Low affinity of heterotrophic bacteria to loose deposits in drinking water distribution systems

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ABSTRACT

Loose deposits (LD) accumulate in drinking water distribution systems (DWDS) and may lead to tap water discoloration incidents upon resuspension. While inconvenient for the consumers and the water companies, discoloration may be accompanied by degradation of the microbiological quality of the water, and possibly to the transport of bacteria. The presence of heterotrophic bacteria towards LD with different characteristics was investigated. Bacterial loads in discoloured water samples and LD concentrated over different settling times were analysed. Total heterotrophic (TH) bacteria numbers did not relate to turbidity or to suspended solids in discoloured waters. Higher affinities of TH were observed for slower-settling LD (<24 h), which were also richer in microbial extracellular polymeric substances. The detection of viable iron-oxidizing bacteria in LD supported their possible roles in LD formation and may be related to microbial growth/regrowth in DWDS. Results suggest that LD may exhibit low affinities to hold and transport bacteria, at least to TH in chlorinated water supplies.

Key words | discoloration, drinking water distribution systems, floc settling times, heterotrophic bacteria, loose deposits

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INTRODUCTION

Tap water discoloration is a frequent reason for customers to complain (Husband & Boxall 2011), particularly when recurrent. Discoloration arises from hydraulic perturbation and consequent resuspension (Vreeburg & Boxall 2007) of loose deposits (LD) that accumulate in drinking water distribution systems (DWDS). LD occurrence is generally associated with cast-iron corrosion (Benson *et al.* 2012), even if also occurring in non-metallic pipes (Vreeburg *et al.* 2008), and is often perceived solely as an aesthetic problem, owing to the yellow to brownish turbidity of discoloured water. However, as LD may adsorb substrates and nutrients, these may also be considered as prime sites for bacterial growth (Batté *et al.* 2003), including the hosting of pathogens, e.g., *Legionella* spp. (USEPA 2001). In fact, many problems in DWDS may be related to microbiological growth/regrowth, e.g., biofilm formation, LD accumulation, microbial influenced corrosion, opportunistic pathogens (Gauthier *et al.* 1999; Zacheus *et al.* 2001; Batté *et al.* 2003; Liu 2013). With respect to LD accumulation and further resuspension, discoloration incidents are often related to the microbial deterioration of drinking waters (Batté *et al.* 2003; Lehtola *et al.* 2004).

Possible sources for LD in DWDS include particles coming with upstream waters, or formed within the distribution network, e.g., through precipitation of bulk water colloidal and dissolved materials, pipe scaling and/or

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scouring, or biofilm detachment or sloughing (Gauthier *et al.* 1999; McNeill & Edwards 2001; Vreeburg & Boxall 2007; Husband & Boxall 2011). In addition, and apart from pipe corrosion, LD may enter the DWDS during pipe repair or rehabilitation procedures. Nonetheless, and despite so many LD origins, even if their formation mechanisms have not yet been comprehensively identified, discoloration LD typically have oxidized forms of iron and organic compounds (i.e., volatile solids (VS)) as main constituents (Gauthier *et al.* 1999; Barbeau *et al.* 2005; Vreeburg & Boxall 2007; Poças *et al.* 2013b).

Microbial extracellular polymeric substances (EPS) have also been reported as important LD constituents (Poças *et al.* 2013a). These, among a diversity of possible origins (e.g., raw water, pipe biofilms), may be produced by iron-oxidizing bacteria (FeOB) growing on appendages impregnated with iron oxides. These bacteria, apart from being associated with discoloration (Ridgway *et al.* 1981), may also produce organic substrates for microbial regrowth (Lehtola *et al.* 2004) during chemolithoautotrophic growth (Emerson *et al.* 2010). While embedded within the polymeric matrices of released or produced EPS' sheaths, micro-organisms may then grow protected against residual disinfectants or other oxidants (Flemming *et al.* 2007). Furthermore, EPS may contribute to the hydrogel floc nature of LD (Poças *et al.* 2013a), including to their high water contents and cohesive and flocculant properties (Vreeburg & Boxall 2007; Husband & Boxall 2010; Abe *et al.* 2012; Douterelo *et al.* 2012), and provide different sorption sites for bacterial development and growth. Thus, given that discoloration LD are hydrogel flocs (Poças *et al.* 2013a) with high interstitial water contents and permeability, discoloration LD may exhibit the ability to hold bacteria, which may differ from 'true particles', such as sand grains or corrosion derivatives.

