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Control of Antibiotic-Resistant Bacteria in Activated Sludge Using Polyvalent Phages in Conjunction with a Production Host

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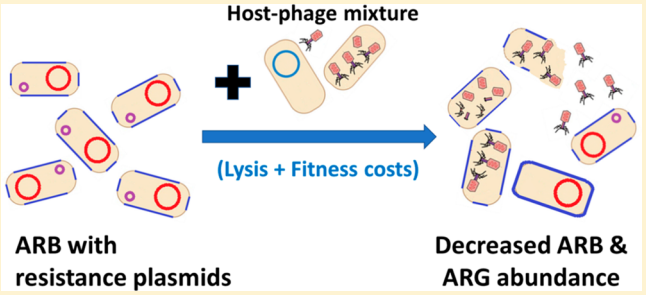
Supporting Information

ABSTRACT: Bacteriophage-based microbial control could help address a growing need to attenuate the proliferation of antibiotic-resistant bacteria (ARB) in wastewater treatment plants (WWTPs). However, the infectivity of commonly isolated narrow-host-range phages decreases quickly upon addition to activated sludge (i.e., plaque-forming units had a half-life of 0.63 h). Here, we show that polyvalent (broad-host-range) phages proliferate and thrive in activated sludge microcosms, especially when added along with their production hosts. Polyvalent phage cocktails (PER01 and PER02) were significantly more effective than narrow-host-range coliphage cocktails (MER01 and MER02) in suppressing a model ARB [β-lactam-resistant *Escherichia coli* NDM-1, initially present at 6.2 ± 0.1 $\log_{10}$ colony-forming units (CFU)/mL]. After 5 days, the NDM-1 concentration significantly decreased to 3.8 ± 0.2 $\log_{10}$ CFU/mL in the presence of the polyvalent phage cocktail, compared to 4.7 ± 0.3 $\log_{10}$ CFU/mL for the coliphage cocktail treatment. Because of the presence of alternative hosts, polyvalent phages reached greater densities, which increased the probability of ARB infection. The fraction of surviving *E. coli* harboring the bla_{NDM-1} resistance gene was also significantly lower for the polyvalent phage cocktail treatment (0.57 ± 0.07) than for the control (0.74 ± 0.08). Therefore, polyvalent phages safely produced with nonpathogenic hosts could offer a novel approach to controlling problematic ARB in WWTPs and mitigating the propagation and discharge of associated resistance genes to the environment.



INTRODUCTION

Phage biocontrol is receiving an increasing level of interest to mitigate the propagation of antibiotic-resistant bacteria.[1] Having co-evolved with bacteria for billions of years, phages possess the capability of infecting their hosts specifically and efficiently, which enables control of problematic bacteria with little impact on the rest of the microbial community.[2] Unlike antibiotics or biocides, whose concentration decreases with time after dosage, phages may continue to replicate and infect the target bacteria.[3] Recently, improved understanding of phage–host interactions has extended phage-based microbial control applications from the medical field to water and wastewater treatment.[4−6]

As with any antimicrobial agent, sufficient phage concentrations [i.e., adequate phage to host ratio, also known as multiplicity of infection (MOI)] must be attained to ensure efficient microbial control.[7,8] However, given the complexity of activated sludge, phages can be removed by adsorption to sludge flocs, suspended particles, and commensal bacteria before infecting their hosts.[9] Furthermore, environmental stresses, such as solar radiation and extreme temperature, salinity, and pH, may also decrease phage numbers and hinder infection.[10,11] Overall, the phage titer is the result of two opposing processes: phage replication after productive infection and phage decay caused by various stressors.[7] This underscores an opportunity to enhance phage proliferation and suppression of antibiotic-resistant bacteria (ARB) in wastewater treatment plants (WWTPs), which is important for mitigating discharge of ARB to the environment.[12]

