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Nitrite production by ammonia-oxidizing bacteria mediates chloramine decay and resistance in a mixed-species community

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Summary

As water distribution centres increasingly switch to using chloramine to disinfect drinking water, it is of paramount importance to determine the interactions of chloramine with potential biological contaminants, such as bacterial biofilms, that are found in these systems. For example, ammonia-oxidizing bacteria (AOB) are known to accelerate the decay of chloramine in drinking water systems, but it is also known that organic compounds can increase the chloramine demand. This study expanded upon our previously published model to compare the decay of chloramine in response to alginate, *Pseudomonas aeruginosa*, *Nitrosomonas europaea* and a mixed-species nitrifying culture, exploring the contributions of microbial by-products, heterotrophic bacteria and AOBs to chloramine decay. Furthermore, the contribution of AOBs to biofilm stability during chloramination was investigated. The results demonstrate that the biofilm matrix or extracellular polymeric substances (EPS), represented by alginate in these experiments, as well as high concentrations of dead or inactive cells, can drive chloramine decay rather than any specific biochemical activity of *P. aeruginosa* cells. Alginate was shown to reduce chloramine concentrations in a dose-dependent manner at an average rate of 0.003 mg l^{-1} h^{-1} per mg l^{-1} of alginate. Additionally, metabolically active AOBs mediated the decay of chloramine, which protected members of mixed-species biofilms from chloramine-mediated disinfection. Under these conditions, nitrite produced by AOBs directly reacted with chloramine to drive its decay. In contrast, biofilms of mixed-species communities that were dominated by heterotrophic bacteria due to either the absence of ammonia, or the addition of nitrification inhibitors and glucose, were highly sensitive to chloramine. These results suggest that mixed-species biofilms are protected by a combination of biofilm matrix-mediated inactivation of chloramine as well as the conversion of ammonia to nitrite through the activity of AOBs present in the community.

Introduction

Current water distribution systems can transport water over 500 km from the point of origin (Harvey *et al.*, 2015), enabling cities to thrive without a local water source. This water is usually first treated via mechanical and chemical methods to remove contaminants such as dissolved and undissolved organic carbon, heavy metals, particulate matter and microorganisms prior to being disseminated through the water network (Ternes *et al.*, 2002; Van der Bruggen *et al.*, 2003). Once contaminants are removed, resulting in 'finished' water, disinfectants such as chlorine and chloramine may be added to prevent microbial recontamination during distribution.

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Chloramines occur when at least one hydrogen atom of an ammonia molecule is substituted with a chlorine atom, yielding monochloramine (NH_2Cl), dichloramine ($NHCl_2$) or nitrogen trichloride (NCl_3). Monochloramine is a weaker disinfectant partly because it exhibits far less reactivity than chlorine. This increased stability allows monochloramine to maintain an effective disinfectant residual, for longer contact times in long-distance water distribution systems (Vikesland *et al*., 2001). The use of chloramine over chlorine decreases the formation of many potentially toxic disinfection by-products, including chlorinated hydrocarbons such as trihalomethanes and haloacetic acid (Brodtmann and Russo, 1979). Chloramine has also been shown to better penetrate bacterial biofilms (Lechevallier *et al*., 1988; Lee *et al*., 2011), which are bacterial communities embedded in a self-produced matrix of extracellular polymeric substances (EPS) that may coat the surfaces of pipelines and cause pitting corrosion in addition to serving as reservoirs for potential pathogens (Wingender and Flemming, 2011). As a result, many drinking water utilities have switched to chloramination. For example, it has been reported that in the United States, more than 20% of the drinking water is treated with chloramines (AWWA Disinfection Systems Committee, 2008).

Despite the enhanced stability of chloramine, there are a number of factors that contribute to its degradation and loss of activity in water distribution systems, including oxidation reactions with reduced organics and iron residuals in the water, hypochlorous ions, auto-catalytic decay and biologically catalysed reactions in biofilms. For the latter, it is known that autotrophic nitrifying (or more specifically, nitritating) microorganisms, such as *Nitrosomonas europaea*, can use ammonia as an energy source by utilizing it as electron donor, fuelling oxidative phosphorylation and ATP generation. In this process, ammonia is oxidized sequentially to hydroxylamine (NH_2OH) then to nitrite, while inorganic carbon from CO_2 is incorporated into organic cell products. The first step of ammonia oxidation is carried out by an ammonia monooxygenase (AMO), encoded by the *amo* genes (Rotthauwe *et al*., 1997), while the second step results from the activity of the hydroxylamine oxidoreductase (HAO), encoded by *hao* (Sayavedra-Soto *et al*., 1994; Batchelor *et al*., 1997).

In nature, nitrifying bacterial communities also commonly contain microorganisms capable of further oxidizing nitrite into nitrate via nitrite oxidoreductase (NXR), thus completing the nitrification process. Nitrite produced during nitritation can be readily and directly oxidized by chloramine, resulting in the formation of nitrate and the decomposition of chloramine into ammonia and free chloride (Vikesland *et al*., 2001). Thus, the organic carbon and energy derived from the oxidation of ammonia produced via the decomposition of chloramine can be coupled with the reduction in chloramine concentration to allow for the proliferation of both nitrifying bacteria and heterotrophic microorganisms in surface-attached mixed-species biofilms (Petrovich *et al*., 2017; Keshvardoust *et al*., 2019).

The structural matrix of biofilms usually consists of diverse natural organic matter (NOM) such as living and dead bacteria, which can readily lead to chloramine decay, in addition to polysaccharides such as alginate (Grobe *et al*., 2001), enzymes (Lu *et al*., 1999), waste products and trace metals (Tsai *et al*., 1992) in addition to extracellular DNA (Flemming and Wingender, 2010) and viruses (Andersson and Banfield, 2008). The biofilm matrix has the potential to form an effective biochemical barrier, with chloramine degraded before reaching viable bacteria within the biofilm (Xue *et al*., 2013). Furthermore, microbial by-products and viable bacteria from these biofilms may also disseminate into the water phase, continuing to reduce disinfectant concentrations and forming more biofilms downstream.

