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Mitigation of membrane biofouling in membrane bioreactor treating sewage by novel quorum quenching strain of *Acinetobacter* originating from a full-scale membrane bioreactor

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Abstract

A novel quorum quenching (QQ) strain, *Acinetobacter guillouiae ST01,* was isolated from a full-scale membrane bioreactor (MBR) and characterized for its QQ activities. Batch reactor studies at lab-scale showed that *A. guillouiae ST01* exhibited higher QQ activity against acyl homoserine lactones (AHLs) with an oxo group compared to those without an oxo group. The organism was then inoculated (10 %) in an MBR (Q-MBR) treating sewage over 48 days and was found to reduce quorum sensing (QS) activity by reducing AHL concentrations in the sludge and the biofilm of the Q-MBR. The concentration of polysaccharides was reduced up to 30% in both the biofilm and sludge relative to the control, whereas protein concentrations were reduced by 40% and 47% in the sludge and biofilm, respectively. The Q-MBR fouling rates were halved. These results indicate that *A. guillouiae ST01* is a promising strain for biofouling reduction in MBR treating real wastewater.

Keywords: Membrane bioreactor, quorum quenching, quorum sensing, acyl homoserine lactone, *Acinetobacter guillouiae.*

1. Introduction

The growth in the use of the membrane bioreactor (MBR) technology is driven by the need for high quality treated effluent that satisfies stricter regulations, and MBR systems can usually provide this more reliably than other biological wastewater treatment technologies

such as the activated sludge process (Melin et al., 2006; Oron et al., 2008). Novel developments in the field of membranes have decreased the capital and operational cost of MBR over the last two decades (Li et al., 2019). However, biofouling of the membranes in MBRs is the most challenging operational problem, limiting the technology from a) becoming widespread; and b) obtaining a more sustainable character. Attachment and growth of cells on the membrane surface leads to biofilm formation, which is the main cause of fouling (Meng et al., 2017). The biofilm build-up is usually attributed to a phenomenon called quorum sensing (QS). In the QS process, bacteria communicate by excreting small chemicals known as auto inducers (AIs) or QS molecules and adopt a different type of social behaviour, including biofilm formation (Mukherjee & Bassler, 2019). Acyl homoserine lactones (AHLs) are one of the main types of AI used by Gram-negative bacteria to orchestrate and adopt social behaviour (i.e. biofilm formation). Higher abundance of Gram-negative bacteria than of Gram-positive bacteria has been previously reported in MBRs. Hence, AHLs have been reported to be one of the main AIs responsible for biofilm formation (biofouling) in the MBR (Aryal et al., 2016; Tabraiz et al., 2020; Yavuztürk Gül et al., 2018).

Different physical (Arefi-Oskoui et al., 2019; Ying & Ping, 2006), chemical (Jiang et al., 2019; Lee et al., 2012) and operational (Tabraiz et al., 2017; Zeeshan et al., 2017) strategies have been previously employed to reduce biofilm formation in MBR, with each of those having its own advantages and disadvantages (Wen et al., 2008). Biological techniques involved; i) blocking of signal molecule production with chemical analogues to prevent precursor synthesis (Rasmussen & Givskov, 2006; Shen et al., 2006); ii) blocking the QS receptor with chemical compounds designed to mimic the native substrate (Khan et al., 2019); and iii) *in situ* degradation of signal molecules using enzymes (quorum quenching enzymes) (Murugayah & Gerth, 2019). Quorum quenching (QQ) enzymes have been successfully employed in MBRs to reduce biofouling. However, due to their limited stability

and retention time in conventional treatment reactors, the application of QQ enzymes requires large enzymatic concentrations/volumes, increasing operational cost. A few studies have been conducted to immobilize QQ enzymes on carrier beads, or even on the membrane itself, to enhance stability and retention time of the enzymes. Although such approaches were limiting fouling, none of those achieved a significant reduction in operational cost (Kim et al., 2013; Yeon et al., 2009).

Direct inoculation of reactors with bacteria able to maintain themselves in the system and to produce QQ enzymes (i.e. QQ bacteria) is potentially a less complex, more cost-effective, and widely applicable approach to regulate biofouling. Previous studies have shown that inoculation with QQ bacteria could reduce MBR biofouling (Ham et al., 2018; Iqbal et al., 2018).

So far, strains from a few QQ bacterial genera (i.e. *Pseudomonas*, *Rhodococcus, Lactobacillus, Delftia*) have been reported to have the potential to reduce MBR biofouling (Gül et al., 2018; Huang et al., 2019; Kaur & Yogalakshmi, 2018; Weerasekara et al., 2016). However, rarely were these QQ bacteria isolated from an MBR sludge before being applied in another MBR for fouling reduction. In the current study, QQ-strains were isolated from a full-scale MBR treating sewage and characterized for QQ activity. After selecting the most promising QQ-strain in screening trials, its ability to reduce biofouling was tested and confirmed in a laboratory-scale MBR treating sewage.

2. Material and Methods

2.1 Sample collection and enrichment of quorum quenching bacteria

Mixed liquor sludge and biofilm samples were collected from two full-scale MBRs treating sewage (Sherwood Forest Nottinghamshire, UK) and washed with phosphate-buffered saline (PBS) (NaCl; 8.0 $g.L^{-1}$, KCl; 0.2 $g.L^{-1}$, Na_2HPO_4; 1.44 $g.L^{-1}$, KH_2PO_4; 0.24) to retain the

bacterial fraction while removing any source of carbon and nitrogen (i.e. organic debris). Briefly, one gram of wet biofilm and 1 mL of sludge were added to the individual tubes containing 10 mL of phosphate buffer solution (PBS), vortexed vigorously, and sonicated 30 seconds using ultrasonic cleaner (USC-TH, VWR, UK). This procedure was repeated three times. The washed fraction was subsequently centrifuged at 3000 × g for 5 seconds to remove debris and large particles, the supernatant was collected and centrifuged again at 4000 × g for 4 minutes. Bacterial pellets were resuspended in PBS and re-centrifuged; this step was repeated three times. The washed bacterial pellets were resuspended in 5 mL of PBS solution. The suspension (200 µL) was used as inoculum in mini bioreactors (Corning®, VWR UK) containing 10 mL minimal media; NaCl (1 $g.L^{-1}$), KCl (0.5 $g.L^{-1}$), $MgCl_2$ (0.4 $g.L^{-1}$), $CaCl_2$ (0.1 $g.L^{-1}$), Na_2SO4 (0.15 $g.L^{-1}$), KH_2PO4 (2 $g.L^{-1}$), Na_2HPO4 (2.25 $g.L^{-1}$), with a single AHL (2.5 mM) as substrate (carbon and nitrogen source); these AHLs were: *N*-Butanoyl-L-Homoserine Lactone (C4-HSL), *N*-3-oxo-Butanoyl-L-Homoserine Lactone (OC4-HSL), *N*-Hexanoyl-L-Homoserine Lactone (C6-HSL), *N*-3-oxo-Hexanoyl-L-Homoserine Lactone (OC6-HSL), *N*-Octonoyl-L-Homoserine Lactone (C8-HSL), *N*-3-oxo-Octonoyl-L-Homoserine Lactone (OC8-HSL), *N*-Decanoyl-L-Homoserine Lactone (C10-HSL), *N*-3-oxo- Decanoyl -L-Homoserine Lactone (OC10-HSL), *N*-Dodecanoyl-L-Homoserine Lactone (C12-HSL), and *N*-3-oxo-Dodecanoyl-L-Homoserine Lactone (OC12-HSL). A volume of 100 µL of sterile trace element solution ($FeCl_3$; 0.10 $g.L^{-1}$, $MnCl_2.4H_2O$; 1.57 $g.L^{-1}$, $ZnCl_2$; 4.60 $g.L^{-1}$, $CoCl_2.6H_2O$; 0.80 $g.L^{-1}$, $CuCl_2.2H_2O$; 0.30 $g.L^{-1}$, H_3BO_3; 0.030 $g.L^{-1}$, $NaMoO_4.2H_2O$; 0.25 $g.L^{-1}$) was also added in each mini bioreactor. The mini bioreactors were incubated at 20 ^{O}C and kept shaking at 220 rpm for three days. Subsequently, 1% (v/v) of the culture was transferred to a fresh medium containing the same single AHL substrate (re-incubation). This procedure was repeated five times to maximize the probability that the resulting microbial community can quench AHL.

