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Effect of pyrolysis on the removal of antibiotic resistance genes and class I integrons from municipal wastewater biosolids

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Wastewater biosolids represent a significant reservoir of antibiotic resistance genes (ARGs). While current biosolids treatment technologies can reduce ARG levels in residual wastewater biosolids, observed removal rates vary substantially. Pyrolysis is an anoxic thermal degradation process that can be used to convert biosolids into energy rich products including py-gas and py-oil, and a beneficial soil amendment, biochar. Batch pyrolysis experiments conducted on municipal biosolids revealed that the 16S rRNA gene, the ARGs *erm*(B), *sul*1, *tet*(L), *tet*(O), and the integrase gene of class 1 integrons (*intI*1) were significantly reduced at pyrolysis temperatures ranging from 300–700 °C, as determined by quantitative polymerase chain reaction (qPCR). Pyrolysis of biosolids at 500 °C and higher resulted in approximately 6-log removal of the bacterial 16S rRNA gene. ARGs with the highest observed removals were *sul*1 and *tet*(O), which had observed reductions of 4.62 and 4.04-log, respectively. Pyrolysis reaction time had a significant impact on 16S rRNA, ARG and *intI*1 levels. A pyrolysis residence time of 5 minutes at 500 °C reduced all genes to below detection limits. These results demonstrate that pyrolysis could be implemented as a biosolids polishing treatment technology to substantially decrease the abundance of total bacteria (*i.e.*, 16S rRNA), ARGs and *intI*1 prior to land application of municipal biosolids.

Water impact

Reuse of wastewater biosolids is critical for sustainable wastewater management. Residual biosolids represent a significant source of antibiotics, antibiotic resistant bacteria, and associated genetic material from biological treatment processes. This research demonstrates that pyrolysis, an anoxic thermal degradation process, could be used to remove antibiotic resistance genes and class 1 integrons from municipal biosolids prior to land application.

Introduction

Wastewater biosolids are a major byproduct from biological treatment processes at water resource recovery facilities (WRRFs). In the United States (U.S.) alone over eight million dry tons of biosolids are produced annually.[1] Biosolids are frequently land applied due to their beneficial soil amendment properties such as high nutrient (N, P) and organic matter content.[2–4] Although biosolids land application has several benefits, this process sends additional pollutants associated with biosolids to the environment, such as organic micropollutants including estrogenic compounds, antimicrobial compounds, and pharmaceuticals and personal care products.[5–7] Residual biosolids also contain elevated levels of antibiotics (*e.g.* tetracycline, sulfonamide), and antibiotic resistance genes (ARGs) are commonly detected in liquid and solid effluent streams from WRRFs and have been detected in agricultural soils amended with biosolids.[8–11]

Antibiotic resistance is a major public health issue,[12] and annual antibiotic resistance-related deaths are expected to increase from 700 000 globally to 10 million by 2050.[13] Unfortunately, the more antibiotics are used the faster antibiotic resistance spreads.[14–16] While antibiotic resistance cannot be stopped, the rate at which it spreads can be slowed by minimizing the release of ARGs into the environment.[12,17] ARGs are considered emerging contaminants[18] because bacteria can acquire them from their environment.[19,20] Additionally, horizontal gene transfer (HGT) of ARGs has been observed between non-pathogenic bacteria and pathogenic bacteria, and even distantly related organisms, such as Gram-positive and Gram-negative bacteria.[21–23] Global efforts should be taken to mitigate the spread of ARGs into the environment.[24] Optimizing antibiotic use in agricultural and clinical settings as well as implementing sanitation and sewage treatment in many developing countries could help mitigate the spread of

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antibiotic resistance.[17,18] Furthermore, residual biosolids represent the effluent stream from WRRFs with the highest concentration of ARGs,[25] and biosolids handling processes could be a control point where the release of ARGs into the environment could be substantially decreased.

WRRFs serve as the primary collection points for commercial, residential, and hospital wastewater effluents that contain a variety of microorganisms and ARGs. The residual solids from the treatment process are of great interest because they contain the vast majority of prokaryotic biomass and ARGs discharged from WRRFs.[26] Several ARGs have been detected in municipal biosolids including, for example, tetracycline resistance genes (*tet*(O), *tet*(W)), sulfonamide resistance genes (*sul*1), and the gene encoding the integrase of class 1 integrons (*intI*1).[10,11] As a result, multiple biosolids handling processes have been investigated with respect to their impacts on ARG removal. Mesophilic anaerobic digestion, air-drying beds, and aerobic digestion processes have all demonstrated the ability to remove ARGs from municipal biosolids to varying extents.[8] However, an increasing demand for higher quality biosolids has driven an interest in more rigorous treatment methods. Alternative methods, such as thermophilic anaerobic digestion,[27] thermal-hydrolysis pretreatment to anaerobic digestion,[28] pasteurization, and lime stabilization[8] have also been analyzed for ARG removal from biosolids. While each of these processes reduce certain ARGs, none have completely eliminated ARGs, and some ARGs even proliferated during anaerobic digestion (*e.g.* *erm*(B), *erm*(F), *tet*(O)).[27,28] Consequently, a biosolids handling process that eliminates ARGs would further mitigate the spread of ARGs in the environment.

