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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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1 Title: Effect of Pyrolysis on the Removal of Antibiotic Resistance Genes and Class I Integrons
2 from Municipal Wastewater Biosolids

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9 **Water Impact Statement**

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

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17 **Key words**

18 biochar, thermal processing, biosolids handling, land application, antimicrobial resistance

19

20   **Abstract**

21

22   Wastewater biosolids represent a significant reservoir of antibiotic resistance genes

23   (ARGs). While current biosolids treatment technologies can reduce ARG levels in residual

24   wastewater solids, observed removal rates vary substantially. Pyrolysis is an anoxic thermal

25   degradation process that can be used to convert biosolids into energy rich products including

26   py-gas and py-oil, and a beneficial soil amendment, biochar. Batch pyrolysis experiments

27   conducted on municipal biosolids revealed that the 16S rRNA gene, the ARGs *erm*(B), *sul*1,

28   *tet*(L), *tet*(O), and the integrase gene of class 1 integrons (*intI*1) were significantly reduced at

29   pyrolysis temperatures ranging from 300-700°C, as determined by quantitative polymerase chain

30   reaction (qPCR). Pyrolysis of biosolids at 500°C and higher resulted in approximately 6-log

31   removal of the bacterial 16S rRNA gene. ARGs with the highest observed removals were *sul*1

32   and *tet*(O), which had observed reductions of 4.62 and 4.04-log, respectively. Pyrolysis reaction

33   time had a significant impact on 16S rRNA, ARG and *intI*1 levels. A pyrolysis residence time of

34   5 minutes at 500°C reduced all genes to below detection limits. These results demonstrate that

35   pyrolysis could be implemented as a biosolids treatment technology to substantially decrease the

36   abundance of total bacteria (i.e., 16S rRNA), ARGs and *intI*1 prior to land application of

37   municipal biosolids.

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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

64   the spread of antibiotic resistance.[17,18] Furthermore, residual biosolids represent the effluent

65   stream from WRRFs with the highest concentration of ARGs,[25] and biosolids handling processes

66   could be a control point where the release of ARGs into the environment could be substantially

67   decreased.

68   WRRFs serve as the primary collection points for commercial, residential, and hospital

69   wastewater effluents that contain a variety of microorganisms and ARGs. The residual solids

70   from the treatment process are of great interest because they contain the vast majority of

71   prokaryotic biomass and ARGs discharged from WRRFs.[26] Several ARGs have been detected in

72   municipal biosolids including, for example, tetracycline resistance genes (*tet*(O), *tet*(W)),

73   sulfonamide resistance genes (*sul*1), and the gene encoding the integrase of class 1 integrons

74   (*intI*1).[10,11] As a result, multiple biosolids handling processes have been investigated with respect

75   to their impacts on ARG removal. Mesophilic anaerobic digestion, air-drying beds, and aerobic

76   digestion processes have all demonstrated the ability to remove ARGs from municipal biosolids

77   to varying extents.[8] However, an increasing demand for higher quality biosolids has driven an

78   interest in more rigorous treatment methods. Alternative methods, such as thermophilic

79   anaerobic digestion,[27] thermal-hydrolysis pretreatment to anaerobic digestion,[28] pasteurization,

80   and lime stabilization [8] have also been analyzed for ARG removal from biosolids. While each of

81   these processes reduce certain ARGs, none have completely eliminated ARGs, and some ARGs

82   even proliferated during anaerobic digestion (e.g. *erm*(B), *erm*(F), *tet*(O)).[27,28] Consequently, a

83   biosolids handling process that eliminates ARGs would further mitigate the spread of ARGs in

84   the environment.

85   Pyrolysis, a thermochemical process that decomposes organic matter at elevated

86   temperatures in the absence of oxygen, is gaining interest for biosolids management

4

87   applications.[29–32] Pyrolysis reduces the total amount of solids that need to be managed by

88   converting a portion of the solids to a liquid fraction (py-oil) and a gas fraction (py-gas), and the

89   remaining solids are converted to biochar, a stable form of carbon similar to activated carbon.[33–

90   35] Py-oil and py-gas can be combusted for energy,[36] and biochar has multiple agricultural

91   benefits including improved soil fertility and nutrient retention.[37,38] Previous research has

92   demonstrated that the energy required for pyrolysis was approximately 5-fold less than the

93   energy required to dry biosolids, therefore a WRRF already using energy to dry biosolids would

94   not significantly increase its energy needs with the addition of pyrolysis treatment[30] In fact,

95   energy can be recovered on-site from the py-gas that is produced. Pyrolysis is best suited as a

96   polishing step after anaerobic digestion and dewatering. For utilities that produce wet biosolids,

97   implementing pyrolysis may be costly due to the energy required to dry the biosolids.[30] Certainly

98   the energy costs associated with pyrolysis increase as the moisture content of the solids

99   increases. Thus, individual WRRFs would need to conduct cost-benefit analyses to determine

100   how the benefits of pyrolysis compare to the energy costs associated with pyrolysis of their

101   specific biosolids.

