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*Research article*

# A novel approach for harnessing biofilm communities in moving bed biofilm reactors for industrial wastewater treatment

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**Abstract:** Moving bed biofilm reactors (MBBRs) are an effective biotechnology for treating industrial wastewater. Biomass retention on moving bed biofilm reactor (MBBR) carriers (biofilm support materials), allows for the ease-of-operation and high treatment capacity of MBBR systems. Optimization of MBBR systems has largely focused on aspects of carrier design, while little attention has been paid to enhancing strategies for harnessing microbial biomass. Previously, our research group demonstrated that mixed-species biofilms can be harvested from an industrial wastewater inoculum [oil sands process water (OSPW)] using the Calgary Biofilm Device (CBD). Moreover, the resultant biofilm communities had the capacity to degrade organic toxins (naphthenic acids—NAs) that are found in OSPW. Therefore, we hypothesized that harnessing microbial communities from industrial wastewater, as biofilms, on MBBR carriers may be an effective method to bioremediate industrial wastewater.

Here, we detail our methodology adapting the workflow employed for using the CBD, to generate inoculant carriers to seed an MBBR.

In this study, OSPW-derived biofilm communities were successfully grown, and their efficacy evaluated, on commercially available MBBR carriers affixed within a modified CBD system. The resultant biofilms demonstrated the capacity to transfer biomass to recipient carriers within a scaled MBBR. Moreover, MBBR systems inoculated in this manner were fully active 2 days post-inoculation, and readily degraded a select population of NAs. Together, these findings suggest that harnessing microbial communities on carriers affixed within a modified CBD system may represent a facile and rapid method for obtaining functional inoculants for use in wastewater MBBR treatment systems.

**Keywords:** biofilm; MBBR; oil sand; wastewater; bioremediation

**Abbreviations**

MBBR = Moving Bed Biofilm Reactor; MBBRs = Moving Bed Biofilm Reactors; OSPW = Oil Sands Process Water; CBD = Calgary Biofilm Device; NA = naphthenic acid; TSB = tryptic soy broth; BH = Bushnell-Haas minimal medium; BH-Y = Bushnell-Haas minimal medium supplemented with yeast extract; SEM = Scanning Electron Microscopy; CLSM = Confocal Laser Scanning Microscopy; DGGE = Denaturing Gradient Gel Electrophoresis; ACA = 1-Adamantane Carboxylic acid.

## 1. Introduction

One of the consequences of this last century's industrialization has been the rapid anthropogenic release of complex pollutants into the environment - threatening the long-term quality of our water resources. Remediation efforts are thus desperately needed to mitigate water pollution. Many biotechnologies exploit the diverse metabolic processes of microorganisms to remediate contaminated wastewater [1,2,3]. One such biotechnology that employs bioremediation principles for treating industrial wastewater is the activated sludge treatment system [4]. Developed in the early 1900's, the conventional activated sludge process uses a suspended culture of microbes to remove contaminants from water sources. Essentially, the microbes both breakdown the contaminants and grow within the primary stage of the reactor. Once the treated water is pumped to the secondary stage, the microbial biomass flocculates out of suspension, and finally settles as "activated sludge" on the bottom of the settler. To prevent complete washout of the activated sludge, as wastewater is cycled through the primary reactor, activated sludge is re-introduced from the settler into the primary reactor. However, issues arise when inadequate flocculation occurs and the mass fails to settle on the bottom of the settler [4]. A contemporary adaptation to the activated sludge system is the moving bed biofilm reactor (MBBR) [4]. The MBBR system incorporates biomass as biofilms (often sourced from activated sludge) on solid carriers within a single reactor. Wastewater is cycled through the system, and the microbial biofilms on the MBBR carriers degrade the contaminants of concern. Advantages of the MBBR system when compared to traditional activated sludge systems include, i) an increased treatment capacity, ii) lack of sludge bulking, and iii) no need for sludge recycling as the biomass is retained on the biofilm carriers [4,5,6]. For these reasons, the MBBR process has had great commercial success and is being used to treat wastewater in more than 22 countries world-wide [7]. The key to the success of the MBBR process is the biofilm carrier. Often made of high-density polyethylene, MBBR biofilm carriers offer a large surface area for biofilm formation despite their small volumetric size, which in turn greatly enhances the effective biomass concentration within the MBBR (as compared to activated sludge) [5,7]. Most often, carrier design is the focal point in optimization attempts for improving the performance of MBBR wastewater treatment systems [6,7,8]. To the best of our knowledge, the capacity to harness a biofilm community for MBBR systems has not been explored in significant detail. However, in 2014, Nakhli et al. evaluated the importance of allowing biofilm communities to acclimate to the targeted wastewater to improve bioremediation efforts [9].

In the early 2000's our research group, the Biofilm Research Group at the University of Calgary, developed a unique method for growing microbial biofilms *in vitro*. Provided commercially as the MBEC™ assay (Innovotech Inc.), the Calgary Biofilm Device (CBD) is a specialized reaction vessel designed to grow multiple equivalent microbial biofilms. It consists of a standard 96-well microtiter plate, the lid of which has been integrated with 96 identical polystyrene pegs—one for each well. In practice, the wells of the device are inoculated with bacteria and growth medium, and upon incubation in the presence of a sheer force, cells adhere to the polystyrene peg and form a microbial biofilm [10]. Historically, the CBD was primarily used for assaying the efficacy of antimicrobials against biofilms [11–14]. However, recently we've discovered that the CBD can be used to grow mixed-species biofilm communities from environmental inoculants. Golby et al. [15], grew mixed-species biofilms directly on the CBD using Alberta oil sands tailings as the inoculum (waste material from the industrial extraction of bitumen petroleum from oil sands). This method allowed for the simultaneous cultivation of approximately 70–80% of the complex microbial community endogenous to the oil sands tailings; far more than what proved culturable by traditional microbiological methods [15]. Through follow-up studies, we determined that mixed-species microbial biofilms grown specifically from oil sands process water (OSPW—the liquid fraction of oil sands tailings waste [16]) demonstrated the capacity to degrade the organic contaminant of principal concern within OSPW—naphthenic acids (NAs) [17,18]. Currently, there is no industry standardized method to treat OSPW, which has become an issue of both environmental and political concern as volumes of oil sands bitumen-extraction waste builds up in tailings ponds [19]. Recently, we demonstrated that environmental mixed-species biofilms grown from an OSPW inoculum in the CBD were capable of degrading NAs -with a wide variety of recalcitrance- below detectable limits within a 14 day time period [18]. As such, it was hypothesized that growing a mixed-species biofilm in the CBD may be a viable way to harness a microbial community for *ex situ* remediation of NAs in OSPW.

