



Quorum sensing inhibition as a promising method to control biofilm growth in metalworking fluids

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Abstract

Microbial contamination in metalworking systems is a critical problem. This study determined the microbial communities in metalworking fluids (MWFs) from two machining shops and investigated the effect of quorum sensing inhibition (QSI) on biofilm growth. In both operations, biofilm-associated and planktonic microbial communities were dominated by *Pseudomonadales* (60.2–99.7%). Rapid recolonization was observed even after dumping spent MWFs and meticulous cleaning. Using *Pseudomonas aeruginosa* PAO1 as a model biofilm organism, patulin (40 µM) and furanone C-30 (75 µM) were identified as effective QSI agents. Both agents had a substantially higher efficacy compared to α -amylase (extracellular polymeric substance degrading enzyme) and reduced biofilm formation by 63% and 76%, respectively, in MWF when compared to untreated controls. Reduced production of putatively identified homoserine lactones and quinoline in MWF treated with QS inhibitors support the effect of QSI on biofilm formation. The results highlight the effectiveness of QSI as a potential strategy to eradicate biofilms in MWFs.

Keywords Metalworking fluids · Biofilm · Quorum sensing inhibition · Patulin · Furanone C-30

Introduction

Metalworking fluids (MWF) are used in machining, grinding, and milling operations for cooling, reducing friction, removing metal particles, and protecting the workpiece and tools. Microbial contamination of MWFs is a fundamental

problem, affecting the quality of the product, the lifetime of MWFs [13, 48], and potentially increasing occupational safety risks [6]. Measures to combat microbial growth in MWFs typically involve the use of biocides, although alternative, more environmentally friendly approaches have also been investigated [1, 2]. These measures are, nonetheless, insufficient at eliminating the microbial load in MWF circulation systems [14, 23, 27, 50]. In fact, certain bacterial strains present in MWFs (e.g., *Mycobacterium* and *Pseudomonas*) are particularly resistant to industrial biocides [11, 48, 49]. Rapid recolonization of freshly recharged MWF systems directly after emptying and extensive cleaning is a strong indication of the ineffective removal of microbial colonizers [18, 50]. The microbial contaminants are believed to persist in the inaccessible parts of these machines in the form of biofilms [48]. While increasing biocide dosage could potentially improve biofilm disruption in MWFs [24], such attempts would be counterproductive to current trends in the MWF industry, by raising costs, increasing occupational safety and environmental risks, and impeding the biodegradation of spent MWFs.

Intercellular communication, a phenomenon termed quorum sensing (QS), has been identified across diverse

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bacterial taxa and enables complex interactions among bacteria [28]. Along with other physiological processes, the formation of biofilm, a well-organized and structured microbial community enclosed in a self-produced polymeric matrix and adherent to a surface, is regulated by QS [29]. Fundamentally, QS is mediated by the production, release, sensing of, and response to small, diffusible signal molecules by bacterial cells (i.e., autoinducers), and is related to cell density [28, 32]. Once the threshold concentration for signaling molecules is reached in the extracellular environment, these molecules diffuse through cell membranes and activate transcription regulators [28]. This dependency on signal molecules to coordinate a translational behavior on a population level provides ideal targets to disrupt bacterial communication and, hence, biofilm formation. While QS systems and their inhibition [e.g., 12, 16, 41] by numerous synthetic [14, 31, 39, 51] and naturally occurring QS disruptive compounds [9, 14, 17, 21, 39, 40, 46] have been studied extensively, limited effort has been directed to the application of QSI [reviewed in 16, 42]. However, some large-scale trials on water treatment systems have demonstrated the applicability of QSI to successfully mitigate biofilm formation and biofouling [19, 22, 30, 44].

Considering that bacteria resistant to biocides are emerging among microbial communities inhabiting MWF circulation systems [11, 48, 49], we assessed whether QSI, a non-biocide treatment, can reduce biofilm formation in these systems. The effect of commercially available QSI compounds on biofilm reduction in a water-soluble MWF were investigated. With *Pseudomonas* spp. frequently being the focus of research on QSI [e.g., 14, 21, 39, 40, 45, 51] as well as representing the bacterial genus that dominates microbial contamination in MFW [10, 13, 27], *Pseudomonas aeruginosa* PAO1 was used as a model organism in the present study.