Pyrolysis, a thermochemical process that decomposes organic matter at elevated temperatures in the absence of oxygen, is gaining interest for biosolids management applications.[29–32] Pyrolysis reduces the total amount of solids that need to be managed by converting a portion of the solids to a liquid fraction (py-oil) and a gas fraction (py-gas), and the remaining solids are converted to biochar, a stable form of carbon similar to activated carbon.[33–35] Py-oil and py-gas can be combusted for energy,[36] and biochar has multiple agricultural benefits including improved soil fertility and nutrient retention.[37,38] Previous research has demonstrated that the energy required for pyrolysis was approximately 5-fold less than the energy required to dry biosolids, therefore a WRRF already using energy to dry biosolids would not significantly increase its energy needs with the addition of pyrolysis treatment.[30] In fact, energy can be recovered on-site from the py-gas that is produced. Pyrolysis is best suited as a polishing step after anaerobic digestion and dewatering. For utilities that produce wet biosolids, implementing pyrolysis may be costly due to the energy required to dry the biosolids.[30] Certainly the energy costs associated with pyrolysis increase as the moisture content of the solids increases. Thus, individual WRRFs would need to conduct cost–benefit analyses to determine how the benefits of pyrolysis compare to the energy costs associated with pyrolysis of their specific biosolids.

Previous research has demonstrated the ability of pyrolysis to remove recalcitrant organic micropollutants such as estrogenic compounds, triclosan, triclocarban, and nonylphenol.[32,35] Pyrolysis of wastewater biosolids at 450 °C removed 75% of polychlorinated biphenyls (PCBs),[39] and greater than 99% reduction of PCBs and dioxins was observed from pyrolysis of contaminated sediment at 800 °C.[40] Moreover, a previous study documented greater than 3-log reduction of *Escherichia coli* after thermal treatment of wastewater sludge at 80 °C.[41] These findings suggest that pyrolysis could provide a means for ARG removal from biosolids prior to land application due to high operational temperatures (typically >450 °C). To our knowledge, no research has been conducted regarding the effects of pyrolysis on the removal of ARGs or class I integrons from wastewater derived biosolids.

The objective of this research was to determine the impact of pyrolysis on the removal of the 16S rRNA gene, ARGs including *erm*(B), *sul*1, *tet*(L), and *tet*(O), and the gene encoding the integrase of class 1 integrons (*intI*1). It was hypothesized that pyrolysis would decrease the abundance of the 16S rRNA gene, ARGs, and *intI*1 following pyrolysis treatment due to decomposition of amplifiable DNA representing these genes. Tetracycline and sulfonamide resistance genes (*tet*(O), *tet*(L) and *sul*1) were quantified in this study due to the prevalent use of tetracycline and sulfonamide as antibiotics in human and veterinary medicine.[42,43] *Sul*1 is also one of the most commonly detected sulfonamide resistance genes in the environment.[44] The *erm*(B) gene is generally found on conjugative genetic elements and encodes resistance to macrolides, lincosamides, and streptogramin antibiotics.[45] Additionally, *intI*1 was quantified in this study as it is considered to be a genetic element substantially contributing to the proliferation and evolution of multiple antibiotic resistant bacteria in the environment.[27,46] Briefly, laboratory-scale pyrolysis experiments were performed on heat-dried biosolids, and the abundance of 16S rRNA, the integrase gene of class 1 integrons (*intI*1), and the ARGs *erm*(B), *sul*1, *tet*(L), and *tet*(O) were quantified *via* quantitative polymerase chain reaction (qPCR).

Methods

Pyrolysis temperature and reaction time experiments

Experiments were set up to determine the effect of pyrolysis on total bacterial 16S rRNA, ARGs, and class I integrons. Pyrolysis of biosolids was performed by adding approximately 10 grams of biosolids to 250 mL flasks in triplicate. The biosolids feedstock was a heat-dried blend of waste activated sludge and anaerobically digested primary solids from a municipal WRRF. These biosolids were chosen over wet biosolids because pyrolysis is ideal as a polishing step to recover energy from already dried biosolids.[30] The flasks were sparged for ten minutes with argon gas, covered with aluminum foil, and heated in a muffle furnace (Fisher-Scientific

Isotemp®, Waltham, MA) similar to previous studies that utilized heat-dried biosolids as a feedstock for pyrolysis.[29,30,32,34,47–50] Flasks were placed in the furnace for a one-hour retention time at temperatures ranging from 100 to 700 °C to determine the effect of temperature on ARG removal. A room temperature control (20 °C) was prepared in the same manner and placed in the oven with no heat for one hour. "Influent" biosolids samples were generated by leaving the flask filled with biosolids on the bench-top for one hour. Biochar yields were determined for each pyrolysis temperature by the following equation: (mass of biochar after pyrolysis (g)/initial biosolids mass (g)) × 100%. The impact of pyrolysis reaction time was determined at 500 °C with reaction times of 2.5, 5, 15, 30, and 60 minutes. Samples were stored at −20 °C until DNA extraction was performed.

DNA extraction

Biosolids samples were homogenized using a sterile mortar and pestle then approximately 0.2 g of biosolids were subsampled for DNA extraction. DNA was extracted using the FastDNA® SPIN Kit (MP Biomedicals LLC, Solon, OH) by manufacture's protocol utilizing 1.0 mL of the CLS-TC lysis buffer with a modified cell lysis procedure instead of bead-matrix homogenization. Cells were lysed by liquid nitrogen freeze thaw cycling (3×) to improve yield.[51] DNA concentrations were determined by microspectrophotometry (NanoDrop™ Lite, Thermo Scientific, Waltham, MA). DNA extracts were stored at −20 °C for further analysis.

qPCR for antibiotic resistance genes and *intI*1 quantification

qPCR was performed for ARGs, the integrase gene of class I integrons (*intI*1), and the 16S rRNA gene. The total reaction volume (20 μL) consisted of 10 μL PowerUp™ SYBR® Green Master Mix, 2 μL each of 10 μM forward and reverse primers, 5 μL of diluted DNA extract, and 1 μL molecular-grade water. DNA extracts were diluted with molecular-grade water to 5 or 10 ng μL^{-1} (total of 25 or 50 ng DNA in qPCR reaction) to remove inhibitor substances and to fall within the range of the qPCR standard curve.