To improve our understanding of the mechanisms of chloramine decay and microbial resistance to chloramination, a microbial model that addresses the ecological role of nitrifying and non-nitrifying organisms, such as those found in chloramine-treated drinking water systems will be invaluable to complement the chemical approaches that have been well-studied. In this study, *Pseudomonas aeruginosa* was used to model a heterotrophic bacterium, while a mixed-species community containing *N. europaea* and other bacteria was used to model mixed-species nitrifying communities (Petrovich *et al*., 2017; Keshvardoust *et al*., 2019). *Pseudomonas aeruginosa* is a model biofilm-forming organism that produces high amounts of alginate (Ochsner and Reiser, 1995; Whiteley *et al*., 2001; Drenkard and Ausubel, 2002; Bazire *et al*., 2005; Barraud *et al*., 2006), that is known to associate with other organisms in mixed-species biofilms (Riedel *et al*., 2001) and can be found in drinking water (Shrivastava *et al*., 2004). Although other species, such as *Nitrosomonas oligotropha*, have been suggested to have a greater affinity for ammonia in weakly chloraminated waters and greater involvement in chloramine decay than *N. europaea* (Regan *et al*., 2002; Regan *et al*., 2003; Krishna *et al*., 2013), *N. europaea* possesses the same ammonia oxidation pathway and has been more thoroughly investigated (Zhang *et al*., 2009), under a range of diverse conditions reflecting environments from soil (Blackmer *et al*., 1980) to drinking water (Maestre *et al*., 2013). Accordingly, this study expands upon a previously published model using a mixed-species nitrifying community containing *N. europaea* and heterotrophic bacteria which varied in community composition in response to growth and culture

conditions (Petrovich *et al*., 2017; Keshvardoust *et al*., 2019), to explore the potential contributions of AOB to biofilms and chloramine decay. This experimental microbial community was comprised of nearly 50% *Nitrosomonasaceae* (AOBs) and approximately 50% of heterotrophic bacteria when grown in nitrification medium. When grown in the presence of glucose, their relative proportion of AOB declined to < 1% and the community was dominated by heterotrophic bacteria such as *Pseudomonas* spp. and *Devosia* spp. which made up between 50% and 65% of the community composition. Since *N. europaea* has a weaker affinity for ammonia than other AOB frequently used in such investigations, the results presented here may show a conservative estimate of the contributions of AOB to the degradation of chloramine compared to those species with greater affinity for ammonia, although other factors such as turnover rates may need to be taken into account. Thus, further work will be needed to directly test the observations reported here using a laboratory-based model community on other AOB species.

In this study, we sought to compare the effects of purified biofilm matrix EPS (alginate) with the effect of active cells to improve our understanding of the role of nitrifying biofilms in mediating chloramine decay in the short-term. Experiments were designed using alginate as a proxy for the biofilm matrix, while the role of bacterial metabolic activity was determined by exposure of chloramine solutions to model nitrifying cultures treated with or without inhibitors of nitrification. Finally, chloramine was added to mixed-species biofilms under various states of carbon supplementation and nitrification inhibition to determine the effects of these parameters on biomass stability after disinfection with chloramine. The supplementation of these cultures with nitrification inhibitors and organic carbon, in the form of glucose, helped to further decouple the heterotrophic bacteria from their reliance on AOB for organic carbon. Under these conditions, it was possible to better assess the role of the heterotrophic bacteria even when in the presence of AOB.

Results

Chloramine decay in the presence of alginate

To first determine the potential impact of biofilm EPS on chloramine, alginate, a common EPS component present in the biofilm matrix, was tested for its effect on chloramine stability *in vitro*. For experimental and quantification purposes, we chose a chloramine concentration, 4.5 mg l^{-1} that is higher than what is typically used in drinking water systems (0.5–4 mg l^{-1}, https://www.epa.gov/dwreginfo/chloramines-drinking-water), to ensure we could measure decay values readily. Additionally, these concentrations are consistent with other studies of chloramine dosing (Pressman *et al*., 2012). We also used a range of concentrations of alginate (1–50 mg l^{-1}), as a proxy for the biofilm matrix, to determine the impact of different concentrations on chloramine decay. The highest concentration of chloramine used is 10-fold lower than the amount of alginate reported for *P. aeruginosa* (Hassett, 1996). When a 4.5 mg l^{-1} chloramine solution was exposed to a 50 mg l^{-1} solution of alginate, representing natural organic matter, significant chloramine decay was observed within 1 day (Fig. 1). The chloramine decayed at an average rate of 0.003 mg l^{-1} h^{-1} per mg l^{-1} of alginate (determined using the maximum and minimum amounts of chloramine measured during the timeframe of the experiment). The decay occurred in a dose-dependent manner, with an increase in both the overall decay and the rate of decay at concentrations of alginate ≥ 20 mg l^{-1}. For example, chloramine exposed to 50 mg l^{-1} alginate decayed at a rate of 0.125 mg l^{-1} h^{-1}, leaving a residual of 1 mg l^{-1} chloramine at 28 h, whereas treatment with 20 mg l^{-1} alginate resulted in a chloramine decay rate of 0.05 mg l^{-1} h^{-1} per mg l^{-1} of alginate, leaving a residual of 3.1 mg l^{-1} of chloramine.

Compared to the 0 mg l^{-1} alginate control solution, concentrations of alginate ≥ 40 mg l^{-1} produced significant chloramine decay within 2 h ($P < 0.05$, $n = 3$), the decay observed with 30 mg l^{-1} alginate was statistically significant after 4 h ($P < 0.05$, $n = 3$) and, although the differences were small, the decay observed with 20 mg l^{-1} alginate was statistically significant after 28 h ($P < 0.05$, $n = 3$). Concentrations of alginate below 20 mg l^{-1} did not result in significant decay within the experimental timeframe (28 h). These results could provide a reference for the process of chloramine decay for

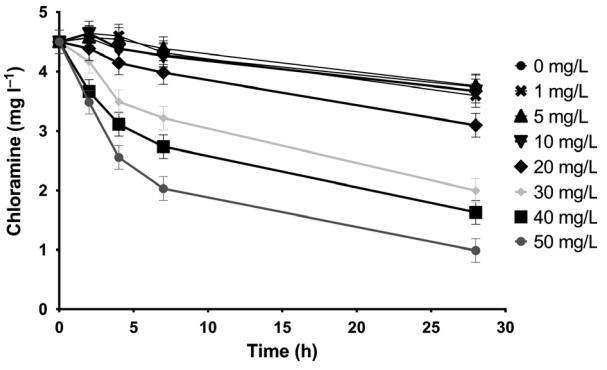

Fig. 1. The biofilm EPS alginate accelerates the decay of chloramine *in vitro*. A ~ 4.5 mg l^{-1} solution of chloramine was co-incubated for 28 h with solutions of alginate at concentrations between 1 and 50 mg l^{-1} to determine the effect of alginate concentration on chloramine decay. Chloramine was quantified by using the DPD assay at 2, 4, 7 and 28 h. Error bars represent SEM ($n = 3$).

comparison to EPS concentrations found in real-world systems, such as drinking water systems.