Materials and methods

Sample collection

Planktonic and biofilm samples were collected from computer numerical control (CNC) machines from two local machine shops in Bozeman, Montana, USA (herein referred to as Shop 1 and Shop 2). MWF (S-500-CF, Hangsterfer's), a water-soluble fluid, was used at both shops. At the time of sample collection, this MWF had been in continuous use under routine fluid management practices for 1 year. Additionally, samples were collected from Shop 2 after a commonly followed industrial dumping, cleaning, and recharging process. Samples from this cleaned and recharged system were collected after 1 day of operation with new fluid. Samples for microbial community analysis from the

MWF reservoir of CNC machines (~ 100 mL) were filtered onto 47 mm Supor® 200 PES membrane filters with a pore size of 0.2 µm using a peristaltic pump. Biofilm from screens and shavings were scraped with a sterile spatula directly from CNC machine compartments into sterile 50-mL Falcon tubes. Mist from inside the CNC machine working chamber during operation in Shop 2 was collected onto 47 mm Supor® 200 PES membrane filters with a pore size of 0.2 µm. Membrane filters were placed into sterile tubes. All samples were stored at – 80 °C until further processing.

Extraction of nucleic acids, DNA amplification, and sequence analyses

DNA was extracted using the PowerWater® DNA Isolation Kit (14900-S, MO BIO Laboratories) following the manufacturer's instructions. Extracted DNA was quantified using the high-sensitivity Qubit DNA Assay Kit with a Qubit® Fluorometer (Thermo Fisher Scientific). The polymerase chain reaction (PCR; see Online Resource 1) was performed using primer pairs specific to the V3–V4 regions of the 16S rRNA gene. Primer complexes included the Illumina adaptor sequences followed by either the universal primers 341F (5'acactctttccctacacgacgtcttccgatctCCTACGGGNGGC WGCAG-3') or 805R (5'gtgactggagttcagacgtgtgctcttccgatct-GACTACHVGGGTATCTAATCC-3') (UW Biotechnology Center, Madison, WI, USA). PCR amplicons were submitted to the University of Wisconsin-Madison Biotechnology Center. A blank sample for the extraction procedure was included in the sequence libraries. Paired end, 250-bp sequencing was performed using an Illumina MiSeq Sequencer and a MiSeq 500 bp (v2) sequencing cartridge.

For joining the forward and reverse sequence libraries QIIME toolkit was used [7]. Quality refinement of contigs was performed using the Mothur platform v.1.34.4 [43; see Online Resource 1]. Operational taxonomic units (OTU) were defined at ≥ 97%. Raw sequences libraries were deposited to the GenBank NCBI database under accession number SRA PRJNA398788.

Agents

Four natural agents, one enzyme, and two chemicals were tested for biofilm reduction and their effect on QSI. Emodin (E7881), gingerol (G1046), patulin (P1639), acylase I from porcine kidney (A3010), and the chemical agents furanone C-30 (53796) and phenyl disulfide (169021) were purchased from Sigma-Aldrich (Table 1). Garlic was extracted and purified adopting the protocol from Rasmussen et al. [39]. To evaluate the efficacy of QS inhibiting agents on biofilm reduction, a comparison was made with α-amylase *Bacillus amyloliquefaciens* (Sigma-Aldrich

Table 1 Compounds tested for reducing *P. aeruginosa* PAO1 biofilm formation grown in 96-well microtiter plates in TSB

Types	Agent	% Biofilm reduction		
		≥ 80	≥ 50	No effect
Natural	Emodin (10–1000 µM)			×
	Garlic extract (0.5–15% v/v)			×
	Gingerol (350 µM)			×
	Patulin (40 µM)*	×		
Enzymes	α-Amylase <i>B. amyloliquefaciens</i> (3.4 U mL ⁻¹)*	×		
	α-Amylase <i>B. amyloliquefaciens</i> (≥ 10 U mL ⁻¹)			×
	Acylase 1 from porcine kidney (~3.6–66.6 U mL ⁻¹)			×
Synthetic	Furanone C-30 (75 µM)*		×	
Chemicals	Phenyl disulfide (0.02–1 mM)			×

Efficacy is presented as percent biofilm reduction in comparison to the spectrophotometric measurements on crystal violet stained biomass in untreated controls

Asterisk identifies agents selected for QSI assays

A7595), an enzyme capable of degrading the extracellular polymeric substance (EPS) matrix of biofilms.