2.2 Quorum quenching bacterial strain - isolation and identification

Bacterial counts in AHL-enriched cultures were determined in each type of AHL sludge using flow cytometry (Brown et al., 2019); the cultures were diluted to a cell concentration of 10 – 30 cells per 20 µL, to harvest the most abundant QQ bacteria (putative potential QQ-strains). The diluted AHL-enriched cultures (20 µL) were streaked on Luria Bertani (LB) agar plates (Sigma Aldrich, UK), and Nutrient Agar (Sigma Aldrich, UK). The plates were incubated for 5 days at 20 ^{O}C. Bacterial colonies were isolated, and their DNA was extracted with Fast DNA Spin Kit for soils (MP Biomedicals, USA) following the manufacturer's instructions. The amplification of the complete *16S rRNA* gene was performed with the universal primer pair 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5′-CGGTTACCTTGTTACGACTT-3′) in a MyCycler Thermal Cycler (BioRad Laboratories, Hercules, CA, USA) using the phusion Flash High-fidelity PCR master mix (ThermoFisher) with the following thermocycler program: (i) 10 sec denaturation at 98 °C, (ii) 35 cycles of 1 sec denaturation at 98 °C, (iii) 5 s annealing at 55°C, (iv) 90 sec elongation at 72 °C, and (v) 90 sec final elongation at 72 °C. The products were separated on 1.5% agarose gel electrophoresis containing SYBR® safe DNA gel stain (Sigma) and visualized using GelDoc (Biorad). Positive PCR products samples were purified by means of the CleanSweep™ PCR Purification (Applied biosystems). Direct sequencing was performed with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The same primers used for PCR were also employed to sequence both strands of the PCR products. Sequencing reactions were analysed in an automatic sequencing system (ABI 3730XL DNA Analyser, Applied Biosystems) with the POP-7 system and carefully reviewed using SnapGene® Viewer 4.1.The resulting sequences were compared against representative genomes using the Microbial Nucleotide Basic Local Alignment Search Tool (BLAST) algorithm. The 25 sequences with the highest Max score and a query cover higher than 99%

were downloaded and aligned using CLUSTALX software. After alignment, sequences were trimmed to a size of 1407 bp; the phylogenetic analysis was conducted with MEGAX software (Kumar et al., 2018), using the neighbour-joining method with 1000 resampling bootstrap analyses.

2.3 Quorum quenching activity of isolated bacteria

The QQ activities of the isolated strains were also determined using *Chromobacterium violaceum CV31532*. Briefly, the separated strains were streaked with *C. violaceum* and incubated at 20 ^{O}C for 4 days. For control *C. violaceum* was streaked with *Escherichia coli*. The strain that showed highest QQ activity against *C. violaceum* was selected and termed as QQ-strain. In addition, batch AHL degradation assays were conducted to further evaluate the selected QQ-strains' potential. To this end, the QQ-strain culture was grown on LB broth, washed, and diluted to OD_{600} = 4.0 in PBS (pH= 6). Solutions of 40 µM of each AHL were also prepared in PBS. The cleaned bacterial culture (OD_{600} = 4.0) and individual AHL solutions (40 µM of each) were mixed in equal proportions in Mini bioreactors (50 mL) (Corning®, VWR, UK) to achieve a 20 µM AHL concentration and QQ-strain absorbance (OD_{600}) of 2.0. A separate reactor was set up for each AHL in duplicate (n = 10 × 2) and incubated for 10 hours. The total reaction volume was 20 mL. Reactors were incubated (20 ºC, 100 rpm), and samples (0.75 mL) were taken from all reactors after 0.5, 1.50, 3.50, 6.50 and 10.0 hours. The control reactors contained only AHL solution (20 µM). Each sample was centrifuged (5424R, Eppendorf, UK) at 8000 × *g* and 5 ºC for 5 minutes. The supernatant (0.65 mL) was removed and mixed with 0.35 mL acetonitrile and 0.1% formic acid solution (UPLC grade, Sigma, UK) and stored at -20 ºC until analysed.

2.4 MBR set-up

Four bioreactors with a working volume of 1 L were established; two were inoculated with the QQ-strain (Q-MBR1 and Q-MBR2), while the other two were the un-amended controls (C-MBR1 and C-MBR2). A 20 L feed tank containing primary settled sewage, collected from Tudhoe Mill wastewater treatment plant (Durham, UK), was placed in a refrigerator at 5 ºC. Content of the wastewater were kept well mixed using a magnetic stirrer (within the refrigerator). The bioreactors were fed with this sewage via a multi-suction head peristaltic pump (520S, Watson-Marlow, UK) at a flow rate of 3.0 $L.d^{-1}$. All reactors were equipped with hollow-fibre polyvinylidene difluoride (PVDF) membranes (Zibo Yingxin Water Treatment Technology, China); the diameter of the fibres was 1.0 mm, with total surface area of 0.008 m^2 and 0.04 micron pore size, which were submerged inside the bioreactor sludge. A permeate flux of 15 $L.m^{-2}.hr^{-1}$ was maintained, resulting in a hydraulic retention time of 8 hours, using a peristaltic pump in suction mode to draw out the treated effluent from each reactor through its membrane. A digital manometer (HD750, EXTECH instruments, UK) was installed in the tubing line between the membrane and the pump to measure the trans-membrane pressure (TMP). To prevent that the manometer would draw water, a check valve was attached between the manometer and the membrane suction pipe. Activated sludge from Tudhoe Mill wastewater treatment plant (Durham, UK) was used as the biomass inoculum for the bioreactors. The reactors were acclimatized at the designed wastewater flow, aeration rate and mixed liquor suspended solids (MLSS) of 3.5 $g.L^{-1}$ for one month prior to the start of the study. For the actual study the MBR reactors were operated continuously for 48 days. Additionally, 10% of the QQ-strain (0.35 $g.L^{-1}$ MLSS) was added to both Q-MBRs in the start of experiment. During the experiment, MLSS was kept between 3.5 - 4 $g.L^{-1}$ and a solid retention time of 20 days was maintained. An air flow of 1.5 $L.min^{-1}$ (intensity ~ 1.15 $m^3.m^{-2}.hr^{-1}$) was supplied in each reactor using a 4 mm pipe (100 mm long) with eight 0.5 mm holes. Dissolved oxygen (DO) concentration in the bioreactors was measured using a DO

meter (HQ30D, Hach, USA) and varied between 3 - 4 mg.L^{-1}. MBR influent and effluent quality was monitored by measuring the following parameters: chemical oxygen demand (COD), total nitrogen (TN), total phosphate (TP) ammonia (NH_3-N), using the kits (Merck Millipore, UK) according to the manufacturer's instructions. The MLSS and volatile suspended solids (VSS) were determined according to standard methods (APHA, 2006). Whenever the membrane TMP reached 35 KPa, an experimental "run" was considered completed. The membrane was detached at the end of each run and the biofilm was removed by manual vigorous shaking in a 50 mL centrifuge tube containing PBS. The membrane was cleaned (section 2.9) and a new run was started.

