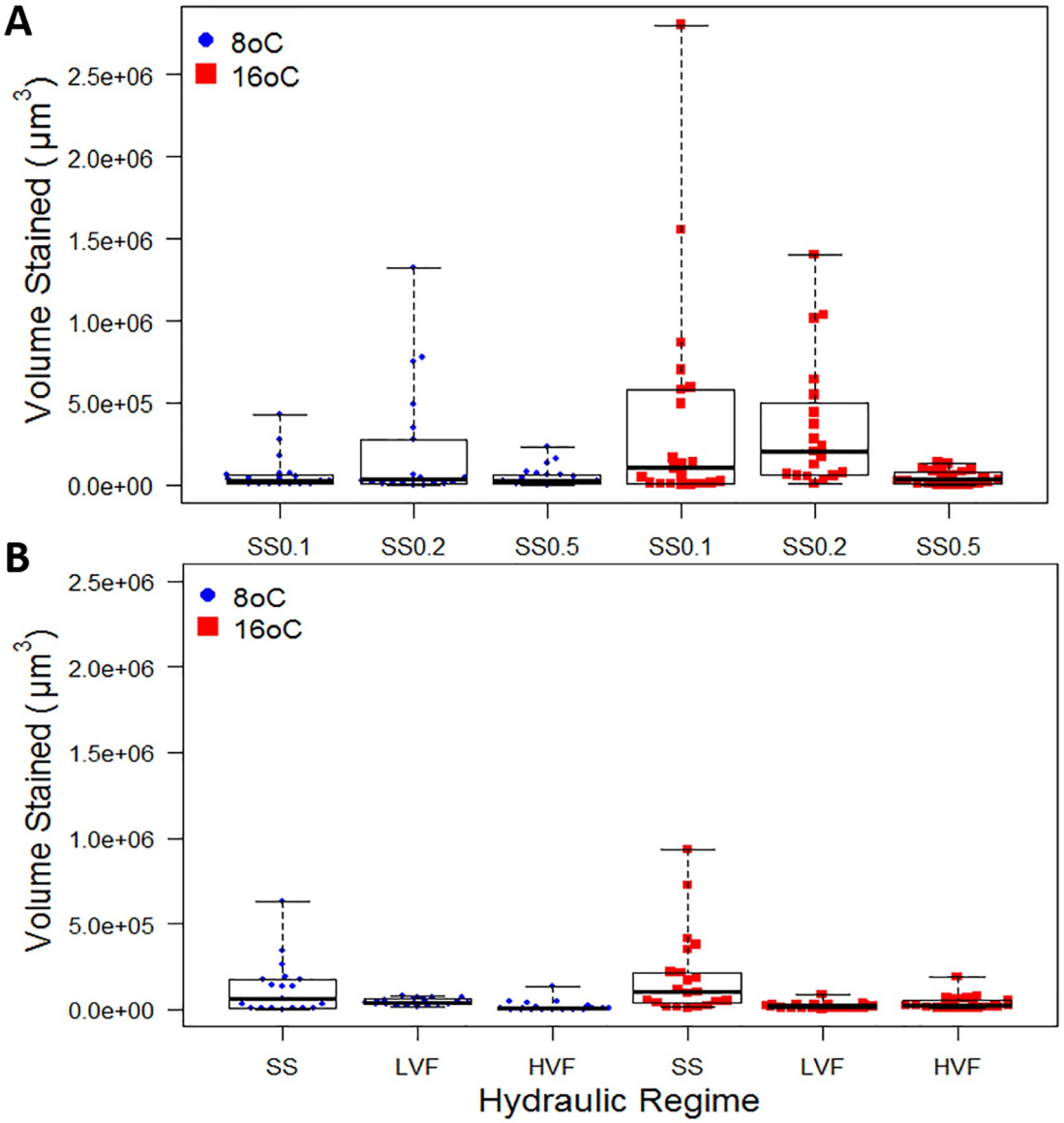

Fig 5. Biofilm cell volumes of drinking water biofilms sampled from pipes conditioned to Steady State (A) or Varied Flow (B) hydraulic regimes, at either 8 or 16˚C. Steady State hydraulics of 0.1 Pa, 0.2 Pa and 0.5 Pa. Varied Flow hydraulics of Steady State (SS), Low Varied Flow (LVF) or High Varied Flow (HVF) profiles. Raw data is plotted (n = 21, apart from SS 8˚C and LVF 16˚C, n = 20 and SS0.2 16˚C, n = 19) with each data point representing a FOV. Box and whisker plots show the range, interquartile range and median.