102   Previous research has demonstrated the ability of pyrolysis to remove recalcitrant organic

103   micropollutants such as estrogenic compounds, triclosan, triclocarban, and nonylphenol.[32,35]

104   Pyrolysis of wastewater biosolids at 450°C removed 75% of polychlorinated biphenyls (PCBs),[39]

105   and greater than 99% reduction of PCBs and dioxins was observed from pyrolysis of

106   contaminated sediment at 800°C.[40] Moreover, a previous study documented greater than 3-log

107   reduction of *Escherichia coli* after thermal treatment of wastewater sludge at 80°C.[41] These

108   findings suggest that pyrolysis could provide a means for ARG removal from biosolids prior to

109   land application due to high operational temperatures (typically >450°C). To our knowledge, no

110 research has been conducted regarding the effects of pyrolysis on the removal of ARGs or class I

111 integrons from wastewater derived biosolids.

112 The objective of this research was to determine the impact of pyrolysis on the removal of

113 the 16S rRNA gene, ARGs including *erm*(B), *sul*1, *tet*(L), and *tet*(O), and the gene encoding the

114 integrase of class 1 integrons (*intI*1). It was hypothesized that pyrolysis would decrease the

115 abundance of the 16S rRNA gene, ARGs, and *intI*1 following pyrolysis treatment due to

116 decomposition of amplifiable DNA representing these genes. Tetracycline and sulfonamide

117 resistance genes (*tet*(O), *tet*(L) and *sul*1) were quantified in this study due to the prevalent use of

118 tetracycline and sulfonamide as antibiotics in human and veterinary medicine.[42,43] *Sul*1 is also

119 one of the most commonly detected sulfonamide resistance genes in the environment.[44] The

120 *erm*(B) gene is generally found on conjugative genetic elements and encodes resistance to

121 macrolides, lincosamides, and streptogramin antibiotics.[45] Additionally, *intI*1 was quantified in

122 this study as it is considered to be a genetic element substantially contributing to the proliferation

123 and evolution of multiple antibiotic resistant bacteria in the environment.[27,46] Briefly, laboratory-

124 scale pyrolysis experiments were performed on heat-dried biosolids, and the abundance of 16S

125 rRNA, the integrase gene of class 1 integrons (*intI*1), and the ARGs *erm*(B), *sul*1, *tet*(L), and

126 *tet*(O) were quantified via quantitative polymerase chain reaction (qPCR).

127 **Methods**

128 **Pyrolysis Temperature and Reaction Time Experiments**

129 Experiments were set up to determine the effect of pyrolysis on total bacterial 16S rRNA,

130 ARGs, and class I integrons. Pyrolysis of biosolids was performed by adding approximately 10

131 grams of biosolids to 250 mL flasks in triplicate. The biosolids feedstock was a heat-dried blend

132 of waste activated sludge and anaerobically digested primary solids from a municipal WRRF

133 (Milorganite®, Milwaukee Metropolitan Sewerage District, Milwaukee, WI). These biosolids

134 were chosen over wet biosolids because pyrolysis is ideal as a polishing step to recover energy

135 from already dried biosolids.[30] The flasks were sparged for ten minutes with argon gas, covered

136 with aluminum foil, and heated in a muffle furnace (Fischer-Scientific Isotemp®, Waltham, MA)

137 similar to previous studies that utilized Milorganite as a feedstock for pyrolysis.[29,30,32,34,47–50]

138 Flasks were placed in the furnace for a one-hour retention time at temperatures ranging from 100

139 to 700°C to determine effect of temperature on ARG removal. A room temperature control

140 (20°C) was prepared in the same manner and placed in the oven with no heat for one hour.

141 "Influent" biosolids samples were generated by leaving the flask filled with Milorganite on the

142 bench-top for one hour. Biochar yields were determined for each pyrolysis temperature by the

143 following equation: (Mass of biochar after pyrolysis (g) / Initial Milorganite mass (g)) x 100%.

144 The impact of pyrolysis reaction time was determined at 500°C with reaction times of 2.5, 5, 15,

145 30, and 60 minutes. Samples were stored at -20°C until DNA extraction was performed.

146 **DNA Extraction**

147   Biosolids samples were homogenized using a sterile mortar and pestle then

148 approximately 0.2 g of biosolids were subsampled for DNA extraction. DNA was extracted

149 using the FastDNA® SPIN Kit (MP Biomedicals LLC, Solon, OH) by manufacture's protocol

150 utilizing 1.0 mL of the CLS-TC lysis buffer with a modified cell lysis procedure instead of bead-

151 matrix homogenization. Cells were lysed by liquid nitrogen freeze thaw cycling (3x) to improve

152 yield.[51] DNA concentrations were determined by microspectrophotometry (NanoDrop™ Lite,

153 Thermo Scientific, Waltham, MA). DNA extracts were stored at -20°C for further analysis.

qPCR for antibiotic resistance genes and *intI1* 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 (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 sec, 60°C for 30 sec, followed by 72°C for 30 sec. 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.