Chloramine decay in the presence of living and inactivated bacterial cultures

Live bacteria can potentially affect chloramine stability via both passive, byse-product-mediated effects and extracellular matrix and active, metabolic effects. To distinguish these two factors, chloramine stability was tested in the presence of either live or heat-inactivated cells of *P. aeruginosa* or mixed-species nitrifying cultures. Chloramine decayed significantly in the presence of *P. aeruginosa*, and the degree and rate of decay was similar for both live and dead cells 2 days (Fig. 2A; $P < 0.05$, $n = 3$). This decay was observed for all *P. aeruginosa* cultures diluted to a final concentration of $\geq 25\%$ of the original inoculum regardless of viability, leaving a maximum chloramine residual of 0.8 mg l^{-1}. In contrast, only the viable cultures of the nitrifying community significantly degraded chloramine (Fig. 2B; $P < 0.05$, $n = 3$), leaving a maximum chloramine residual of 0.5 mg l^{-1}. The inactivated mixed-species nitrifying community had no effect on the chloramine residual except for the vials containing the undiluted, inactivated culture, although those samples retained a high chloramine residual of 2.5 mg l^{-1}. This suggests that the nitrifying cultures require metabolic activity to degrade the chloramine.

Nitrite secretion from N. europaea *nitrifying cultures induces chloramine decay*

Since chloramine decay upon exposure to nitrifying cultures appeared to be mostly dependent on metabolic activity in the experiments above, pure cultures of *N. europaea* in the absence of other bacterial species were tested to determine the role of nitritation by *N. europaea* in chloramine decay. The cultures were exposed to 2 mg l^{-1} chloramine, which was completely degraded after 2 days of incubation with *N. europaea*, in comparison with similar control cultures still retaining 1.5 mg l^{-1} of chloramine when ammonia oxidation was inhibited by the addition of 2-ethynylpyridine ($P < 0.05$, $n = 3$; Fig. 3). This level of decay was not observed when the nitrite-containing supernatant was removed and replaced with fresh medium ($P < 0.05$, $n = 3$), leaving a chloramine residual of 1.4 mg l^{-1} compared to 1.7 mg l^{-1} in the control, which suggested that the culture supernatant contained a secreted product responsible for the degradation.

We determined that *N. europaea* released 1.4 g l^{-1} of nitrite into the culture supernatant when grown with 2 mg l^{-1} chloramine. Therefore, to test if the nitrite produced by *N. europaea* from the oxidation of chloramine could also play a direct role in the degradation of chloramine, the addition of nitrite to both sterile media and washed cells was tested. Indeed, supplementation with

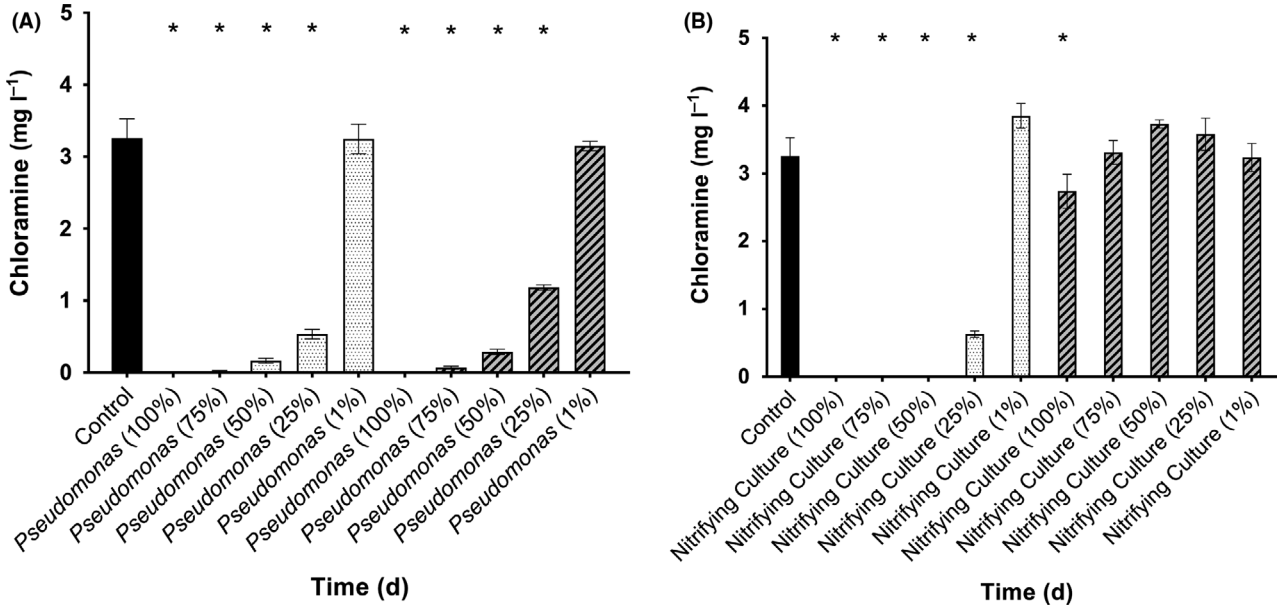

Fig. 2. Chloramine is degraded by both live and dead *P. aeruginosa* heterotrophic bacteria, yet only by living mixed nitrifying cultures. Chloramine was added to dilutions of living (dotted) and heat-inactivated (diagonal lines) *P. aeruginosa* (A), mixed nitrifying culture (B) or deionized water (Control) to a final concentration of 4.5 mg l^{-1} and the chloramine residual was measured after 2 days. Error bars represent SEM ($n = 3$). Statistically significant differences in comparison with the control ($P < 0.05$) as determined by two-way ANOVA and Tukey's multiple comparisons test are indicated by *.