2.5 EPS extraction and measurement of proteins and polysaccharides

The fouled membrane was removed from the reactor, detached from the pipe and placed in 50 mL tubes containing PBS (KCl; 0.2 g.L^{-1}, NaCl; 8 g.L^{-1}, KH2PO_4; 0.24 g.L^{-1} Na2HPO_4; 1.44 g L^{-1}) with pH adjusted to 6.5. The solution was then shaken well to completely disperse the biofilm. This suspension as well as the reactor sludge (10 mL) were separately centrifuged at 6000 × g for 5 minutes (4 ºC). The supernatants from the top were removed and filtered through cellulose acetate 0.2 µm filter (Millipore, Merk). The filtrates represent the soluble microbial product (SMP)/soluble EPS. The pellets were re-suspended in 10 mL PBS. The solutions were subjected to sonication for 2 minutes, and then shaken for 10 minutes at 150 rpm. Then, the solutions were further centrifuged at 8000 × g for 10 minutes. The supernatants were removed and the EPS present in it were loosely bound EPS (LB-EPS). For the tightly bound EPS (TB-EPS), the pellets were re-suspended in 10 mL PBS and sonicated for 3 minutes. Hydrated cation exchange resin (2g) (Dowex® Marathon® C sodiumform, Sigma- Aldrich, Germany) was washed twice with phosphate buffer (15min; 10 mL.g^{-1} Dowex) and added to the suspensions. The suspensions were centrifuged at 12000 × g for 30 minutes. The supernatants obtained contained the TB-EPS (Jiang et al., 2013).

The quantitative analysis of the total proteins in the EPS samples was carried out using the Lowry method. Briefly, Folin-ciocaleu phenolic reagent was used and the color intensity was measure using a spectrophotometer (Spectramax M3 spectrophotometer ; Molecular Devices, USA) at a wavelength of 750 nm (Lowry et al., 1951). To measure the total polysaccharides the phenol-sulfuric acid method was adopted. A sample volume of 1 mL was taken, and 1 mL phenol solution (5%) was added, followed by the addition of 5 mL concentrated sulfuric acid (96%). The color intensity of the solution was read in a spectrophotometer at 490 nm. A standard curve was prepared using glucose at different concentrations (0 - 150 $mg.L^{-1}$) (Dubois et al., 1956).

2.6 AHL extraction, identification and quantification from biofilm and sludge

The remaining 35 mL volume containing biofilms or 35 mL of sludge was used for AHL extraction. AHLs were extracted from the biofilm and sludge using a modified Lade et al. (2014) method (Lade et al., 2014). The suspensions in PBS was centrifuged at 10000 × g for 10 minutes, and the supernatant was mixed with equal volume of ethyl acetate. The mixture of supernatant and ethyl acetate was shaken at 180 rpm for 2 hours. The upper organic layer was harvested and then dried via N2 gas (99.9%) purging. The residue was dissolved in solution (0.75 mL) of acetonitrile and formic acid (0.1%).

2.7 AHL identification and quantification method

Standard AHL solutions were obtained by dissolving commercial AHL (Chemodex, Switzerland) in acetonitrile to achieve an AHL concentration of 1 $mg.mL^{-1}$. These AHL solutions were diluted (acetonitrile + 0.1% formic acid solution) to obtain standard solutions of 1 µM, 0.5 µM, 0.25 µM, 0.125 µM, 0.0625 µM, 0.03125 µM, 0.015 µM and 0.0075 µM for a calibration curve. Quantification and identification of AHL was carried out using ultra performance liquid chromatography coupled with triple quadrupole mass spectrometry

(UPLC-MS/MS) (Waters, Xevo TQ-S, UK). An Aquity (UK) BEH C18 (2.1 x 100 mm; 1.7 µm particle size) column was used at 20 ^{0}C for these analyses. Two mobile phases were used: a) water + formic acid (0.1 %); and b) acetonitrile + formic acid (0.1 %). The solvent gradients (time: % B) used were; (0.0: 30), (5.0:30), (12.0: 90), (12.5: 90), (15: 30), (17: 95), (18, 30), (20, 30). Samples were injected at a rate of 0.25 mL min^{-1}. The MS settings were: ionisation source: electrospray ionisation; ionisation mode: positive; capillary voltage: 3.0 kV; cone voltage: 30 V; source offset: 50 V; desolvation temperature: 350 ^{0}C; desolvation gas flow: 650 L hour^{-1}; cone gas flow: 150 L hour^{-1}; nebuliser gas pressure: 7.0 bar; collision gas flow: 0.2 mL min^{-1}; collision energy: 2 eV. The effluent was analysed using the multiple reaction monitoring approach. Specific liquid chromatography retention time, appearance of precursor ions (m/z) and two transition ions (m/z = 102, m/z = 74), relative intensity of two transition ions were used to identify AHL compounds. The transition ions with highest intensity were used for the preparation of the standard curves.

2.8 Membrane resistance and fouling rates measurements

Dead-end filtration was employed to determine the total resistance, cake resistance, pore resistance and intrinsic resistance. Prior to the start of a run, a cleaned membrane was placed in the water tank and the feed pump was operated at the same flux as had been maintained in the study. The pressure developed across the membrane was observed and denoted as intrinsic pressure (Pi). When the TMP had reached 35 kPa (Pt = total pressure) and the run had been stopped, the cake was removed from the membrane by shaking the membrane vigorously by hand in a 50 mL tube containing PBS. The membrane was placed in tap water tank and the pump was operated at the same flux. The pressure that developed across the membrane was denoted as (P_i + Pp), intrinsic pressure plus pores blockage pressure. The pressure developed due to pores (P_p) was calculated by subtracting P_i from (Pi + P_p).

Similarly, the ($Pi + P_p$) was subtracted from Pt to calculate the pressure developed due to cake (P_c).

The cake resistance, pore resistance, intrinsic resistance, resistance percentages, biofilm mass per unit area and biofilm accumulation rates were calculated by using various equations (supplementary information). The solution of biofilm in PBS was used to measure the MLSS and VSS of biofilm.

2.9 Membrane cleaning

To restore the intrinsic TMP of the fouled membrane, it was drenched in a cleaning solution (2% NaOH + 4 $g.L^{-1}$ NaOCl) for 30 minutes, followed by filtration with tap water for 30 minutes. Cleaned membranes were used in the next run after measuring the intrinsic pressure (Pi).

2.10 Particle size distribution

For particle size distribution measurements, the sludge from the reactors was diluted in effluent to give an absorbance of 0.1 (OD_{600}) to avoid quick settling of particles during the measurement. The particle sizes were measured using a Zetasizer Nano (Malvern, UK). The particles size distribution was measured immediately after pouring the sample in a cuvette and after 5 minutes of settling. The readings with and without settling time were taken to investigate the settling behaviour of the sludge flocs of C-MBR and Q-MBR.