Although described as prime sites for bacterial regrowth in DWDS (Batté *et al.* 2003), the ability of LD leading to discoloration events to hold and transport bacteria is not clearly understood yet. With the purpose of evaluating the potential of discoloration LD for bacterial attachment and transport, the presence of total heterotrophic (TH) bacteria in LD with different characteristics (e.g., settling times, composition) was studied. The presence of viable FeOB in LD, due to their role in LD formation (Ridgway *et al.* 1981), and possible impacts in the bio-stability of DWDS water (Emerson *et al.* 2010), was also investigated.

MATERIALS AND METHODS

LD sampling

Samples were collected from the Lisbon DWDS. The water is mainly of surface origin (86%) and undergoes conventional treatment. A minor fraction of groundwater undergoes chlorination only. Samples were collected from pipes targeted by the water company cleaning programme, designed to respond to discoloured water complaints or reports. Sampling sites covered pipes from different and dispersed district metered areas, thus allowing for the sampling of pipes supplied with waters of different make-up and residence times.

Sampled locations (12) included dead-end and distribution pipes of different materials (asbestos cement (AC), ductile iron (DI) and high-density polyethylene (HDPE)) and ages (from 1 to 27 years). Cast-iron pipes, although present in the network (~19%), were not part of the sampled LD sites. Of the 14 samples collected within the 12 locations, two were taken from the same pipes: one made of DI and the other of HDPE. Over about 2 months, large volume samples were collected in sterilized cylindrical containers (30 L) from each sampling site, along with unidirectional pipe routine discharges (Poças *et al.* 2013a, 2013b). Sampling velocities, rather than predefined, were those achieved by the hydrant's valve opening, thus allowing for selective and representative discoloration LD collection and characterization (Poças *et al.* 2013b).

LD separation

At the laboratory (Figure 1), 1 L aliquots of the large volume samples (W_{0h}) were used for turbidity, conductivity, pH, total suspended solids (TSS) and TH enumeration analyses. For LD separation, the remaining volume of the large volume samples (c. 29 L) was left to settle at 4 °C for 24 h. Afterwards (Figure 1), 1 L of the top-supernatant water

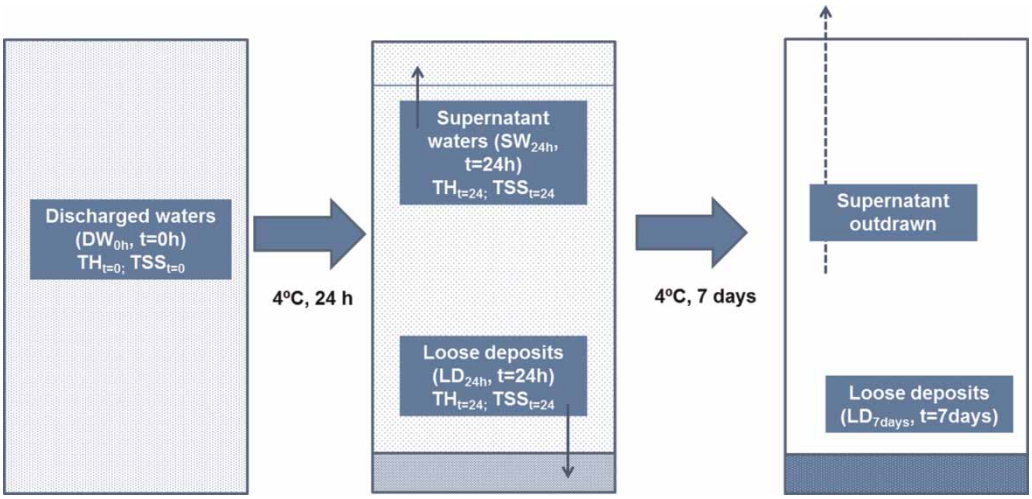

Figure 1 | Sample separation: initial sampled waters (W_{0h}), (SW_{24h} and LD_{24h}) fractions over 24 h of settling, and the concentrated slurries (LD_{7days}), over 7 days of settling.

(SW_{24h}) and 2 L of the bottom settled concentrates (LD_{24h}) were collected with a peristaltic pump at a flow rate of 6 L/h. The SW_{24h} and LD_{24h} fractions were then analysed for TSS and TH. The remaining volume (c. 26 L) was left to decant at 4 °C (Poças *et al.* 2013a, b). After 7 days of settling (Figure 1), the supernatant volume was removed and c. 1–2 L of the enriched slurries (LD_{7days}) was collected for LD physical–chemical characterization (Poças *et al.* 2013a, b). In this fraction, TH were not analysed, as bacterial proliferation is insignificant at 4 °C.