We further hypothesize that harnessing environmental microbial communities—derived from industrial wastewater—as biofilms using the methodology developed for the CBD, is an optimal strategy for *ex situ* treatment of contaminated wastewater. Therefore, this experimental endeavor targets the previously un-resolved issue of transferability from CBD-scale (150 μL) to industry approved, larger-scale MBBR systems. In attempting to resolve this issue (within the context of an OSPW case study), our current work introduces a novel method for harnessing biofilm communities for industrial wastewater treatment in MBBR systems, and specifically focuses on 3 main goals: 1) demonstrating that OSPW-derived mixed-species biofilms can grow on industry approved wastewater biofilm support carriers designed for MBBR systems, 2) demonstrating that biofilm carriers developed using the CBD approach are logistically practical seeds for starting-up MBBR systems, and 3) verifying that OSPW-derived biofilms grown on carriers, and can be used to inoculate an MBBR system that retains the ability to rapidly degrade NAs as observed in our prior studies.

## 2. Materials and Methods

All methods utilized in this study are described in complete detail in 2 chapters in the Springer Protocols Handbook, Hydrocarbon and Lipid Microbiology Protocols (2015) [20,21].

*2.1. Biofilm carriers and modified Calgary biofilm device*

For this study a larger scale, modified CBD was designed such that biofilms were grown on MBBR biofilm carriers commonly used in the wastewater treatment industry. A detailed methodology on how to setup and use the CBD, also referred to as the MBEC™ device, can be found at Innovotech's website: (http://www.innovotech.ca/products_use.php). MBBR biofilm carriers (biofilm support materials) used in this study include the K1, K3 and K5 series carriers, available commercially as the AnoxKaldnes™ biofilm carriers (Veolia Water Technologies), along with the Peenox™ carrier (Mabarex) (Figure 1).

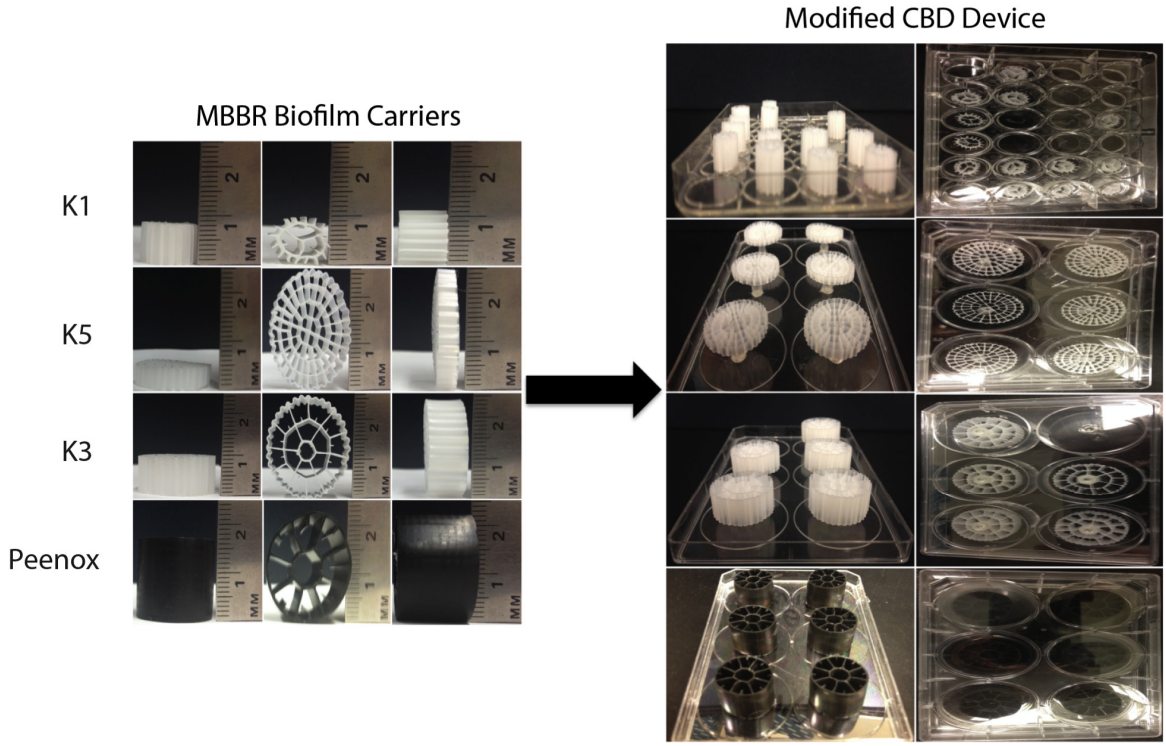


**Figure 1. Fabricating a modified Calgary Biofilm Device (CBD) from conventional polyethylene moving bed biofilm reactor (MBBR) carriers. K1 (0.8 × 1.0 × 1.1 cm) carriers are fixed to the lid of a 24-well plate, while K5 (0.5 × 2.6 × 2.5 cm), K3 (1.0 × 2.5 × 2.4 cm), and Peenox (1.7 × 2.3 × 2.2 cm) carriers are fixed to the lids of 6-well plates. K1, K3 and K5 series carriers are manufactured by Veolia Water Technologies, while Mabarex manufactures Peenox carriers.**

Using a hot glue dispenser, carriers were fixed to the lids of 6-well (K3, K5 and Peenox) and 24-well (K1) plates in order to fabricate modified CBDs (Figure 1), which were subsequently sterilized with ethylene oxide gas prior to use. Mixed species microbial biofilms were grown and evaluated directly within this modified CBD system, or for some experiments, once a biofilm was established individual carriers were aseptically removed from the modified CBD lid and used to inoculate a simple 1 L volumetric flask reactor as a model MBBR. Carrier selection varied throughout different elements of this study, and was influenced by both carrier availability, as well as carrier performance.