Thermal cycling and fluorescence detection were conducted on a Roche LightCycler® 96 (Roche Molecular Diagnostics, Pleasanton, CA). Thermal cycling conditions were as follows: 2 min at 50 °C to activate the uracil-DNA glycosylase (UDG), 10 min at 95 °C to inactivate UDG and activate the DNA polymerase, 40 cycles of 95 °C for 30 s, 60 °C for 30 s, followed by 72 °C for 30 s. Following each qPCR, melting curves were generated and analyzed to verify specific amplification based on the positive control (standard). Gene concentrations for each sample were quantified in triplicate, and the mean value was used for subsequent statistical analysis. If only two of three replicates yielded positive detections on the qPCR assay then the mean value of the two positive replicates was used in subsequent analyses.[52] In the event that positive quantification was found for only one replicate or no replicates then the detection limit was used as the reported value. The final reported values for gene copies per g of biochar were a function of the detection limit for qPCR as well as the DNA yield from the biochar sample and amount of biochar extracted. Thus, if experiments from two temperatures such as 500 °C and 700 °C resulted in qPCR reads below detection limit the 700 °C result could be higher because of differences in DNA yield and biochar extracted.

The quantity of the target gene in unknown samples was calculated based on a standard curve generated using known quantities of plasmids bearing the target gene (either the pUC19 or pGEM-T Easy (Promega, Madison, WI) plasmids). The primers and probes along with the annealing temperatures used for resistance genes were previously developed.[53–56] Standard curves (five-point minimum) for qPCR were produced by ten-fold serial dilution of plasmid DNA yielding 10^8 to 10^0 target gene copies per reaction. R^2 values were greater than 0.99 for all standard curves used to quantify target genes in this study and no template controls were included in each assay. To compare absolute reductions of target genes, gene quantities are presented normalized to grams of dry biosolids. Specific primer sets, annealing temperatures, efficiencies, and detection limits are described in Table 1.

Data analysis

Copy number of the target gene were $\log_{10}$ transformed to meet the assumptions of normality for statistical analysis.[8,53] The absolute copy numbers of each gene are presented in this study rather than normalized to 16S rRNA since target genes were reduced to below detection limits in most experiments. GraphPad Prism (V 7.02, La Jolla, CA) was used to perform analysis of variance (one-way ANOVA) and *t*-tests. Tukey's *post hoc* multiple comparisons test was used to determine significant differences between each pyrolysis condition.

Results and discussion

Pyrolysis temperature experiments

The impact of pyrolysis temperature on the removal of the bacterial 16S rRNA gene, ARGs, and the *intI*1 gene from municipal wastewater biosolids was determined in batch pyrolysis experiments. Pyrolysis reactions were successful as confirmed by quantifying biochar yields (Fig. 1). Increasing pyrolysis temperatures resulted in a significant decrease in biochar yield ($p < 0.0001$). At 500 °C, biochar yield was approximately 43%, which is congruent with previous biochar yield from pyrolysis of biosolids.[34] Previous studies have reported that the decrease in biochar yield as temperature rises is likely due to the destruction of organic matter such as cellulose, hemicellulose, and lignin.[57] Cellulose drastically reduces weight from 275–350 °C, and lignin reduces weight linearly with increasing temperature from 250–500 °C.[58] The reduction in biochar yield at pyrolysis temperatures of 300–700 °C likely resulted in the concomitant destruction of prokaryotic biomass and genetic material such as DNA.

Table 1 Primers, annealing temperatures, efficiencies, and detection limits for qPCR analysis of target genes

Gene	Annealing temperature (°C)	Forward primer & reverse primer	Efficiency average range (%)	Detection limit (copies per μL)	Detection limit (copies per g)	Reference
16S rRNA	60	F-(5′-CCTACGGGAGGCAGCAG-3′) R-(5′-ATTACCGCGGCTGCTGG-3′)	95.5–104.5%	500	8.2×10^4	73
erm(B)	60	F-(5′-AAAACTTACCCGCCATACCA-3′) R-(5′-TTTGGCGTGTTTCATTGCTT-3′)	104–104.5%	5	8.2×10^2	55
*intI*1	60	F-(5′-CCTCCCGCACGATGATC-3′) R-(5′-TCCACGCATCGTCAGGC-3′)	104.5–108.5%	5	1.6×10^3	54
*sul*1	60	F-(5′-CCGTTGGCCTTCCTGTAAAG-3′) R-(5′-TTGCCGATCGCGTGAAGT-3′)	95.5–97.5%	50	8.2×10^3	53
tet(L)	60	F-(5′-TCGTTAGCGTGCTGTCATTC-3′) R-(5′-GTATCCCCACCAATGTAGCCG-3′)	92.5%	50	8.2×10^3	56
tet(O)	60	F-(5′-AAGAAAACAGGAGATTCCAAAACG-3′) R-(5′-CGAGTCCCCAGATTGTTTTTAGC-3′)	99–100%	5	8.2×10^2	55

Quantification of the 16S rRNA gene (Fig. 2) was performed to determine the impact of pyrolysis on the removal of total bacterial biomass from municipal biosolids. There was no significant difference in 16S rRNA gene abundance between the biosolids feedstock (*i.e.* the influent biosolids), the room temperature samples (20 °C) ($p > 0.98$), or the 100 °C samples ($p > 0.53$). There was a significant 4.62-log reduction in the 16S rRNA gene observed for 300 °C biochar compared to the biosolids feedstock ($p < 0.0001$). Approximately 6-log reduction in the 16S rRNA gene was observed for biochar produced at 500 °C ($p < 0.0001$) and 700 °C ($p < 0.0001$). Both pyrolysis conditions removed the 16S rRNA gene to levels below the detection limit (8.2×10^4 copies per g biochar). Compared to other biosolids treatment processes, the reduction in total bacterial biomass (*i.e.*, 16S rRNA) observed in this study was approximately five orders of magnitude greater than removal observed in air-drying beds and thermophilic anaerobic digestion employed for treatment of municipal biosolids.[8,53] These results indicate that pyrolysis of wastewater biosolids could decrease the amount of total bacterial biomass released to the environment when land applying biosolids-derived biochar relative to land application of biosolids.