176   The quantity of the target gene in unknown samples was calculated based on a standard

177   curve generated using known quantities of plasmids bearing the target gene (either the pUC19 or

178   pGEM-T Easy (Promega, Madison, WI) plasmids). The primers and probes along with the

179   annealing temperatures used for resistance genes were previously developed.[53–56] Standard

180   curves (five-point minimum) for qPCR were produced by ten-fold serial dilution of plasmid

181   DNA yielding 10^8 to 10^0 target gene copies per reaction. R^2 values were greater than 0.99 for all

182   standard curves used to quantify target genes in this study and no template controls were

183   included in each assay. To compare absolute reductions of target genes, gene quantities are

184   presented normalized to grams of dry biosolids. Specific primer sets, annealing temperatures,

185   efficiencies, and detection limits are described in Table 1.

186 **Data Analysis**

187   Copy number of the target gene were $\log_{10}$ transformed to meet the assumptions of

188   normality for statistical analysis.[8,53] The absolute copy numbers of each gene are presented in

189   this study rather than normalized to 16S rRNA since target genes were reduced to below

190   detection limits in most experiments. GraphPad Prism (V 7.02, La Jolla, CA) was used to

191   perform analysis of variance (one-way ANOVA) and t-tests. Tukey's post hoc multiple

192   comparisons test was used to determine significant differences between each pyrolysis condition.

193 **Results and Discussion**

194 **Pyrolysis Temperature Experiments**

195   The impact of pyrolysis temperature on the removal of the bacterial 16S rRNA gene,

196   ARGs, and the *intI*1 gene from municipal wastewater biosolids was determined in batch

197   pyrolysis experiments. Pyrolysis reactions were successful as confirmed by quantifying biochar

198 yields (Figure 1). Increasing pyrolysis temperatures resulted in a significant decrease in biochar

199 yield ($p < 0.0001$). At 500°C, biochar yield was approximately 43%, which is congruent with

200 previous biochar yield from pyrolysis of biosolids.[34] Previous studies have reported that the

201 decrease in biochar yield as temperature rises is likely due to the destruction of organic matter

202 such as cellulose, hemicellulose, and lignin.[57] Cellulose drastically reduces weight from 275-

203 350°C, and lignin reduces weight linearly with increasing temperature from 250-500°C.[58] The

204 reduction in biochar yield at pyrolysis temperatures of 300-700°C likely resulted in the

205 concomitant destruction of prokaryotic biomass and genetic material such as DNA.

206   Quantification of the 16S rRNA gene (Figure 2) was performed to determine the impact

207 of pyrolysis on the removal of total bacterial biomass from municipal biosolids. There was no

208 significant difference in 16S rRNA gene abundance between the biosolids feedstock (i.e. the

209 influent biosolids), the room temperature samples (20°C) ($p > 0.98$), or the 100°C samples ($p >$

210 0.53). There was a significant 4.62-log reduction in the 16S rRNA gene observed for 300°C

211 biochar compared to the biosolids feedstock ($p < 0.0001$). Approximately 6-log reduction in the

212 16S rRNA gene was observed for biochar produced at 500°C ($p < 0.0001$) and 700°C ($p <$

213 0.0001). Both pyrolysis conditions removed the 16S rRNA gene to levels below the detection

214 limit (8.2×10^4 copies/g biochar). Compared to other biosolids treatment processes, the reduction

215 in total bacterial biomass (i.e., 16S rRNA) observed in this study was approximately five orders

216 of magnitude greater than removal observed in air-drying beds and thermophilic anaerobic

217 digestion employed for treatment of municipal biosolids.[8,53] These results indicate that pyrolysis

218 of wastewater biosolids could decrease the amount of total bacterial biomass released to the

219 environment when land applying biosolids-derived biochar relative to land application of

220 biosolids.

221   Quantification of four ARGs and the *intI*1 gene was performed to determine the impact of

222   pyrolysis on the removal of various classes of resistance genes (Figure 3). All genes quantified in

223   this study were selected due to their frequent detection in municipal biosolids.[27,53] All four ARGs

224   and the *intI*1 gene significantly decreased in abundance compared to the biosolids feedstock as

225   pyrolysis temperatures increased above 300°C (p < 0.0001). Observed reductions in ARG

226   quantities ranged from 2.2 to 4.2-log at pyrolysis temperatures of 500°C and greater compared to

227   the biosolids feedstock.