*2.2. Bacterial inoculant and biofilm growth conditions*

All biofilm carriers mounted in modified CBDs were inoculated directly with a 1:1 mixture (offered a workable ratio of inoculant:nutrient) of OSPW and growth medium to a total volume of 2 mL for 24-well plates, and 6 mL for 6 well plates. Media used in this study included tryptic soy broth (TSB) (rich and undefined), Bushnell-Haas minimal salts (BH) (defined with no additional carbon source) [22] and BH medium supplemented with 1 g/L yeast extract (BH-Y) (semi-rich, undefined). When cultivating biofilms in the modified CBDs, spent medium was replenished every 2 days. Flask reactors (MBBR proxies) consisted of 1 L Erlenmeyer flasks containing 500 mL of growth medium, 15 sterile biofilm carriers (gas sterilized), and were inoculated with a single seed carrier previously grown for 5 days in the modified CBD system. Both modified CBD and flask systems were incubated under aerobic conditions at 25 °C and 125 rpm [20].

*2.3. Qualitative assessment of biofilm growth*

Biofilm growth on the surface of the carriers was visually confirmed using either scanning electron microscopy (SEM) or confocal laser scanning microscopy (CLSM). SEM was performed using a Philips ESEM XL-30 microscope. Prior to visualization by SEM, carrier biofilms were fixed using 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2), dehydrated with 70% ethanol, mounted, and finally powder-coated with a platinum and gold coating [20]. CLSM was performed using a Leica DM IRE2 microscope, employing a Texas Red filter and 64x water immersion objective. Biofilms were fixed to the carriers using 5% glutaraldehyde in phosphate buffered saline, and stained with Syto® Red 62 nucleic acid stain [excitation⁄emission 649⁄680 (nm)] in preparation for visualization [20].

Biofilm community diversity was fingerprinted using denaturing gradient gel electrophoresis (DGGE). DGGE was conducted using bacterial 16S rRNA universal primers following previously established methodology [15,17,21]. Note: that biofilm microbes were removed from carriers using sonication in the presence of a 0.1% Tween 20 solution, after which genomic DNA was extracted using a FastDNA® Spin Kit from MP Bio.

*2.4. Quantitative assessment of biofilm growth*

To quantitatively measure bioflm biomass, the Bradford protein assay was adapted for high-throughput use in 96-well microtiter plates as previously described [20]. Notable elements of this procedure include the removal of biofilm microbes from carriers using sonication in the presence of 0.1% Tween 20. This sonicate (2 mL) was centrifuged for 10 min at 10,000 g in a 15 mL conical tube. The pelleted cells were then resuspended in 100 μL of a cell storage buffer and boiled for 10 min to solubilize the proteins. Each well of the microtiter plate received 20 μL of Bradford reagent, 2 μL of the protein sample and dd$H_2O$ up to 100 μL. Absorbance was measured in a plate reader at 595 nm, and compared to a standard curve from 1 to 100 μg of bovine serum albumin.

*2.5. Evaluation of biofilm functionality – naphthenic acid degradation*

NA degradation by carrier-bound biofilms was evaluated in the MBBR flask system. In summary, seed biofilms were grown for 5 days in modified CBDs containing BH-Y medium with 1 g/L yeast extract. Single seed carriers were subsequently used to inoculate 1 L flasks containing 15 sterile carriers and 500 mL of BH-Y (1g/L) medium spiked with a synthetic mixture of 8 commercially available NAs at a total NA concentration of 200 mg/L (Supplementary material: Table S1). At various time points, 0.2 mL of the medium from the MBBR flask reactors was aseptically removed, from which the NA fraction was extracted using dichloromethane. NA extracts were derivatized into trimethylsilylates with N,O-Bis(trimethylsilyl)trifluoroacetamide, and quantified using gas chromatography (Agilent HP-5 30 m column) coupled to a flame ionization detector as described previously [17,20].

## 3. Results and Discussion

*3.1. Evaluation of biofilm growth on MBBR carriers within a modified CBD system*

The first objective of this study was to confirm and evaluate the growth of OSPW-derived biofilms on MBBR carriers grown within our modified CBD system. False colored SEM images of K1 carrier-bound biofilms imaged 5 days post OSPW inoculation visually confirmed that OSPW mixed-species biofilms were capable of growing on the MBBR carriers using our modified CBD system (Figure 2).

Figure 2B shows limited biofilm growth, which was to be expected as no additional nutrients were added to BH media, in that instance the only carbon source available was from the OSPW inoculum itself. Figure 2C represents a biofilm grown using semi-rich BH-Y (1 g/L) medium on a K1 support, and exhibits more biofilm growth than what was observed without yeast extract amendment, although not as confluent as biofilms grown using rich TSB medium (Figure 2D). Note: CLSM was used to visually confirm biofilm growth on the other 3 carriers used throughout parts of this study (data not included). These SEM images were taken at the 5 day time point, which roughly corresponds to the optimal growth point of these biofilms as shown in Figure 3.

Biofilm-biomass was measured using soluble protein concentration to semi-quantify microbial growth on the MBBR carriers [23,24]. Data are reported as mg protein/carrier, and is thus representative of total biomass present on a single carrier. Biofilm populations quantified in this manner complimented the SEM observations. Under conditions of no supplemental nutrients aside from BH minimal salts (Figure 3A), less than 4 mg of total protein was observed at any particular time point. Growth on rich TSB medium (Figure 3C) allowed the biofilm to reach peak density approximately 2 days before biofilms grown on BH-Y (Figure 3B), and protein concentrations of TSB-fed biofilms (14 mg/carrier) were nearly double of BH-Y-fed biofilms (6 mg/carrier). Analyzing spent medium for total protein from planktonic populations (resulting from the shedding of cells from the biofilm), also demonstrated an increase in population size as more rich media were utilized (Figure 3). These results mirror previous observations from our research group in which qPCR of 16S rRNA genes confirmed that more populated biofilms resulted when OSPW microbes were grown in the traditional CBD system [17].