Culture conditions

Pseudomonas aeruginosa PAO1 (ATCC strain: 15692), a model biofilm forming organism, was grown in tryptic soy broth (TSB) at 37 °C while shaking at 125 rpm for 16 h. Cultures were harvested and centrifuged at 10,000×g for 2 min. Cell pellets were resuspended as applicable in either TSB or autoclaved municipal drinking water. Municipal drinking water was used for MWF assays, as the local machine shops use this water to dilute MWFs to the recommended operational concentration.

Screening for compound applicability

P. aeruginosa PAO1 enrichments were transferred into 96-well microtiter plates at an optical density (OD) of 0.03 at 600 nm. Wells were supplemented with TSB (1× final concentration) and the agents shown in Table 1. Eight replicates per agent and control were tested. Plates were incubated at 37 °C for 48 h. Quantitative analysis in microtiter plates was performed following the crystal violet method [35]. At selected time points (i.e., 0, 24, 48 h), plates were washed five times in deionized water (DIW) to remove planktonic cells. Wells were stained with 300 µL of 1:3 diluted crystal violet solution for 20 min at 22 °C. Excess stain was washed off five times in DIW. Following washing, 300 µL of 95% ethanol was added to each well, and de-colorization of the wells was allowed for 15 min. Light absorbance data were read at 540 nm in a BioTek FL600 micro-plate reader.

CDC biofilm reactors

Compounds that demonstrated the highest percentage of biofilm reduction (i.e., α-amylase, patulin, and furanone C-30) were further investigated. CDC Biofilm Reactors (Biosurface Technologies, Bozeman, MT) were assembled with stainless-steel coupons and autoclaved for 30 min. Each reactor contained 300 mL of 1× TSB medium, 2 mL of an overnight *P. aeruginosa* PAO1 culture, and a biofilm treatment agent (Table 1). Experiments were performed as batch reactors on a stir plate at 125 rpm at 37 °C for 48 h. After 24 and 48 h of incubation, one rod holding three stainless-steel coupons was removed from untreated and treated reactors. Coupons were dipped in 1× phosphate-buffered saline (PBS) to remove unattached, planktonic cells. Each coupon was then placed into sterile falcon tubes filled with 5 mL of 1× PBS and vortexed for 10 s. Tenfold dilution series were prepared in sterile 1× PBS. Five drops (10 µL each) per dilution were pipetted onto TSA plates [15], using an electronic pipette (Thermo Labsystems) for accuracy. Colony forming units (CFUs) of biofilm were enumerated after 16 h of incubation at 37 °C. Resulting CFUs were log₁₀-transformed. A log reduction (LR) was calculated for each experiment by subtracting the mean of the three log-densities for the treated coupons from the mean of the three log densities for the concurrent controls.

Multi-well plate assays with MWF

Overnight enrichments of *P. aeruginosa* PAO1 were harvested by centrifugation at 10,000×g for 2 min, washed twice in autoclaved municipal drinking water, and transferred into 12-well microtiter plates (08-772-3A, Falcon™ Polystyrene Microplates) at an OD of 0.03 at 600 nm. Wells were supplemented with 7% (final concentration) MWF in

autoclaved municipal drinking water (i.e., recommended operational concentration, Hangsterfer's Laboratories Inc.) and the agents shown in Table 1. A stainless-steel coupon in each well served as the biofilm growth surface. Coupons were autoclaved for 30 min prior to the experiment. Agents and abiotic controls were tested in triplicate. Plates were incubated at 37 °C for 48 h. Biofilm formation was investigated after 24 and 48 h by applying the drop-plating method described above.