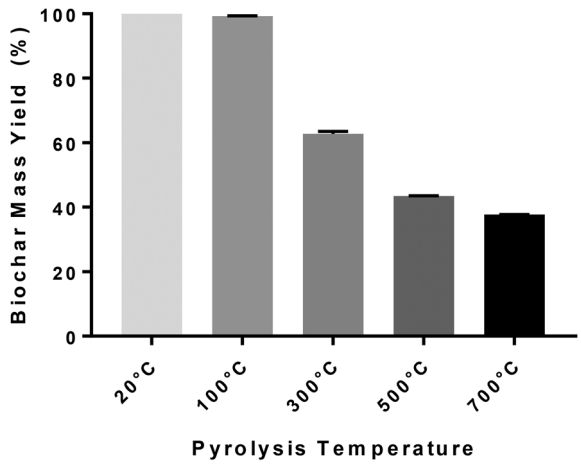

Fig. 1 Impact of pyrolysis temperature on biochar mass yield. Bars represent the average yield and error bars represent the standard deviation among triplicate experiments ($n = 3$).

Quantification of four ARGs and the *intI*1 gene was performed to determine the impact of pyrolysis on the removal of various classes of resistance genes (Fig. 3). All genes quantified in this study were selected due to their frequent detection in municipal biosolids.[27,53] All four ARGs and the *intI*1 gene significantly decreased in abundance compared to the biosolids feedstock as pyrolysis temperatures increased above 300 °C ($p < 0.0001$). Observed reductions in ARG quantities ranged from 2.2 to 4.2-log at pyrolysis temperatures of 500 °C and greater compared to the biosolids feedstock.

ARGs with the highest observed removals were the *sul*1 and *tet*(O) genes, which had observed reductions of 4.20 and 4.04-log, respectively. Sulfonamide resistance genes, such as *sul*1, are frequently detected in residual biosolids.[25,53] The *sul*1 gene is generally associated with class 1 integrons on conjugative plasmids and is a good indicator of HGT and multiple antibiotic resistance.[45] The *tet*(O) gene is commonly

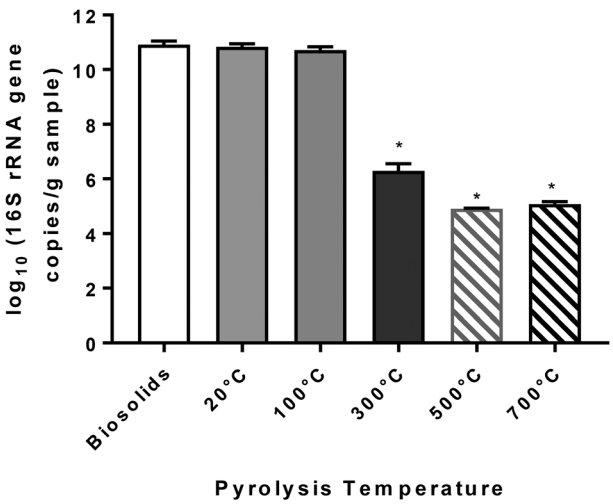

Fig. 2 Impact of pyrolysis temperature on the removal of the 16S rRNA gene. Bars represent the average concentration and error bars represent the standard deviation among triplicate experiments ($n = 3$). Solid fill bars indicate values detected within the standard curve and striped bars indicate values below the detection limit. Values that were below the detection limit were reported as the detection limit. Statistical differences from the biosolids control are indicated with a star ($p < 0.05$).