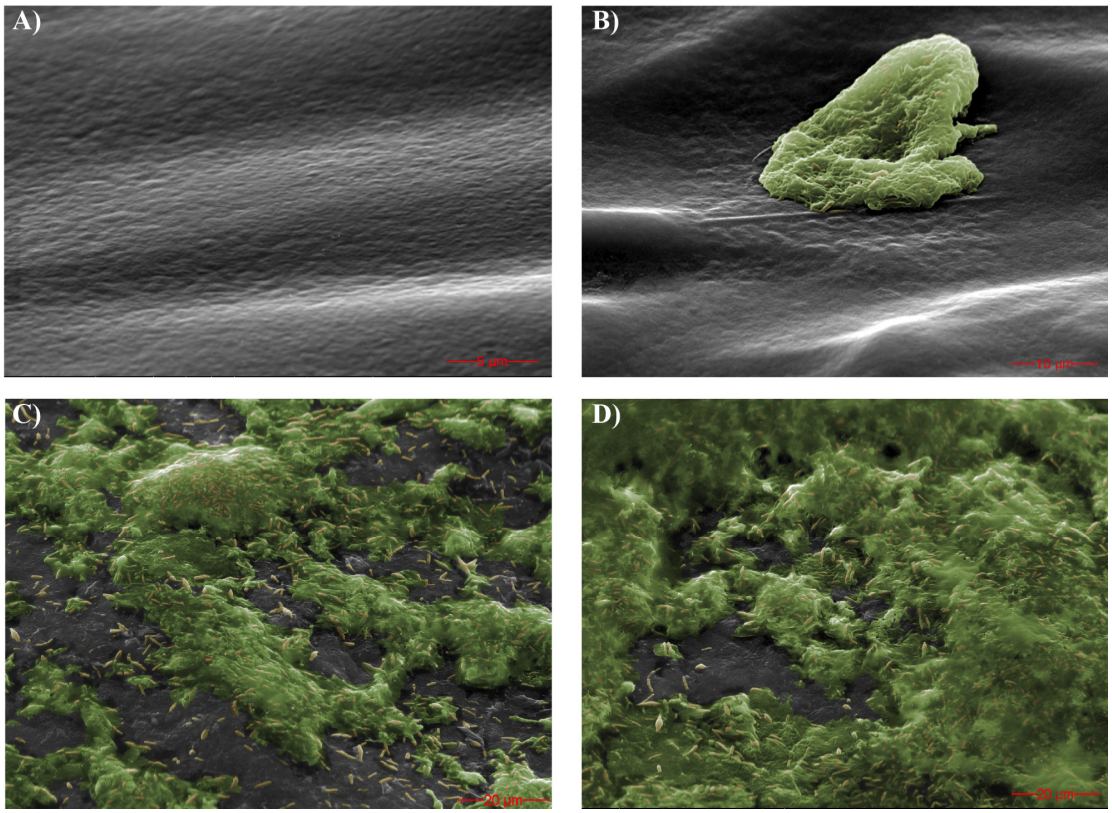


**Figure 2. Scanning electron micrographs of OSPW-derived mixed species biofilms growing on K1 series carriers in a modified CBD system. Biofilms were incubated for 14 days at 25 °C and 125 rpm under the following media conditions, A) sterile control, B) Bushnell-Haas minimal medium, C) Bushnell-Haas supplemented with yeast extract (1 g/L) and D) tryptic soy broth. Images are falsely colored with cells in yellow, and extracellular matrix in green.**

Having established that OSPW-derived biofilms had the capacity to grow on MBBR carriers in our modified CBD system, our subsequent goal was to determine if these biofilm communities varied in composition from the original OSPW inoculum, and if they were dynamic or static in species richness. Although the resolution is limited to no more than 1% of the most populous microbial species within the carrier-bound biofilms, DGGE was an effective tool to fingerprint the community diversity [25]. The DGGE gels in Figure 4 demonstrated variability between the original OSPW community, and that of the K1 carrier-bound biofilms and their associated planktonic populations (cells shed from the biofilm).

Moreover, populations were dynamic—Figure 4 red boxes highlight species that changed with respect to incubation time. Results with K3, K5 and Peenox carriers are much the same (Supplementary material: Figure S1). All of the carrier-bound biofilms evaluated in these experiments were grown on BH-Y (1 g/L) media, under aerobic conditions without exposure to NAs. Based on the DGGE and next-generation sequencing community profiling of oil sands tailings microbes grown in a traditional CBD system by Golby et al. (2012), we can confidently speculate that manipulation of abiotic factors such as oxygen tension, and nutrient supplementation would result in unique biofilm communities on our MBBR carriers [15]. This may have important

ramifications regarding the tailoring/conditioning of a biofilm community grown on carriers in a modified CBD system to serve as an inoculant in an MBBR system.