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Table 3. Median cell volume (to nearest μm^3) of biofilms sampled from pipes conditioned to different hydraulic regimes and water temperatures.

Hydraulic Dataset	Hydraulic Regime[A]	Temperature	
		8˚C	16˚C
Steady State	**SS0.1**	27,215	102,270
	SS0.2	31,380	205,295
	SS0.5	24,256	33,399
Varied Flow	**SS**	61,733	103,606
	LVF	38,821	15,327
	HVF	3,088	23,156

[A] Codes refer to the conditioning hydraulic regime during the growth phase, as set out in Methods 2.1: Steady State 0.1Pa / 0.2Pa /0.5Pa, Low Varied Flow and High Varied Flow.

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3.2.2. Biofilm cell accumulation across Varied Flow hydraulics at different temperatures. When considering just the Varied Flow hydraulic experiments, cell volume increased at 16˚C compared to 8˚C (Fig 5; Table 3 and area distribution plots in S2 Fig), in both the SS and HVF regimes although this was only statistically significant for the HVF regime ($W = 78$, $p<0.001$). This trend was not reflected in the LVF biofilms. At 8˚C biofilm cell volumes decreased with increasing variation or peak flow in the hydraulic regime (($\chi^2 = 18.85$, $df = 2$, $p<0.001$) such that HVF had the lowest cell volume and SS the greatest. However, at 16˚C LVF had the lowest biofilm cell volume, followed by HVF and SS again had the greatest ($\chi^2 = 19.47$, $df = 2$, $p<0.001$).

3.2.3. Comparing the influence of Steady State and Varied Flow hydraulics. Pipes with varied shear stress during the Accumulation phase had lower cell volumes compared to the experiments performed under steady state conditions. This was most obvious at 16˚C, for which LVF and HVF had fewer cells than the highest steady state profile of SS0.5 Pa, despite having a lower average (0.4 Ls^{-1}, 0.2Pa) and peak (0.75 Ls^{-1}, 0.40 Pa) flow rate and shear stress than the SS0.5 regime. Overall, a hydraulic regime with a shear stress above 0.2Pa and/or with any variation in hydraulics led to a magnitude reduction in cell volume compared to profiles operating at 0.2Pa or less (Table 3).

3.3. Comparisons of results from the repeated control condition of 0.2Pa Steady State hydraulics

The 0.2Pa steady state hydraulic experimental conditions were intended to allow a degree of comparison between the hydraulic repeats at different temperatures, close to a control. The turbidity responses were similar between the repeats at both temperatures and manganese was comparable (very similar at 16C, SS double that of VF at 8C), however iron was not comparable (approximately five times greater at 8C and seven time greater at16C, again with SS results great that VF). There were no statistically significant differences in volume of DNA at 8˚C ($W = 201$, $p = 0.979$) or 16˚C ($W = 143$, $p = 0.131$). The number of samples is greatest for the turbidity response ($1199 \geq n \leq 6728$), then DNA staining ($n = 21$), and then iron and manganese with least replication ($n = 3$), this gives some confidence in integration and comparison of results across hydraulic conditions at each temperature. However, the different in metals analysis suggests there is a need for some caution in doing this, particularly for these parameters.

4. Discussion

Results presented herein show how water temperature (UK summer vs. winter) and hydraulic regime impact upon both discolouration response (as assessed by turbidity, iron and manganese concentrations) of DWDS pipes and microbial cell accumulation at the pipe wall. After 28-days, the scale of the discolouration response, the volume of cells at the pipe wall and the mobilisation profile of the accumulated material differed, depending upon the temperature and hydraulic regimes during the accumulation phase. This demonstrates the importance of the pipe environment in governing the physical, chemical and microbial interactions occurring within it; better understanding of these interactions is critical to proactively and sustainably manage DWDS systems and mitigate water quality degradation. These results provide the first direct causal association of temperature on discolouration (as other variables were controlled), supporting previous correlation observations from field data [13, 18], where multiple variables were changing simultaneously. The results also add to the body of evidence that confirms biofilms are integral in the process of material accumulation within DWDS, leading to discolouration.