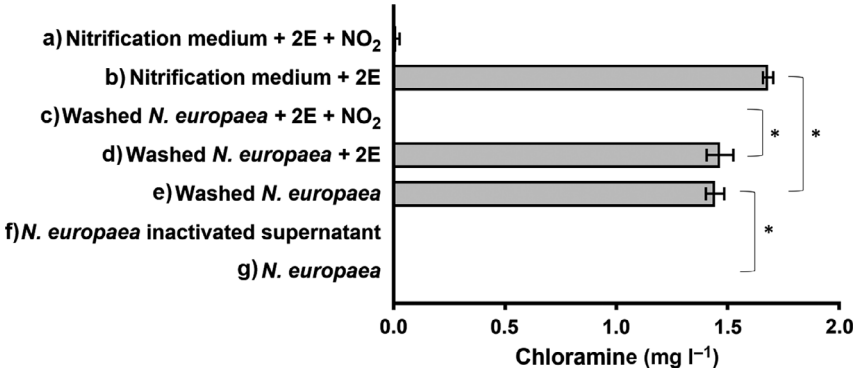

Fig. 3. Chloramine decay in the presence of *N. europaea* cells and filtrate, supplemented with nitrite and a nitrification inhibitor. Chloramine was added at a final concentration of 2 mg l^{-1} to 10 ml of the following samples as represented on the *y*-axis: (A) sterile NM supplemented with 2-ethynylpyridine and sodium nitrite ('Nitrification medium + 2E + NO_2'); (B) sterile NM supplemented with 2-ethynylpyridine ('Nitrification medium + 2E'); (C) washed *N. europaea* cells resuspended in sterile NM, supplemented with 2-ethynylpyridine and sodium nitrite ('Washed *N. europaea* + 2E + NO_2'); (D) washed *N. europaea* cells resuspended in sterile, fresh NM, supplemented with 2-ethynylpyridine ('Washed *N. europaea* + 2E'); (E) washed *N. europaea* resuspended in sterile NM ('Washed *N. europaea*'); (F) cell-free NM filtered to remove *N. europaea* ('*N. europaea* inactivated supernatant'); (G) *N. europaea* in spent NM ('*N. europaea*'). These supplemented samples contained 2-ethynylpyridine at final concentration of 10.31 mg l^{-1}, (designated as 2E) and/or sodium nitrite at a final concentration of 1.31 g l^{-1} (designated as NO_2). Chloramine was quantified after 2 days. Error bars represent SEM ($n = 9$). Statistically significant differences between treatments ($P < 0.001$) as determined by Tukey's multiple comparisons test after one-way ANOVA are indicated by *.

nitrite restored the degradation of chloramine (Fig. 3). Further, addition of nitrite to sterile medium also resulted in the complete decay of chloramine within 2 days.

The similarity between remaining chloramine in the washed cultures of *N. europaea* and the washed cultures of *N. europaea* supplemented with 2-ethynylpyridine ($P > 0.05$, $n = 3$) demonstrated that any loss of chloramine potentially arising from additional growth after inoculation in the non-inhibited cultures is negligible, as chloramine rapidly inactivates bacteria before significant nitrite production takes place. The results also show that most of the activity associated with chloramine decay was from the nitrite in the supernatant and was not dependent on the presence of biomass.

The nitrification inhibitor, 2-ethynylpyridine, does not chemically cause chloramine decay, as determined experimentally, and therefore was included in subsequent controls. When the washed culture was supplemented with 1.31 g l^{-1} of sodium nitrite, equivalent to the concentration of nitrite produced in the unwashed cultures, significant and complete chloramine decay was once again observed with a residual that was below the detectable limit, as was observed in the unwashed *N. europaea* culture. Complete chloramine decay was also observed when chloramine was added to either the nitrite-containing filter-sterilized *N. europaea* supernatant or to sterile NM supplemented with nitrite (Fig. 3). In all treatments, significant chloramine decay only occurred in the presence of nitrite, either as a product of nitritation or when added exogenously. This suggests that the production of nitrite by a bacterial community may protect the bacterial cells from the inhibitory effects of chloramine. Comparison of chloramine decay with nitrite at concentrations found in real-world systems would also help to extrapolate these results to such systems.

The effect of chloramine on the biomass of established mixed-species nitrifying biofilms

To determine if nitrification activity could also protect mixed-species, nitrifying communities against chloramine, established biofilms of the mixed-species nitrifying cultures were stressed by exposure to 2 mg l^{-1} chloramine, a sub-growth-inhibitory concentration, for 1 h. The growth medium was then replenished and incubated for another 3 days, after which time the biomass was quantified. As an additional treatment, biofilms were also supplemented with glucose which would select for a community dominated by the heterotrophs present in the community.

Biofilms of the nitrifying cultures grown in nitrification medium and replenished in the same medium showed a significant reduction in biomass after chloramine treatment (CV stain OD_{550} of 0.17) compared to the untreated control (OD_{550} of 0.52; Fig. 4). However, when these biofilms were grown in, and replenished with, nitrification medium supplemented with glucose, an increase in biomass was observed (OD_{550} of 1.28) without significant reduction in biofilm after treatment with 2 mg l^{-1} of chloramine (OD_{550} of 1.06; Fig. 5). The presence of the nitrification inhibitor, 2-ethynylpyridine, in glucose-supplemented wells treated with 2 mg l^{-1} of chloramine significantly reduced biomass (OD_{550} of 0.31) compared to chloraminated samples supplemented with glucose in

the absence of the nitrification inhibitor ($P < 0.05$, $n = 9$). This reduced biomass was similar to the amount of biomass of the chloraminated samples grown in, and replenished with, nitrification medium (OD_{550} of 0.17). However, the inclusion of 2-ethynylpyridine in the glucose-supplemented wells (without chloramine treatment) did not inhibit biofilm formation (OD_{550} of ~ 1.3) and hence the 2-ethynylpyridine alone was not growth-inhibitory towards heterotrophic bacteria at this concentration, in the presence of glucose.

Discussion

In this study, we have demonstrated a role for AOB in the decay of chloramine and the protection of mixed-species biofilms from chloramination. It was shown that the dominant contributor to the rapid loss of chloramine was the metabolic activity of the AOB, with the nitrite produced by AOB directly causing significant chloramine decay. Thus, the biochemical nitrite barrier formed by AOBs within biofilms may enable the persistence of multispecies nitrifying biofilms after chloramination. Additionally, inhibition of nitrite production through the addition of 2-ethynylpyridine greatly diminished the resistance of these biofilms to chloramination. The effect of nitrite on chloramine decay was far greater and more rapid than the effect of alginate, representing organic microbial by-products or the extracellular matrix on their own.

The use of alginate as a representative for the polysaccharide component of the biofilm matrix demonstrated the non-metabolic contribution of biopolymers produced by microorganisms (Boyd and Chakrabarty, 1995), especially during biofilm development, to chloramine decay. Further, by using the purified form of alginate, it is possible to separate the putative biofilm matrix material's effect on chloramine decay from other products or components of bacterial cells. It is also possible that other natural organic materials (NOM) present in drinking water systems could have a similar effect as alginate in mediating the decay of chloramine. It has been previously shown that a mucoid strain of *P. aeruginosa* produced between 200 and 500 μg mg^{-1} alginate per cell dry weight, which is almost 10-fold higher than what we tested here, 50 μg ml^{-1}. Thus, the concentrations tested in this study are realistic relative to the amounts of polysaccharide present in the biofilm matrix of *P. aeruginosa* (Hassett, 1996). The overproduction of organic matter has previously been observed in alginate-overproducing mucoid *P. aeruginosa* strains, where the production of alginate was protective against oxidative stress (Mathee *et al*., 1999; Cochran *et al*., 2000; Hentzer *et al*., 2001; Periasamy *et al*., 2015). However, further investigations into the amounts of EPS found in real-world biofilms and identification of the specific biopolymers found in those would be important to better understand their role in chloramine decay in those systems.