In this study, a nonpathogenic production host (*Pseudomonas putida*) was used to grow polyvalent phages (PER01 and PER02), and the host–phage mixture was tested for its ability to suppress multidrug (β-lactam)-resistant *Escherichia coli* NDM-1 in activated sludge microcosms. Narrow-host-range coliphage cocktails (MER01 and MER02) were also tested to discern the benefits of phage polyvalence. We propose a novel approach to mitigating ARB proliferation in WWTPs, based on adding polyvalent phage cocktails with their benign production host. This strategy would simplify phage production and improve phage survival and ARB control efficacy.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions. The bacterial strains used in this study are listed in Table 1. *E. coli* NDM-1 carrying the plasmid-encoded bla_{NDM-1} gene is resistant to β-lactam antibiotics.[13] M9 medium was used for the measurement of bacterial and viral growth parameters. Activated sludge

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Table 1. Host Range of Isolated Phages against *E. coli* NDM-1

bacterium	ATCC #	infectivity (EOP)[a]			
		PER01	PER02	MER01	MER02
E. coli NDM-1	BAA-2452	0.89 ± 0.10	0.94 ± 0.07	1.00	1.00
E. coli J53	BAA-196	0.46 ± 0.09	0.43 ± 0.11	*b*	*b*
E. coli K-12	10798	0.82 ± 0.12	0.48 ± 0.09	*b*	*b*
E. coli C3000	15597	0.71 ± 0.08	0.79 ± 0.10	0.57 ± 0.09	0.23 ± 0.11
P. putida F1	700007	1.00	1.00	*b*	*b*

[a]Means ± the standard deviation are results of triplicate measurements. [b]No infection.

(Table S1) for phage isolation and microcosm studies was obtained from the aeration tank of the 69th Street WWTP in Houston, TX.

Isolation of Bacteriophages against *E. coli* NDM-1. A culture-independent phage stock was obtained from activated sludge as previously described.[14] Polyvalent phages (PER01 and PER02), which can infect both *E. coli* strains and *P. putida* F1, were isolated by sequential multihost method A,[14] according to the following sequence: *E. coli* NDM-1 > *P. putida* F1 > *E. coli* K-12 > *P. putida* F1. Coliphages (MER01 and MER02) were isolated by enrichment in *E. coli* NDM-1 liquid monoculture.[15] Isolated phages were purified by the standard procedure and stored in SM buffer.[15] The phages were characterized in terms of morphology, adsorption rate constant, latent time, and burst size (Supporting Information).

Bacterial Challenge Tests in Activated Sludge Microcosms. Microcosm studies were conducted using 1.0 L bottles containing 400 mL of activated sludge with 0.2% glucose at 25 °C. Group 1 microcosms were free of antibiotics, and group 2 microcosms were supplemented with 50 μg/L (each) ampicillin and kanamycin. *E. coli* NDM-1 was introduced at 10^6 colony-forming units (CFU)/mL and incubated for 6 h before being treated with phages. Microcosm treatments were as follows: (a) MER01 and MER02 [5×10^6 phage-forming units (PFU)/mL each], (b) PER01 and PER02 (5×10^6 PFU/mL each), and (c) SM buffer as a control. *P. putida* F1 (10^6 CFU/mL) was also added to all microcosms. During the 5 day experiment, 20 mL sludge samples were collected at 24 h intervals, and 20 mL of M9 medium with 4% glucose was added to sustain volume and nutrient levels.

Isolation and Characterization of Bacteriophage-Insensitive Mutants (BIMs). The isolation of BIMs was performed as previously reported.[16] Briefly, double-layer agar plates were seeded with 10^9 PFU/mL phage and a series of 10^8, 10^7, or 10^6 CFU/mL *E. coli* NDM-1 as the host. After 24 h at 30 °C, the ratio of surviving colonies on the confluent lysis assay plate and the initially inoculated bacteria was calculated as the BIM frequency.[16] Surviving colonies were picked and further purified by co-culture with phages in the soft agar overnight. The specific growth rate and biofilm formation of BIMs were measured to assess the fitness costs of phage resistance.[16] Phage-resistant *E. coli* NDM-1 were challenged with *P. putida* F1 to test the stability of bla_{NDM-1} in various phenotypes of *E. coli* under interspecies competition.