When performing laboratory tests, care was taken to reproduce the real system conditions as far as possible, including the LD physical features (e.g., shape, behaviour) and water matrix make-up.

Water and LD characterization

Turbidity (NTU) was measured in 25 mL borosilicate glass cells in a nephelometer (Turb 555–600100, WTW). Conductivity and pH were measured in a multi-parameter potentiometer (Consort, model C863). TSS were determined by the gravimetric method (APHA 1998). Total solids and VS were determined by drying at 110 °C to constant weight and ignition at 550 °C (APHA 1998). Total iron (Fe^t) was determined by the phenanthroline method (APHA 1998), with adaptations (Poças *et al.* 2013a, b). Polysaccharides (PL) and total protein (PT) were determined by the anthrone (Daniels *et al.* 1994) and Bradford (Bradford 1976) methods, as described in Poças *et al.* (2013a).

Microbiological analyses

In W_{0h}, SW_{24h} and LD_{24h}, TH were determined by the most probable number (MPN) method in liquid R2A medium at 22 °C incubated for 7 days. The MPN method, which is appropriate for LD samples (Schaule *et al.* 1992), was not influenced by background turbidity, as it is distinguishable in colour and aspect from that due to TH growth. To preserve LD behaviour, and for comparison of results with previous publications (Zacheus *et al.* 2001), no sample pretreatment was used, other than gravitational settling.

An adaptation of the gel-stabilized gradient tubes method (Emerson & Floyd 2005) was used to detect FeOB growth on Fe(II)-EDTA in O_2 gradient cultures (Kumaraswamy *et al.* 2006). The presence of grown FeOB in the turbidity bands was further confirmed by microscopic observations of Prussian Blue (Pellegrin *et al.* 1999) stains. Bands' micro-organisms were also observed in fresh wet mounts under phase contrast microscopy and tested for Gram staining. Similarly treated smears of fresh *Escherichia coli* (ATCC 25922) cultures were used as controls. Tested inocula ($n = 7$ samples) were LD collected from a tap with discoloured water in a household with corroded DI plumbing and from the discharges collected from different DI ($n = 4$) and HDPE ($n = 2$) network pipes.

RESULTS AND DISCUSSION

Sampled waters

Large volume samples ($n = 14$) were collected during routine pipe cleaning at velocities from 0.07 to 0.32 m/s (Table 1) from the 12 sampling sites. Neither turbidity (from 1 to 46 NTU) nor TSS (from 0.3 to 35.8 mg/L) was influenced by the sampling velocities, the pipe materials, or the water characteristics, as shown by the variations in electrical conductivity (from 169 to 500 μs/cm) and in pH (from 7.5 to 9.9). This suggests that sampled waters were of different make-up and residence times.

TH were detected in 11 out of 14 sampled waters at levels from 2.0×10^2 to 5.4×10^6 per L. Overall, sampled waters did not carry high TH loads, as these were within those typically found for drinking waters, where the average is around $5–10 \times 10^6$ per L (Zacheus *et al.* 2001; van der Kooij 2003; Allen *et al.* 2004), either with or without residual disinfectant.

TH loads were not related to sample turbidity or TSS levels (Figure 2). In fact, the highest loads (in the order of 10^6 per L) occurred in sampled waters with both low (<10 NTU) and high (>10 NTU) turbidity levels. In addition, at the locations where LD were sampled twice (Table 1), higher TH loads occurred in the less turbid samples, thus supporting the conclusion that TH loads were not related to sample turbidity or TSS levels.

These observations are consistent with those from previous studies where no clear relationship was found between turbidity and TH numbers (Liu 2013). On the other hand, a linear correlation ($r^2 = 0.82$ and p-value <0.05) could be found between turbidity and TSS, which may suggest a similar LD behaviour in the collected samples. Therefore, and taking into account the observed different ranges of TH, LD bacterial content may have not varied due to different water turbidities (Figure 2), but possibly because LD were different in composition and/or age.