3. **Results and discussion**

3.1 Identification of the isolated strain and it's in vitro QQ evaluation

The isolated QQ-strain was identified by sequencing its *16S rRNA* gene and was evaluated *in vitro* for its QQ potential. The strain was given the name of ST01. The *16S rRNA* gene identified the bacterium as a member of the genus *Acinetobacter*. The alignment against the

271 microbial nucleotide database indicated that the isolate's closest relative is *Acinetobacter*
272 *guillouiae* (Figure 1). The isolated strain shared 99.6% sequence identity with the *16S rRNA*
273 of *A. guillouiae.* Hereafter the new isolate will be referred to as *A. guillouiae ST01.*

274 To evaluate its *in vitro* QS signal degradation potential, the *A. guillouiae ST01* strain was
275 streaked with *C. violaceum* (CV31532). The *A. guillouiae ST01* quenched the OC6-HSL QS
276 signal molecules and reduced the indigo pigmentation of *C. violaceum* (CV31532).
277 Moreover, batch reactor experiments with individual AHLs showed that the degradation rates
278 of the long chain AHL signal molecules (C10-HSL, C12-HSL, OC10-HSL and OC12-HSL)
279 were higher than the degradation rates of short chain AHLs (C4-HSL, C6-HSL, C8-HSL,
280 OC4-HSL, OC6-HSL and OC8-HSL). The long chain AHLs with 10 and 12 carbons (with
281 and without oxo groups) were completely degraded within six hours while for short chain
282 AHLs (with and without oxo groups) the concentration approximately halved within four
283 hours. This difference is probably due to the higher bioconcentration factors and organic
284 carbon adsorption coefficients of the long chain AHLs (Decho et al., 2011). Plausibly, these
285 two factors were also responsible for the rapid reduction in AHL levels at the start of the
286 experiment (supplementary information). It may seem that AHLs without oxo groups had
287 slightly higher degradation rates than corresponding AHLs with oxo groups (Figure 2a & b),
288 however, degradation rates calculated based on measurements after the first half hour (given
289 the assumption that the reduction in AHL level during first half hour was due to the
290 aforementioned factors, and thus not true degradation) showed that AHLs with an "oxo"
291 group had the higher degradation rates, which can be tentatively attributed to their higher
292 solubility compared to AHLs without an "oxo" group (supplementary information) (Decho et
293 al., 2011). Importantly, these results proved the QQ ability of *A. guillouiae ST01* against all
294 AHLs. The concentration of AHLs in the control reactor decreased up to 5.0 – 25%. This can
295 be attributed to ring opening (Yates et al., 2002). The C10-HSL and C12-HSL concentrations

were reduced more in the control at pH = 6.0 and 20 ℃ compared to other AHLs used in study which can be attributed to their poor solubility.

No *Acinetobacter* strain has previously been reported to bring about any reduction in biofouling in MBR. However, several *Acinetobacter* stains isolated from the marine environment have been reported to show some QQ activity against different types of AHLs and a strain of *Acinetobacter baumannii* with a clinical origin was reported as having the *AiidA* QQ gene (López et al., 2017). In addition, seven types of lactonase enzymes and QS activity have been reported in various *Acinetobacter baumannii* strains (Muras et al., 2018). Similarly, a strain of *Pseudomonas sp. 1A1* was identified as having QQ activity and shown to contain AHL-acylase synthesizing genes (Cheong et al., 2013).

3.2 MBR fouling rates; C-MBR vs Q-MBR

An increase in TMP is a sign of membrane fouling. The TMP profile of the Q-MBR reactors implied a noticeable fouling reduction by the *Acinetobacter ST01* as QQ-strain compared to the C-MBR. The operational time of the C-MBR were 10 and 12 days (average 11 days) before reaching the 35 kPa threshold that completed a run whilst the two Q-MBR ran for 20 and 25 days (average 22.5 days) until reaching the same TMP (Figure 3a). Thus, application of QQ-strain resulted in a doubling of the operational time of the MBR.

It is clear from (Figure 3a) that the biofilm maturation phase (the phase between lag and jump phase; e.g. the first 15 days in the first run of the QQ reactors) was prolonged by the presence of the QQ-strain (*A. guillouiae ST01*) in the Q-MBR, leading to a longer operational run-time. Total fouling rates in the Q- MBR were significantely lower ($p < 0.05$) than in the C-MBR (Figure 3b). The main contributing factor to the overall lower fouling rate reduction of the Q-MBR was that the maturation phase fouling rates were 3 to 4-fold lower in the Q-MBR than in the C-MBR. The extension of the length of the maturation phase (i.e. presence of low

fouling rates) indicates that the *A. guillouiae ST01* application inhibited excessive biofilm formation on the surface of membrane. In the maturation phase, the EPS starts attaching to and on the surface of the membrane, which facilitates the attachment of bacteria on the membrane. After the membrane surface reaches to a certain bacterial cell density the QS mechanism activates which initiates the biofilm formation and the jump phase commences (Gao et al., 2013). Therefore, it can be inferred that bioaugmentation with QQ *A. guillouiae ST01.* retarded the biofilm formation process by interfering with and degradation of the QS signal molecules (AHLs). This is deduced from the biofilm acccumulation rates per unit area (Figure 3c) which were less in Q-MBRa. However, the average biofilm biomass per unit area of membrane showed no significant difference between Q-MBR and C-MBR reactors at the end of the operation period (Figure 3d). Previous QQ studies in MBRs have reported a similar extension of the maturation phase (Iqbal et al., 2018;Yu et al., 2016). In studies with synthetic wastewater, augmentation of MBRs with *Pseudomonas* sp. 1A1 and *Rhodococcus* sp. BH4 has previously been shown to reduce fouling and doubled membrane operational times (Cheong et al., 2013), findings comparable to that of the current study using actual sewage.

3.3 Effect of the QQ-strain on membrane resistance

Total hydraulic resistance (Rt) of the C-MBR was higher than that of the Q-MBR at the end of operational period (Figure 4a). Cake resistance (Rc) was the main component of the total resistance (60 – 63 %) in both systems. It was slightly higher in C-MBR (62.9 % in both controls) than in the Q-MBR, 60.4 and 61.2 % (average 60.8 %). This could be linked towards the low cake (biofilm) formation rates (section 3.2) and EPS production in the biofilm and sludge of the Q-MBR (Section 3.4). In contrast, the pore resistance was slightly higher in the Q-MBR membrane than in the C-MBR membrane (Figure 4b). However, the pore and cake resistance (Rp and Rc) development rates were higher in C-MBR than Q-MBR

(Figure 4c). The intrinsic resistance (Ri) increased over time in both Q-MBR and C-MBR, yet the rate was higher in the latter (Figure 4d). The difference in the cake layer development can be attributed to QQ.

The higher rates of intrinsic resistance increase of the membrane in the C-MBR compared to Q-MBR can be attributed to more frequent washing (quick run completion due high fouling rates) and deterioration of the membrane surface plausibly due to the oxidation effect of the NaOCl, which could have activated some functional groups on the membrane surface (Puspitasari et al., 2010; Wang et al., 2010). Thus, higher accumulation of inorganic compounds in the C-MBR membrane could have been due to their bonding with functional groups (Yan et al., 2012). The inorganic compounds binding on the membrane surface could cause irreversible fouling (Yamamura et al., 2007). These observations indicate that the QQ-strain also plays a role in enhancing the working lifespan of the membranes.