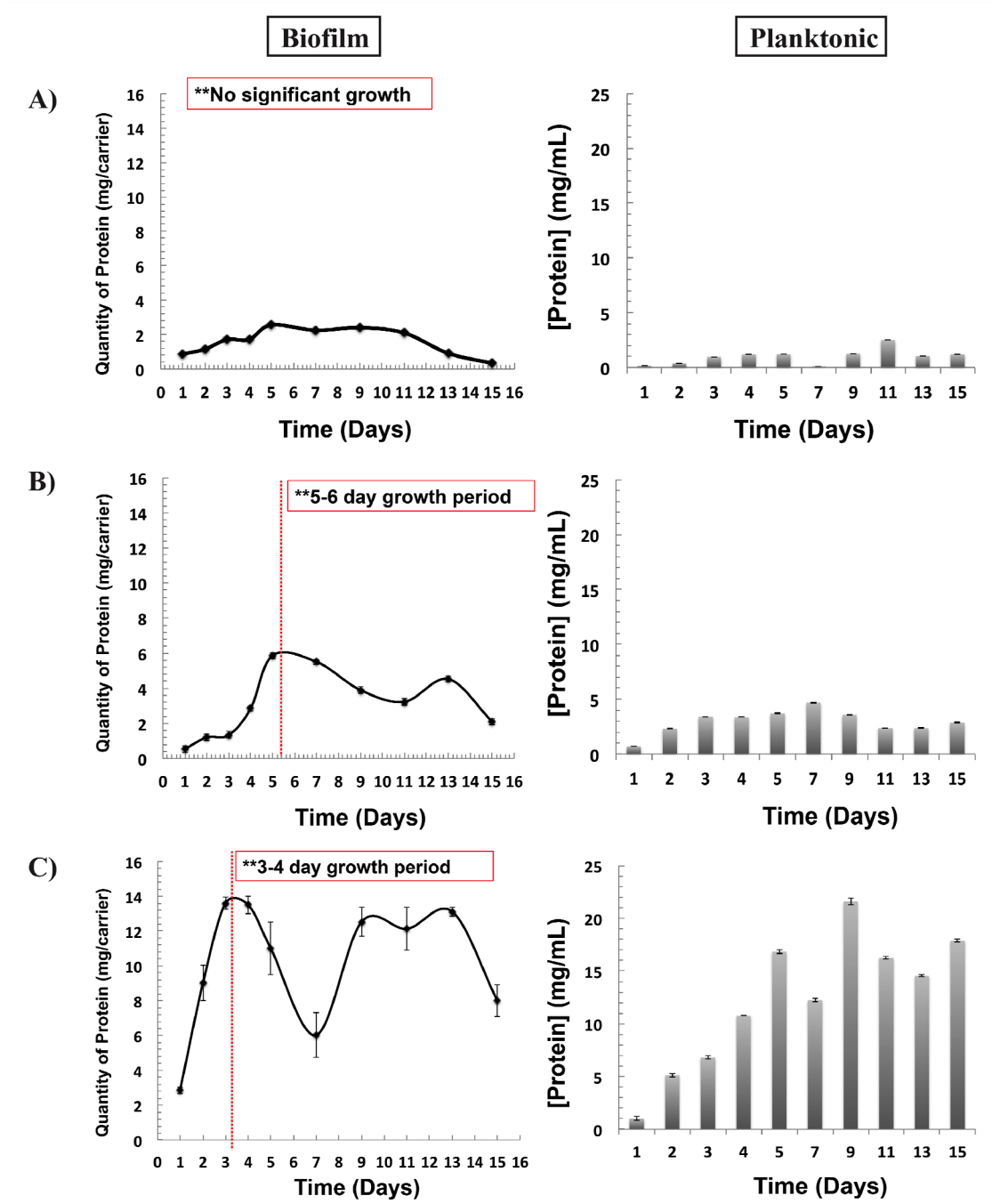


**Figure 3. Biofilm and planktonic biomass quantification of OSPW-derived cultures grown on K1 carrier modified CBD systems as determined by the Bradford protein assay. Biofilm protein concentrations are mg protein/carrier. Cultures were incubated for 14 days at 25 °C and 125 rpm under the following media conditions, A) Bushnell-Haas minimal medium, B) Bushnell-Haas supplemented with yeast extract (1 g/L) and C) tryptic soy broth. Values are presented as the average ±SD, n = 4.**

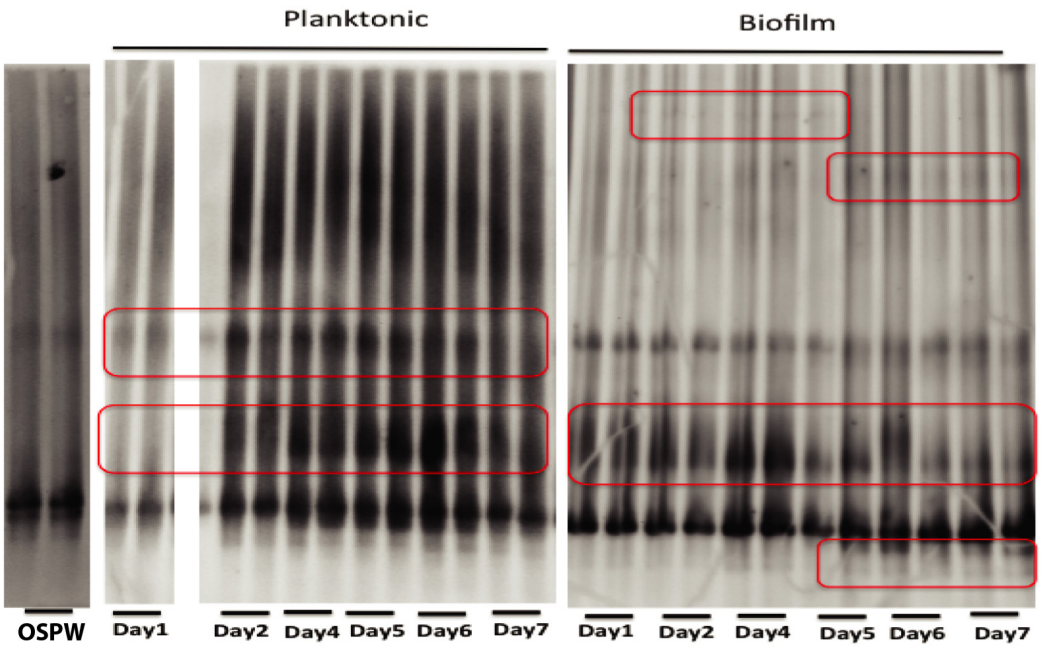


**Figure 4. Denaturing gradient gel electrophoresis (DGGE) was used as a means to examine the biofilm and planktonic population diversity of OSPW-derived cultures grown on K1 carrier modified CBD systems over a 7 day period at 25 °C and 125 rpm using Bushnell-Haas minimal medium amended with yeast extract (1 g/L). DGGE was also performed on the original OSPW inoculum in order to fingerprint the original community for comparative purposes. Note: the red boxes highlight changes in the population over time.**

### *3.2. Logistical considerations of harnessing microbial communities in a modified CBD system for use in seeding moving bed biofilm reactors*

Currently, we have established that our modified CBD system can successfully grow OSPW mixed-species biofilms on commonly used MBBR biofilm carriers. The second objective of this study was to examine various practical aspects towards exploring the feasibility of using our proposed approach to harness environmental bacteria for wastewater bioremediation treatment in an MBBR system. Specifically, we wished to address which biofilm carrier supports the most biomass, what concentration of supplemental nutrients is appropriate, and finally whether biofilms grown on MBBR carriers in a modified CBD system serve as a convenient means to generate an inoculant and start-up a new MBBR.

#### 3.2.1. Comparing biomass on MBBR carriers

Three different MBBR carriers (K1, K5 and Peenox) were compared for their ability to develop OSPW-derived biofilms in the modified CBD system. (K3 carriers were excluded due to limiting resources). The Bradford protein assay was used to monitor biofilm biomass over a 15 day period—all biofilms were grown in BH-Y (1 g/L) media (Figure 5).