We observed that the AOB were observed to be poor biofilm formers on their own, as has been previously

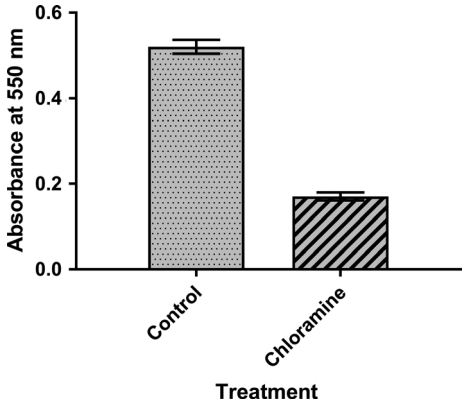

Fig. 4. The addition of chloramine to pre-formed biofilms of mixed-species nitrifying cultures in NM results in the loss of biomass. Four-day biofilms grown in NM were treated with 2 mg l^{-1} of chloramine for 1 h. The supernatant was removed and fresh NM was added before incubation for 3 days at 25°C with shaking at 100 r.p.m, followed by measurement of biofilm biomass. Error bars represent SEM ($n = 9$). Statistically significant difference from the untreated control ($P < 0.0001$) as determined by a two-tailed unpaired *t*-test is indicated by *.

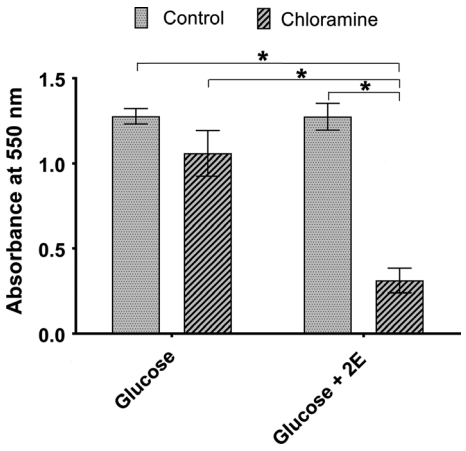

Fig. 5. Biofilms of glucose-supplemented mixed-species nitrifying cultures grown with or without 2-ethynylpyridine, a nitrification inhibitor, before and after exposure to chloramine. Biofilms grown for 4 days in NM with 200 mg l^{-1} of glucose, with and without 10.31 mg l^{-1} of 2-ethynylpyridine were treated with 2 mg l^{-1} of chloramine for 1 h. The supernatant was then removed and fresh NM with glucose, with and without 2-ethynylpyridine (final concentration of 10.31 mg l^{-1}) was added before incubation for a further 3 days at 25°C with shaking at 100 r.p.m., followed by measurement of biomass. The designation of 'Glucose' refers to NM with 200 mg l^{-1} of glucose, and the designation of '2E' refers to the inclusion of 2-ethynylpyridine to a final concentration of 10.31 mg l^{-1}. Error bars represent the SEM ($n = 9$). Statistically significant differences ($P < 0.001$) as determined by Tukey's multiple comparisons test following two-way ANOVA are indicated by *.

reported (Tsuneda *et al*., 2001), but readily became incorporated into biofilms when grown in the presence of heterotrophic bacteria, e.g. *P. aeruginosa* (Keshvardoust *et al*., 2019). It is possible that the heat treatment used to inactivate *P. aeruginosa* or the AOB culture may have altered the proteins or other cellular components, e.g. through denaturation, which may affect chloramine decay. However, the chloramine decay patterns for the heat-inactivated and live *P. aeruginosa* cultures are similar and therefore the heat treatment appears to have had little overall impact on this effect. Future work could further address the impact of the purified biofilm matrix components from *P. aeruginosa* and to investigate what, if any, EPS is produced by AOB when they become incorporated into a biofilm. To further separate the role of polysaccharides from other cellular components on chloramine decay, future work could also take advantage of *P. aeruginosa* mutants that do not produce detectable amounts of polysaccharide matrix. As noted above, the specific matrix biopolymers associated with real-world drinking water biofilms may be chemically distinct from alginate, and therefore, it will be important to identify and test those matrix components for their impact on chloramine decay.

In addition to this non-metabolic contribution of heterotrophic bacteria, bacterial metabolism has an overwhelmingly significant role in the removal of chloramine. The decay of chloramine in the presence of nitrite is well-supported in the literature; for example, Vikesland *et al*. (2001) calculated that this decay produces one molecule of nitrate and one molecule of ammonia for every molecule of nitrite that reacts with chloramine. While it is not possible to directly compare chloramine degradation rates between the *P. aeruginosa* and mixed-species cultures, the results none the less highlight that biochemically mediated removal of chloramine is greater than the abiotic removal process. Here, it has been shown that incubation of the mixed-species nitrifying culture with chloramine resulted in the significant loss of chloramine, even though the total cell density of the nitrifying culture was much lower than that of the non-nitrifying culture of *P. aeruginosa*. This effect was generally not observed when the nitrifying culture was heat-inactivated, suggesting a significant role for metabolic activity in chloramine decay by this community. Experiments with washed, pure cultures of *N. europaea* supplemented with nitrite indicated that this effect was almost entirely due to the reaction of chloramine with nitrite, the product of ammonia oxidation.

When supplemented with glucose during growth and after exposure to 2 mg l^{-1} of chloramine for 30 min, biofilms of the mixed-species nitrifying culture appeared to be insensitive to chloramination, as there was no reduction in biofilm biomass. This protection was lost upon inclusion of 2-ethynylpyridine, which blocks the production of nitrite from ammonia oxidation, in the glucose-supplemented wells. This suggests a mechanism by which ammonia-oxidizing bacteria protect microbial communities from chloramine treatment is through the production of nitrite. Interestingly, it has previously been shown that when the mixed-species biofilm was grown in the presence of glucose, members of the *Nitrosomonadaceae* decreased from approximately 50% to < 1% of the community while maintaining the same nitrite concentrations (Keshvardoust *et al*., 2019). The maintenance of nitrite production levels would suggest that this reduction in the relative amount of *Nitrosomonadaceae* is likely due to the increased numbers of heterotrophs rather than the reduction in absolute numbers of *Nitrosomonadaceae* (Petrovich *et al*., 2017; Keshvardoust *et al*., 2019).