Real-Time Quantitative Polymerase Chain Reaction (qPCR) Analysis. The *E. coli*-specific universal stress protein gene A (*uspA*),[17] bla_{NDM-1} gene,[18] toluene degradation gene (*todC1*),[19] and bacterial 16S rRNA gene[20] were chosen as biomarkers to assess the abundance of total *E. coli*, β-lactam antibiotic-resistance gene, *P. putida*, and total bacteria, respectively (Table S2). Standard curves were prepared using bacterial CFUs (plate assay) and Ct values (qPCR) (Figure S1).

Statistical Analysis. A Student's *t* test (unpaired, two-tailed) was used to determine the significance of the differences between treatments in end point samples. Differences were significant at the 95% level ($p < 0.05$).

RESULTS AND DISCUSSION

Isolation, Enrichment, and Characterization of Bacteriophages. Consistent with previous studies,[21,22] enrichments using high-density, nutrient-rich liquid monocultures of *E. coli* resulted in the isolation of narrow host-range coliphages (MER01 and MER02), while a sequential multiple-host approach preferentially selected for phages with a broader host range (PER01 and PER02) (Table 1). Specifically, optimal foraging theory posits that host discrimination (narrowing of host range) is beneficial under conditions of high host abundance.[23] Phages with rapid propagation rates in the dominant host are more likely to outcompete those with slow propagation rates under planktonic conditions with high host densities.[24] Our sequential multiple-host approach ensures spatial separation of multiple hosts, which dilutes specialist phages and enriches generalist phages in each step.[14]

On the basis of electron microscopy, phage PER01 belongs to the Myoviridae family, PER02 to the Podoviridae family, MER01 to the Myoviridae family, and MER02to the Siphoviridae family (Figure S2). Phages PER01 and PER02 were capable of infecting all isolation hosts (*E. coli* NDM-1, *E. coli* K-12, and *P. putida* F1) as well as *E. coli* C3000 and *E. coli* J53 without a dramatic decrease in the efficiency of plating (0.43−1.00), while coliphages MER01 and MER02 showed a narrower host range, infecting only two of the four tested *E. coli* strains (Table 1). The coliphages (MER series) displayed adsorption rate constants higher than (Figure S3) and latent times shorter than (Figure S4) those of the polyvalent phages (PER series). The estimated growth parameters (Table S3) indicate faster propagation of the coliphages in *E. coli* NDM-1, which is consistent with previous reports that narrow-host-range (specialist) phages generally outcompete polyvalent (generalist) phages in liquid monoculture.[25−27] However, polyvalent phages may more effectively suppress target bacteria in mixed cultures with alternative hosts that facilitate their proliferation. Both phage cocktails (MER and PER series) exerted a significant inhibitory effect on the growth of *E. coli* NDM-1 in monoculture (Figure S5), indicating their potential application as antimicrobial agents.

Phage Polyvalence and the Presence of a Production Host Facilitated Control of ARB in Activated Sludge Microcosms. When added with production host *P. putida* F1, polyvalent phage cocktails (PER01 and PER02) were more effective at suppressing *E. coli* NDM-1 than coliphage cocktails

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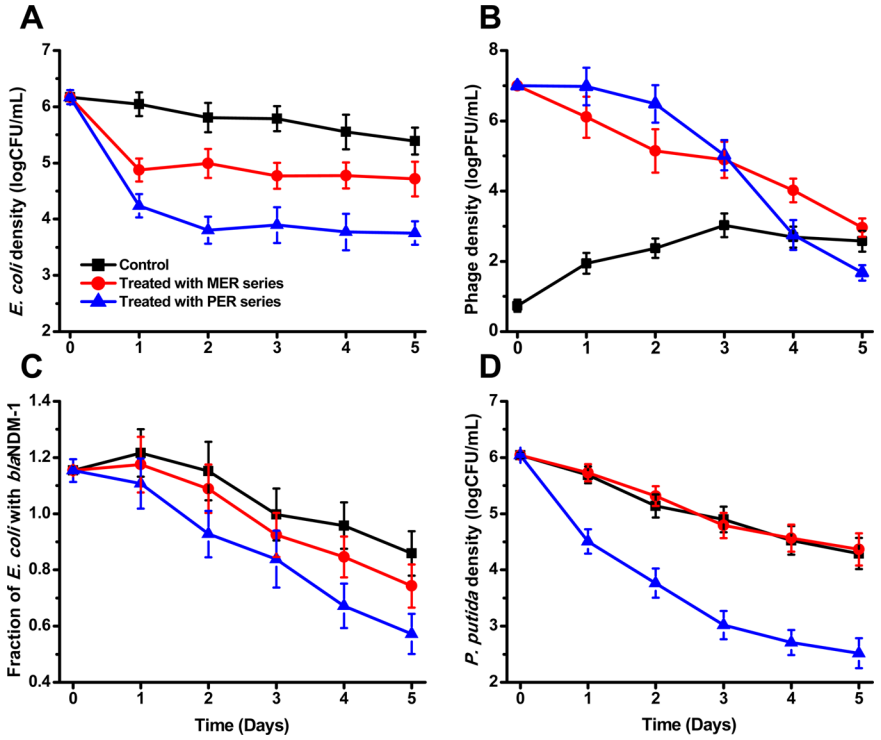