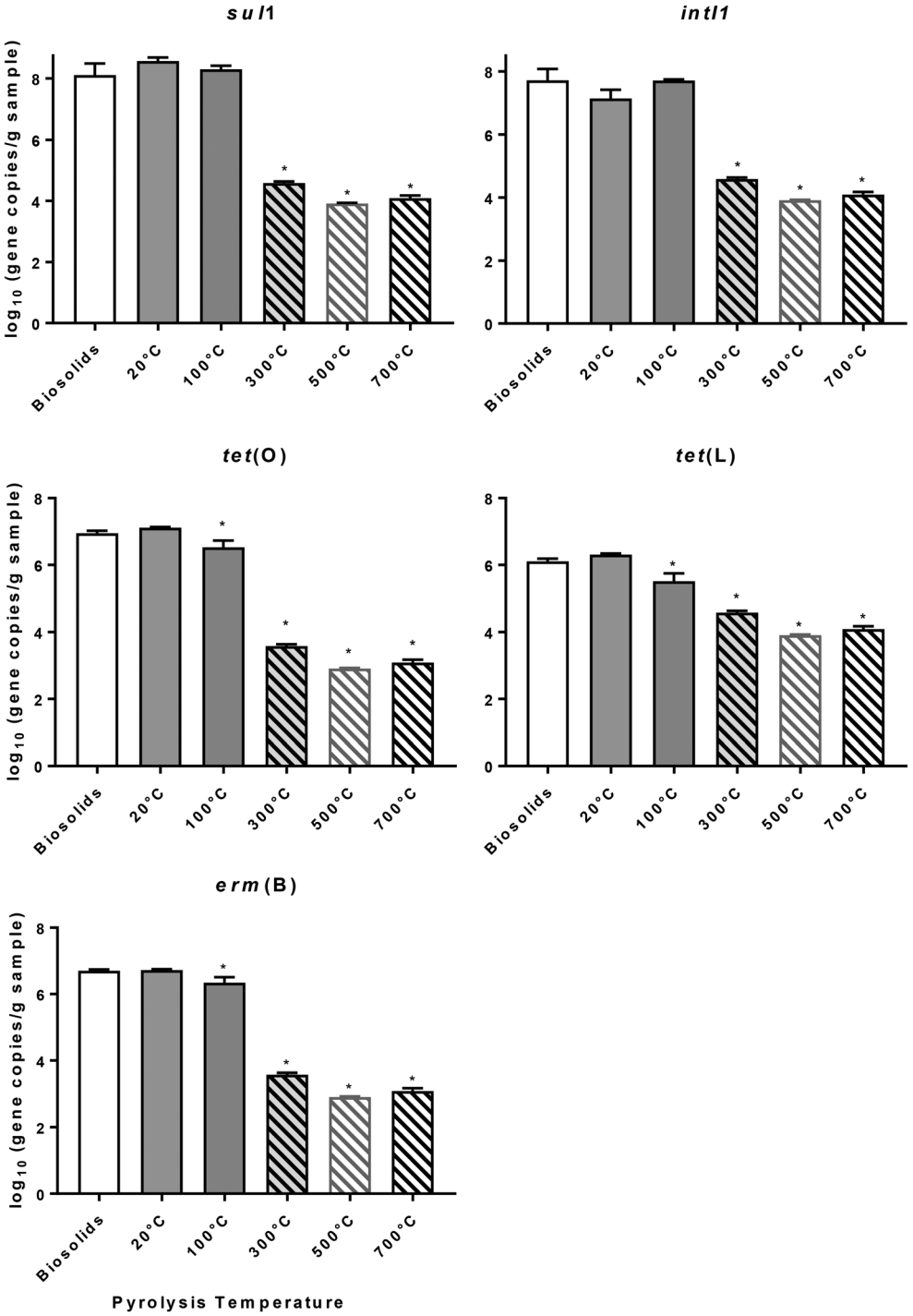

Fig. 3 Impact of pyrolysis temperature on the removal of ARGs and *intI*1. Bars represent the average concentration and error bars represent the standard deviation among triplicate experiments (n = 3). Solid fill bars indicate values detected within the standard curve and striped bars indicate values below the detection limit. Values that were below the detection limit were reported as the detection limit. Statistical differences from the biosolids control are indicated with a star ($p < 0.05$).

associated with ribosomal protection in aerobic bacteria, and can be found in conjugative plasmids or in the chromosome.[59] Tetracycline resistance genes such as *tet*(L) and *tet*(O) are commonly detected in influent and effluent streams in WRRFs and have been shown to increase in abundance with increasing concentrations of influent tetracycline.[60] The *tet*(L) gene encodes for an efflux pump, and has been found in Gram-positive and Gram-negative bacterial isolates.[43] In the current study, the *tet*(L) gene was removed to a lesser degree compared to *tet*(O), with an observed 2.2-log reduction compared to the biosolids feedstock. The abundance of the *tet*(L) gene, however, was lower than that observed for the *tet*(O)

gene in the biosolids feedstock, which contributed to higher observed removal for the *tet*(O) gene relative to the *tet*(L) gene. Additionally, the detection limit for *tet*(L) was one order of magnitude higher than that observed for *tet*(O), which also contributed to the lower observed removal of the *tet*(L) gene compared to *tet*(O).

Similar to tetracycline resistance genes, pyrolysis temperatures ≥300 °C significantly decreased the abundance of the *erm*(B) and *intI*1 genes in the resultant biochar ($p < 0.0001$) compared to the biosolids feedstock. Observed reductions in gene quantities were 3.79 and 3.80-log for the *erm*(B) and *intI*1 genes, respectively. Both genes were removed to levels below the detection limit in biochar produced from 300 to 700 °C. As all genes quantified in this study were reduced below the detection limit of the qPCR assays at temperatures greater than 300 °C, log removal was dependent on the initial abundance of target genes in the municipal biosolids feedstock. We expect that the reduction in ARG and *intI*1 genes during pyrolysis was due to the destruction of DNA (intracellular and extracellular).

Previous studies have documented that anaerobic digestors can physically destroy extracellular DNA through hydrolysis and biodegradation processes.[28] However, ARGs may also be harbored by host bacterial cells and subject to amplification *via* cell growth or HGT.[61,62] Similarly, other biosolids treatment technologies such as pasteurization and alkaline stabilization are known to aggressively inactivate pathogens, but failed to significantly decrease levels of the *erm*(B), *sul*1, and *intI*1 genes in wastewater solids prior to being applied to soil microcosms.[8] The current study demonstrates that pyrolysis can be used as a biosolids treatment technology to substantially reduce levels of ARGs and the *intI*1 gene in municipal biosolids prior to land application.

Pyrolysis reaction time experiments

Quantification of the 16S rRNA gene (Fig. 4) was performed to determine the impact of pyrolysis reaction time on total bacterial abundance in wastewater biosolids. Greater than 85% of 16S rRNA gene copies were removed from biosolids with a pyrolysis reaction time of only 2.5 minutes at 500 °C ($p < 0.05$). A significant 3.5-log reduction was observed for biosolids with a 5 minute pyrolysis retention time with respect to the biosolids feedstock ($p < 0.0001$). There was not a significant difference in 16S rRNA abundance in biochar produced at 15 and 30 minutes ($p > 0.55$). Compared to the biosolids feedstock, there was a significant 4.05 and 4.39-log reduction in 16S rRNA for biochar pyrolyzed for 15 and 30 minutes, respectively (p values <0.0001). Similarly, a significant 4.87-log reduction in bacterial 16S rRNA was observed for biosolids pyrolyzed for 60 minutes ($p < 0.0001$). These results indicate that pyrolysis reaction time has a substantial impact on the quantity of bacterial gene markers present in the resultant biochar.