LD

Over the 24 h of settling, the average turbidity at the supernatant fraction was 6 ± 3 NTU, while that in the settled LD

Table 1 | Sampling conditions and results observed for the 14 large volume samples

Pipe material	Pipe age (years)	Velocity (m/s)	pH	Conductivity (μs/cm)	Turbidity (NTU)	TSS (mg/L)	TH counts/L	
AC	27	0.25	9.9	169	17	12.6	1.10×10^6	
DI	10	0.09	8.4	415	21	16.6	2.70×10^4	
DI	13	0.19	7.9	386	25	11.1	5.40×10^3	[a](1st)
DI	10	0.21	8.0	386	26	21.5	2.00×10^2	
DI	11	0.08	8.0	496	6	1.5	3.50×10^6	
DI	17	0.09	7.5	248	1	1.1	$< 2.00 \times 10^2$	
DI	10	0.08	7.9	483	43	35.8	1.10×10^4	
DI	13	0.09	7.9	472	1	0.5	4.90×10^3	[a](2nd)
DI	14	0.09	7.9	500	2	2.0	4.50×10^2	
DI	8	0.07	7.5	298	5	4.2	$< 2.00 \times 10^2$	
DI	12	0.32	8.8	253	1	0.3	2.10×10^6	
HDPE	6	0.18	7.8	438	7	9.0	9.20×10^5	[a](1st)
HDPE	1	0.18	7.8	373	46	19.0	5.40×10^6	
HDPE	6	0.20	7.9	392	27	21.0	$< 2.00 \times 10^2$	[a](2nd)
Average	11	0.15	8.1	379	16	11.2	1.19×10^6	
St. dev.	6	0.08	0.6	102	16	10.6	1.80×10^6	
Max.	27	0.32	9.9	500	46	35.8	5.40×10^6	
Min.	1	0.07	7.5	169	1	0.3	2.00×10^2	

[a]Refers to the pipes sampled twice: a DI pipe at a 20 day interval and an HDPE pipe at a 49 day interval.

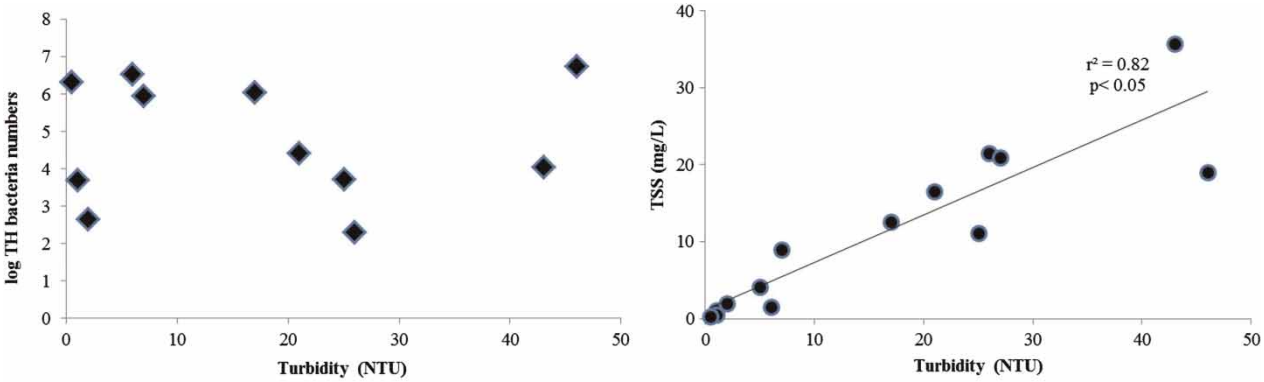

Figure 2 | Turbidity and logs of TH bacteria numbers (on the left) and TSS (on the right) in sampled waters.

fraction was 91 ± 182 NTU. This shows there were still deposits settling after the first 24 h, which is consistent with previous studies where it is suggested that complete clarification of discoloured water samples takes place over several days (Poças *et al.* 2013b).

With respect to the bacterial loads in TH per gram, values found in SW_{24h} and LD_{24h} were compared to those measured in the collected water samples W_{0h} (Figure 3). Relative to the changes in bacterial counts over the first 24 h, these should not have been significant, as the similarities on readings from the W_{0h} and SW_{24h} fractions show.

The two log difference in the unsettled (SW_{24h}) and the settled (LD_{24h}) LD fractions suggested that the TH loads were higher in the LD with the slowest settling rates, i.e., the SW_{24h} fraction. Despite the fact that Figure 3 refers only to the averaged TH, that same trend was observed between W_{0h} and SW_{24h}, and, except in one case where TH were about four times higher in the LD_{24h} fraction, between SW_{24h} and LD_{24h}. As TH per gram in SW_{24h} and in W_{0h} were also at comparable levels to those reported by Zacheus *et al.* (2001), which results were also obtained in R2A medium ($\leq 10^9$ TH/g of LD) and with the same sample treatment, it can be suggested that higher bacterial loads may be found in younger or in 'softer' LD (Zacheus *et al.* 2001). The different LD settling characteristics may have been due to dissimilarities in LD age and, likewise, in LD composition. This is in line with Liu (2013), who observed that the type of bacteria present in LD differed over accumulation and may be dependent on the amounts of LD used for characterization. Therefore, the differences in bacterial counts per mass of deposit may differ with LD settling rates and/or sampling sites, owing to different LD accumulation levels.