Microscopy

Epi-fluorescence microscopy (Nikon Eclipse E-800) was performed to visualize biofilm on untreated and treated sample coupons. Coupons were pulled from CDC Biofilm Reactors after 48 h and dipped in filter sterilized 1× PBS to remove planktonic cells from the coupon surface. Biofilm was stained with the fluorescent dye SYTO[®] 9 (L7012, Invitrogen by Thermo Fisher Scientific) in the dark at 22 °C for 30 min. Sample coupons were carefully rinsed with filter sterilized DIW prior to imaging. Samples were imaged under 60× magnification with the MetaVue version 7.8.13.0 (Molecular Devices, LLC.) software package. Randomly selected images ($n = 10$) were taken per coupon. Interference between the MWF and epi-fluorescent signals prevented imaging of biofilm grown in MWF.

Metabolite extraction

Biofilm was grown on stainless-steel coupons in 12-well plates in TSB or MWF at 37 °C for 24 h. Trials were prepared in triplicate for untreated controls, and patulin (40 µM) and furanone C-30 (75 µM) amendments. Triplicate stainless-steel coupons were pooled in sterile falcon tubes containing 6 mL of 1× PBS buffer and vortexed for 10 s. Subsequently, cell pellets were collected by centrifugation at 10,000×g for 2 min. Supernatant was discarded and cell pellets were resuspended in 1 mL 1× PBS.

Metabolites were extracted according to a modified procedure from Carlson et al. [8; see Online Resource 1]. Samples were kept on ice at all times. Extracts were dried in a speed vacuum (Vacufuge plus, Eppendorf) for 2.5 h, resuspended in 50% methanol and stored at − 80 °C until liquid chromatography–mass spectrometry (LC–MS) was performed.

Metabolomic profiling and data processing

Extracted metabolites were analyzed using an Agilent 1290 UPLC system connected to an Agilent 6538 Q-TOF mass spectrometer (Agilent, Santa Clara, CA, USA). The samples were run in normal phase, using a Cogent Diamond Hydride HILIC 150 × 2.1 mm column (MicroSolv, Eatontown, NJ,

USA). Output files were converted to MZxml format using the Masshunter Qualitative software provided with Agilent instruments (Agilent, Santa Clara, CA, USA). Analysis of LC–MS data was done using the features and identification function in MZmine version 2.31 [36]. See Online Resource 1 for more details.

Statistical analysis

Linear mixed-effects models were applied to determine the repeatability of the untreated control log densities across experiments. Welch 2-sample t tests were performed on log densities in single experiments to assess the efficiency of individual treatments compared to untreated controls. All pairwise comparisons of the mean LR among the different agents, for each combination of media (MWF or TSB) and time points (24 or 48 h), were performed by Welch t tests with a Benjamini–Hochberg correction. Linear mixed-effects models and follow-up t tests using Kenward–Roger degree of freedom approximation were applied to determine the repeatability of experiments [47]. Standard deviations (SD) for repeatability of ≤ 0.5 are accepted [33]. The pairwise comparisons of LR were performed by Welch t tests with a Bonferroni multiple comparison correction for each agent. All statistical analyses were performed using the statistical software R [37].

Results

Determination of microbial communities

Quality refinement (including removal of blank OTUs and singletons) resulted in $98,162 \pm 40,475$ sequences per library, which clustered into 46 OTUs. All samples collected tested positive for the presence of bacteria and were dominated by *Pseudomonadales* (60.2–99.7%; Fig. 1). MWF was used at two different shops over a similar period; however, compared to Shop 1 dominated by *Pseudomonadales* ($92 \pm 4\%$), the MWF circulation system in Shop 2 was colonized by both *Pseudomonadales* ($74 \pm 9\%$) and *Xanthomonadales* ($23 \pm 9\%$). After dumping and cleaning the MWF circulation system at Shop 2, infection of the newly recharged MWF occurred within the first day, with *Pseudomonadales* driving recolonization.