3.4 Effect of quorum quenching on sludge / biofilm properties and treatment efficiencies

The concentrations of polysaccharides and proteins in the SMP, LB-EPS and TB-EPS fractions of sludges and biofilms were measured to determine the effect of quorum quenching on the presence of these constituents. The concentration of polysaccharides in the sludge and biofilm of the Q-MBR was 30% lower (in both) than in the C-MBR; similarly, the protein concentrations in the Q-MBR sludge and biofilm were 40% and 47.4% lower than in the C-MBR, respectively (Figure 5a, b & c). The average concentration of proteins in the sludge of the Q-MBR and C-MBR were ~ 1.8 $mg.g^{-1}VSS$ and ~ 3 $mg.g^{-1}VSS$, respectively, whilst the biofilms contained ~ 10 $mg.g^{-1}VSS$ and ~19 $mg.g^{-1}VSS$, respectively. The biofilms had a five to six-fold higher protein concentration than the sludges in both systems (C-MBR and Q-MBR). The polysaccharide concentrations were higher than the protein concentrations in both biofilm and sludge for both C-MBR and Q-MBR. Specifically, the polysaccharide concentrations in the sludge of the C-MBR and Q-MBR were: 4.5 – 5 $mg.g^{-1}VSS$ and 3 – 3.8

370 mg.g^{-1}VSS respectively, whereas the biofilms of these reactors had ~ 86 mg.g^{-1}VSS and ~ 60
371 mg.g^{-1}VSS, respectively. However, the biofilms in both types of reactor (C-MBR and Q-
372 MBR) had 16 to 17-fold higher concentrations of polysaccharides than the sludges, which can
373 be attributed to higher microbial density and subsequently high QS activities in the biofilm
374 (Gao et al., 2013). A recent study reported 50% reduction of carbohydrates in the EPS and
375 SMP of MBR augmented with a QQ-strain (Kampouris et al., 2018). Therefore, the results of
376 the current study are consistent with these previously reported studies. The SMP plays the
377 main role in the fouling of the MBR, directly as well as indirectly. It interacts with other
378 organic and inorganic compounds present in the reactor through complexation and chelation
379 and gives rise to the formation of colloids and macromolecules which cause membrane
380 fouling (Wang et al., 2013).
381 Reduced levels of EPS also affected floc size and sludge properties. Overall, the floc size of
382 the sludge from Q-MBR was smaller than that of the C-MBR, but after allowing 5 minutes
383 settling time, the floc size of Q-MBR sludge was found to have increased (supplementary
384 information). This shows that the sludge flocs' settleability improved due to the reduced
385 levels of EPS, a plausible scenario attributed to the reduced zeta potential of flocs, a concept
386 previously described by (Jiang et al., 2013). The lower level of EPS and reduced interaction
387 of the flocs in the sludge of Q-MBR could possibly have prolonged the maturation phase seen
388 in the Q-MBR, by preventing the attachment of flocs on the membrane surface of Q-MBR,
389 which would have delayed biofilm formation. The fouling rates observed in the maturation
390 phase (Figure 3b) strengthen this argument.

391 The influent sewage had medium strength (COD; 443.6 ± 8.5, TN; 42.7 ± 2.1 and TP; 18.4 ±
392 1.2). The COD, TN and TP removal efficiencies were slightly lower in the Q-MBR than in
393 the C-MBR, but these differences were not significant (Figure 5e). Overall, the COD

394 removal efficiencies were more than 95%, while the TN and TP removal efficiencies were 30
395 – 35% and 38 – 44%, respectively.

396 ***3.5 AHL status in sludge and biofilms***

397 AHL concentrations in the sludge and biofilm of the Q-MBR were lower than in the C-MBR.
398 The total AHL concentration in the sludge of the Q-MBR was 58% lower than in that of the
399 C-MBR, whereas for biofilm the Q-MBR had 75% lower total AHL than the C-MBR. Out of
400 the ten AHLs (with and without oxo group) studied, seven were found in the biofilms and
401 sludge of both reactors; C4-HSL, 3-oxo-C4-HSL, C6-HSL, 3-oxo-C6-HSL, C8-HSL, 3-oxo-
402 C8-HSL and 3-oxo-C10-HSL. In the sludge of C-MBR, 3-oxo-C8-HSL concentrations were
403 highest followed by C4-HSL and C6-HSL whereas biofilm had highest concentration of C4-
404 HSL followed by the 3-oxo-C8-HSL and C6-HSL (Figure 6a & b). In the biofilm and sludge
405 of the Q-MBR, the C4-HSL concentration was highest followed by 3-oxo-C8-HSL and C6-
406 HSL.

407 A recent study reported C6-HSL as the main AHL (~ 1000 $ng.g^{-1}$ VSS) followed by 3-oxo-
408 C8-HSL (~550 $ng.g^{-1}$ VSS) in the sludge of MBR reactors (Waheed et al., 2020), which is
409 consistent with the results of the current study. However, their study reported less types of
410 QS molecules (C6-HSL, C7-HSL, C8-HSL, C10-HSL and 3-oxo-C8-HSL) compared to the
411 current study (C4-HSL, C6-HSL, C8-HSL, C10-HSL, 3-oxo-C4-HSL, 3-oxo-C6-HSL, 3-
412 oxo-C8-HSL). This difference in the types of AHLs observed can be attributed to the type of
413 wastewater used, or perhaps to the specific enzymatic activity of the augmented QQ-strains.
414 Similarly, the concentrations of AHLs in the sludge of the present study agree closely with
415 those reported in other studies (Xiao et al., 2018).

416 The *A. guillouiae ST01* strain isolated in the current study proves to be a potential QQ option.
417 It doubled the operating time of an MBR treating sewage. However, long term pilot-scale

studies to evaluate the time span of its effectiveness after inoculation are recommended before testing full-scale application of *A. guillouiae ST01*. In addition, monitoring QQ strains and community structure in MBR over time shall be illuminating. It has been reported that addition of QQ strains, or of AHLs directly, can disturb the microbial community structure of the biomass in the reactor (Li et al., 2017). Other studies have reported reduced abundance of Gram-negative organisms in the bacterial community due to quenching of AHLs in the MBR (Kim et al., 2013; Waheed et al., 2020). Further studies are needed to find the optimal inoculum concentration of *A. guillouiae ST01* for fouling reduction in MBRs. Moreover, optimizing the operational conditions for long time effectiveness of QQ-strain is one of the challenges to be met for successful implementation of this green technology in full-scale MBRs.

4. Conclusions

A novel *A. guillouiae ST01* was isolated from the biomass of a full-scale MBR and its potential for quorum quenching was assessed. The *A. guillouiae ST01* showed promising QQ activity in AHL degradation assays. Specifically, it reduced biofouling in MBRs by degrading AHLs. This was linked to reduction in EPS concentrations in the sludge and biofilm, and delay in biofilm formation on the surface of the membrane in the MBRs (as suggested by slower development of TMP). These results indicate that the *A. guillouiae ST01* represents a novel and promising strain for biofouling reduction in MBRs treating real wastewater.

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588

589 **Figure captions**

590 Figure 1 Phylogenetic tree of *A. guillouiae ST01* and close relatives

591 Figure 2 Acyl homoserine lactones (AHL) a) with and b) without "oxo" group degradation rates of *A.*
592 *guillouiae ST01* AHL abbreviations are; C4: C4-HSL; C6: C6-HSL; C8: C8-HSL; C10: C10-HSL; C12: C12-
593 HSL; OC4: 3-oxo-C4-HSL; OC6: 3-oxo-C6-HSL; OC8: 3-oxo-C8-HSL; OC10: 3-oxo-C10-HSL; OC12: 3-oxo-
594 C12-HSL, (error bars represent the standard deviation , n = 2)

595 Figure 3 a) Trans membrane pressure (TMP) profile of all MBRs , b) Average fouling rate in the maturation
596 phase and fouling rates during total periods ($kPa.d^{-1}$), c) Average biofilm accumulation rates on the membrane
597 surface, d) average biomass accumulation (VSS) of biofilm per unit area at the end of each run. Error bars show
598 the standard deviation (n = 2) of last two run of C-MBR and Q-MBR; Q-MBR is the MBR augmented with QQ-
599 strain whilst C-MBR is a control without QQstrain added

600 Figure 4 a) Average cake (Rc), pore (Rp) and intrinsic (Ri) resistances measured at the end of each run, b)
601 percentage of cake resistance (Rc), pore resistance (Rp) and Intrinsic resistance (Ri) to total resistance (Rt), c)
602 cake and pore resistance development rate during the run, d) increase in the intrinsic resistance (Ri) over the
603 cyle run time

604 Figure 5 a) Protein concentration per unit biomass ($mg.g^{-1}$VSS) in the sludge in C-MBR and Q-MBR, b)
605 Polysaccharide concntrations per unit biomass ($mg.g^{-1}$VSS) in the sludge in C-MBR and Q-MBR, c) Protein and
606 polysaccharides concentration per unit biomass ($mg.g^{-1}$VSS) in the biofilm measured at the end of each
607 operation

608 Figure 6 Acyl homoserine lactone (AHL) concentration per unit biomass ($ng.g^{-1}$ VSS) in (a), sludge and (b),
609 biofilms of C-MBR and Q-MBR. The concentrations shown are the average of samples of sludge and biofilms
610 taken at the end of last run (n = 2) and error bars represent standard deviation. AHL abbreviations are; C4: C4-
611 HSL; C6: C6-HSL; C8: C8-HSL; C10: C10-HSL; C12: C12-HSL; OC4: 3-oxo-C4-HSL; OC6: 3-oxo-C6-HSL;
612 OC8: 3-oxo-C8-HSL; OC10: 3-oxo-C10-HSL; OC12: 3-oxo-C12-HSL.

