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Biological removal of benzalkonium chlorides from wastewater by immobilized cells of *Pseudomonas* sp. BIOMIG1 in an up-flow packed bed reactor

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**A R T I C L E I N F O**

**A B S T R A C T**

Quaternary ammonium compounds (QACs) are active ingredients of many disinfectants used against SARS-CoV-2 to control the transmission of the virus through human-contact surfaces. As a result, QAC consumption has increased more than twice during the pandemic. Consequently, the concentration of QACs in wastewater and receiving environments may increase. Due to their antimicrobial activity, high levels of QACs in wastewater may cause malfunctioning of biological treatment systems resulting in inadequate treatment of wastewater. In this study, a biocatalyst was produced by entrapping *Pseudomonas* sp. BIOMIG1 capable of degrading QACs in calcium alginate. Bioactive 3-mm alginate beads degraded benzalkonium chlorides (BACs), a group of QACs, with a rate of 0.47 \( \mu \text{M-BACs/h} \) in shake flasks. A bench-scale continuous up-flow reactor packed with BIOMIG1-beads was operated over one and a half months with either synthetic wastewater or secondary effluent containing 2–20 \( \mu \text{M} \) BACs at an empty bed contact time (EBCT) ranging between 0.6 and 4.7 h. Almost complete BAC removal was achieved from synthetic and real wastewater at and above 1.2 h EBCT without aeration and effluent recirculation. The microbial community in beads dominantly composed of BIOMIG1 with trace number of *Achromobacter* spp. after the operation of the reactor with the real wastewater, suggesting that BIOMIG1 over-competed native wastewater bacteria during the operation. This reactor system offers a low cost and robust treatment of QACs in wastewater. It can be integrated to conventional treatment systems for efficient removal of QACs from the wastewater, especially during the pandemic period.

1. Introduction

The emergence of the SARS-CoV-2 pandemic by late 2019 has changed the routine daily life in society. Disinfection has become an essential process in all daily activities. As a result, the consumption of disinfectants has sharply increased. Among many other formulations, disinfectants with quaternary ammonium compounds (QACs) are very popular (Schrank et al., 2020). More than 50% of the disinfectants that the U.S. Environmental Protection Agency has short-listed for use against SARS-CoV-2 contain QACs, particularly benzalkonium chlorides (BACs) (Hora et al., 2020). A recent study analyzing the QACs in indoor dust collected before and after the SARS-CoV-2 pandemic has confirmed that QAC consumption has doubled during the pandemic (Zheng et al., 2020).

Due to their surfactant properties, QACs easily dissolve lipids and agglomerate proteins; therefore, they used to be consumed widely before the pandemic and will still be utilized after the pandemic to control both viral and bacterial contamination on the human body and human contact surfaces (Dev Kumar et al., 2020; Lin et al., 2020; Merchel Piovesan Pereira et al., 2019; Schrank et al., 2020). Human exposure to QACs is mainly through the mouthing mediated ingestion of dust bound and surface residual QACs (Li et al., 2020). Given the fact that QACs are associated with reproductive (Maher, 2008; Melin et al., 2016, 2014) and developmental problems in animals (Hrubec et al., 2017), and induce disruption of vital cellular processes (Datta et al., 2017; Herron et al., 2016, 2019), amplified consumption of QACs may pose a health risk to humans during the pandemic (Hrabe et al., 2020).

On the other hand, a significant amount of QACs consumed indoors reach the environment mainly via water. Water contaminated with QACs is treated in wastewater treatment plants then discharged to nearby surface water. The occurrence of QACs in raw and treated wastewater, surface water, and sediment has been comprehensively studied.
reviewed (Hora et al., 2020; Tezel and Pavlostathis, 2015). All those studies imply that although conventional wastewater treatment plants are efficient in removing QACs from the wastewater, there is still a continuous flux into the environment. This may be due to uncertainties in the operation of treatment plants, inhibition because of shock loads, and fluctuations in the microbial community. QAC flux into the environment will increase as the demand for the disinfectants increases as it happens during the SARS-CoV-2 pandemic (Hora et al., 2020).

Biological treatment systems play a critical role in controlling QAC pollution and QAC related implications, such as ecotoxicity and antimicrobial resistance, in the environment. However, conventional biological treatment systems are high-entropy systems composed of diverse microbial communities and functions, receiving many different pollutants at temporally variable concentrations. Therefore, process control is critical for the efficient operation of those systems. The expected high concentrations of QACs in wastewater during the pandemic may result in operational problems as well as a shift of microbial community to a state with high antibiotic resistance (Kim et al., 2018; Mercel Piovesan Pereira et al., 2019; Tezel and Pavlostathis, 2015) which may proliferate in the environment and cause serious health problems in the near future. The conventional wastewater treatment plants play a significant role on proliferation and dissemination of antibiotic resistance genes (Rizzo et al., 2013). Given the strong link between QACs and antibiotic resistance (Tandukar et al., 2013), efficient and specialized treatment systems that can easily be integrated into conventional treatment systems to mitigate QACs especially in these days of the pandemic, are on demand (Lu and Guo, 2021).