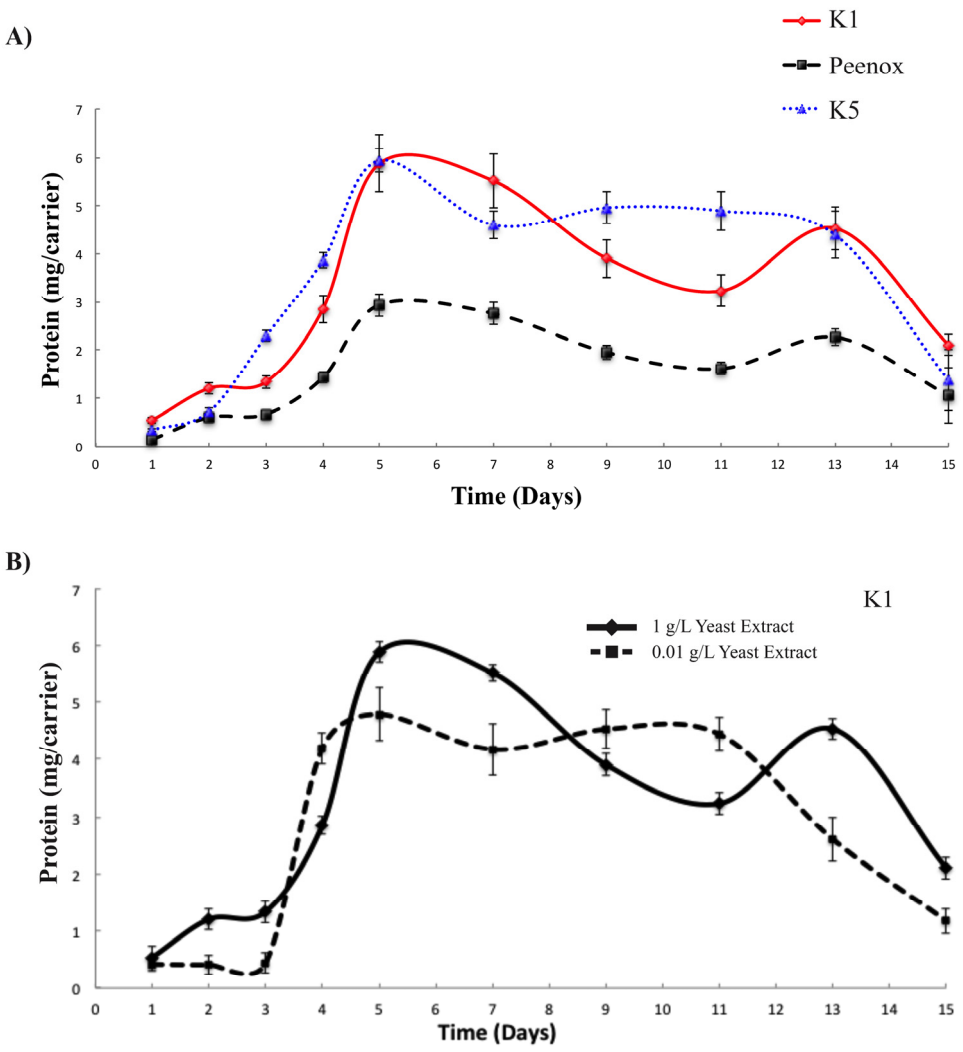


**Figure 5. Biofilm-biomass analysis (via Bradford protein assay) of OSPW-derived cultures grown on A) K1, K5 or Peenox carriers in a modified CBD system using Bushnell-Haas yeast extract (1 g/L) medium, and B) biofilms grown on K1 carriers in a modified CBD system using Bushnell-Haas yeast extract supplemented at either 1 or 0.01 g/L. Values are presented as the average ±SD, n = 4.**

As indicated in Figure 5A, both K1 and K5 significantly outperformed the Peenox carriers. K1 and K5 reached a maximum protein concentration of 6 mg/carrier, while Peenox only achieved 3 mg/carrier. For all carriers, protein concentrations significantly dropped at day 15. We suspect this may be a result of the biofilm reaching a maturation characterized by a greater proportion of quiescent and even dead cells [26,27].

One of the main advantages of the MBBR system when compared to other wastewater treatment systems (for example, activated sludge reactors), is that a high organic loading rate in a comparatively smaller reactor vessel is achievable because of the high concentration of microbial biomass grown on MBBR carriers [4,5]. Thus, the success of a MBBR system is inherently linked to the ability of the chosen carrier to harness biomass [5,9]. In the context of the results presented in this study, we therefore predict that K1 and K5 carriers would be more effective than Peenox carriers in a theoretical MBBR system designed to treat OSPW, on the basis that K1 and K5 carriers

supported the establishment of greater quantity of biomass (Figure 5A). Carrier design elements including effective surface area, presence of protective surface features (fins), surface roughness and surface hydrophilicity can have a significant effect on the ability of the carrier to establish biomass [6]. Effective surface area for biofilm growth has been the focus of previous studies [8], and subsequently greatly enhanced by manufacturers of MBBR carriers over the past couple decades. For example, the K1 AnoxKaldnes carrier has an effective surface area of 500 $m^2/m^3$, while its more recent counterpart, the K5 series AnoxKaldnes carrier, has an effective surface area of 800 $m^2/m^3$. Potential performance ramifications of K5 vs. K1 carriers as a result of effective surface are discussed in section 3.3.

### 3.2.2. Effect of supplemental nutrients concentration on biofilm biomass

The cost-effectiveness and thus feasibility of our modified CBD method for harnessing microbial communities for MBBR wastewater treatment systems is arguably affected by the use of supplemental nutrients to stimulate microbial growth. Our past studies have demonstrated that varying media conditions results in differences and diversity of the microbial communities [15]. In addition, concentrations as low as 0.001 g/L of supplemental nutrients were sufficient to maintain growth and pollutant degradation in OSPW-derived biofilms grown in traditional CBD systems [17]. Here we assessed the effect of supplemental nutrient concentrations on the ability of the community to populate an MBBR carrier. To this end, we examined K1 carriers under media conditions of 1 and 0.01 g/L yeast extract amended BH medium (Figure 5). The use of 100-fold less nutrient supplementation resulted in less biomass on the K1 carrier; 1 g/L amended biofilms maxed out at 6 mg protein/carrier, while 0.01 g/L amended biofilms peaked at 5 mg/carrier (Figure 5B).

### 3.2.3. Verifying transfer of biomass from seed to recipient carriers

Logistically we aimed to decipher whether or not biomass from a single seed carrier could efficiently transfer biomass to virgin recipient carriers. This process is necessary for MBBR startup, and requires cells shed from the seed carrier adhering to, and proliferating new biofilms on sterile recipient carriers.

To this end, K1 seed carriers were grown for 5 days in a modified CBD, and used to inoculate a 1 L flask containing 500 mL of BH-Y (1 g/L) media and 15 virgin K1 recipient carriers. A single K1 seed carrier was used to inoculate this bench-scale MBBR. Seven days post inoculation CLSM was used to evaluate the presence of biofilm biomass on recipient carriers (Figure 6).

Figure 6 confirms that biofilm existed on both inner and outer surfaces of the K1 seed carrier prior to the MBBR inoculation event. Seven days post inoculation, the recipient carrier also had biofilm biomass attached to the inside and outside of the carrier as observed by CLSM (Figure 6).

Protein quantification using the Bradford protein assay for biofilm-biomass was used to determine the timeframe associated with population of recipient carriers. K1, K5 and Peenox recipient carriers were assessed for biomass transfer over a 7 day period (Figure 7).