4.1. Temperature and biology impact discolouration

The temperature during the accumulation phase had a demonstrable impact upon the discolouration response and microbial accumulation, for all hydraulic regimes. Greater discolouration responses were consistently observed at 16˚C than 8˚C. Our finding that temperature increases discolouration response is supported by previous studies that reported higher discolouration complaints, iron concentrations [48] and manganese release [49] during warmer temperatures. Greater biofilm cell volumes were also seen at 16˚C compared to 8˚C. Temperature has previously been shown to influence planktonic microbial community composition [50] and activity in tap water [51], as well as the composition of biofilms in chlorinated [30, 44] and unchlorinated systems [52]. Preciado *et al.* [44], using an evolution of the facility reported here and similar water qualities, demonstrated that for the same LVF diurnal flow as studied here the discolouration response during flushing was greater at 24˚C than 16˚C. Additionally, quantitative analysis via scanning electron microscopy suggested more cells and inorganics at the pipe wall under 24˚C than 16˚C, with the microbial community changing in its complexity and composition [44]. Combining the results reported here with those of Preciado *et al.* [44] suggests a continuum of increasing biological activity, biofilm growth and discolouration accumulation as water temperature increases from 8 to 16 to 24˚C. Although it is possible that the 8˚C tests represent a lower bound, as this is below the reported threshold for an increase in biological activity within drinking water [31]. Thus, with changes in climate and anthropogenic activity (increasing urbanisation can lead to man-made structures acting as heat sources [53]) likely to increase temperatures, there is a very real likelihood of DWDS experiencing higher temperatures and a greater rate or magnitude of biofilm induced discolouration events in the future.

Higher water temperatures increased the propensity for biological activity and discolouration within the DWDS pipes. These results, uniquely generated under tightly controlled conditions allowing dominance of single factors, add valuable evidence that biological processes are intimately associated with material accumulation and hence discolouration risk within DWDS. Importantly the results presented here go beyond the correlations found and presented previously. Prior field based correlation (as presented in the introduction) suggest temperature changes may be influential upon discolouration, but other seasonal differences could be responsible for the increased turbidity, such as changes in customer demand, which effects various hydraulic related variables such as shear stress, hydraulic residence time or nutrient

and disinfectant concentrations within the water column. These variables are eliminated in the current study, by maintaining the same water supply and water quality matrices between experiments, thus demonstrating the direct impact of temperature.

The evidence that the accumulation of discolouration material in DWDS is mediated by biofilms is further supported by the finding that there were no differences in the quantity of cellular material attached to the coupons taken from the crown, side or invert of the pipe. Fish *et al.* [45] also demonstrated no significant spatial variation in the physical characteristics or community composition of DWDS biofilms sampled from around the circumference of DWDS pipes. If the accumulation processes where dominated by gravity self-weight, and with biofilms also know to form on particle surfaces, we would expect increase mass on the invert coupons, as is observed in wastewater networks where the quantity of material and bacterial compositions on the invert are distinct from those on the crown [54]. Further, under the low hydraulic conditions studied, we would expect a greater bias towards invert accumulation, decreasing as the hydraulic forces increase (either steady state or due to the simulated patterns). These trends are not observed, rather any differences between replicates was attributed to the heterogenic development of biofilms.

4.2. Temperature and hydraulics interact

Neither temperature nor hydraulic conditions dominated as a driver for discolouration or biofilm accumulation, for the ranges studied here. Rather there is a continuum that overlaps, with non-linear effects. Pipes that experience varied shear stress during the accumulation phase resulted in lower cell volumes compared to the experiments performed under steady state conditions. As demonstrated in Sharpe *et al.* [43], this trend is observed even when the Steady State shear stress is greater than the peak of a Varied Flow regime. In the current study, this trend was most obvious in the 16˚C experiments.