In this study, we designed and tested a low-entropy system to remove QACs from wastewater solely by biotransformation. The system is a continuous up-flow reactor packed with alginate entrapped Pseudomonas sp. BIOMIG1 cells, which can degrade QACs even at very high concentrations. Alginate was chosen as the immobilization matrix for bacterial cells due its low cost and performance consistency on different wastewater compositions (Cassidy et al., 1996). The reactor system was operated at various hydraulic retention times within a ten-fold concentration range without aeration and effluent recirculation over one and a half months, and its performance was continuously monitored.

QAC concentrations in the wastewater will increase due to their high consumption during the pandemic period. For the first time in the literature, we tested removal of QACs from wastewater at environmentally relevant concentrations in a continuous reactor system. This reactor system made of a single species of bacteria specialized in QAC biotransformation, offers a flexible and low-cost complete QAC removal version to BDMA but lacks a 22 kb gene cluster that catalyzes BDMA utilization. Given the fact that BDMA is not an adsorbable organic solute and cannot be easily degraded by other microorganisms, this dead-end metabolite produced by strain BIOMIG1BDMA was used as a tracer molecule to evaluate the extent of BAC biotransformation by BIOMIG1 in the reactors and differentiate removal by biotransformation from adsorption to alginate beads. BAC biotransformation by BIOMIG1BAC and BIOMIG1BDMA is comparable; therefore, any conclusions made in this study related to BAC removal in the reactors are also applicable to the reactors using BIOMIG1BAC. BIOMIG1 contains antibiotic and disinfectant resistance genes in its genome but cannot grow temperatures above 35 °C which makes it a non-pathogenic strain. Given that BIOMIG1 is an antibiotic and biocide resistant but non-pathogenic strain, it is an ideal microorganism for any biotechnological application. In addition, BIOMIG1 can utilize BACs at pH between 4 and 9, and threshold dissolved oxygen concentration as low as 0.83 mg O2/L.

Dodecyl benzyl dimethyl ammonium chloride (C12BAC, C23H36NCl, 340 g/mole) and tetradecyl benzyl dimethyl ammonium chloride (C14BAC, C25H42NCl, 368 g/mole) obtained from Tokyo Chemical Industry Co., Japan were used as target BACs for continuous flow packed bed reactor experiments. Other chemicals used in the preparation of culture medium, synthetic wastewater, and alginate beads were purchased at their analytical quality from either Merck Chemical Co. or Sigma-Aldrich Inc.

2.2. Preparation of alginate beads

A single colony of the strain BIOMIG1BDMA grown on the Luria Bertani (LB) agar containing 50 mg/L (c.a. 140 μM) BACs was incubated in 200 mL LB broth with 50 mg/L BACs overnight at 28 °C. The culture was centrifuged at 10,000 rpm for 10 min, washed with 0.85% NaCl saline solution, and resuspended in 4 mL volume. Whole culture suspension was added into 200 mL of 3% Na-alginate solution, which was autoclaved for 15 min at 121 °C. The alginate solution containing bacteria culture was dripped into a 500 mL 0.15 M CaCl2 solution from a 20 cm height via peristaltic pump operated at 2.5 mL/min flow rate through a glass Pasteur pipette aseptically under a laminar flow. The resulting beads had a 3 mm diameter and entrapped about 5 × 10⁶ colony forming units (CFU)/bead.

BAC biotransformation activity of beads was evaluated in 250-mL Erlenmeyer flasks. Four parallels and a control were set in 3-N-morpholino propane sulfonic acid (MOPS) buffered mineral salt medium (M-SM) containing 10 μM C12BAC and 10 μM C14BAC. M-SM was composed of 2.1 g/L MOPS, 0.5 g/L NaCl, 1.00 g/L NH4Cl, 0.25 g/L MgSO4·7H2O, 0.015 g/L CaCl2·2H2O, 0.5 mg/L ZnCl2, 0.30 mg/L MnCl2·4H2O, 3.0 mg/L H3BO3, 2.0 mg/L CoCl2·6H2O, 0.10 mg/L CaCl2·2H2O, 0.20 mg/L
2.3 Reactor set-up and operation

Two 32-mL glass chromatography columns with 15 mm diameter and 200 mm length were used as reactors (Fig. 1). Each column was sterilized via autoclaving at 121 °C for 15 min. Sterile glass beads occupying 4 mL of the effective column volume were transferred into each column as the supporting medium. Then columns were filled with alginate beads. One column received beads with BIOMIG1BDMA cells (a total of 3 × 10^9 CFU), whereas the other was packed with beads containing no microorganism. Each column was attached to a stand. A bottle containing a liquid medium with BACs was connected to the columns through a peristaltic pump via sterile Teflon tubing penetrating through the bottle’s cap. The liquid entered the reactors from the bottom port, packed with glass beads for homogeneous delivery of the influent. The exit of the column packed with BIOMIG1BDMA beads was connected to a fraction collector to get samples at desired time intervals, whereas the effluent of the control reactor was collected in a beaker. Flow rate in each reactor was adjusted using a calibrated peristaltic pump.