Quantification of the ARGs *erm*(B), *sul*1, *tet*(L), *tet*(O), and the integrase gene of class 1 integrons (*intI*1) was performed to determine the impact of pyrolysis reaction time on the removal of various resistance genes (Fig. 5). All four ARGs and the *intI*1 gene significantly decreased in abundance after a pyrolysis reaction time of 2.5 minutes ($p < 0.0005$). Biosolids with a pyrolysis residence time of 5 minutes resulted in the reduction of all ARGs and the *intI*1 gene to below detection limits ($p < 0.0001$), with log removals ranging from 2.14 for the *tet*(L) gene to 4.62 for the *sul*1 gene. Similar to the results from pyrolysis temperature experiments, the ARG with the highest observed removal rate was the *sul*1 gene. In the current study, the abundance of the *sul*1 gene in the biosolids influent samples was 2.56×10^8 copies per g dry weight on average. The results observed in this study are congruent with previous studies that have reported *sul*1 as one of the most prevalent ARGs detected in municipal WRRFs.[18,25,63]

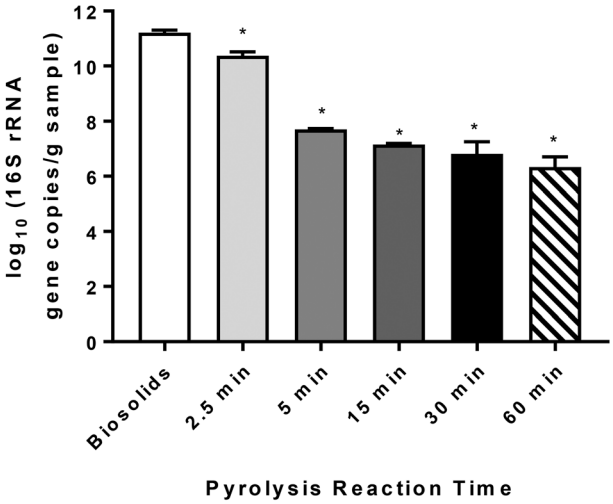

Fig. 4 Impact of pyrolysis reaction time on removal of the 16S rRNA gene from biosolids at 500 °C. Bars represent the average concentration and error bars represent the standard deviation among triplicate experiments (n = 3). Solid fill bars indicate values detected within the standard curve and striped bars indicate values below the detection limit. Values that were below the detection limit were reported as the detection limit. Statistical differences from the biosolids control are indicated with a star ($p < 0.05$).

ARGs such as sulfonamide resistance genes can proliferate in biological processes at WRRFs and previous studies have reported *sul*1 concentrations of up to 10^{11} copies per g dry weight in dewatered sludge.[25] The *sul*1 gene is generally harbored in class 1 integrons containing multiple resistance genes, and encodes dihydropteroate synthase that is not inhibited by sulfonamides.[64] The *intI*1 gene was also detected at elevated levels in municipal biosolids in the current study, with an observed abundance of 6.66×10^7 copies per g dry weight on average. This is congruent with previous research that observed a positive correlation between *sul*1 and *inti*1 in the feed and effluent of anaerobic and aerobic digesters.[28] The enrichment of class 1 integrons and ARGs such as *sul*1 in biological treatment processes at WRRFs underscores the need for rigorous biosolids treatment technologies that can