Data presented shows that discolouration decreased with increases in daily flow rate and shear stress, and with increased variation in diurnal flow, and that during lower conditioning shear stresses temperature can have a greater influence on discolouration risk. While the increased shear stress reduced material accumulation, this effect can be counteracted by an increase in temperature. Temperature effects at 8˚C were less obvious that for 16˚C, where once hydraulic conditions are above SS0.2 or have a varied flow, trends are similar. However, the impact of hydraulics on cells is different from the impact on discolouration. The LVF flow data is an interesting example to explore in more detail. LVF biofilm cell volume did not follow the trend for increasing discolouration and biofilm with temperature; whilst discolouration was greatest at 16˚C, cell volume did not increase between 8˚C and 16˚C. Fish *et al.* [35], studied the impact of chlorine concentrations on biofilms and discolouration and demonstrated that discolouration does not necessarily correlate with the biofilm cell concentrations that accumulate or are mobilised. A higher chlorine residual during accumulation resulted in greater discolouration in the subsequent mobilisation phase. This was despite high-chlorine biofilms having fewer bacterial cells than the lower chlorine residual regimes. The high-chlorine biofilms had a distinct EPS and inorganic composition (possibly synthesised to offer protection from the bulk water), hence the authors hypothesise that discolouration is driven by the mobilisation of EPS and associated particles (*e.g.* iron and manganese) from the pipe wall into the bulk-water [35]. In the current study chlorine concentration did not differ consistently between experiments, so was not a driving factor in biofilm dynamics. However, EPS is credited with providing mechanical stability to the biofilm and facilitating the concentration of trace inorganics/organics at the pipe wall. Therefore, it is possible that the LVF regime biofilms had similar cell volumes but a different type or amount of EPS at 16˚C than at 8˚C, which

would not have been identified as the current study only quantified cells. Impacts of temperature on EPS within DWDS are unstudied. However, temperature has been shown to modulate EPS expression in idealised studies of other habitats. For instance, the expression of EPS genes in *Clostridium perfringens* has been reported to be temperature regulated and defines the morphology of the biofilm that develops [55]. Higher temperatures promoted EPS synthesis in bench-top studies of single culture biofilms of *Pseudomonas putida* in a rhizosphere context [56] and in multi-species biofilms seeded from a freshwater pond ecosystem ([57]; grown at 10 and 26˚C). Temperature has also been reported to impact the composition of EPS in biofilms of *P.putida* [56] and *U.confervicolum* (a phototroph), in which protein-to-polysaccharide ratios altered with an increase in temperature [58]. These changes in EPS quantity and content could occur in DWDS biofilms, potentially impacting the EPS function and hence biofilm resilience properties, such as stability / coherence, disinfection protection—as shown by Chaumet *et al.* [57], where temperature impacted EPS composition and herbicide sensitivity. Greater EPS production or a change in composition could also promote the accumulation of other particles such as trace inorganics/organics at the pipe wall for subsequent mobilisation. Hallam *et al.* [30] demonstrated that water temperature directly influenced biofilm activity, using biofilm potential monitors and reactors, reporting around a 50% increase in ATP at 17˚C compared to 7˚C. This increase in activity could be indicative of cell replication, cell growth and/or EPS production. Potentially the increase in temperature combined with the LVF regime selected for a biofilm that put more energy into EPS production than cell growth, hence the increase in discolouration but little or no change in cell volumes.

