COMMUNICATION TO THE EDITOR

BIOTECHNOLOGY and BIOENGINEERING

Influence of Adhesion to Activated Carbon Particles on the Viability of Waterborne Pathogenic Bacteria Under Flow

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ABSTRACT: In rural areas around the world, people often rely on water filtration plants using activated carbon particles for safe water supply. Depending on the carbon surface, adhering microorganisms die or grow to form a biofilm. Assays to assess the efficacy of activated carbons in bacterial removal do not allow direct observation of bacterial adhesion and the determination of viability. Here we propose to use a parallel plate flow chamber with carbon particles attached to the bottom plate to study bacterial adhesion to individual carbon particles and determine the viability of adhering bacteria. Observation and enumeration is done after live/dead staining in a confocal laser scanning microscope. *Escherichiae coli* adhered in higher numbers than *Raoultella terrigena*, except to a coconut-based carbon, which showed low bacterial adhesion compared to other wood-based carbon types. After adhesion, 83–96% of the bacteria adhering to an acidic carbon were dead, while on a basic carbon 54–56% were dead. A positively charged, basic carbon yielded 76–78% bacteria dead, while on a negatively charged coconut-based carbon only 32–37% were killed upon adhesion. The possibility to determine both adhesion as well as the viability of adhering bacteria upon adhesion to carbon particles is most relevant, because if bacteria adhere but remain viable, this still puts the water treatment system at risk, as live bacteria can grow and form a biofilm that can then be shedded to cause contamination.

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Introduction

In rural areas around the world, people often drink untreated ground or surface water (Bifulco et al., 1989; Percival and Walker, 1999), resulting in potential human health impacts, despite preventive measures aimed at removing pathogenic microorganisms. Indeed, water treatment methods cannot completely eliminate or inactivate opportunistic pathogens, like *Aeromonas* spp. and *Pseudomonas aeruginosa*, *Raoultella terrigena*, and *Escherichia coli* since these strains have even been detected within filtration plants in normal potable water supply systems (Payment et al., 1988). Microorganisms in potable water supplies grow as a biofilm on the surfaces of a system, where they are protected against disinfectants and contaminate the water when occasionally shed. Particularly troublesome in this respect is, that shedding is episodic and frequently the microorganisms are not detectable by current methods.

Microorganisms attach to activated carbon particles through strong Lifshitz-Van der Waals forces despite electrostatic repulsion between negatively charged cells and carbon surfaces (Jucker et al., 1996). In low ionic strength solutions, such as potable water systems, electrostatic interactions can be sizable and at the same time offer possibilities to enhance the efficacy of activated carbons to remove microorganisms from water by charge modification of the carbon surfaces. Once there is charge reversal, the electrostatic attraction between negatively charged microbial cell surfaces and positively modified carbon particles will be strong (Bos et al., 1999). This strong interaction has also been shown to have detrimental effects on microbial growth and reproduction (Gottenbos et al., 2001). Also positively charged pDADMAC-coatings of glass surfaces resulted in nearly complete loss of

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viability of bacteria as indicated by membrane disruption upon adhesion (Van der Mei et al., 2008).

There are various systems in use to study bacterial adhesion to surfaces, such as parallel plate and stagnation point flow chambers, batch assays and packed bed reactors. Each of these has its specific advantages and disadvantages. The advantage of a parallel plate flow chamber (Busscher and Van der Mei, 2006) is that adhesion occurs under well controlled mass transport conditions. Previously, we concluded that batch adhesion assays (Busscher et al., 2006), in which carbon particles and bacteria are suspended together under shaking to facilitate adhesion are mass transport limited and not interaction controlled. For interaction controlled assays, sufficient transport of bacteria toward a collector surface is required. Packed bead reactors constitute such a system and indeed packed bed reactors have been applied in bacterial removal by carbon particles (Paramonova et al., 2006), but reproducible packing of the reactor system is difficult. Moreover, the bacterial fate in terms of growth and reproduction upon adhesion to a carbon particle cannot be determined, as generally only breakthrough curves of bacteria passing the system are measured.