Empty bed contact time (EBCT), which was calculated by dividing the total volume of the empty glass column to flow rate, was used to represent the hydraulic residence time in the reactors. Also, mean residence time, which is the average time that the fluid spent in the packed reactor at each flow rate, was measured by slug-dosing the reactor with BDMA as a tracer solute as follows: Reactor packed with beads containing no microorganisms was fed with M-SM containing 20 μM BTMA for 10 EBCTs. A solution containing 60 mM BDMA was introduced into the reactor for 1 min, 30 s, and 15 s when the reactor was operated at 0.1 mL/min, 0.2 mL/min, and 0.4 mL/min flow rates, respectively. Samples were collected at the exit of the reactor for 24 h and analyzed by HPLC.

Three sets of experiments with different wastewater compositions were performed on the continuous flow reactor. In the first experiment, the effect of EBCT on the removal of 20 μM BACs from M-SM synthetic wastewater was investigated. In this experiment, reactors were fed with synthetic wastewater containing 10 μM C_{12}BAC and C_{12}BAC at 0.1, 0.2, 0.4, and 0.8 mL/min flow rates. BAC removal performance of the reactor was determined both using the difference between the influent and effluent BAC concentrations, and the amount of BDMA produced. In the second experiment, the reactors were operated with synthetic wastewater at low BAC concentrations (1 μM C_{12}BAC and C_{12}BAC) at a minimum EBCT that achieved over 90% BAC removal in the first experiment. In the third tier, the reactors were operated with secondary effluent taken from ISKI Pasakoy Advanced Wastewater Treatment Plant, Istanbul, Turkey (41°00′30.5″N, 29°16′50.7″E), and fortified with 10 μM C_{12}BAC and C_{12}BAC. Before use, the wastewater samples were filtered through Whatman paper (11 μm pore size). The wastewater had the following characteristics measured following the Standard Methods (Eaton et al., 2005): pH was 8.63 ± 0.02, and total organic carbon (TOC) concentration was 14.1 ± 1.5 mg C/L. Concentrations of Ni^{2+}, Si^{4+}, and Al^{3+} were 40 ± 0.6 μg/L, 42 ± 2 mg/L, and 25 ± 2 μg/L, respectively. Concentrations of Mo, Zn, Pb, Co, Cd, Fe, Mn, Cr, and Cu were below the detection limit, whereas concentrations of K^{+}, Na^{+}, NO_{3}^{-}, PO_{4}^{3-}, Cl^{-}, and SO_{4}^{2-} were 21 ± 0.4 mg/L, 9.2 ± 0.14 mg/L, 9.2 ± 0.14 mg/L, 0.28 ± 0.09 mg/L, and 104 ± 1.5 mg/L, and 0.28 ± 0.09 mg/L, respectively.

In the reactor experiments, both influent and effluent concentrations of BACs and BDMA were measured using the HPLC method described below. As quality control and quality assurance measures, the influent medium was spiked with 20 μM benzyl trimethyl ammonium chloride (BTMA), a non-retaining, non-biodegradable solute for the system, as a surrogate standard. Further degradation of BACs in the samples was avoided by collecting the effluent with the fraction collector into vials containing half sample volume of HPLC mobile phase composed of 60:40 acetonitrile and 50 mM phosphate buffer at pH 2.5.

2.4 Microbial community analysis with 16S rRNA amplicon sequencing

After the operation with real wastewater, about 50 beads were collected randomly from the reactor and solubilized with phosphate buffer. The content was centrifuged at 10,000g, and the pellet was washed several times with a saline solution (0.85% NaCl). The MO BIO PowerSoil DNA Isolation Kit was used to extract genomic DNA from the pellet. Then, the V1-V3 regions of the bacterial 16S rRNA gene were amplified and sequenced on the Roche FLX 454 platform by MacroGen Europe Inc. A total of 37,015 reads above 30% quality, and 300 bases length were processed using SILVANgs Pipeline (Quast et al., 2013). Phylogenetic analysis was performed on sequences using Geneious version 7.0 (Biomatters Inc., Auckland, New Zealand).

2.5 Analytical methods

BACs, BDMA, and BTMA were measured using an Agilent 1260 high-performance liquid chromatography unit equipped with a diode array detector (Agilent Technologies, Waldbronn, Germany). Separation of the analytes was performed on Phenomenex Luna SCX column (250 mm × 4.6 mm, 5 μm particle size, Phenomenex, Inc., Torrance, CA) followed by a Polaris C18 A column (50 mm × 4.6 mm, 3.2 μ particle size, Agilent Technologies Inc., Waldbronn, Germany) with a mobile phase composed of a 60:40 (vol/vol) mixture of acetonitrile and 50 mM phosphate buffer (pH 2.5) at a flow rate of 1.0 mL/min. The columns were maintained at 35 °C, and detection of analytes was performed at 210 nm wavelength. Before injection, every sample was centrifuged at 10,000 rpm for 10 min in microcentrifuge tubes, and 0.6 mL of supernatant was used for HPLC analysis. BACs and BDMA were quantified using a calibration curve constructed based on the ratio of peak area of surrogate standard (BTMA) to the area of analyte standards at a concentration range between 0.05 and 100 μM.