It is known that chloramine penetrates bacterial biofilms up to 170-fold faster than free chlorine, although this does not correlate to an immediate loss of cell viability (Lee *et al*., 2011; Pressman *et al*., 2012). As chloramine is less reactive than free chlorine, treatment with chloramine may result in the retention of chloramine within the biofilm matrix, even after the replenishment of media in the test wells. Although the biofilm matrix in biofilms reacts with chloramine and facilitates the decay of the disinfectant, this decay is not instantaneous, as shown by the alginate co-incubation experiments. Any residual disinfectant within the matrix would also have rapid deleterious effects on cells and negatively affect the stability of the biofilm. These results suggest that the biofilm matrix alone cannot fully protect biofilms against chloramine, and a biofilm capable of maintaining an active population of AOB would possess an intrinsic chloramine removal pathway in the biofilm matrix through the steady production of nitrite, which may rapidly react with chloramine to release ammonia (Vikesland *et al*., 2001). By increasing chloramine demand in this way, AOB may help to increase biofilm stability and this effect would be extended to other species present within the biofilm. This would be supported by the spatial organization of nitrifying biofilms and aggregates, as AOB may be found throughout the biofilm but tend to localize at the surface where nutrient exchange and oxygen availability are best supported (Schramm *et al*., 1998; Okabe *et al*., 1999).

Although a study by Pressman *et al*. (2012) clearly demonstrated a role for nitrification in resistance to disinfection by chloramine, Pressman *et al*. (2012) suggested that the role of free ammonia was to increase the persistence of viable biomass and thus allow for faster recovery after cessation of chloramination. These results highlight the importance of considering chloramine decay as a microbial community-based problem rather than

being limited to the activity of a single population of bacteria.

Therefore, to expand on the conclusions offered by Pressman *et al*. (2012), we offer here a laboratory-based model where it is suggested that mixed-community biofilm formation is protective from the effects of chloramine by two intertwined mechanisms (Fig. 6). First, the secretion of biofilm matrix polymers, such as alginate, that will react with chloramine. This mechanism may also release free ammonia from chloramine that would then increase the concentration of substrate available to ammonia-oxidizing organisms present. In doing so, this would further enhance the second mechanism, the release of nitrite, which contributes to rapid chloramine decay. If the nitrite is concentrated in the biofilm matrix where it is produced before diffusion, this would represent a dual active, protective barrier to prevent chloramine from inhibiting the community members. Thus, the novelty of our study is indirectly linking biofilm structure and nitrification to a localized protective effect from AOBs within multispecies biofilms, rather than to just biofilm recovery or systemic chloramine decay. Future work based on this hypothetical model could directly test various aspects, such as whether nitrite and ammonia indeed become concentrated in the matrix and if the amount of ammonia produced can further support ammonia oxidation by the AOB within the biofilm community. While it remains to be experimentally demonstrated, the retention of nitrite in the matrix may overcome the dilution effects in high flow-through systems such as water distribution pipes. As nitrite-oxidizing bacteria frequently co-localize with AOBs in biofilms to better facilitate the exchange of nitrite (Schramm *et al*., 1996; Okabe *et al*., 1999), this suggests a significant localized concentration of available nitrite in the regions of a biofilm housing AOBs. The use of micro-electrode-based sensors to quantify chloramine and oxygen (Lee *et al*., 2011) in future studies would allow precise determination of chloramine penetration and activity between conditions of active and inhibited nitrification.

The limitations of the model biofilm system used in this study have previously been discussed (Keshvardoust *et al*., 2019), particularly in relation to shear stress forces normally occurring in water pipelines. The experiments conducted here only assessed short-term responses, as these may provide more immediate correlation with relatively sudden changes, such as fluctuating concentrations of ammonia. Future work should consider the longevity of such changes in microbial communities and their impact on disinfection practices. Although future work should consider expansion of this experimental model system to include continuous flow culture conditions for enhanced applicability to real-world water distribution systems, the model allowed for small-scale testing and screening of interactions between chloramine, AOBs and heterotrophic bacteria, while providing avenues for decoupling these interactions for further study. As this work presents a model for initial consideration and evaluation of mechanisms of chloramine decay by biofilm, it is expected that further study of specific mechanisms would eventually be conducted in systems with nutrient and flow characteristics more representative of drinking water distribution systems, such as in actual testing pipelines. Additionally, detailed biochemical studies focused on the specific rates of auto-oxidation, reaction kinetics under different substrate concentrations etc. would be invaluable to develop a detailed and potentially predictive model of this process.

This work highlights the importance and effect of AOBs in laboratory biofilm systems and the potential of AOBs to protect mixed-species biofilms from disinfection by chloramination. It is therefore suggested that future water disinfection strategies could incorporate

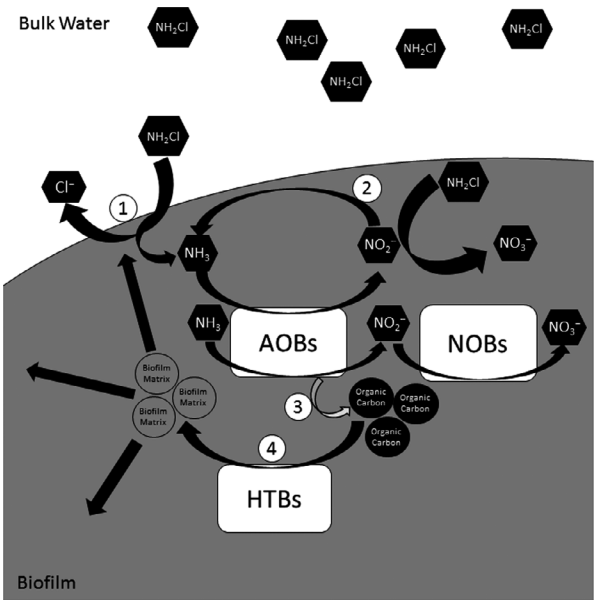

Fig. 6. Multiple pathways result in the decay of chloramine. Multiple pathways result in the decay of chloramine. (1) Chloramine from bulk water reacts with polysaccharides that comprise the biofilm matrix (shaded section), forming free ammonia and chloride. The ammonia may then be oxidized by AOBs, generating nitrite. (2) Chloramine that penetrates the biofilm reacts with nitrite (produced by AOBs), forming free ammonia and nitrate. Importantly, this reaction may also occur outside of the biofilm as nitrite leaves the biofilm. The ammonia may then be oxidized by AOBs, generating more nitrite. (3) AOBs assimilate inorganic carbon, producing organic carbon compounds and nitrite as part of their life cycle. The organic carbon may then be released, either through secretion or during cell turnover, and becomes available to HTBs. (4) HTBs may consume the organic carbon compounds released by AOBs, producing polysaccharides necessary for the development of the biofilm matrix, which in turn provides protection from disinfection by chloramine. AOBs, ammonia-oxidizing bacteria; HTBs, heterotrophic bacteria; NOBs = nitrite-oxidizing bacteria.

mechanisms to specifically target AOBs and ammonia oxidation/nitritation, whether through nutrient limitation or active inhibition, to diminish chloramine demand in drinking water distribution systems.