Figure 1. Abundance of (A) *E. coli*, (B) phage (infecting *E. coli* NDM-1), (C) bla_{NDM-1} gene, and (D) *P. putida* in the microcosms spiked with antibiotics. Activated sludge microcosms were spiked with ampicillin and kanamycin (50 μg/L each). The initial *E. coli* density was 6.2 ± 0.1 $\log_{10}$ CFU/mL, and phage treatment used 7.0 $\log_{10}$ PFU/mL phage cocktails amended with 6.0 $\log_{10}$ CFU/mL *P. putida*. Error bars indicate ±one standard deviation from the mean of triplicate independent experiments.

(MER01 and MER02, which were also added with *P. putida* F1 to facilitate comparison). For example, the viable concentration of *E. coli* NDM-1 decreased by 2.4 ± 0.2 orders of magnitude after 48 h in microcosms treated with the polyvalent phage cocktails, which is 1.2 ± 0.1 orders of magnitude lower than that for the coliphage cocktail treatment at a similar time, and represents >99% inhibition (Figure 1A). Treatment with polyvalent phage cocktails was significantly enhanced by co-amendment with the production host, because viable *E. coli* NDM-1 concentrations were 1.1 ± 0.2 orders of magnitude higher in treatments with phages alone after 48 h (Figure S6A). This reflects enhanced phage proliferation in the presence of the production host, which increases the probability of productive infection. Accordingly, the polyvalent phage titer was significantly higher (i.e., by 1.1 ± 0.3 orders of magnitude) than that of the coliphages (Figure 1B) or polyvalent phages in control microcosms without *P. putida* F1 within the first 48 h (Figure S6B). Eventually, as is commonly observed after successful phage treatment,[28] an unsustainable high phage to host ratio was reached, followed by a rapid decrease in polyvalent phage titer after 72 h (Figure 1B,D).

Enhanced polyvalent phage proliferation in nontarget hosts (e.g., *P. putida* F1) can offset the relatively fast phage decay in the activated sludge. In the absence of hosts, PER01 and MER01 decay rates followed first-order kinetics (Figure S7). Decay rate constants were 1.03 ± 0.06 h^{-1} for PER01 and 1.12 ± 0.08 h^{-1} for MER01, which are similar to reported values for laboratory-scale activated sludge systems.[5] When *P. putida* F1 (production host for PER01 but not MER01) was co-amended (MOI = 1.0), the phage density of PER01 increased within the first 2 h and then fluctuated around 6.2 ± 0.2 $\log_{10}$ PFU/mL, while that of MER01 continued to decay in the same pattern (Figure S7).

Phage Cocktails Accelerated the Loss of bla_{NDM-1}. Antibiotic-resistant genes (ARGs) enhanced the survival of multidrug-resistant *E. coli* NDM-1 in the presence of ampicillin and kanamycin (50 μg/L each). In antibiotic-spiked microcosms, *E. coli* densities were significantly higher than those in antibiotic-free microcosms at each comparable exposure time (Figure 1A and Figure S8A). At the end of the 5 day experiment, the *E. coli* density was 5.5 ± 0.2 $\log_{10}$ CFU/mL and the fraction of *E. coli* with bla_{NDM-1} 0.86 ± 0.09 in antibiotic-spiked microcosms, while the corresponding *E. coli* density in antibiotic-free microcosms was 4.0 ± 0.2 $\log_{10}$ CFU/mL and the fraction of *E. coli* with bla_{NDM-1} 0.61 ± 0.07 (Table 2).