Cell concentration in the effluent of the packed-bed reactor columns loaded with cell beads and empty beads, and in the influent reservoir were analyzed during the reactor operation. Samples were diluted with saline solution and spread over either Chromagar Pseudomonas (Chromagar Microbiology, Paris, France), LB or LB-BAC agars. Colonies formed were counted, and cell concentration was reported as colony-forming units per mL (CFU/mL). All cell counts were done at least with three replicates. In addition, viable cell concentration in the alginate beads was determined as follows: A bead was transferred into a 1.5 mL microcentrifuge tube and washed twice with saline solution. After washing, the bead was dissolved in phosphate buffer composed of 7.4 g/L KH_{2}PO_{4}, 3.0 g/L KH_{2}PO_{4}, under vigorous agitation over a vortex. The content was then appropriately diluted and spread over Chromagar Pseudomonas or LB-BAC agars. The number of colonies on the plate after overnight incubation at 28 °C was reported as CFU/bead.

2.6 Visualization of data and statistical analysis

The data were visualized and statistically evaluated using Igor Pro version 6.37 (WaveMetrics Inc., Oregon, USA). Removal efficiencies achieved during three experiments were calculated using either BACs or BDMA in the effluent as in Eqs. (1a and 1b), respectively.

\[
R_{\text{BAC}} = \left(1 - \frac{\text{BAC}_{\text{eff}}}{\text{BAC}_{\text{inf}}}\right) \times 100
\]

(1a)

\[
R_{\text{BDMA}} = \left(\frac{\text{BDMA}_{\text{eff}}}{\text{BDMA}_{\text{inf}}}\right) \times 100
\]

(1b)
shake flasks containing M-SM with 10 continuous flow reactors. In alginate beads (bacteria cells about 2 mean); and (C) major BAC removal mechanisms by BIOMIG1 entrapped in BDMA formation profile in synthetic media inoculated by BIOMIG1 entrapped

Fig. 2. Camera and scanning electron microscope images of (A1) alginate beads, (A2) Pseudomonas sp. BIOMIG1 cells, (A3) single bead cross-section and (A4) alginate entrapped BIOMIG1 cells in bead pores; (B) BAC utilization and BDMA formation profile in synthetic media inoculated by BIOMIG1 entrapped in alginate beads (n = 3, whiskers represent one standard deviation of the mean). and (C) major BAC removal mechanisms by BIOMIG1 entrapped in alginate beads (BACliquid phase BAC; BACadsorbed BAC; ↔: adsorption-desorption; →: biotransformation).

where $\text{R}_{\text{BAC}}$ (%) is BAC removal efficiency calculated based on the effluent BAC concentration ($\text{BAC}_{\text{eff}}, \mu\text{M}$), whereas $\text{R}_{\text{BDMA}}$ is BAC removal efficiency calculated based on the effluent BDMA concentration ($\text{BDMA}_{\text{eff}}, \mu\text{M}$). $\text{BAC}_{\text{tot}}$ is the total BAC concentration measured in the influent of the reactor. $\text{R}_{\text{BDMA}}$ was used to refer BAC removal efficiency solely by biotransformation, whereas $\text{R}_{\text{BAC}}$ was the total BAC removal efficiency both by adsorption and biotransformation.

Statistical significance of the difference between removal efficiencies calculated using BAC and BDMA at the same reactor operating conditions was determined using paired t-test after the data were evaluated for normal distribution and randomness using Jarque-Bera, Serial Randomness tests at a significance level of 0.01, respectively. Statistical comparison of removal efficiencies at two different operating conditions was performed using Wilcoxon Rank test after distribution, randomness and variance of the data were evaluated with Jarque-Bera, Serial Randomness, and F tests at a significance level of 0.01, respectively.

3. Results and discussion

3.1. Cell entrapment and BAC biotransformation activity of beads

On average 3-mm beads synthesized in 0.15 M CaCl$_2$ were porous (Fig. 2A1 and A3), and each bead was able to entrap 4.6 ± 1.4 × 10$^6$ bacteria cells about 2–4 $\mu$m length (Fig. 2A2 and A4). Beads were durable and did not get sheared during the operation of both batch and continuous flow reactors.