Conclusions

This study showed that:

- Organic microbial by-products from heterotrophic bacteria (represented by alginate) slowly degraded chloramine in a dose-dependent manner, but the dominant contributor to the rapid loss of chloramine was the metabolic activity of the AOB biofilm.
- Active nitritation allowed for the persistence of laboratory biofilms after chloramination.
- Inhibition of nitrite production from the oxidation of ammonia greatly diminished the resistance of laboratory biofilms to chloramination.

Experimental procedures

Bacterial strains, cultures and growth media

Ammonia-oxidizing bacteria (AOBs) were grown in minimal nitrification medium (NM; Stein and Arp, 1998a,b) at 30°C with agitation at 100 r.p.m. The medium contained final concentrations of 3.3 g l^{-1} $(NH_4)_2SO_4$, 5.85 g l^{-1} KH_2PO_4, 0.48 g l^{-1} NaH_2PO_4, 90 mg l^{-1} $MgSO_4$, 22 μg l^{-1} $CaCl_2$, 1.5 mg l^{-1} $FeSO_4$/5 mg l^{-1} EDTA (a chelated iron mixture), 80 ng l^{-1} $CuSO_4$ and 400 μg l^{-1} Na_2CO_3, at a pH of 8.5.

Nitrosomonas europaea (ATCC 19718) pure cultures or a commercial nitrifying culture (Bio-Culture; AquaSonic™, Sydney, NSW, Australia), containing ammonia-oxidizing bacteria, nitrite-oxidizing bacteria and heterotrophic bacteria, were used in this study, expanding upon a modelling system described previously (Keshvardoust *et al*., 2019). This community and biofilm model was used as an experimental laboratory system to enable relatively rapid experiments, that incorporate the biochemical functionality of AOB coupled with the biofilm-forming ability of co-occurring species. For chloramine decay experiments with *N. europaea* cultures and biofilm experiments with the mixed-species nitrifying community, nitrifying bacterial cultures were inoculated directly from glycerol stocks into 100 ml of NM in aerated Erlenmeyer flasks (500 ml), incubated in the dark at 30°C with agitation at 100 r.p.m. for either 14 days (mixed-species nitrifying culture) or 30 days (*N. europaea* pure cultures). Preliminary studies determined that this incubation time allowed the mixed-species culture to form aggregates and the measurable nitrite concentrations increased to between 0.69 g l^{-1} and 1.73 g l^{-1} (representing 10 and 25 mM, respectively), with maximum nitrite concentrations of approximately 2 g l^{-1}, suggesting that nitritation was occurring. Previous studies have indicated that the toxic effect of nitrite on ammonia monooxygenase occurred only at concentrations greater than 30 mM and that such effects were reversible upon washing the cells (Stein and Arp, 1998a, b). After 14 days, the bacterial culture was mixed by vortexing to more evenly distribute the floccular biomass before 1 ml was subcultured into 100 ml of fresh NM in aerated flasks, which were then incubated for another 3 days (designated as the 3 day pre-culture), until aggregates were visible and the concentration of nitrite produced was again between 46 and 115 mg l^{-1}. The presentation of aggregates was used as a visual cue for the maturity of the culture, as this coincided with significant nitrite production.

Pseudomonas aeruginosa PAO1 (Holloway, 1955), which is an opportunistic pathogen and has been associated with cases of contamination in water distribution systems (Xue *et al*., 2013), was chosen as a model heterotrophic bacterium for chloramine decay studies. *P. aeruginosa* was grown in M9 minimal medium with glucose (0.68 g l^{-1} Na_2HPO_4, 0.3 g l^{-1} KH_2PO_4, 50 mg l^{-1} NaCl, 99 mg l^{-1} NH_4Cl, 0.24 g l^{-1} $MgSO_4$, 11 mg l^{-1} $CaCl_2$, 0.2 g l^{-1} glucose, pH 7.0) at 37°C as previously described (Kukavica-Ibrulj *et al*., 2008).

Chloramine formation

Chloramine was prepared freshly for each experiment as described (Hach Company, 2014). Briefly, 813 μl of 55 g l^{-1} sodium hypochlorite (45 mg of available chlorine; Sigma-Aldrich, USA) was added to a 50 ml solution of 763.6 mg l^{-1} ammonium chloride in chlorine demand-free deionized water adjusted to pH 8.5. After gently mixing for 1 min, deionized water was added to a final volume of 100 ml and stirred for a further 1 min. The resulting solution represented a 100× stock of chloramine (450 mg l^{-1} final concentration) that was used within 1 h. The concentration of chloramine was measured using the N,N-diethyl-p-phenylenediamine (DPD) colorimetric method (Rice *et al*., 1999). Briefly, 50 μl of phosphate buffer (800 mg l^{-1} of ethylenediaminetetraacetic acid (EDTA), 24 g l^{-1} of Na_2HPO_4 and 46 g l^{-1} of KH_2PO_4) was added to a 1-ml plastic cuvette, followed by 50 μl of the DPD colorimetric reagent (8 ml l^{-1} of 25% H_2SO_4, 200 mg l^{-1} of disodium-EDTA, 1.1 g l^{-1} of N,N-Diethyl-p-phenylenediamine [DPD]). One millilitre of the sample was then added and mixed by inversion of the cuvette three times. The absorbance of the sample was immediately measured using a NovaSpec II visible light spectrophotometer (Pharmacia Biotech, Sweden) at 515 nm (Reading A). To measure monochloramine, the cuvette was immediately removed, supplemented with

10 μl of a 1 g l^{-1} stock of potassium iodide, inverted three times and re-inserted into the spectrophotometer (Reading B). The monochloramine concentration was determined as (Reading B–Reading A), compared against a standard curve using $KMnO_4$.

Chloramine decay in the presence of heterotrophic bacteria and polysaccharides

To simulate the polysaccharide components of the biofilm matrix, a 100 mg l^{-1} stock solution of alginic acid (alginate; Sigma-Aldrich) in deionized water was diluted to 0 (control), 1, 5, 10, 20, 25, 30, 40 or 50 mg l^{-1} with deionized water and 10 ml of each concentration was added to three replicate screw-capped sterile scintillation vials. Chloramine was added to a final concentration of 4.5 mg l^{-1} before incubation in the dark at 25°C with shaking at 100 r.p.m.