Efficacy of agents on biofilm reduction

Various enzymes, natural, and chemical compounds were screened for their effect on biofilm formation (Table 1). Using crystal violet staining as an indicator for biofilm biomass, emodin (10–1000 µM), garlic extract (0.5–15% v/v), gingerol (350 µM), α -amylase (≥ 10 Units mL^{−1}), acylase 1

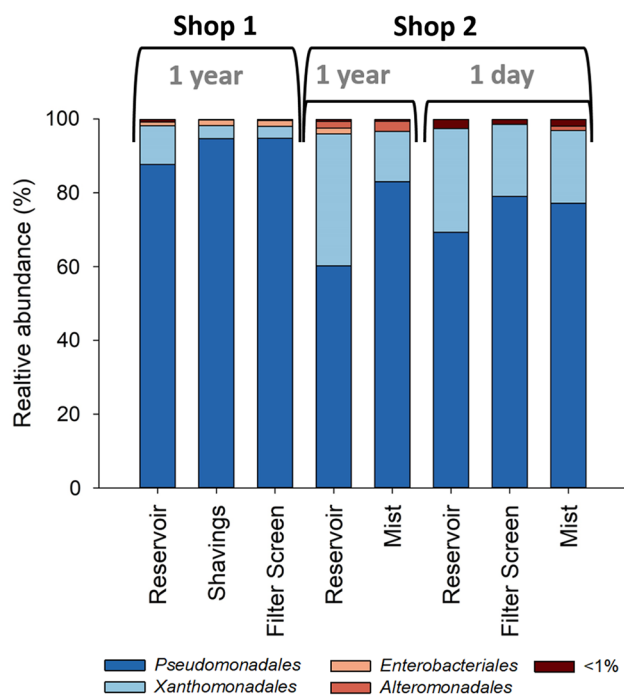


Fig. 1 Relative abundance of the microbial contamination (order level) present in CNC equipment at two machine shops (Bozeman, MT, USA)

from porcine kidney (~ 3.6 – 66.6 Units mL^{-1}), and phenyl disulfide (0.02 – 1 mM) showed no effect on eradication of *P. aeruginosa* PAO1 biofilms when compared to the untreated control. Conversely, patulin (40 μM), furanone C-30 (75 μM), and α -amylase from *B. amyloliquefaciens* (~ 3.4 Units mL^{-1} ; herein referred to as α -amylase) were effective in reducing biofilm formation (with reductions ≥ 50 – 80%). These three agents were selected for further investigation of their effectiveness in reducing *P. aeruginosa* PAO1 biofilm formation.

When testing *P. aeruginosa* PAO1 biofilm grown in TSB in CDC batch reactors (Fig. 2a), patulin ($p=0.034$) and furanone C-30 ($p=0.002$) demonstrated a statistically significant treatment effect after 24 h when compared to an untreated reactor. Although incubation over 48 h in TSB resulted in an increase in CFUs by 30.8 and 28.5% on average for patulin and furanone C-30, respectively, differences were not statistically significant from the previous time point ($p \geq 0.160$). Biofilm reduction efficiency between α -amylase, a biofilm matrix degrading enzyme, and the two QS inhibitors were similar, and no significant differences were found after both 24 and 48 h between compounds ($p \geq 0.146$). Calculated standard deviations of untreated control log densities ($N=18$, $\text{SD}=0.245$) suggests acceptable repeatability of the three treatments [33, 47]. Treatment effects on *P. aeruginosa* PAO1 biofilm formation were also visualized by epi-fluorescent microscopy and SYTO[®] 9 staining. Biofilm