BAC biotransformation activity of BIOMIG1-beads was evaluated in shake flasks containing M-SM with 10 $\mu$M C$_{12}$BAC and 10 $\mu$M C$_{14}$BAC. The experiment lasted 50 h. The pH was constant and equal to 7.2 during the incubation period. The concentration of both C$_{12}$ and C$_{14}$BACs suddenly drop within 1 h without any significant formation of BDMA (Fig. 2B). Initially, the concentration of C$_{12}$BAC was 11.4 $\mu$M, then it dropped to 7.7 $\mu$M. Likewise, the concentration of C$_{14}$BAC decreased from 10.4 to 3.7 $\mu$M at the end of 1 h of incubation. However, only 0.3 $\mu$M BDMA, which was significantly lower than the expected amount, i.e. 10.4 $\mu$M, was produced in that period. Given that BACs are hydrophobic molecules, we attributed the initial sharp drop in the BAC concentration to the adsorption of BAC molecules on the alginate beads (Fig. 2C). Similar removal of BACs was also reported in the control flask containing the same amount of beads without BIOMIG1 (see Supporting Information, Fig. S1A). The significant difference between the concentrations of C$_{12}$ and C$_{14}$BAC after 1 h of incubation verifies that adsorption was the main mechanism of BAC removal from the wastewater initially since C$_{14}$BAC is significantly more hydrophobic than the C$_{12}$BAC. After an hour, BDMA concentration started to increase while BAC concentration was decreasing gradually. Within 48 h, all BACs were converted to BDMA in the flask (Fig. 2B). Since BDMA does not adsorbs on the alginate beads (Fig. S1B) and get degraded by the strain BIOMIG1$^{BDMA}$, the rate of BAC biotransformation was estimated based on BDMA formation by fitting a trendline to BDMA data. Such interpretation of rate of assumes that BAC biotransformation follows a zero-order reaction kinetics. As a result, the BAC biotransformation rate constant of BIOMIG1-beads was 0.47 $\mu$M/h, 8 × 10$^{-4}$ $\mu$M/h bead or 1.6 × 10$^{-10}$ $\mu$M/h cell ($r^2 = 0.99$).

Alginates is a conventional low-cost carrier for cell and enzyme entrapment. Typical alginate concentration used in cell entrapment ranges between 1% and 8%, whereas 0.05–2.0% CaCl$_2$ is preferred for solidification while preparing biocatalytic alginate beads for environmental applications (Cassidy et al., 1996). Entrapment of cells into alginate makes cells resistant to shock loads of inhibitory chemicals, adverse environmental conditions such as extreme temperature, pH, and salinity; facilitates metabolic activity; increases plasmid stability, and extend cellular life span. However, limitations in solute and oxygen diffusion into the beads, which depends on the structure and the composition of the bead, may decrease the biotransformation rates (Zur et al., 2016). Given the similar BAC transformation rate achieved with alginate entrapped cells of BIOMIG1 compared to suspended cells (Ertek et al., 2016), bead structure and composition did not affect the activity of BIOMIG1 cells in the alginate beads.

3.2. Effect of EBCT and influent concentration on BAC removal from synthetic wastewater

An up-flow reactor system packed with Ca-alginate entrapped BIO- MIG1$^{BDMA}$ cells was operated continuously with synthetic wastewater and secondary effluent containing two different BACs at a concentration of either 1 or 10 $\mu$M (c.a. 0.350–3.5 mg/L). The low concentration agrees with nominal concentrations of QACs detected in wastewater, whereas the high concentration would be a maximum concentration expected in domestic and hospital wastewaters during the SARS-CoV-2 pandemic. Given that BIOMIG1’s oxyBAC enzyme is very specific on the cleavage of the bond between quaternary nitrogen and alkyl alpha carbon, when expressed, its BAC biotransformation rate follows a zero-order reaction kinetics that does not depend on BAC concentration. Also, the BAC biotransformation activity of oxyBAC is not inhibited by other non-toxic organic substrates. Therefore, the results achieved under the conditions tested in this study could easily be reproduced in the field no matter the wastewater characteristics are.

The first course of experiments testing the effect of EBCT on the BAC removal efficiency of the reactor lasted for 30 days. During that period, both control and test reactors were fed with synthetic wastewater containing two BACs at approximately 10 $\mu$M concentration. BAC and BDMA concentrations in the influent and the effluents of the test and control reactors were monitored. C$_{12}$ and C$_{14}$BAC concentrations in the
influent synthetic wastewater were stable at 9.3 ± 0.3 and 9.7 ± 0.4 μM (n = 49), respectively, suggesting no biotic or abiotic transformation happening in the feed reservoir. We also confirmed that the feed solution was not contaminated during the reactor operation by routinely plating a sample on PS and LB agar.

The reactor was first operated at 4.7 h EBCT for almost 10 days. The mean residence time of fluid in the reactor was 1.3 h at that EBCT. For 5 days, the effluent concentrations of both BACs and BDMA were not stable (Fig. 3A). During that period, the total BAC concentration in the influent changed between 8.2 and 9.0 μM. The highest C12 BAC concentration detected in the effluent was 6.5 μM, whereas it was 2.2 μM for C12 BAC during that start-up period. The removal efficiency of C12 BAC was between 30% and 100%, with a mean equal to 82 ± 23% (n = 60). At the same time, C13 BAC removal was between 77% and 100%, with a mean of 94 ± 7% (n = 60) (Fig. 3B1). On the contrary, BAC removal efficiency calculated using the effluent BDMA concentration, assuming that all removal was achieved via biotransformation (RBDMA) was in a range of 6.4–119.1% with a mean of 84 ± 32% (n = 60). Although total BAC removal (Rbac) in the reactor was always higher than 56%, a significantly low removal, i.e., 6.4%, was calculated based on the effluent BDMA (Fig. 3B1). This result suggested that the removal of BACs in the reactor during the initial stages of the start-up period was due to adsorption rather than biotransformation. At the later stages of the start-up period, the removal efficiencies greater than 100% was recorded. Such high RBDMA’s were attributed to the conversion of adsorbed BACs to BDMA when biotransformation became the dominant removal mechanism in the reactor. Likewise, we observed low BAC concentrations in the effluent of the control reactor that was packed with empty beads. For instance, the mean C12 and C13 BAC concentrations in the effluent of the control reactor were 8.7 ± 0.7 and 4.8 ± 2.9 μM during the first 5 days of operation. Since BDMA was not detected in the effluent of the control reactor, low BAC concentrations suggested that Ca-alginate beads adsorb BACs, and adsorption affinity is high for BACs having longer alkyl chain length.