For all 3 biofilm carrier types, transfer was evident within 24 h, and at least 10 mg protein/carrier had been established by 48 h (Figure 7). In the case of K1 recipient carrier biofilms, the 10 mg protein/carrier observed was 4 mg/carrier more than that observed on equivalent K1 seed carriers (Figure 3).

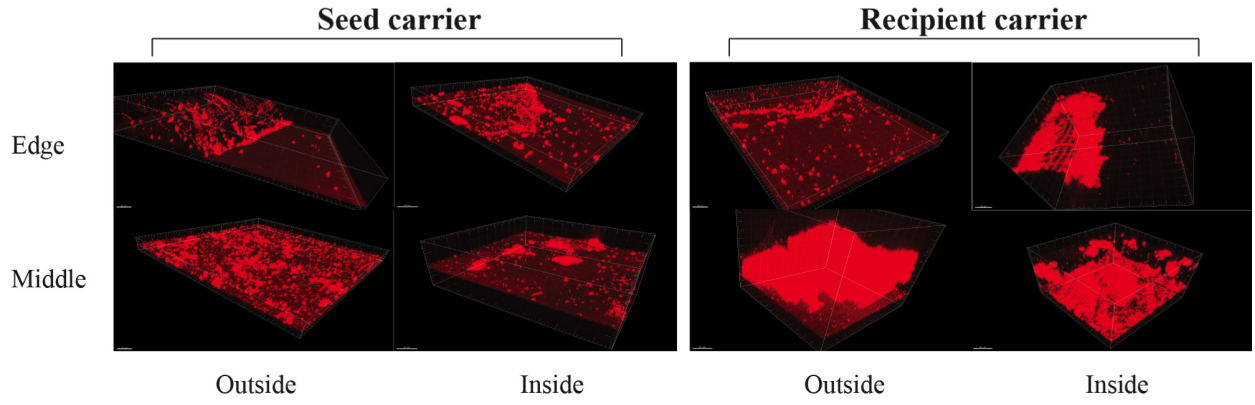


**Figure 6. Confocal laser scanning microscopy images demonstrating biofilm presence on K1 seed carriers grown in a modified CBD system for 5 days, and the resulting biofilms established on sterile recipient K1 carriers in a scaled MBBR-flask system 7 days post inoculation with a seed carrier. Biofilms were grown using Bushnell-Haas yeast extract (1 g/L) medium at 25 °C and 125 rpm. Images were taken at both the inside (protected) and outside (exposed) areas of the K1 biofilm carrier.**

*3.3. Verifying MBBR functionality*

Having established that we can culture mixed-species biofilms on MBBR carriers using a modified CBD system, and that seed carriers developed in this manner can be used to inoculate a bench scale MBBR, it was necessary to confirm that the resultant MBBR system had retained the ability to degrade the OSPW organic contaminants of concern (NAs) as previously observed [17,18]. MBBR flasks were setup and inoculated using OSPW-derived biofilms on K1, K5 and K3 carriers. MBBR media (BH-Y) was supplemented with a synthetic mixture of 8 commercially available model NAs at 200 mg/L total NA concentration (roughly twice the concentration reported in OSPW) [19]. Using GC-FID to monitor NA levels in the MBBR system, we observed a steady decrease in total NA concentration (Figure 8).

By day 28, approximately 80% of the NAs had been degraded by all of the three different carrier/MBBR systems tested (Figure 8). Loss of the NAs can be attributed to microbial metabolism as abiotic, sterile controls exhibited no loss of the NAs (Supplementary material: Figure S2A). Moreover, of the 8 NA structures tested, all exhibited various levels of degradation -save for one particularly recalcitrant NA, adamantane carboxylic acid (ACA)- which correlates with the findings of our earlier studies (Supplementary material: Figure S2B-D) [18].

Curiously, the K5 MBBR system actually degraded 2% more NAs than did the K1 MBBR system over the same 28 day time period; each reactor had an equivalent number of biofilm carriers (1 seed, 15 recipient). This may be attributable to the aforementioned fact that K5 series carriers have an effective biofilm surface area of 800 $m^2/m^3$, while K1 carriers only have an effective biofilm surface area of 500 $m^2/m^3$. The relationship between carrier effective biofilm surface area and extent of contaminant removal has been well documented [6,8].

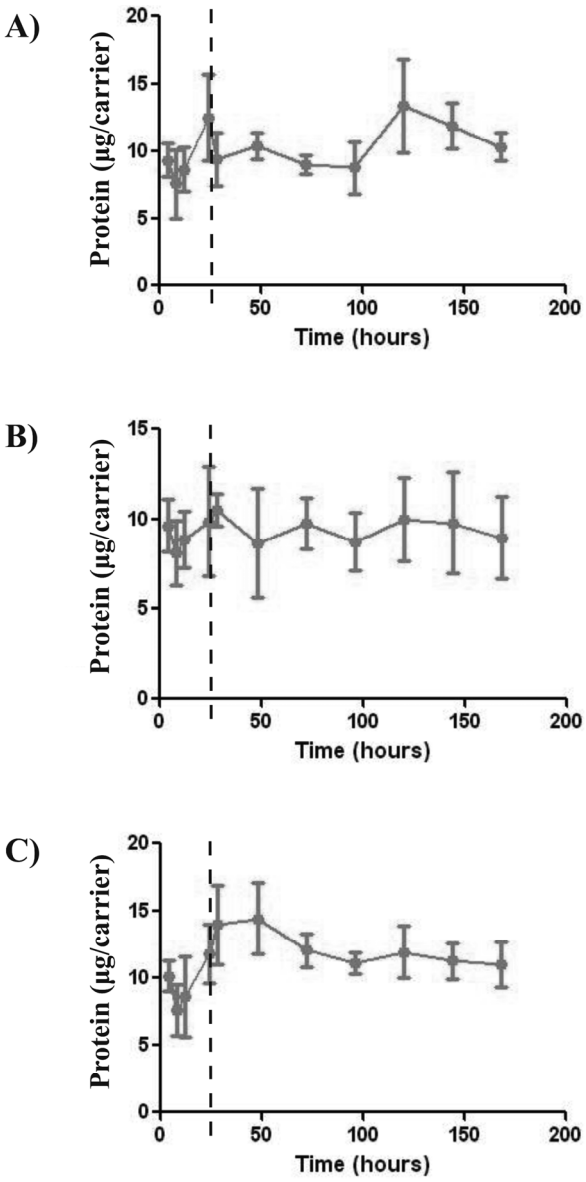


**Figure 7. Biofilm-biomass analysis (measured via the Bradford protein assay) of A) K1, B) K5, and C) Peenox MBBR recipient carriers post inoculation with a same-series seed carrier. Biofilm-biomass was recorded every 4 h until the 48 h time point, after which 24 h elapsed between measurements. All biofilms were grown in scaled MBBR-flask systems using Bushnell-Haas yeast extract (1 g/L) medium. For all three-carrier types, biomass was successfully transferred and established from seed to recipient carriers within 48 hours. Values are presented as the average ±SD, n = 3.**

*3.4. Comparing conventional and modified-CBD MBBR inoculation methods*

In this study we hypothesized that harnessing microbial communities under controlled conditions from contaminated wastewaters directly as biofilms on MBBR carriers may be a choice method to acquire an inoculum to startup an MBBR system to treat the wastewater in question. The data obtained from this study lend support to this hypothesis.