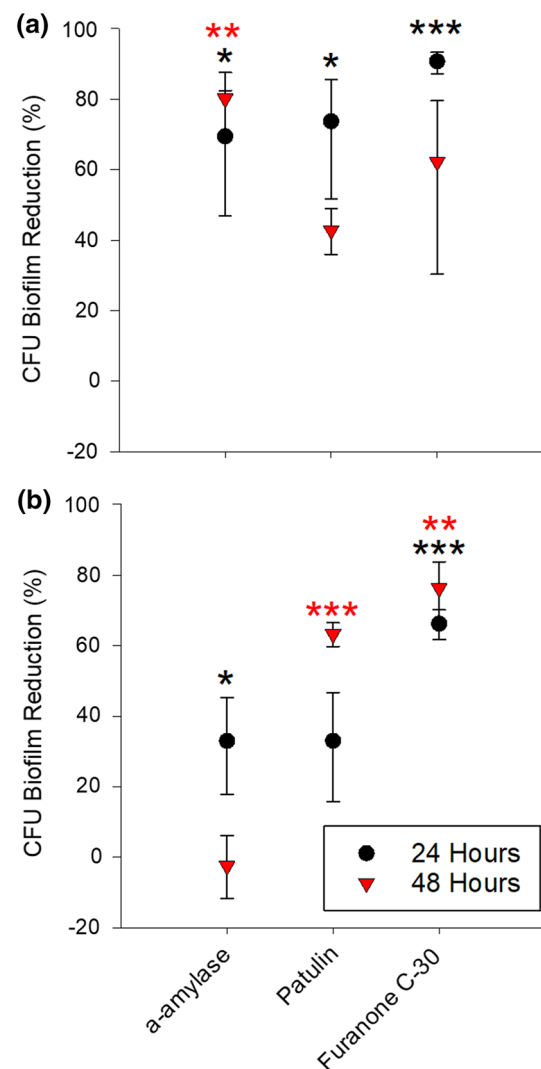


Fig. 2 Effect of quorum sensing inhibitors on *P. aeruginosa* PAO1 biofilm formation in **a** $1 \times$ TSB in CDC batch reactors, and **b** 7% (final concentration) water-soluble MWF in 12-well plates. Data points represent CFU biofilm reduction (%) at 24 and 48 h. Error bars indicate 95% confident intervals. Percent reductions that met significant levels are shown as * ≤ 0.05 , ** ≤ 0.01 , *** ≤ 0.001

on stainless-steel coupons treated with patulin and furanone C-30 was reduced in comparison to a denser growth on untreated coupons (Fig. 3). Green fluorescent cells indicated that the biofilm-associated cells on the coupon surfaces were viable.

The same agents were applied to multi-well plate assays, but in MWF over a period of 48 h (Fig. 2b). In 7% MWF, both patulin and furanone C-30 led to significant biofilm reduction over 48 h ($p=0.001$ and $p=0.012$), respectively, decreasing CFUs by 63% and 76%. In comparison, α -amylase had little effect on biofilm reduction in MWFs after 48 h when compared to the untreated control ($p=0.386$) and differed significantly from the assays with

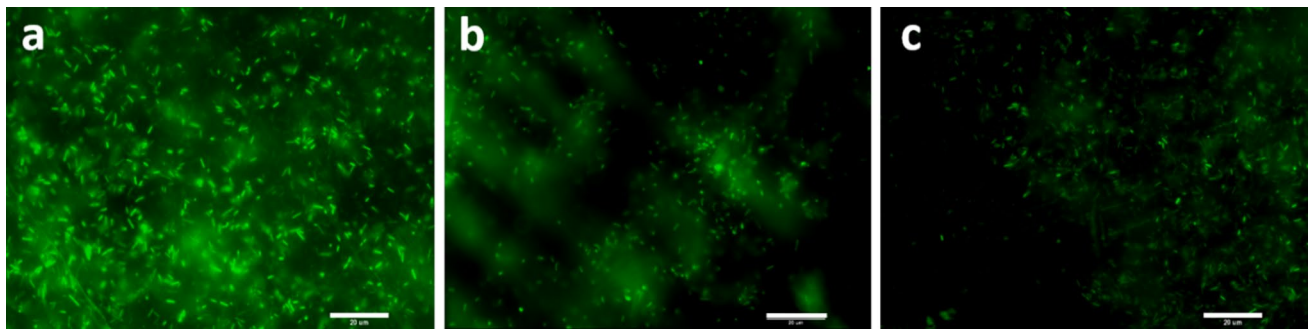


Fig. 3 Epi-fluorescent images of *P. aeruginosa* PAO1 biofilm stained with SYTO® 9 on stainless-steel coupons after 48 h in TSB. Biofilm was imaged under 60× magnification. Scale bar = 20 µm. **a** CDC control reactor, **b** patulin, and **c** furanone C-30 treatment