Concentration fluctuations in the effluent decreased, and the reactor reached a steady-state after 5 days, which was equal to 25 EBCTs. After 5 days, none of the BACs were detected in the reactor’s effluent at 4.7 h EBCT (Fig. 3A). The mean BDMA concentration in the effluent was 17.2 ± 0.3 μM (n = 58). Although BAC removal at this EBCT was 100%, when effluent BDMA concentration was taken into account, the removal by biotransformation was 89.9 ± 3.1% (Fig. 3B1). The difference between Rbac and RBDMA was statistically significant (t = 24.9 > tc = 2.7 at α = 0.01, n = 58). Therefore, about a 10% difference between the removal efficiencies was attributed to the adsorption of BACs to the alginate beads.

Subsequently, the flow rate of the feed was elevated to 0.2 mL/min, which maintained an EBCT equal to 2.3 h in the reactors. The corresponding mean residence time of the fluid was 0.83 h. The reactors were operated for 7.5 days at this EBCT. Total BAC concentration in the effluent of the control reactor was 17.8 ± 1.2 μM, which was similar to 18.8 ± 0.9 μM in the influent. This minor difference in the BAC concentration was due to the adsorption of BACs to the beads rather than biotransformation; since BDMA was not detected in the control effluent.

The mean concentration of C12 and C13 BAC in the effluent of the test reactor was 0.30 ± 0.21 μM and 0.01 ± 0.07 μM, respectively. The concentration of both BACs was significantly lower than the BACs in influent. Besides, 17.2 ± 0.6 μM BDMA was detected in the effluent during the operation period (Fig. 3A). Like the operation at 4.7 h EBCT, Rbac was 98.4 ± 1.2%, whereas RBDMA was 91.5 ± 4.4% (n = 93), suggesting that significant removal of BACs in the reactor was due to biotransformation (Fig. 3B1). When removal efficiencies of the reactor while operating at 4.7 and 2.3 h EBCTs were compared, the difference in the RBDMA’s was statistically significant (p < 0.001). On the contrary, no significant difference was observed on Rbac’s (p = 0.061). Equal BDMA production at both EBCTs suggests that removal by biotransformation did not get affected by the EBCT; however, the adsorption efficiency of the beads decreased.

In the next 7.5 days, the reactors were operated at 1.2 h EBCT. The mean residence time of the fluid in the reactors at this EBCT was 0.33 h. The influent concentration and the effluent concentration of BACs in the control reactor was 18.8 ± 0.8 μM and 19.0 ± 1.4 μM, respectively, verifying that any biological contamination resulting in potential BAC removal was avoided during the operation period. On the other hand, the mean total BAC and BDMA concentration in the effluent of the test reactor was 1.5 ± 0.5 μM and 16.0 ± 0.6 μM (Fig. 3A), which corresponded to an Rbac of 91.8 ± 2.7 and 85.7 ± 5.0% (Fig. 3B1), respectively. Although the relative difference between Rbac and RBDMA was low compared to the difference between the removal efficiencies achieved in previous EBCTs, still the difference is statistically significant (t = 13.3 > tc = 2.6 at α = 0.01, n = 34), suggesting that both adsorption and biotransformation were two independent mechanisms of BAC removal in the reactor. Moreover, the difference between the Rbac and RBDMA’s obtained at 2.3 and 1.2 h EBCTs was also statistically significant (p < 0.001), which implies that reactor efficiency was affected by the last switch of the EBCT from 2.3 to 1.2 h. In fact, the removal efficiency was still above 90%, which was promising.

When we started to operate the test reactor at 0.6 h EBCT, the concentration of both BACs increased, and the BDMA concentration in the effluent decreased. The mean total BAC and BDMA concentrations recorded in the effluent at this EBCT was 5.7 ± 1.6 μM and 12.0 ± 2.0 μM, respectively (Fig. 3A). Given the fact that the difference between Rbac and RBDMA, which were 70.2 ± 8.5 and 62.1 ± 10.8%, respectively (t = 9.0 > tc = 2.7 at α = 0.01, n = 34), was statistically significant, both adsorption and biotransformation were still active in the reactor even at low EBCT (Fig. 3B1).