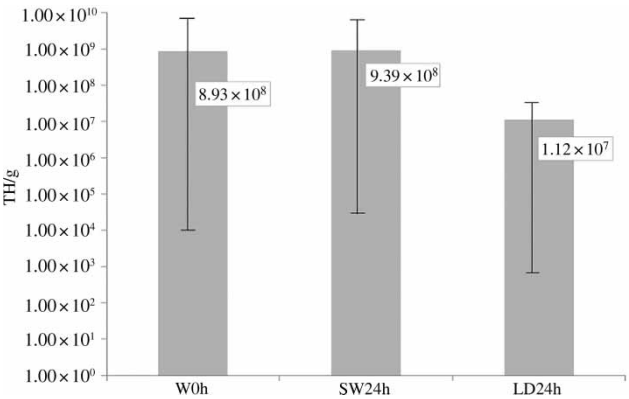

Figure 3 | Average TH bacteria per gram of LD in sampled waters (W_{0h}), and in the unsettled (SW_{24h}) and the settled deposits (LD_{24h}) after 24 h of settling. The error bars represent the maximum and minimum TH/g values, respectively.

After the 7 days of settling, the settled LD fractions were used for physical–chemical characterization. Since only the slowest LD were left to settle after the first 24 h, LD characterization results mostly refer to the supernatant LD fraction (SW_{24h}). On average, VS contents were 179 ± 103 mg/g and Fe^t contents 126 ± 56 mg/g (Table 2). This confirms that iron-rich LD may occur in all pipe types of the DWDS, given that sampled locations did not include pipes made of cast iron. Relative to the main constituents of EPS (Flemming & Wingender 2001), PT was only detected in four samples, while PL was found in all samples at considerable amounts (up to 268 mg/g). Although no correlations could be observed between LD components and TH (results not

Table 2 | LD constituents and TH bacteria

Pipe material	VS (mg/g)	Fe^t (mg/g)	PT (mg/g)	PL (mg/g)	TH/g
AC	131	141	N.D.	135	6.2×10^8
DI	157	83	N.D.	82	4.1×10^6
DI	220	141	N.D.	105	2.8×10^5
DI	133	119	N.D.	141	2.9×10^4
DI	43	244	N.D.	INT.	6.6×10^9
DI	263	N.A.	2	268	N.D.
DI	148	68	N.D.	56	5.9×10^4
DI	125	N.A.	N.D.	INT.	2.4×10^6
DI	168	93	4	INT.	2.5×10^5
DI	67	73	N.D.	74	N.D.
DI	471	N.A.	16	148	N.D.
HDPE	250	N.A.	1	35	4.1×10^8
HDPE	155	192	N.D.	152	1.8×10^9
HDPE	171	107	N.D.	60	N.D.
Average	179	126	6	114	9.4×10^8
St. dev.	103	56	7	65	2.1×10^9
Max.	471	244	16	268	6.6×10^9
Min.	43	68	1	35	2.9×10^4

N.D. = not detected; N.A. = not analysed; INT. = interferences.

shown), these showed a potential relation with Fe^t and with EPS-PL components (Table 2), thus suggesting that LD composition may have had an influence on TH loads in LD.

The different LD settling rates suggested that slower-settling LD could be closer to soft pipeline young deposits, which is in agreement with what had already been suggested by Zacheus *et al.* (2001). Likewise, slower LD may be closer to hydrogel flocs, i.e., flocs with fibrillar matrices and densities approaching water (Poças *et al.* 2013a), apart from having a fractal three-dimensional structure. These hydrogel flocs, unlike heavier 'true particles' that can settle more rapidly (e.g., pipe and encrustation derivatives, sand particles), may be easily transported throughout the network upon resuspension and reach consumers' taps during discoloration events. Therefore, to analyse the potential health risks occurring upon LD accumulation, not only are the microbial identification and quantification required, but also the probability of sampled LD reaching the consumers' taps needs to be evaluated. Thus, as in water safety plan methodologies (Bartram *et al.* 2009), both the microbial hazards and the probability of LD reaching the consumers' taps need to be evaluated when assessing LD potential risks that may occur upon LD resuspension.