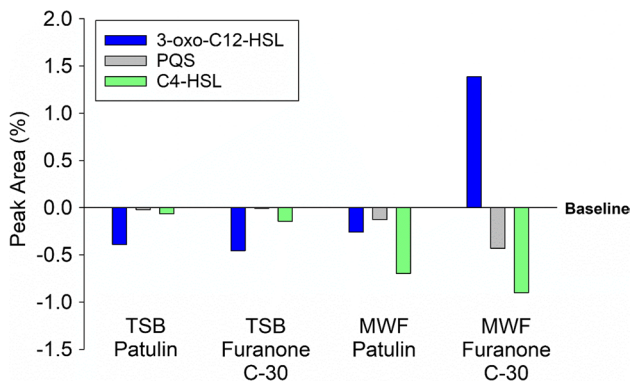


Fig. 4 Relative production of putatively identified QS molecules found in *P. aeruginosa* PAO1 biofilm in the presence of QSI after 24 h in TSB and MWF. The baseline represents detection of putatively identified QS molecules identified in the untreated controls

QS inhibitors ($p \leq 0.043$ using Welch t test). The untreated control log densities exhibited excellent repeatability across the three experiments ($N = 18$, $SD = 0.159$).

Liquid chromatography–mass spectroscopy: screening for quorum sensing signals

LC–MS data were analyzed to identify the three major QS signaling molecules produced by *P. aeruginosa* PAO1. Putative identification showed the presence of C4-HSL (*N*-Butyryl-DL-homoserine lactone; expected $[M + H]^+$: 172.10, observed $[M + H]^+$: 172.09), 3-oxo-C12-HSL (*N*-(3-Oxo-dodecanoyl) homoserine lactone; expected $[M + H]^+$: 298.20, observed $[M + H]^+$: 298.21), and PQS (*Pseudomonas* quinolone signal; expected $[M + H]^+$: 259.16, observed $[M + H]^+$: 259.15). Higher production of 3-oxo-C12-HSL was identified in *P. aeruginosa* PAO1 biofilm assays treated with furanone C-30 in MWF when compared to the untreated control (Fig. 4). Detection of C4-HSL and PQS by *P. aeruginosa* PAO1 growing in both TSB and MWF was reduced in the presence of QS inhibitors.

Discussion

Planktonic and biofilm samples collected from two local machine shops tested positive for microbial contamination and were dominated by *Pseudomonadales* (Fig. 1). Previous studies on microbial contaminants in MWFs confirm the dominance of this Order [3, 13]. *Pseudomonas* spp. may account for up to ~90% of bacterial sequence libraries [10] with biomass as high as $> 10^8$ CFUs mL⁻¹ in some MWFs [27]. Of significance in the present study was the rapid recolonization after a commonly practiced dumping, cleaning, and recharge process at Shop 2. With residual microbes residing in inaccessible parts of MWF circulation systems escaping these hygiene practices, recolonization is a fundamental issue in the MWF industry [18, 26, 50]. Most sequences classified within the Order *Pseudomonadales* were phylogenetically related to a single OTU. This OTU had 99% sequence identity to the 16S rRNA gene of *Pseudomonas stutzeri*, a species frequently associated with biodegradation activity in MWFs [10, 13]. In preliminary experiments, *P. stutzeri* was unable to form stable biofilm in a CDC reactor. Thus, *P. aeruginosa*, another known MWF contaminant [10] was selected for QSI experiments. Specifically, *P. aeruginosa* POA1, a model strain for biofilm formation, was chosen [20].

Although biofilms in MWFs are pervasive, data concerning measures on biofilm removal are limited. Biocides are commonly applied to reduce the microbial load in MWFs; yet, a recent study investigating more than 60 machines operated with MWFs found no evidence for changes in the size of microbial populations in biofilms between untreated MWFs and those containing biocides [49]. Conversely, the QSI compounds patulin and furanone C-30 investigated in this study had a strong negative effect ($p \leq 0.012$) on biofilm formation of *P. aeruginosa* PAO1 on stainless-steel coupons in MWF, reducing CFUs in biofilm by ~63% and 76% on average, respectively (Fig. 2). These efficiencies were higher when compared to previous reports on the inhibition of biofilm formation by patulin and furanone [21, 53] and