In addition to BAC and BDMA analyses during the reactor operation, we also measured the number of active BIOMIG1BDMA cells in the effluent of the test reactor at different EBCTs. Effluent cell concentrations were 8.3 ± 1.0 × 10^5 CFU/mL, 2.2 ± 0.2 × 10^5 CFU/mL, and
2.0 ± 1.5 × 10^5 CFU/mL in the effluent of the reactor at 2.3 h, 1.2 h, and 0.6 h EBCT, respectively, that is about 1.5 × 10^5 cells were released from the reactor in every minute. Considering that BIOMIG1 cells in the beads reproduce as they utilize BACs and a bead has a holding capacity, elution of cells from an operating reactor was expected.

To understand the effect of BAC concentration on the packed bed reactor’s performance, both control and test reactors were operated at 1.2 h EBCT with a feed containing 2 µM BACs (~1 µM C_{12}BAC + 1 µM C_{14}BAC) for 7 days. The BAC concentration in the influent was stable and equal to 1.9 ± 0.2 µM. The BAC concentration in the control effluent was similar to the influent BAC concentration suggesting that biotic and abiotic transformation processes were not present in the absence of BIOMIG1 BDMA. On the other hand, none of the BACs were detected in the effluent of the test reactor during the operation period. Moreover, the mean BDMA concentration in the effluent was 1.9 ± 0.3 µM, which was almost equal to the total amount of BACs fed into the reactor. The overall BAC removal efficiency of the reactor at low BAC concentration was 100% (Fig. 3B2). Similarly, R_{BDMA} was 97.7 ± 11.7% (n = 73). This set of experiments performed with synthetic wastewater verified that our reactor system efficiently removes BACs within at least a ten-fold concentration range at and below 2 h EBCT. Additionally, about 4.1 ± 0.6 × 10^4 CFU/mL cells were detected in the test reactor’s effluent. A significantly lower amount of cell concentration at the effluent of the test reactor operated at 2 µM total BAC concentration compared to the reactor operated at 20 µM was attributed to less yield of bacteria due to lower amount of substrate introduced.

When the reactor performance at a certain EBCT was evaluated, we observed that higher BAC removal efficiency was achieved at low BAC concentration. Given that fewer BAC molecules enter the reactor at low concentration than high concentration, efficiency would be greater if the reactor removes a constant number of molecules per unit time that the liquid spends in the reactor. The Eq. (2) derived from the steady-state plug-flow equation simulated the removal efficiency in the reactors with a good agreement to the experimental data (Fig. 4).

\[ R_{BAC} = 1 - e^{-k_{obs} \times \frac{EBCT}{BAC_{inf}}} \]  

where \( R_{BAC} \) is total BAC removal efficiency (%); \( k_{obs} \) is observed BAC removal rate constant (µM/h); \( EBCT \) is applied empty bed contact time (h), and \( BAC_{inf} \) is total influent BAC concentration (µM).

BAC removal performance of the system was simulated for different influent concentrations between 0.5 and 50 µM (c.a. 0.175–17.5 mg/L) at a range of EBCT. Simulations showed that almost 100% BAC removal can be achieved at and below 20 µM BACs less than 2 h EBCT (Fig. 4A). The hydraulic retention time of conventional plug-flow activated sludge reactors treating domestic wastewater is between 4 and 8 h (Tchobanoglous et al., 2003). Therefore, our reactor system can completely remove QACs from wastewater less than half of the volume of the activated sludge reactors even at concentrations up to 7 mg/L. EBCT, as well as the volume of the reactor, significantly decreases as the desired removal efficiency decreases. For instance, the reactor should be operated at 2.4 h EBCT to remove 99.99% of 10 µM BACs from the...
wastewater. On the other hand, 1.6 h and 0.5 h EBCT is enough to remove 99.9% and 90%, respectively (Fig. 4B).

3.3. Operation with real wastewater and evaluation of microbial competition

We also tested the reactor’s performance with real wastewater spiked with 10 µM C₁₂BAC and 10 µM C₁₄BAC at 1.2 h EBCT. The reactors were operated on for 7 days which was equal to 140 retention times. Given that operation period was significantly longer than 3 retention-times, which is the threshold value for reaching steady-state in a continuous reactors, it is assumed that efficiencies reported here are reliable to justify long-term performance stability of the reactor. The mean total BAC concentration in the influent was 18.1 ± 1.0 µM. In the first 2 days of operation, there was a course of adaptation of BIO-MIG1 to incoming wastewater (Fig. 5A). After those days, the mean total BAC and BDMA concentration in the effluent of the reactor was 0.2 ± 0.3 µM and 17.3 ± 1.0 µM, respectively, throughout the operation period (Fig. 5A). The reactor removed 99.2 ± 1.5% of the BACs and converted 92.3 ± 5.9% BACs in the influent to the BDMA (Fig. 5B). Both R_BAC and R_BDMA obtained during the operation of the reactor with real wastewater were comparable to the removal efficiencies achieved in the synthetic wastewater. As a result, we demonstrated that the reactor system could easily be implemented to alleviate BACs in wastewater treatment plants without any problem. In addition, the effluent of the test reactor contained 1.2 ± 1.1 × 10⁶ CFU/mL cells, which was comparable to the operation at 1.2 h EBCT with the synthetic wastewater.