The aim of this article is to demonstrate the use of a parallel plate flow chamber to quantify bacterial adhesion to individual carbon particles as well as the fate of the adhering bacteria, that is, whether or not they survive adhesion to the carbon particle. Experiments were conducted with two waterborne pathogens, *R. terrigena* and *E. coli*, and a variety of activated wood-based and coconut carbon particles.

Materials and Methods

Strains and Culture Conditions

R. terrigena ATCC 33257 and *E. coli* ATCC 25922 were cultured in nutrient broth (NB, OXOID, Basingstoke, Great Britain). For each experiment, a preculture was inoculated from nutrient agar into NB and cultured for 24 h. A second culture was inoculated and grown for 16 h. Bacteria were harvested by centrifugation (5 min at 10,000*g*), washed twice with ultrapure water and re-suspended in stabilized water to a concentration of 3×10^8 bacteria per mL. For stabilization, the water was weakly buffered with 0.00025 M KH_2PO_4/K_2HPO_4, yielding an ionic strength of 0.001 M and pH 6.8.

Carbon Particles

A basic wood-based activated carbon, RGC (MeadWestvaco Corp., Carbon Department, Covington, VA), an acidic wood-based activated carbon, CA-10 (Carbochem, Inc., Ardmore, PA), and coconut-based carbon (Calgon Carbon Corp., Pittsburgh, PA) were used as received. Part of RGC was furthermore coated with polyvinyl amine (PVAM) to a level of about 4–5% (w/w) as measured by thermogravimetric analysis. CA-10 and RGC are carbon particles with a total pore volume of around 1.26 mL/g and a volume of pores with diameter larger than 2 nm of about 0.64 mL/g. Coconut-based carbon (total pore volume 0.77 mL/g) had a volume of pores with diameter larger than 2 nm of about 0.15 mL/g. Based on the pore volumes, coconut-based carbon can be classified as microporous and all other carbons used are mesoporous. Coating the RGC carbon with PVAM did not affect the mesoporous character of the carbon particles, but did invert the charge on the carbon particle from −43 to +33 mV in stabilized water.

Carbon particles were sieved upon receipt to obtain a fraction with size between 25 and 50 μm, as determined using the Mastersizer by Malvern Instruments Ltd. (Malvern, UK). Zeta potentials of the carbon particles were previously (Busscher et al., 2006) measured in a 0.001 M (KNO_3) ionic strength suspension by electrophoretic light scattering and laser doppler velocimetry (Brookhaven Zeta Plus Analyzer).

Bacterial Adhesion to Carbon Particles

A stainless steel, parallel plate flow chamber (175 mm × 17 mm × 0.75 mm) was used, with the bottom and top plates made of glass. Five carbon particles were fixed on the bottom plate of the chamber with a micro-manipulator using Pattex Superglue. Care was taken to half-dry the glue, before mounting the carbon particles to prevent spreading of the glue over the surface of the carbon particles. Particles were fixed perpendicular to the direction of flow with a distance of more than 3 mm between particles, to eliminate disturbances of the flow by neighboring particles. Prior to each experiment, all tubes and the flow chamber were filled with stabilized water, while care was taken to remove air bubbles from the system. Flasks, containing the bacterial suspension and stabilized water, were positioned at the same height with respect to the chamber to ensure that immediately after the flows were started, all fluids would circulate through the chamber at the desired wall shear rate of 15 s^{-1} (0.025 mL/s), which yields a laminar flow (Reynolds number of 1.4). The suspension was circulated through the system for 30 min, after which the flow was switched for 15 min to stabilized water without bacteria to remove unbound organisms from the tubes and the flow chamber at the same flow rate. The carbon particles with adhering bacteria were stained with a solution of live/dead *bac*light™ bacterial viability stain (Molecular Probes, Breda, The Netherlands). After staining, fluorescent images (187.5 μm × 187.5 μm) were taken of each particle with a confocal laser scanning microscope (CLSM, Leica TCS SP2, Heidelberg, Germany). Images were taken while scanning over the visible top half of a particle with *z*-increments of 0.5 μm. Bacteria were excited with 488 and 543 nm light and emitted light with wavelength 495–535 nm, arising from viable bacteria, was assigned the color green, while the light