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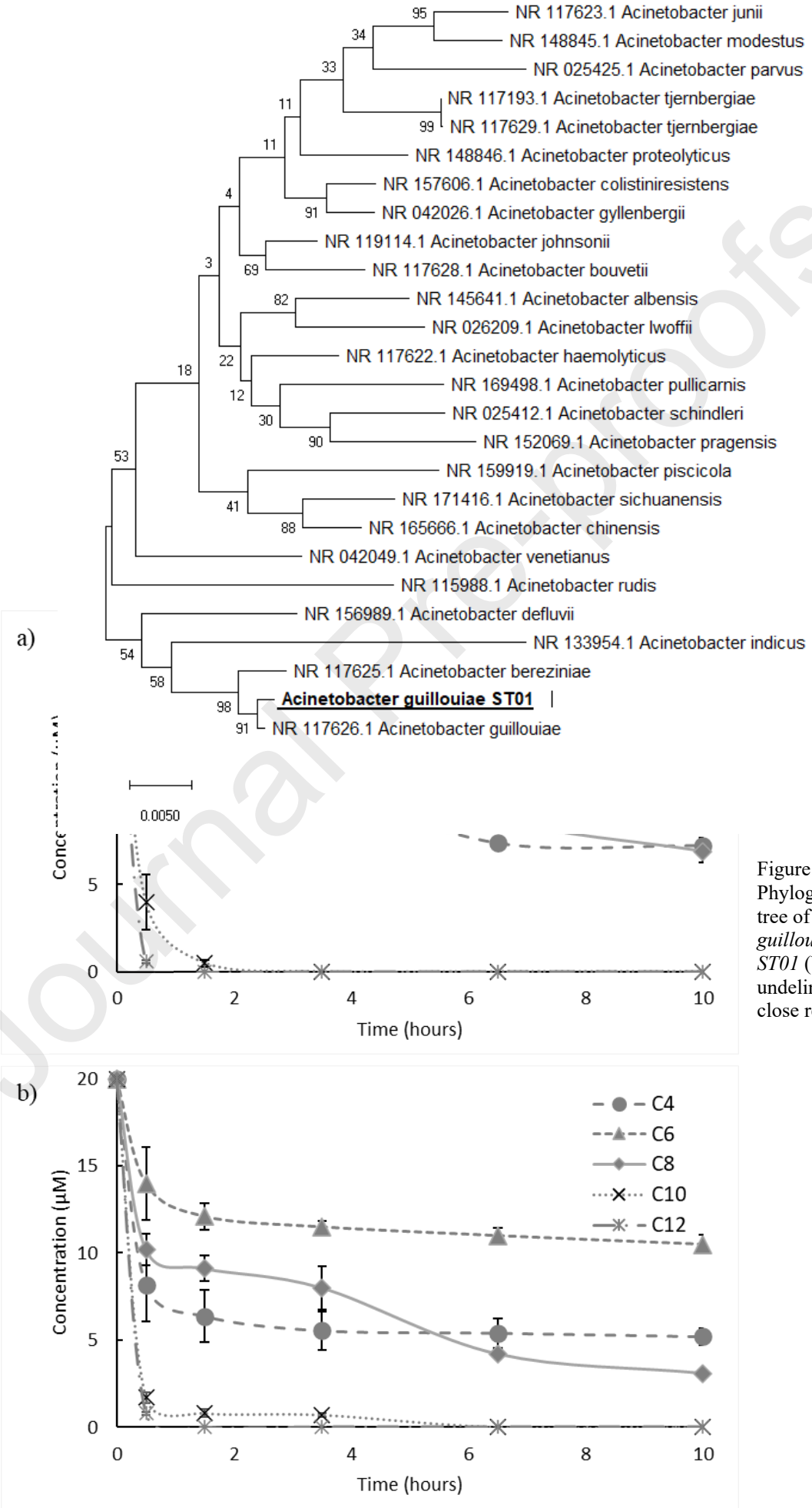

Figure 1 Phylogenetic tree of *A. guillouiae ST01* (bold, undelined) and close relatives

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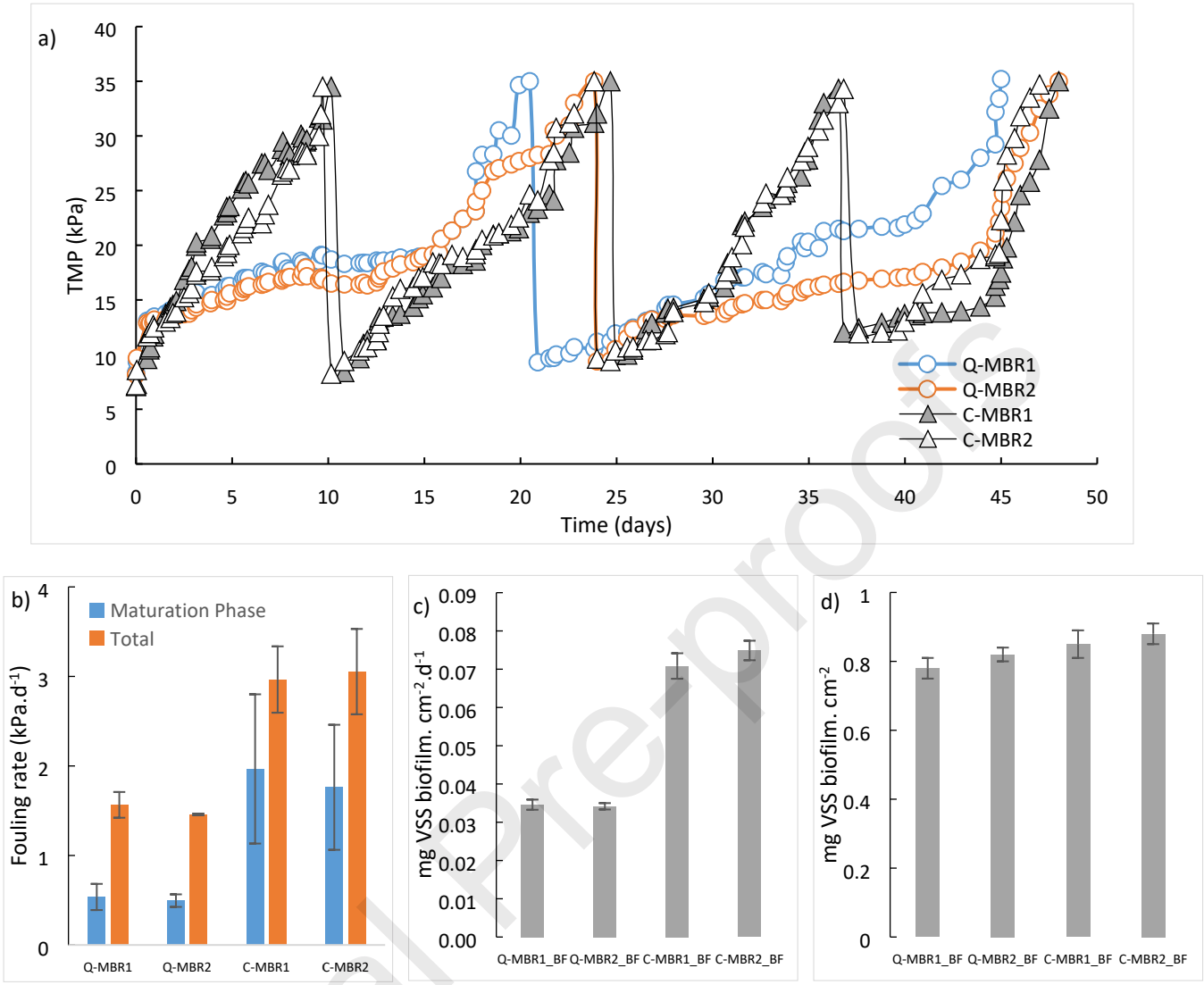