The incremental increases in the mobilisation phase were designed to enable comparison of the material mobilised at each shear stress and thus determine if there were similar mobilisation profiles between temperatures and hydraulics. Irrespective of temperature or hydraulic regime the discolouration responses were not linear, which is in contrast to previous studies [35, 42, 44] and suggests that material of different cohesive strengths developed. In both the Steady State and Varied Flow experiments the greatest increases in turbidity occurred earlier in the Mobilisation phase for systems conditioned to 16˚C, compared to those conditioned to 8˚C. For instance, in the Varied Flow experiments at 8˚C turbidity mainly increased after 2.00 Pa, whereas at 16˚C the turbidity increased primarily between 0.5–1 Pa. This suggests that, regardless of hydraulics, there was more, weaker adhered, material accumulated at 16˚C than at 8˚C, potentially because cell growth and replication was promoted at the higher temperatures rather than EPS production and so the biofilms were less mechanically stable, or potentially because there was more biomass. However, it should be noted that the increments of the flow increases imposed here were far finer resolution, and that the changes and deviations from overall approximately linear trends where often near limits of detection and resolution and close to the accuracy of repeats. The finding does however suggest the biofilm derived layer strength may not be linear at this resolution, at least not for young biofilm mediated material accumulated for 28 days. It should also be recognized that hydraulic transients (also termed water hammer or hydraulic shock) persist in DWDS [59, 60]. Despite their prevalence, the impact of regular transients on the growth and characteristics of drinking water biofilms remains unstudied. However, research in a full-scale pipeline (50mm internal diameter MDPE) has shown that transients are able to mobilise adhered particles where steady-state hydraulics could not [61]. Thus demonstrating the potential for transients to impact biofilm dynamics and dynamics of biofilm-mediated processes, such as discolouration. In the current study, both flow rate and pressure were continually monitored and all hydraulic changes were undertaken slowly to effectively eliminate transients as an environmental variable and isolate the effects of "controlled" flow variation. The overall observation of a sequential mobilisation of material, rather than a threshold release response (as would be anticipated if material was

being remobilised when the external shear stress exceeded the self-weight of the accumulated material) was discussed in detail in Sharpe *et al.* [43]. These results further support the importance of biological processes in governing discolouration material accumulation and subsequent release into the bulk-water.

4.3. Implications for managing DWDS

This study supports the assumptions of the cohesive layer concept for describing and understanding discolouration within DWDS, such as in the PODDs/VCDM models. Namely, that material with variable cohesive strengths develops simultaneously and is responsible for the discolouration behaviour that is observed. The data presented here provides new evidence that biological processes, namely biofilms, are key drivers in this process. Results showed that temperature was influential upon discolouration and biofilm microbial volumes, acting in combination with the shear stress imposed by the hydraulic regime. Accumulation and mobilization dynamics were not determined purely by temperature, average flow rate (SS0.2, SS, LVF and HVF had the same daily flow rate and different responses) or low flow rates (LVF, HVF and SS0.1 had the same low flow but different responses). This is the 'conditioning' effect of hydraulic conditions, the application of which has been shown effective for managing discolouration risk in large diameter strategic trunk mains [16]. Trends between temperature and hydraulics are complex and suggest a "tipping point" between the two parameters with respect to which exhibits the dominant behaviour in governing microbial accumulation and discolouration response.

With biological processes observed to have a fundamental impact on discolouration risk, discolouration management should take this into consideration. Results demonstrate that the best practice to reduce biofilm cell accumulation and hence associated discolouration risk is via a varied flow regime at lower temperatures. However, as turbidity and cells do not necessarily correlate, microbial cells and the microbiome may not be the best monitoring approach to consider the biological aspects of discolouration, rather sensors and monitoring devices are needed that detect or quantify the entire biofilm. Developing innovative solutions in this area could provide early warnings to suppliers of a need for cleaning or maintenance of a main ahead of a discolouration event occurring. If water utilities know a pipe is likely to experience low steady flows and high temperatures, such areas should be prioritised as being high risk and appropriate management practices put in place. Flushing return rates could be increased during warmer months and where pipes have a low daily shear profile. Likewise, a pipe that is not considered a high discolouration risk in normal temperature conditions due to it having a high peak flow, may still be a discolouration risk if there is an increase in temperature. A review of management practices and the prioritising of cleaning should therefore be continuously updated to take account of any network or seasonal changes.

Increasing urbanisation and climate change are important trends impacting DWDS temperature and thus water quality. However, it is difficult to control water temperature, especially as soil temperature is credited as the main factor governing DWDS water temperatures [21–23]. Other variables, such as hydraulics are more easily controlled, thus increasing our understanding of the impacts of interacting variables such as temperature and hydraulics is critical as combining this with increased water temperature monitoring could ensure that management practices are more appropriately deployed within networks. This concept was captured in 'flow conditioning' to manage discolouration in trunk mains, as presented by Sunny *et al.* [62] and Husband and Boxall [16], although no biofilm assessment was undertaken in these studies. The results presented herein confirm that interventions that cause regular changes in hydraulic conditions in DWDS pipelines are far more effective at managing and reducing

biological cell accumulation and biologically mediated discolouration processes, rather than simply net increasing the flow.