bracketed by efficacies reported for α -amylase [17]. With biofilm formation being highly sensitive to the chemical and biological makeup of an environment, these comparisons should, nonetheless, be viewed conservatively. While α -amylase, an enzyme that has been shown to significantly reduce biofilm formation and increase biofilm detachment [17], delayed initial biofilm development, it had no effect on biofilm reduction in MWF after 48 h in the present study. It is noteworthy that as a mycotoxin, patulin could exhibit antibiotic properties and impair growth when administered at high concentrations. However, in both our work and others, inhibitory effects of patulin on cell growth of *P. aeruginosa* PAO1 were not detected for the applied concentrations [data not shown; 40].

It is well established that *P. aeruginosa* uses QS to collectively coordinate motility, virulence factors, and biofilm formation [28]. Its QS system consists of a multilayered, interconnected network, the LasI/R and RhlI/R system, with the binding of the autoinducers 3-oxo-C12-HSL and C4-HSL to their cognate LasR and RhlR regulatory proteins, respectively, activating the transcription of QS controlled genes [28]. LasR not only regulates the expression of C4-HSL, but also genes involved in the synthesis of a large array of 4-hydroxy-2-alkylquinoline (HAQ) QS molecules. The best characterized HAQ is PQS, which is directly involved in cell-to-cell signaling [28].

While patulin and furanone C-30 do not interfere with the synthesis of 3-oxo-C12-HSL, per se, in silico analysis shows a high affinity of patulin and furanone C-30 to the LasR receptor site [45], the protein that regulates genes involved in the synthesis of C4-HSL and PQS. Patulin mimics the lactone moiety of HLSs with the ability to bind to LasR via H-bonds. Furanone C-30, on the other hand, blocks the formation of the hydrophobic core of LasR and the breaking of internal H-bonds further destabilizes the protein [4]. The expression of C4-HSL and PQS in this study was consistently lower in all samples treated with patulin and furanone C-30, particularly in MWFs. Most importantly, these two QS molecules are critical to biofilm development as they activate, among others, genes encoding elastase, rhamnolipids, phenazines, carbohydrate-binding lectins, and EPS secretion [29, 34, 38, 52]; genes which are downregulated by patulin and furanone C-30 [14, 39]. While QS signaling molecules are produced and released at a low baseline level, the activation of the QS system is auto-regulated and depends on a threshold concentration of signaling molecules related to cell density [28]. In fact, an excess of exogenous signaling molecules is insufficient to induce a QS response when cell densities are low [14]. In agreement with these reports, the detection of putatively identified QS molecules (i.e., 3-oxo-C12-HSL, C4-HSL, and PQS) corresponded with fewer CFUs in the present study (Fig. 3) and was, with one exception, lower in samples treated with QS inhibitors (Fig. 4).

By manipulating the QS circuitry on a transcriptional level and by interfering with the synthesis and accumulation of QS molecules, patulin and furanone C-30 may have affected a cascade of genes, including those involved in biofilm formation. The application of these QS inhibitors has also been shown to render *P. aeruginosa* biofilm more susceptible to biocide treatment [14, 39]. With biocides commonly used to combat microbial growth in MWFs, their combination with QS inhibitor agents awaits investigation for preventing biofilm formation. Further, while patulin and furanone C-30 were administered only at the beginning of the 48 h of incubation, improved biofilm reduction may be achieved by periodic amendments of QS inhibitors [14, 51]. A recent review of environmental applications of QSI predicts that in the future wastewater treatment will indispensably rely on QS-based manipulations [25]; findings that may greatly encourage research on the applicability of QSI in other systems. As such, data presented herein highlight QSI as a promising approach for battling microbial contaminants in MWFs and warrants future fundamental and large-scale trials on QS-QSI to optimize alternative strategies that could replace or supplement the usage of biocides in MWFs. Brinksmeier et al. [5] provided an overview of the development of MWF, the underlying mechanisms behind various additives, and the state of the field regarding improvements to MWF performance. Aspects including complex chemistry, regulatory requirements, and effect of such additives on MWF formulations would need to be addressed before QSI could be fully implemented into the day to day operations of the real-world environment.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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