from 580 to 700 nm, arising from non-viable bacteria, was assigned the color red. Note that green for alive and red for dead is an over simplification of the staining method. SYTO 9 labels bacteria with both intact and damaged membranes. In contrast, propidium iodide penetrates only bacteria with damaged membranes, competing with SYTO 9 stain for nucleic acid binding sites, when both stains are present. Red stained bacteria have a damaged phospholipid layer and it is supposed that these bacteria are non-viable. From the literature it is known that certain percentages of these red stained bacteria, depending on the bacterial strain, can be cultured (Auty et al., 2001). For practical purposes we define red as dead and green as alive. To prevent negative effects of the laser on bacterial viability, images were taken immediately after image selection and never twice of the same area.

Subsequently, adhesion was expressed as the total number of dead and live bacteria adhering to a carbon particle per unit area. To this end, bacteria were enumerated from an overlayer image of all CLSM images taken per particle, while the area of the visible top half of a particle was approximated as the one of a half sphere. The radius of the sphere was determined in five directions from the width of a particle as visible in the bottom CLSM image, and its height, that is, the *z*-value in the CLSM optical sectioning at which the particle disappeared out of focus.

Results and Discussion

Figure 1 shows two examples of bacteria adhering to a carbon particle. As can be clearly seen, a percentage of the bacteria lose membrane integrity and potentially viability when adhered and appeared red. Note that analysis of the initial bacterial suspension yields 95% viable bacteria in the culture. A quantitative summary of the results is given in Table I.

First, it can be seen that *R. terrigena* adheres in significantly ($P < 0.05$, Student's *t*-test) smaller numbers to wood-based carbons than *E. coli*, but not to coconut-based carbon, which showed a similarly low adhesion for both strains. Whereas the differences in bacterial adhesion of each strain to other acidic, basic, and positively charged wood-based carbons were small, there were major differences in the viability of adhering bacteria. After 30 min of adhesion, 83–96% of the adhering *R. terrigena* and *E. coli* adhering to an acidic carbon were not viable by method, compared to basic carbon at 54–56%. A positively charged, basic carbon yielded 76–78% loss of viability, while on coconut-based carbon 32–37% were stained red upon adhesion.

It is of interest from a methodological point of view, to compare the results of a previous batch test (Busscher et al., 2006) employing the same strains and carbon particles with the current results. The log-reductions achieved by 2,800 cm^{-2} of carbon particles in one liter of bacterial suspension (*R. terrrigena* and *E. coli*; 5×10^8 bacteria per liter) was used in that paper, but can be recalculated to number of bacteria per cm^2, which will be around 1.8×10^5 per cm^2 for both bacteria to all carbon particles. If we calculate the potential bacterial removal by carbon particles based under the current interaction controlled conditions, we arrive at 3–42 times higher numbers depending on the bacterial strain and carbon particle, than measured in the batch assay. In fact, the current results