Drinking water temperature is an important global concern, regardless of source water, treatment train or network characteristics. Despite the expectation that water temperatures will rise, DWDS water temperature is rarely accurately recorded either for regulatory purposes [21] or during microbial studies of operational systems. Increasing systematic monitoring of water temperature could guide potential operational changes and inform future, sustainable, water quality policies and research.

5. Conclusions

This study has conclusively shown that temperature and hydraulics interact and affect discolouration within DWDS pipes. The evidence presented confirms that the processes by which discolouration material accumulates within DWDS are mediated by biofilms.

A higher temperature (16˚C) resulted in an increased quantity of cells prior to flushing and a greater magnitude of discolouration compared to a lower temperature (8˚C), which consistently had less of a discoloration response. Thus a direct impact or causal relationship between temperature and discolouration was established, which previous studies have only inferred based on correlated observational data. Increasing the shear stress of Steady State hydraulics reduced discolouration, but Varied Flow regimes (with the same 24 hours total flow as the medium steady state flow) reduced this further, including in cases where the peak in a varied flow profile was lower than the steady state. These hydraulics were also demonstrated to impact on biofilm cell volume: as shear stress experienced during growth increased there was less material accumulated which was more likely to be more strongly adhered. These effects of temperature and hydraulics interact, with a tipping point between which is dominant depending on the variation in the daily hydraulic profile.

A link between temperature and microbial accumulation around the circumference of the pipe-wall is confirmed. Thus refuting the likelihood that discolouration processes are self-weight driven sedimentation, providing new evidence that microbiology, and biofilms specifically, are key drivers in discolouration behaviour within DWDS. The correlation between cell volume and discolouration across most of the temperature and hydraulic conditions emphasises the need to consider the microbial aspect of discolouration as well as bulk-water physical and chemical parameters.

The full-scale controlled experiments have ensured transferability of data and understanding to live DWDS. Thus impact of hydraulics provides a tangible variable that can be managed within operational DWDS to manage discolouration, with the interactions with temperature informing when such controls will have maximum benefit (*i.e.* in the summer). This study highlights the importance of integrated interdisciplinary research, and how considering discolouration as a microbiologically driven processes can inform flexible monitoring and management strategies. So doing will safeguard against the impacts of more extreme hydraulic events and temperature increases, thus aiding proactive and sustainable provision of safe drinking water.

Supporting information

S1 Table. Water quality parameters during the accumulation phases of the Steady State and Varied Flow experiments at 8˚C and 16˚C. Medians (and ranges) are shown, based on water utility supply data and weekly discrete sampling.
(PDF)

S1 Fig. Area coverage of cells within Steady State conditioned biofilms from 8˚C (A) and 16˚C (B) experiments. Quartiles and median are plotted based on n = 21, "0 normalized Z-stack depth" indicates the maximum cell coverage location. Area coverage fraction refers to the proportion of each XY image of the Z-stack covered by the stained cells.
(PDF)

S2 Fig. Area coverage of cells within Varied Flow conditioned biofilms from 8˚C (A) and 16˚C (B) experiments. Quartiles and median are plotted based on n = 21, "0 normalized Z-stack depth" indicates the maximum cell coverage location. Area coverage fraction refers to the proportion of each XY image of the Z-stack covered by the stained cells.
(PDF)

Author Contributions

Conceptualization: Katherine E. Fish, Rebecca L. Sharpe, Catherine A. Biggs, Joby B. Boxall.

Formal analysis: Katherine E. Fish.

Funding acquisition: Joby B. Boxall.

Investigation: Katherine E. Fish, Rebecca L. Sharpe, Catherine A. Biggs, Joby B. Boxall.

Methodology: Katherine E. Fish, Rebecca L. Sharpe.

Project administration: Joby B. Boxall.

Resources: Catherine A. Biggs, Joby B. Boxall.

Supervision: Catherine A. Biggs, Joby B. Boxall.

Visualization: Katherine E. Fish.

Writing – original draft: Katherine E. Fish.

Writing – review & editing: Katherine E. Fish, Rebecca L. Sharpe, Catherine A. Biggs, Joby B. Boxall.

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