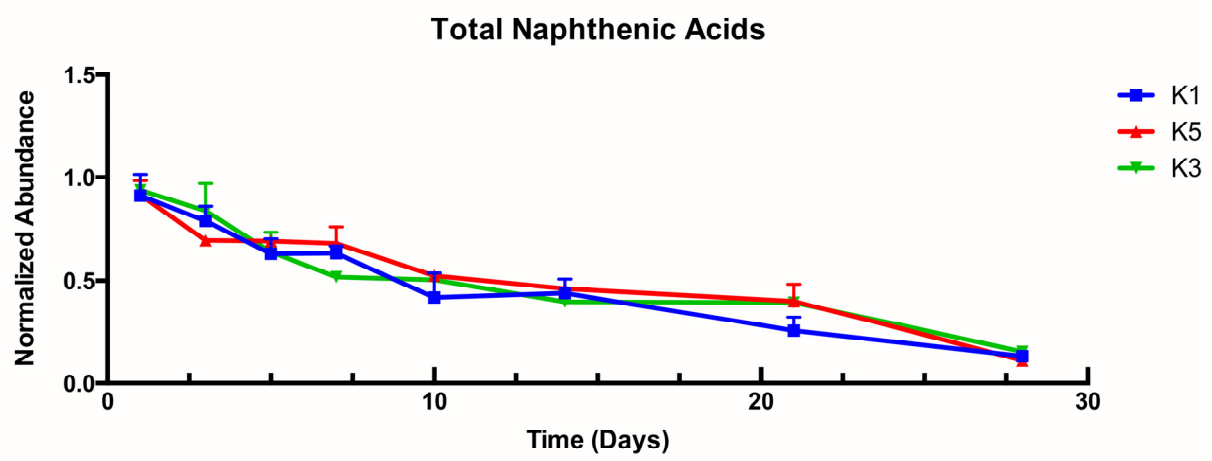


**Figure 8. Naphthenic acid (NA) degradation profiles of MBBR-flask systems utilizing K1, K3 or K5 carriers. For all 3 carrier types NA degradation exhibits no discernable lag phase following inoculation of the MBBR with a seed carrier (day 0), and by day 28 approximately 80% of the model NAs had been removed. Values are presented as the average ±SD, n = 2.**

Using OSPW as a specific case study in which the target pollutant was NAs, this study demonstrated that the modified CBD method could be used to grow biomass on biofilm seed carriers (Figures 2–5), and that this biomass is easily transferable to recipient carriers within an MBBR system (Figures 6–7). Moreover, the freshly inoculated MBBR was fully operational and effective at its task of degrading NAs in as little as 2 days (Figure 8). This relatively quick startup period is in stark contrast to those reported in studies where more conventional MBBR inoculation procedures were employed. MBBR systems are commonly inoculated using waste sludge from an activated sludge system treating similar wastewater (especially when an activated sludge system is being converted to an MBBR), or passively inoculated by simply incubating the wastewater in an MBBR charged solely with virgin carriers. Startup periods ranging from 25–90 days have been reported in the literature for MBBR systems inoculated with activated sludge [4,9,28]. When passively inoculated using wastewater and virgin carriers alone, acclimation and startup periods as long as 6 months have been reported [6,7]. A possible explanation for the quick startup period demonstrated by our modified CBD inoculation method may be related to the fact that we are not reverse-engineering the biofilm community. Activated sludge communities are specifically targeted and adapted for use in activated sludge wastewater treatment systems. Activated sludge microbial processes may start with the wastewater community as a whole, but select for communities containing a low proportion of filamentous microbes, and produce large amounts of extracellular matrix to promote flocculation, and reduce the effect of sludge bulking [5]. The MBBR process does not select for, or require these same characteristics to operate efficiently. Thus, it may be inferred that inoculation of an MBBR system with activated sludge effectively requires a specially adapted sub-community of the whole to reverse its adaptations, and re-adapt for use in an MBBR system. Alternatively, our proposed method of using a carrier-modified CBD to grow MBBR inoculant generates the inoculant directly from the whole wastewater community, and not a previously specialized sub-community.

Although not specifically examined in this study, our proposed modified CBD method for generating MBBR inoculant has other potential advantages that should be explored. Potential exists to cryogenically store seed carriers, which we speculate could allow for quick recovery after reactor spoilage events. Furthermore, cryogenically stored seed carriers charged with specially designed and adapted communities could conceivably become a marketable product. Lastly, the ability to quickly startup a new MBBR may allow for a new inoculum to be periodically harnessed for wastewaters whose characteristics readily change. OSPW for example varies drastically in terms of the prevalent organics and co-contaminating inorganics as different ores and geology are mined for bitumen oil [19].

## 4. Conclusion

In conclusion, the present study demonstrated that OSPW-derived mixed-species microbial biofilms could be grown on high-density polyethylene MBBR carriers, within a modified CBD system, and could degrade NAs. Biofilm growth was manipulated/affected by both nutrient conditions as well as MBBR carrier design characteristics. Moreover, biofilms grown in this manner may be used to seed a bench-scale MBBR containing virgin/sterile carriers. Lastly, evidence of efficient biomass transfer and MBBR functionality presented in the OSPW/NA case study suggests that harnessing microbial communities on carriers affixed within a modified CBD system may represent a facile and rapid method for obtaining functional inoculants for use in wastewater MBBR treatment systems. These advantages presented here in our study, are in contrast to other biological wastewater treatment systems that take months to establish a functional bioreactor.

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## Conflict of Interest

The author declares no conflicts of interest in this paper.

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