ARGs and plasmids exert a metabolic burden associated with their energy requirements for expression, maintenance, and replication[29] and can be prone to segregational instability under growth-limiting conditions or phage infection.[30,31] Accordingly, the plasmid-borne ARG bla_{NDM-1} gene was lost to a significantly greater extent ($p < 0.05$) when *E. coli* NDM-1 was treated with the polyvalent phage cocktails (Figure 1C). In these antibiotic-spiked microcosms, the fraction of *E. coli* containing bla_{NDM-1} was significantly lower upon treatment with polyvalent phage cocktails (0.57 ± 0.07) than that of the control group without phages (0.86 ± 0.09) (Table 2). Overall, the phage cocktails inhibited the growth of ARB (*E. coli* NDM-1) and decreased the fraction of target bacteria harboring bla_{NDM-1}, resulting in a significant reduction in the overall abundance of this ARG.

Development of Phage Resistance (BIM) Incurred Fitness Costs and Promoted Plasmid Curing. The frequency of *E. coli* NDM-1 resistant to both MER01 and MER02 was (1.2 ± 0.4) × 10^{-7}, while the corresponding

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Table 2. Abundance of *E. coli* NDM-1 after a 5 Day Treatment with Various Phage Biocontrol Approaches

microcosm	treatment	*E. coli* density[a]	bla_{NDM-1} gene concentration[a]	fraction of *E. coli* with bla_{NDM-1}[b]
antibiotic-spiked	control	5.54 ± 0.24	5.47 ± 0.24	0.86 ± 0.09
	MER series	4.72 ± 0.31	4.59 ± 0.29	0.74 ± 0.11
	PER series	3.75 ± 0.21	3.50 ± 0.24	0.57 ± 0.07
antibiotic-free	control	3.97 ± 0.23	3.76 ± 0.21	0.61 ± 0.07
	MER series	3.26 ± 0.28	3.01 ± 0.25	0.56 ± 0.07
	PER series	2.72 ± 0.27	2.37 ± 0.25	0.45 ± 0.06

[a]Units are $\log_{10}$ CFU/mL for *E. coli* density and $\log_{10}$(copy number/mL) for bla_{NDM-1} gene concentration. [b]Calculated as the ratio of resistant gene bla_{NDM-1} to *E. coli*-specific gene *uspA*.

frequency resistant to both PER01 and PER02 was $(6.2 \pm 0.5) \times 10^{-7}$. All ($n$ = 50) of the *E. coli* NDM-1 BIMs were bla_{NDM-1} positive as determined by colony PCR and survived exposure to 10 μg/mL kanamycin or ampicillin, suggesting that development of phage resistance alone did not result in resistance plasmid loss. However, development of phage resistance was associated with fitness costs, as observed in previous studies[15,19] (and section 1 of the Supporting Information). The specific growth rate of *E. coli* NDM-1 during exponential growth decreased by 28.5 ± 1.5% because of MER series resistance and by 19.2 ± 2.3% because of PER series resistance. MER series BIMs exhibited a biofilm formation capability decreased by 25.7 ± 1.7%, while PER series BIMs showed an 18.6 ± 1.9% decrease (Figure 2A).

ARGs may be retained by bacteria long after antibiotic exposure stops.[32] In the absence of antibiotics, ARG maintenance and reproduction exerts a metabolic burden that can be exacerbated by interspecies competition and the fitness cost imposed by the development of phage resistance.[29] For example, under interspecies competition with *P. putida* F1, the plasmid-borne ARG bla_{NDM-1} was lost to a significantly greater extent ($p < 0.05$) from phage-resistant *E. coli* NDM-1 than from wild-type *E. coli* NDM-1 (Figure 2B). This suggests that phage biocontrol may accelerate resistance plasmid curing in activated sludge.