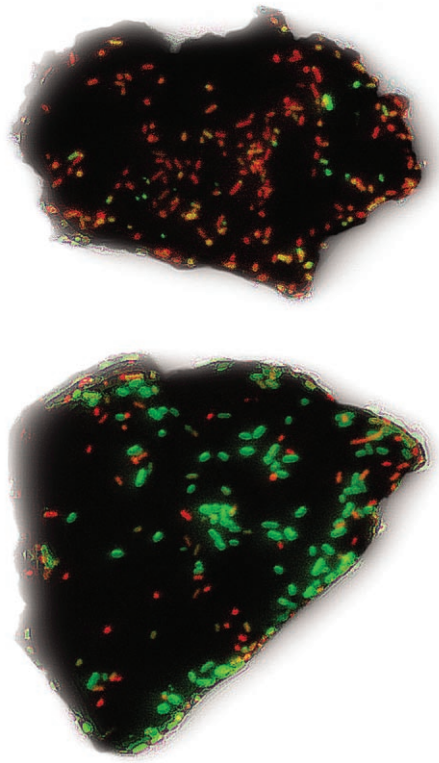

Figure 1. Overlay CLSM images of live/dead stained *R. terrigena* ATCC 33257 adhering to a CA-10 (**top**) and to a coconut (**bottom**) carbon particle. Bar denotes10 μm.

Table I. Number of bacteria adhering to different activated carbon particles (CA-10, acidic carbon; RGC, basic carbon; PVAM, positively charged carbon; coconut, coconut based carbon) after 45 min (30 min adhesion phase and 15 min flowing with stabilized water) of flow, together with the % dead.

	R. terrigena ATCC 33257		*E. coli* ATCC 25922	
Carbon type	N (10^6 cm^{-2})	% Dead	N (10^6 cm^{-2})	% Dead
CA-10	1.8 ± 1.7^{a}	83 ± 26^{a}	7.6 ± 3.1^{a}	96 ± 3^{a}
RGC	$1.1 \pm 0.6^{a,b}$	56 ± 26^{b}	5.6 ± 1.5^{b}	54 ± 6^{b}
PVAM	$1.5 \pm 0.9^{a,b}$	78 ± 27^{a}	4.5 ± 3.2^{b}	76 ± 23^{c}
Coconut	$1.0 \pm 0.9^{a,b}$	37 ± 22^{c}	0.6 ± 0.1^{c}	32 ± 12^{d}

All experiments were conducted for 10 different carbon particles of each type, and in total involved duplicate cultures of bacteria. Data were compared pair-wise using a Student's *t*-test. $a \neq b \neq c \neq d$ at $P < 0.05$ (Student's *t*-test).

suggest that based on the number of bacteria and the available carbon surface area available in the batch assay, complete removal of all bacteria in suspension should have been feasible. This discrepancy confirms that the batch assay is mass transport limited and thus does not properly reflect influences of carbon surface modifications, as this requires an interaction controlled assay (Elimelech, 1994).

At this point it should be emphasized, however, that a batch assay involves considerably more individual particles and bacteria than can be involved in the proposed assay. For the present study, it was chosen to study 10 individual carbon particles of each type, and although a higher number could have been used, this would not have decreased the standard deviations in Table I in any meaningful way (standard deviations only decrease with the square root of the number of observations). Yet, based on observations on 10 particles, we could draw statistically significant conclusions, most notably also on the viability of the bacteria after adhesion.

Both acidic as well as positively charged carbons yield high bacterial viability reduction upon adhesion. These carbon types are therefore to be preferred above other carbon types on which adhering bacteria remain viable and can grow to form a biofilm, since acidic and positively charged carbons are less likely to shed viable organisms in water treatment systems.

Conclusion

(1) Opposite to a batch assay, the proposed assay to study bacterial adhesion to individual carbon particles under flow is interaction controlled and yields the additional advantage of quantifying bacterial viability after adhesion. As a drawback, the number of carbon particles that can be included, is small.
(2) *E. coli* adheres better to wood-based carbons than *R. terrigena*, while both strains adhere in similarly low numbers to a coconut-based carbon.
(3) Both strains adhere in highest numbers to an acidic, wood-based carbon. Moreover, adhesion to the acidic carbon causes the highest loss of viability. However, also the creation of a positive charge on a basic wood-based carbon causes increased loss of viability.

In addition, the methodology allows to evaluate mixtures of different pathogens, which would be especially interesting when combined with the use of specific FISH-probes (Fluorescence in situ hybridization probes).

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