At the end of 1.5 months of operation, we removed BIO-MIG1-beads from the reactor and measured the viable cell concentration in 100 of the beads. The beads contained 1.4 ± 1.1 × 10⁶ CFU/bead. This value is lower than the initial cell concentration of the beads. However, the reactor was very consistent within the operation period without any problem and substantial maintenance.

Overall, 18 operational taxonomic units (OTUs) were identified on the beads recovered from the reactor at the end of the operation period (Fig. 6). As expected, BIO-MIG1_BDMA was the dominant species of the microbial community. Out of 37,015 reads, 32,884 reads were assigned to OTU1, which has more than 98% similarity to 16S rRNA of strain BIO-MIG1_BDMA. The other OTUs identified on the beads included strains of *Stenotrophomonas maltophilia*, *Serratia proteamaculans*, *Achromabacter xylosoxidans*, *Herbaspirillum autotrophicum*. Some of the OTU belonging to uncultured species of gamma and beta proteobacterium class of bacteria (Fig. 6). Since the OTUs of other bacteria accounted for only 11.2% of the whole microbial community, we can conclude that BIO-MIG1 over-competed the other bacteria in the wastewater while treating the BACs, and the reactor system can perform undisturbed despite minor bacterial contamination.

There are applications of immobilized cells in the pharmaceutical, food, and chemical industry, yet this technology is recently emerging for the treatment of organic (Ha et al., 2009) and inorganic (Ito et al., 2012) pollutants in the environment (Oas and Adholeya, 2015). In one study similar to ours, Bergero and Lucchesi (2015) tested the biodegradation of two hydrophobic BACs, i.e., C₁₂BAC and C₁₄BAC, in batch reactors inoculated with alginate entrapped *Pseudomonas putida* A. Either synthetic or industrial wastewater containing c.a. 100–1000 µM BACs were used in the experiments. They achieved a maximum of 90% total
removal of BACs in the reactors within 24 h, and the removal efficiency did not change with prolonged incubation. They suggested that the removal was due to biotransformation by the bacteria-containing beads, but the interplay between adsorption and biotransformation in the reactor was not clear. Moreover, the reason why the removal ceased after a certain period of incubation needed an explanation to verify the factors affecting the biotransformation extent in the batch reactors (Bergero and Lucchesi, 2015).

In another study, the biological removal of hexadecyl trimethyl ammonium bromide from synthetic wastewater in a continuous flow oxygen-based membrane biofilm reactor inoculated with activated sludge was investigated (Lai et al., 2017). The reactors were set up with two different membranes and operated at QAC concentration between 400 and 1100 µM at 24 h hydraulic retention time. Almost a complete removal of the QAC was achieved in both composite fiber and polypropylene membrane reactors at 400 µM concentration. In both reactors, certain species of bacteria belonging to Pseudomonas, Stenotrophomonas, and Achromobacter genera were dominated, a very common microbial community profile observed in our study and other studies as well (Ertekin et al., 2016; Lai et al., 2017). Authors mentioned that antibiotic resistance genes proliferated in the biofilm of the reactors but were not transferred to the effluent (Lai et al., 2017).

In addition, recently, synthetic and industrial wastewater containing BACs up to 3 mM was treated in continuous up-flow aerobic biofilm reactors utilizing either PVC or lightweight expanded clay aggregate attached Pseudomonas sp. isolated from a lake (Fortunato et al., 2019). Over 99% BAC removal was achieved in both reactors at a hydraulic retention time of 8 days. Given the high removal efficiency of the reactors, the removal mechanism was not clear in the study.

Efficient removal of QACs has been achieved in different treatment systems employing either a single strain of bacteria or a microbial community. However, all of those systems were operated at very high QAC concentrations, which are not environmentally relevant, and have long hydraulic retention times that cannot compete or be physically implemented to a conventional treatment system. The reactor system introduced in this study efficiently removes BACs from wastewater at environmentally relevant concentrations predominantly via biotransformation.

4. Conclusions

The continuous up-flow packed bed reactor system defined in this study utilizes a very-well characterized non-pathogenic species of bacteria that is highly specialized in QAC degradation. The system achieved around 100% BAC removal from wastewater within 1 h hydraulic retention time without aeration and effluent recirculation. Therefore, the reactor system offers a promising low maintenance alternative for water treatment and after the discharge of the effluent into the environment.

Declaration of Competing Interest

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.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2021.126210.

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CRediT authorship contribution statement

Fahri Koray Sakarya: Investigation, Formal analysis, Data curation, Visualization, Writing - original draft. Berat Zeki Haznedaroğlu: Writing - review & editing. Ulas Tezel: Conceptualization, Formal analysis, Resources, Visualization, Supervision, Project administration, Funding acquisition, Writing - review & editing.

Declaration of Competing Interest

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.
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