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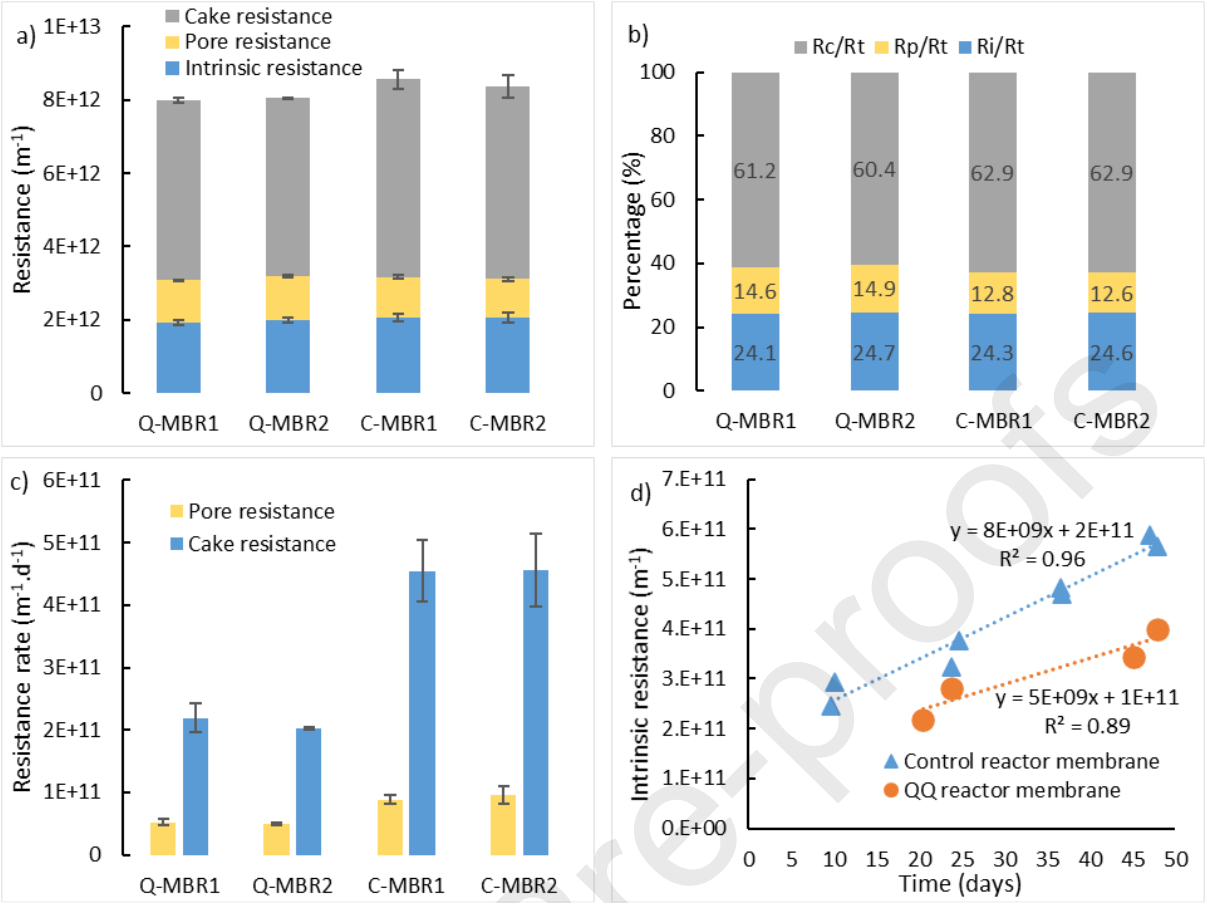

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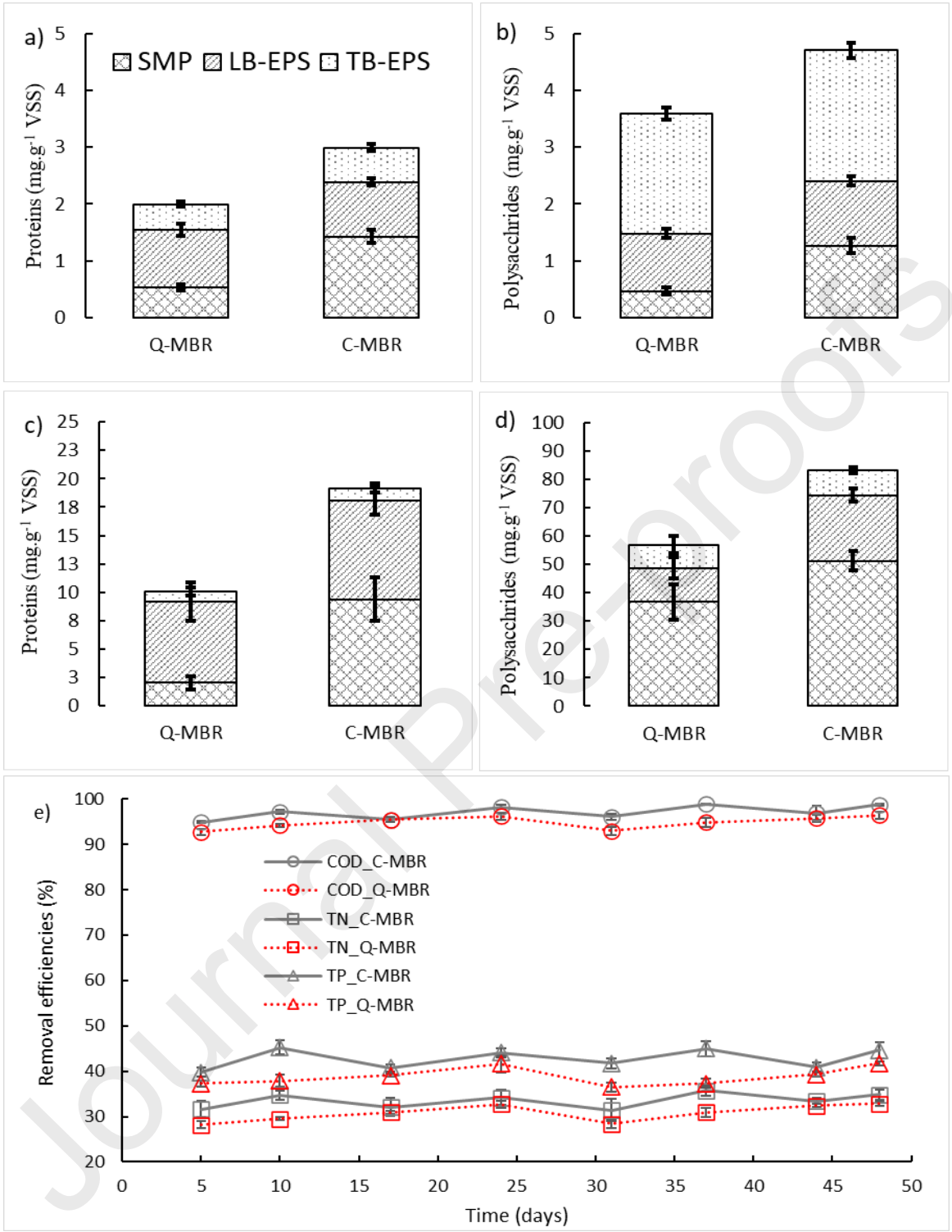