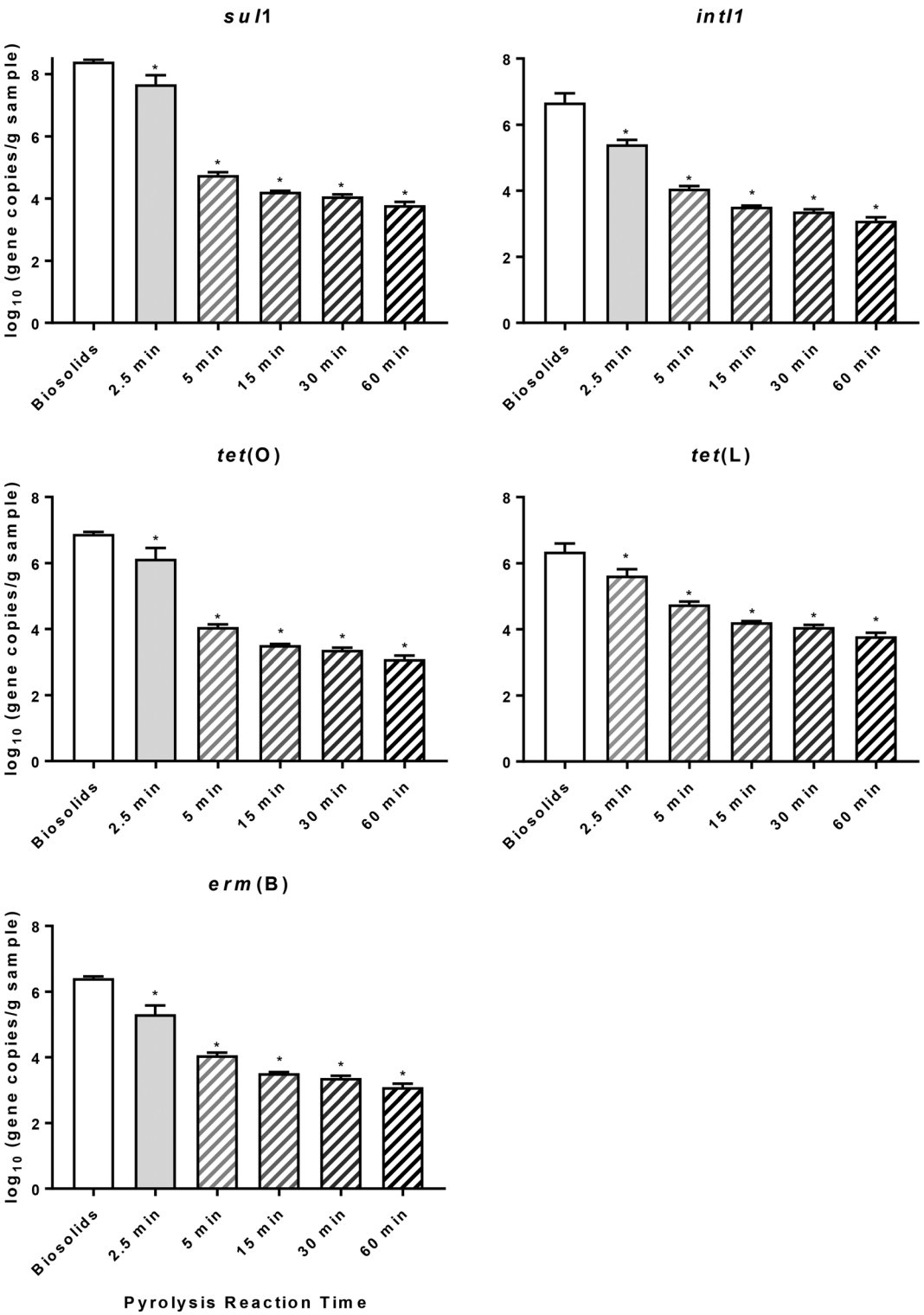

Fig. 5 Impact of pyrolysis reaction time on removal of ARGs and the *intI*1 gene from biosolids at 500 °C. Bars represent the average concentration and error bars represent the standard deviation among triplicate experiments (n = 3). Solid fill bars indicate values detected within the standard curve and striped bars indicate values below the detection limit. Values that were below the detection limit were reported as the detection limit. Statistical differences from the biosolids control are indicated with a star ($p < 0.05$).

significantly reduce levels of ARGs in biosolids prior to land application. The results of the current study demonstrate that pyrolysis can effectively reduce the levels of total bacterial biomass, ARGs, and class 1 integrons in municipal biosolids and could provide WRRFs with a means of mitigating the discharge of ARGs to the environment.

Comparison of pyrolysis to other biosolids handling processes for removal of ARGs

There is a growing body of literature regarding existing biosolids handling processes and their influence on ARG removal from residual biosolids. Biosolids handling processes are currently designed to reduce pathogenic microorganisms, water content, and organic carbon content.[8] Commonly used biosolids handling processes such as air-drying, aerobic digestion, and anaerobic digestion can significantly reduce the abundance of various ARGs and class 1 integrons in wastewater sludge and residual biosolids (Table 2). However, Table 2 illustrates the fact that removal of ARGs through typical biosolids handling processes is highly variable, and multiple studies have observed enrichment of ARGs (*e.g.* *erm*(B), *erm*(F), *tet*(O)) during anaerobic digestion of municipal biosolids.[27,28]

Table 2 Comparison of biosolids handling processes for ARG removal

Reference	Technology	Temperature (°C)	Observed removal of target genes (Log_{10} scale)					
			16S rRNA	*intI*1	*sul*1	*erm*(B)	*tet*(O)	*tet*(L)
Burch *et al.* 2013 (ref. 53)	Air drying beds	(10–30)	1	1	1	4		
Burch *et al.* 2013 (ref. 70)	Aerobic digestion	20	1	1	1	2		
Diehl and LaPara 2010 (ref. 27)	Anaerobic digestion	22		0.5			0	0
	Anaerobic digestion	37		1.5			0.5	0.5
	Anaerobic digestion	46		1			0.25	0
	Anaerobic digestion	55		2			0.5	0.25
	Aerobic digestion	22		0			0	−0.1
	Aerobic digestion	37		0.25			0.1	−0.1
	Aerobic digestion	46		0.1			−0.25	0
	Aerobic digestion	55		0.5			0.1	0.5
Ma *et al.* 2011 (ref. 28)	Anaerobic digestion	35		1	1.3	−0.5	0.4	
	Anaerobic digestion	47		0.75	1.1	1	0.25	
	Anaerobic digestion	52		0.75	1.1	0.9	0.75	
	Anaerobic digestion	59		0.5	1.1	0.8	0.7	
	Thermal hydrolysis process (THP)	150		2.6	2.2	1.5	2.1	
	THP pretreatment + anaerobic digestion	37		2.4	2.1	−0.5	1.2	
	THP pretreatment + aerobic digestion	32		0.2	0.4	0.5	1.8	
Burch *et al.* 2017 (ref. 8)	Air-dried	(10–30)	0.2	0.4	1	2.6		
	Aerobic digestion	17	−0.4	−0.9	−0.4	0.8		
	Anaerobic digestion	38	0	0.5	0.6	0.8		
	Anaerobic digestion	55	−0.1	0.6	1.1	1.5		
	Anaerobic digestion	63	0.1	0.4	1	1.2		
	Anaerobic digestion	69	0.4	0.8	1.4	0.9		
	Pasteurization	70	0.4	0.3	−1.6	−1.3		
	Alkaline stabilization	20	0.2	0.2	−1.1	−1.4		
Ghosh *et al.* 2009 (ref. 71)	2 stage anaerobic digestion (full scale)	(50–60) (35–37)		0.7–1.3			0.3–0.7	
Jang *et al.* 2018 (ref. 65)	Aerobic digestion	55	0.33	1.11	1.22	0.10		
Zhang *et al.* 2016 (ref. 67)	Sludge bio-drying		0.30	0.86	0.56	0.99		
Zhang *et al.* 2016 (ref. 68)	Sludge composting	(20–60)	−0.37	−0.72	−0.61	0.87		
	Sludge composting + zeolite addition	(20–60)	−0.07	−0.7	−0.33	1.55		
	Sludge composting + DMPP addition	(20–60)	−0.3	−0.72	−0.66	0.88		
Tong *et al.* 2016 (ref. 72)	MW pretreatment	(20–100)	0.3	0			0.2	
	MW pretreatment + anaerobic digestion	38	−0.2	0			−0.5	
	MW + HCl pretreatment	(20–100)	1	1.1			1	
	MW + HCl + anaerobic digestion	38	−0.5	−0.4			−1	
	MW + H_2O_2 pretreatment	(20–80)	0.2	0			0	
	MW + H_2O_2 + anaerobic digestion	38	−0.3	0			−0.2	
Current study	Pyrolysis	100	0.2	0	−0.19	0.36	0.42	0.59
	Pyrolysis	300	4.62	3.13	3.53	3.11	3.37	1.53
	Pyrolysis	500	6.0	3.8	4.2	3.79	4.04	2.2
	Pyrolysis	700	5.8	3.62	4.02	3.61	3.86	2.02