Proposed Modified Phage Biocontrol Approach. Our results demonstrate that polyvalent phages may allow the specific control of target bacteria, while having little impact on activated sludge activity [measured as oxygen consumption (Figure S9)]. Importantly, polyvalent phages can be produced using multiple hosts and therefore may circumvent the need to utilize pathogenic or difficult-to-culture hosts during production.[33] Conventional phage biocontrol relies on narrow-host-range bacteriophages that must be produced using the bacterial target, which may create major safety or logistical concerns.[34] Additionally, because polyvalent phages can be produced using benign hosts, there may be no need to purify them from the host prior to use (Figure 3), leading to significant savings in capital and operating costs.

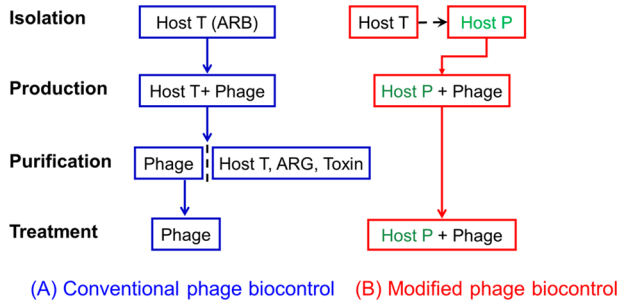

Figure 3. (A) Conventional phage biocontrol approach based on narrow-host-range phages and (B) modified phage biocontrol approach based on polyvalent phages. Host T represents the target host, which is used for phage production in conventional phage biocontrol. In the modified approach, the phage production process is surrogated by benign production hosts (Host P), and the host−phage mixture is used for microbial control.

Whereas polyvalent phages may target multiple resistance carriers and utilize alternative hosts to increase phage titer, not all ARBs present in activated sludge are necessarily susceptible to a specific polyvalent phage. This challenge might be addressed by using trained polyvalent phage cocktails[35] or additional microbial control methods such as effluent disinfection[36,37] or liming of biosolids.[38]

In summary, polyvalent phages can be safely grown and introduced with nonpathogenic hosts to enhance their proliferation in activated sludge, thus enhancing suppression

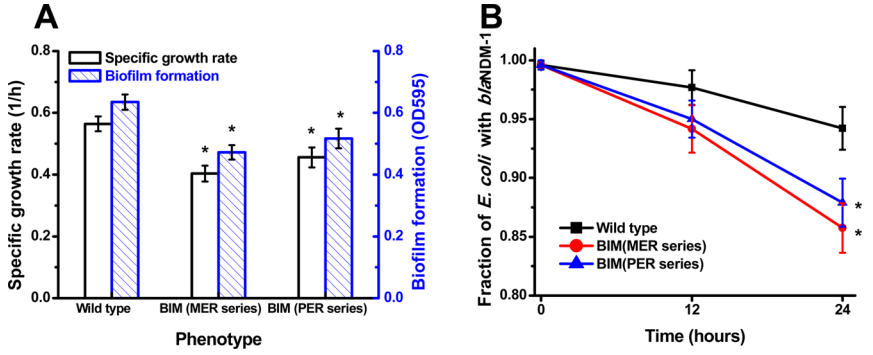

Figure 2. *E. coli* fitness costs associated with phage resistance. Panel A shows decreased specific growth rates and biofilm formation capacity of *E. coli* NDM-1 caused by the development of phage resistance. Panel B shows the faster loss of plasmid-borne antibiotic-resistant gene bla_{NDM-1} (in the absence of antibiotics) during interspecies competition with *P. putida* F1; the initial densities of *E. coli* and *P. putida* were both 6.0 $\log_{10}$ CFU/mL. Error bars represent ±one standard deviation from the mean of 10 replicates. The asterisks denote significant decreases ($p < 0.05$) compared with the value of wild-type *E. coli* NDM-1.

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Environ. Sci. Technol. Lett. 2017, 4, 137−142

of target bacteria. This approach offers the potential to serve as a supplement or alternative to biocides and disinfectants for microbial control of problematic bacteria in activated sludge and may also hold promise for microbiome engineering.[39]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.estlett.7b00045.

Activated microcosm studies; isolation, purification, and characterization of bacteriophages; verification and characterization of BIMs; and PCR and qPCR of selected biomarkers (PDF)

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Notes

The authors declare no competing financial interest.

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Environ. Sci. Technol. Lett. 2017, 4, 137−142