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710 Figure 5 a) Protein concentration per unit biomass (mg.g^{-1}VSS) in the sludge of C-MBR and Q-MBR, b)
711 Polysaccharide concntrations per unit biomass (mg.g^{-1}VSS) in the sludge of C-MBR and Q-MBR, c) Protein
712 concentration per unit biomass (mg.g^{-1}VSS) in the biofilm measured at the end of each run of C-MBR and Q-
713 MBR, d) Polysaccharides concentration per unit biomass (mg.g^{-1}VSS) in the biofilm measured at the end of
714 each run of C-MBR and Q-MBR, and e) COD, TN, TP removal efficiencies of the C-MBR and Q-MBR (error
715 bars represent the standard deviation , minimun number of sample = 2). Abbreviation are; soluble microbial
716 product (SMP), loosely bound extracellular polymeric substance (LB-EPS), tightly bound extracellular
717 polymeric substance (TB-EPS).

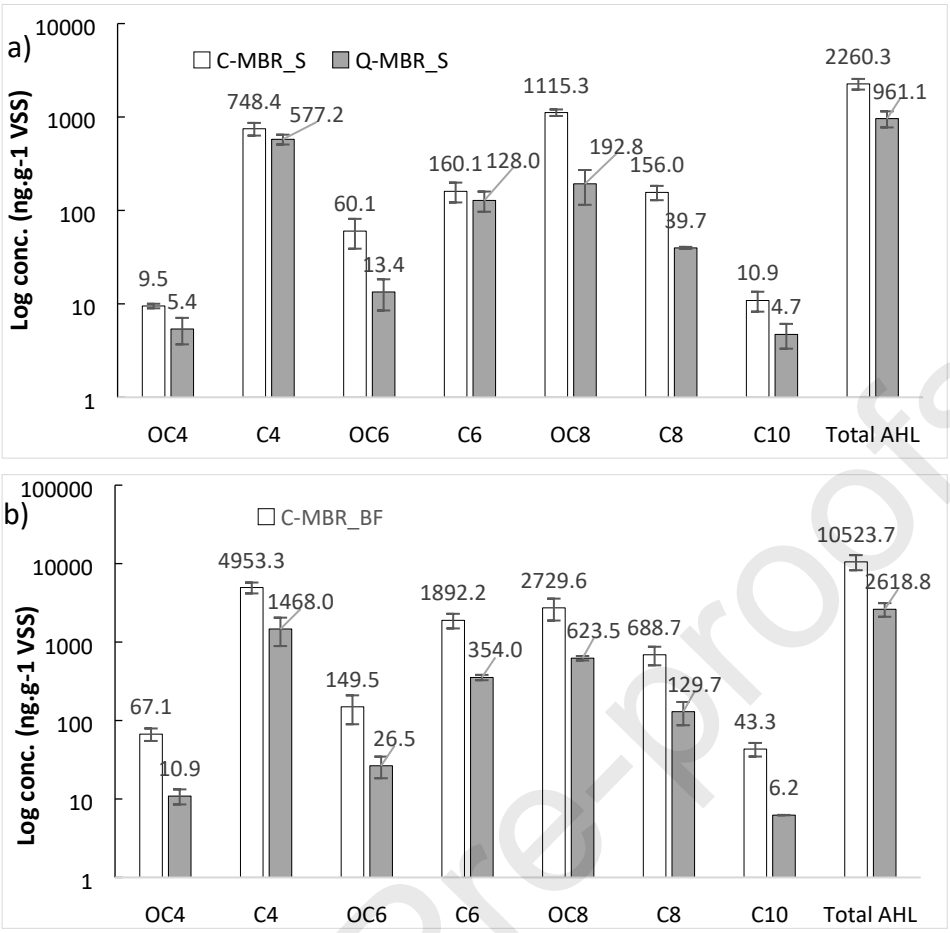

Figure 6 Acyl homoserine lactone (AHL) concentration per unit biomass (ng.g^{-1} VSS) in (a), sludge and (b), biofilms of C-MBR and Q-MBR. The concentrations shown are the average of samples of sludge and biofilms taken at the end of last run (n = 2) and error bars represent standard deviation. AHL abbreviations are; C4: C4-HSL; C6: C6-HSL; C8: C8-HSL; C10: C10-HSL; C12: C12-HSL; OC4: 3-oxo-C4-HSL; OC6: 3-oxo-C6-HSL; OC8: 3-oxo-C8-HSL; OC10: 3-oxo-C10-HSL; OC12: 3-oxo-C12-HSL.

Credit author statement

Shamas Tabraiz: Conceptulization, Methodology, Experimentation, Formal Analysis, Resources, Writing – original draft preparation. **Burhan Shamurad:** Data Curation. **Evangelos Petropoulos**: Writing-Editing**, Marcos Quintela-Baluja:** Visualization**. Alex**

Charlton: Chemical Analysis. **Jan Dolfing:** Writing – Review & Editing. **Paul J. Sallis:** Supervision**,** Writing – Review & Editing.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

none

Highlights

- Quorum Quenching (QQ) strains were isolated from a full-scale MBR treating sewage
- The predominant QQ strain was *Acinetobacter guillouiae*
- This QQ strain can degrade short, medium, and long chain AHL
- It quenched the AHL concentrations of the MBR biofilm and mixed liquor
- This QQ strain halved the fouling rates of MBR treating sewage

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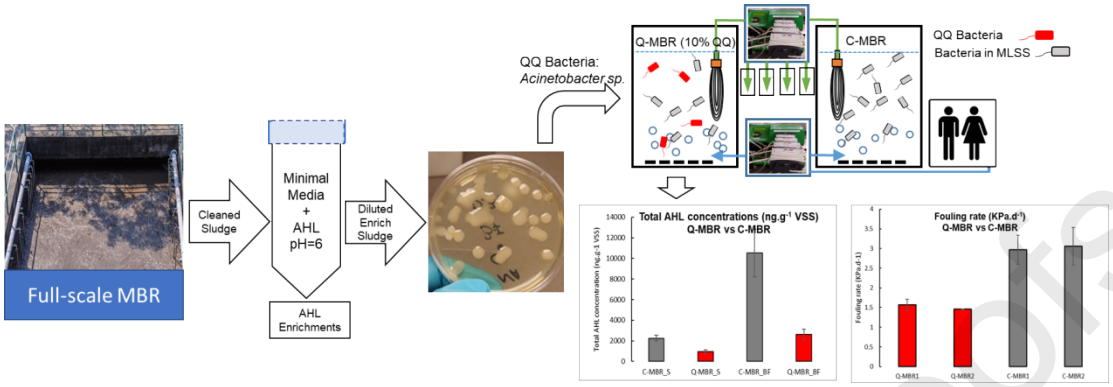

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