Iron bacteria

The occurrence of Gram-negative motile rods with the ability for microaerophilic growth coupled to Fe(II) oxidation at circumneutral pH was observed in the analysed LD samples from DI pipes ($n = 5$). This suggests a possible involvement of FeOB in discoloration. Likewise, since chemolithoautotrophic *Galionella* spp. may be associated with discoloration LD (Ridgway *et al.* 1981; Li *et al.* 2010), carbon fixation products may be formed within the LD matrices, then allowing for increases in the overall AOC budget and, consequently, to bacterial regrowth. As detected FeOB grow under low oxygen concentrations (Emerson *et al.* 2010) it is also possible that, similarly to within the pipe-wall biofilm EPS matrix, other micro-organisms, including anaerobes, survive within the LD floc microenvironments (Liu 2013), where they are protected against residual disinfectants. The assessment of the potential health risks occurring during LD resuspension and tap water discoloration may need, therefore, to be complemented with the enumeration of specific bacterial species, other than TH only.

Bacterial affinity towards LD with different characteristics

In this study, TH loads were low in the sampled waters ($<10^7$ TH/L) and did not correlate with discoloration intensity (Figure 2). This is consistent with previous studies on LD, where TH were not associated with turbidity or TSS, possibly due to particle-associated bacteria and, thus, to TH underestimations (Liu 2013). Likewise, TH numbers may only refer to a small fraction (<0.1%) of the total quantity of microbes present, thus adding difficulty to the finding of correlations.

Overall, these results, although obtained from a single water supply system, which is also chlorinated, contrast with descriptions of LD as prime sites for bacterial growth (Zacheus *et al.* 2001; Batté *et al.* 2003; Liu 2013), either from chlorinated or non-chlorinated systems. This may be due to the different water characteristics, the analytical

methods, the sample treatment (Liu 2013), or the levels of residual disinfectants (Camper 2004). On the other hand, investigations into the microbiological quality of drinking waters usually disregard the different accumulation times of sampled LD. In addition, overestimations of LD potential health hazards may be led by the collection of non-discoloration representative LD. Comparative studies on three-stage samples (i.e., biofilms, LD and the bulk water) should also address possible differences within sample residence times and behaviour (e.g., LD with different settling rates), as well as describe differences in the sampling procedures in use (e.g., pipe velocities and sampled volumes).

As Table 1 shows, the finding of trends between the water characteristics and turbidity or TSS may be difficult to achieve, possibly due to the differences in the water and the LD residence times. However, while LD behaviour and sampling velocities may be quantified, sample residence times are more difficult to assess. As hypothesized herein, lighter LD may refer to the younger LD fraction. If confirmed, the relation between LD behaviour and age could help to estimate sample residence times and, therefore, contribute to a better understanding of the risks associated with tap water discoloration. In terms of DWDS' operational and maintenance procedures, indications could also be provided on how to assess the potential health hazards of discoloration LD samples with different settling rates, which, in turn, may be an indication of the LD resuspension potentials.

CONCLUSIONS

The presence of TH in LD from DWDS was investigated. TH loads in discoloured water samples and in LD that were concentrated over different settling times were analysed. Higher affinities were observed for slower-settling LD, which were also richer in microbial EPS. Overall, results showed that the intensity of discoloration was not related to high TH loads in a chlorinated DWDS. Despite this, different bacterial loads and types of bacteria may be expected in LD with different characteristics (e.g., settling times and behaviour, composition or residence times) and collected using different sampling methods. Furthermore, the detection of chemolithoautotrophic iron-oxidizing bacteria in LD, apart from the role of FeOB in discoloration, suggests a possible association with bacterial regrowth in DWDS.

On the whole, the assessment of microbial health hazards of LD accumulation should rely not only on microbial identification and quantification data, but also on sample residence times and LD behaviour (e.g., settling and resuspension), as well as on LD sampling procedures (e.g., pipe velocities, sampled volumes). Also, as lighter LD may correspond to younger LD, better knowledge on the association of LD behaviour with age (e.g., through LD settling/resuspension velocities and EPS contents) could help the assessment of the potential health risks arising with tap water discoloration on consumers. Similarly, it could provide indications of the necessary pipe cleaning frequencies, thus supporting water companies in designing LD control programmes.

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