Notes: DMPP – 3,4-dimethylpyrazole phosphate (nitrification inhibitor). MW – microwave. Negative values indicate an increase in gene abundance. Results were obtained from reported values in source reference text and/or approximated from figures.

Previous studies have demonstrated that temperature plays an important role in ARG removal, and removal of ARGs is typically greater under thermophilic conditions compared to mesophilic conditions.[27,65] Treatment technologies such as thermophilic anaerobic digestion and thermal hydrolysis pretreatment (THP) have demonstrated increased removal of ARGs compared to traditional methods (*e.g.* mesophilic anaerobic digestion, aerobic digestion).[8,28,66] The high temperature (130–170 °C) and pressure of THP sterilizes sludge, destroys cell walls, and releases readily degradable components.[28] Ma *et al.* (2011) performed thermal hydrolysis of sewage sludge and documented over 2-log removal of *intI*1, *sul*1, and *tet*(O) genes. Comparatively, biosolids pyrolysis achieved greater than 3.5-log removal for *inti*1, *sul*1, and *tet*(O) genes in the current study. It should also be noted that most ARGs (excluding the *sul*1 and *tet*(G) genes) were observed to rebound during subsequent anaerobic and aerobic digestion following THP pretreatment.[28]

Biosolids handling processes such as sludge bio-drying and sludge composting have also been investigated for ARG removal. Sludge bio-drying of municipal biosolids effectively reduced levels of the 16S rRNA, *intI*1, *sul*1, and *erm*(B) genes by 0.3 to 0.99-log.[67] Similarly, composting of sewage sludge (20–60 °C) substantially reduced the *erm*(B) gene levels by 1.55-log, but failed to reduce levels of the *sul*1 and *intI*1 genes.[68] Previous studies have suggested that more rigorous technologies such as biosolids incineration are zero-risk solutions for the reduction of ARGs, although there are trade-offs with air quality and the loss of value-added soil amendment products.[62] Therefore, processes with operating temperatures exceeding those typically used for biosolids handling, such as pyrolysis and incineration, could potentially provide additional removal of ARGs compared to existing biosolids treatment technologies. In the current study, pyrolysis of municipal biosolids at operating temperatures ≥300 °C significantly reduced the abundance of total bacteria (*i.e.*, 16S rRNA), ARGs, and class 1 integrons.

It should be noted that the influent feed to many of the processes referenced in Table 2 was undigested sludge which is different than the digested, heat-dried biosolids used for feed in this study. It is possible that the production of heat-dried biosolids could also reduce ARGs. Nevertheless, ARGs were present in the feed biosolids used in the pyrolysis experiments and these experiments demonstrated that pyrolysis could reduce ARGs to below detection limits.

Conclusions

Biosolids pyrolysis has potential to contribute to future sustainability plans of WRRFs because it produces valuable products (py-gas, py-oil, and biochar). The current study demonstrated that pyrolysis of dried municipal biosolids at operating temperatures of ≥300 °C resulted in ARG and the *intI*1 gene levels that were below the detection limit of the qPCR assays (*i.e.*, similar to negative controls). The significant reduction in all genetic biomarkers quantified in this study likely corresponded with the destruction of prokaryotic genetic material and ARGs. This research makes a vital contribution to new knowledge by identifying a potentially sustainable biosolids handling approach to help mitigate the spread of antibiotic resistance. In the U.S., over 8 million tons of biosolids are produced annually,[1] and this study identified an approach to significantly reduce the levels of total bacteria (*i.e.*, 16S rRNA), ARGs, and class 1 integrons in municipal biosolids prior to land application. Additionally, the resultant biochar from biosolids pyrolysis represents a valuable source of organic carbon, nutrients (N, P), and energy that can be recovered from the pyrolysis process itself to help offset operating costs and power requirements.[30]

The investigation of ARG removal from wastewater biosolids is an important issue in controlling the dissemination of antibiotic resistance in the natural environment. Traditional biological treatment methods may result in the selective increase of antibiotic resistant bacteria and ARGs due to conditions present in WRRFs that appear to foster HGT and the development of multidrug-resistant bacteria.[62,69] As a result, advanced biosolids treatment technologies, such as pyrolysis, could provide WRRFs with a method of further decreasing ARG levels in municipal biosolids prior to land application.

Conflicts of interest

There are no conflicts of interest to declare.

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