To determine a potential role for the metabolism of bacteria in chloramine decay, a flask containing either a 3-day pre-culture of the mixed-species nitrifying community or an overnight culture of *P. aeruginosa* in M9 minimal medium (OD_{600} of 0.5) was divided into two equal aliquots, which were either kept intact ('live' culture) or inactivated by autoclaving at 121°C for 15 min ('inactivated'). The live and dead cultures were then diluted to 0 (control), 1%, 25%, 50% or 75% of their initial concentrations with deionized water, and 10 ml of each combination was transferred to 20-ml scintillation vials. Chloramine was then added to a final concentration of 4.5 mg l^{-1} before incubation in the dark at 25°C with gentle agitation. Chloramine was quantified daily over 3 days, from cell-free supernatants passed through a 0.22-μm pore-size filter (Millex®, Merck Millipore, United States).

Chloramine decay in the presence of ammonia-oxidizing bacteria

A flask containing a 14-day-old culture of *N. europaea* (nitrite concentration of 1.31 g l^{-1}) in nitrification medium (NM) was divided into separate aliquots which were either (i) kept intact (whole culture), (ii) filtered through 0.22-μm pore-size filters (cell-free supernatants) or (iii) bacterial cells were washed twice with one volume of sterile NM and then resuspended in one volume of NM (pH 8) in order to lower the concentration of nitrite below the limit of detection (nitrite-free culture). Ten millilitres of each solution were transferred into 20-ml scintillation vials and treated with the nitrification inhibitor, 2-ethynylpyridine (final concentration of 10.31 mg l^{-1}; Chen *et al*., 1995), and/or sodium nitrite (final concentration of 1.38 g l^{-1}, equal to that of the unwashed culture reflecting the amount of nitrite naturally made through ammonia oxidation under the conditions used here). 2-ethynylpyridine inhibits nitrification by specifically binding to the copper-based active site of the AMO enzyme to inactivate its function (McCarty, 1999). This concentration of 2-ethynylpyridine was experimentally determined to not cause chloramine decay for the duration of the experiment while still completely inhibiting nitrification and nitrite production (data not shown). Each condition was tested in three independent experiments.

Experiments included the following samples: (i) *N. europaea* in spent NM; (ii) cell-free NM filtered to remove *N. europaea*; (iii) washed *N. europaea* resuspended in sterile NM; (iv) washed *N. europaea* resuspended in sterile, fresh NM, supplemented with 2-ethynylpyridine; (v) washed *N. europaea* resuspended in sterile NM, supplemented with 2-ethynylpyridine and sodium nitrite; (vi) sterile NM supplemented with 2-ethynylpyridine; and (vii) sterile NM supplemented with 2-ethynylpyridine and sodium nitrite. Chloramine was then added to each vial to a final concentration of 2 mg l^{-1} before incubation in the dark at 25°C with shaking at 100 r.p.m. Chloramine was measured after 2 days, allowing time for decay to occur through the various potential pathways investigated.

Biofilm assays with mixed-species, nitrifying cultures

For biofilm experiments, mixed-species nitrifying cultures were used as part of a previously described modelling system (Keshvardoust *et al*., 2019). Biofilms were grown from a 1:100 dilution of a 3-day pre-culture in 2 ml of fresh NM into 12-well microtitre plates that were then sealed with Parafilm M® (Bemis, USA), covered with aluminium foil and incubated at 25°C on an orbital shaker with agitation at 100 r.p.m for an initial time of 3 days. The medium was carefully removed and replaced with fresh NM, and the plates were incubated for an additional 24 h, before analysing the biofilm or testing its resistance to chloramine treatment.

These conditions were determined here to be optimal for the growth of mixed-species nitrifying biofilms in the absence of any treatment and provided the best yield of biomass (data not shown). After the final incubation, biofilm biomass was analysed by using crystal violet (CV) staining (O'Toole, 2011). Briefly, the supernatant was removed and biofilms were washed once with phosphate-buffered saline (PBS, 2 ml) to remove non-attached bacteria, before adding 2 ml of a CV stain solution consisting of a 10 times dilution in deionized water of a commercial CV solution (Becton Dickinson, USA). The plates were then incubated at 25°C for 15 min, before washing the wells twice with PBS. Finally, CV remaining in the wells was solubilized with 2 ml of 96% ethanol (15 min incubation) and quantified

(OD_{550}) using a Wallac 1420 Victor^2 spectrophotometer (PerkinElmer, USA).

To test the effect of various treatments on biofilm formation, mixed-species nitrifying biofilms were cultivated as described above and compounds were added to the growth medium for the first 3-day culture phase as well as for the last 24-h incubation, including glucose to a final concentration of 200 mg l^{-1}, or 2-ethynylpyridine to a final concentration of 10.31 mg l^{-1}. Glucose was added to promote the growth of heterotrophic bacteria, particularly in the presence of nitrification inhibitors. As it was previously demonstrated that the growth of heterotrophic bacteria in this system was dependent on nitritation by AOBs, this provided an avenue to decouple this metabolic reliance.

For chloramine resistance tests, biofilms were developed for the initial 3-day phase in the absence of chloramine, then the supernatant was removed and replaced with 2 ml of sterile deionized water containing 2 mg l^{-1} of freshly prepared chloramine, or water only for control wells, and the plates were incubated for a further 1 h, giving a contact time value of 120 mg min l^{-1}. This contact time value was intentionally below the value needed for the 3 log inactivation of *Giardia* cysts (750 mg min l^{-1}) or 4 log inactivation of viruses (497 mg min l^{-1}; USEPA, 1998; benchmarks used for water disinfection), to stress, but not eradicate, the biofilms. Deionized water was used instead of NM during treatment with chloramine to reduce any potential confounding effect by ammonia in the NM. After 1 h, the supernatant was removed and replaced with fresh NM, with or without glucose and 2-ethynylpyridine, and the plates were incubated for a further 3 days. For these experiments, a duplicate set of plates was used to enable measurement of biomass before and after treatment with chloramine.

Statistical analysis

Statistics analyses were performed using PRISM 6.04 graphing software (GraphPad Software, USA). *T*-tests, one-way ANOVA and two-way ANOVA were performed as appropriate, followed by Tukey's multiple comparisons test. Results with a *P* value of less than 0.05 were deemed statistically significant.

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Conflict of interest

None declared.

Author contributions

P.K., N.B. and S.R. conceived the initial experimental plan, which was further developed and refined by M.C. and V.A.A.H, and N.B. and S.R. contributed to the funded grant for support for the project. P.K. conducted the experimental work with help from V.A.A.H. All authors contributed to data analysis and writing of the manuscript.

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