228   ARGs with the highest observed removals were the *sul*1 and *tet*(O) genes, which had

229   observed reductions of 4.20 and 4.04-log, respectively. Sulfonamide resistance genes, such as

230   *sul*1, are frequently detected in residual biosolids.[25,53] The *sul*1 gene is generally associated with

231   class 1 integrons on conjugative plasmids and is a good indicator of HGT and multiple antibiotic

232   resistance.[45] The *tet*(O) gene is commonly associated with ribosomal protection in aerobic

233   bacteria, and can be found in conjugative plasmids or in the chromosome.[59] Tetracycline

234   resistance genes such as *tet*(L) and *tet*(O) are commonly detected in influent and effluent streams

235   in WRRFs and have been shown to increase in abundance with increasing concentrations of

236   influent tetracycline.[60] The *tet*(L) gene encodes for an efflux pump, and has been found in gram-

237   positive and gram-negative bacterial isolates.[43] In the current study, the *tet*(L) gene was removed

238   to a lesser degree compared to *tet*(O), with an observed 2.2-log reduction compared to the

239   biosolids feedstock. The abundance of the *tet*(L) gene, however, was lower than that observed

240   for the *tet*(O) gene in the biosolids feedstock, which contributed to higher observed removal for

241   the *tet*(O) gene relative to the *tet*(L) gene. Additionally, the detection limit for *tet*(L) was one

242   order of magnitude higher than that observed for *tet*(O), which also contributed to the lower

243   observed removal of the *tet*(L) gene compared to *tet*(O).

244   Similar to tetracycline resistance genes, pyrolysis temperatures ≥300°C significantly

245   decreased the abundance of the *erm*(B) and *intI*1 genes in the resultant biochar (p < 0.0001)

246   compared to the biosolids feedstock. Observed reductions in gene quantities were 3.79 and 3.80-

247   log for the *erm*(B) and *intI*1 genes, respectively. Both genes were removed to levels below the

248   detection limit in biochar produced from 300 to 700°C. As all genes quantified in this study were

249   reduced below the detection limit of the qPCR assays at temperatures greater than 300°C, log

250   removal was dependent on the initial abundance of target genes in the municipal biosolids

251   feedstock. We expect that the reduction in ARG and *intI*1 genes during pyrolysis was due to the

252   destruction of DNA (intracellular and extracellular), indicated by the presence of non-amplifiable

253   DNA in the resultant biochar.

254   Previous studies have documented that anaerobic digestors can physically destroy

255   extracellular DNA through hydrolysis and biodegradation processes.[28] However, ARGs may also

256   be harbored by host bacterial cells and subject to amplification via cell growth or HGT.[61,62]

257   Similarly, other biosolids treatment technologies such as pasteurization and alkaline stabilization

258   are known to aggressively inactivate pathogens, but failed to significantly decrease levels of the

259   *erm*(B), *sul*1, and *intI*1 genes in wastewater solids prior to being applied to soil microcosms.[8]

260   The current study demonstrates that pyrolysis can be used as a biosolids treatment technology to

261   substantially reduce levels of ARGs and the *intI*1 gene in municipal biosolids prior to land

262   application.

263   **Pyrolysis Reaction Time Experiments**

264   Quantification of the 16S rRNA gene (Figure 4) was performed to determine the impact

265   of pyrolysis reaction time on total bacterial abundance in wastewater biosolids. Greater than 85%

266   of 16S rRNA gene copies removed from biosolids with a pyrolysis reaction time of only 2.5

267   minutes at 500°C (p < 0.05). A significant 3.5-log reduction was observed for biosolids with a 5-

268   minute pyrolysis retention time with respect to the biosolids feedstock (p < 0.0001). There was

269   not a significant difference in 16S rRNA abundance in biochar produced at 15 and 30 minutes (p

270   > 0.55). Compared to the biosolids feedstock, there was a significant 4.05 and 4.39-log reduction

271   in 16S rRNA for biochar pyrolyzed for 15 and 30 minutes, respectively (p values < 0.0001).

272   Similarly, a significant 4.87-log reduction in bacterial 16S rRNA was observed for biosolids

273   pyrolyzed for 60 minutes (p < 0.0001). These results indicate that pyrolysis reaction time has a

274   substantial impact on the quantity of bacterial gene markers present in the resultant biochar.

275   Quantification of the ARGs *erm*(B), *sul*1, *tet*(L), *tet*(O), and the integrase gene of class 1

276   integrons (*intI*1) was performed to determine the impact of pyrolysis reaction time on the

277   removal of various resistance genes (Figure 5). All four ARGs and the *intI*1gene significantly

278   decreased in abundance after a pyrolysis reaction time of 2.5 minutes (p < 0.0005). Biosolids

279   with a pyrolysis residence time of 5 minutes resulted in the reduction of all ARGs and the *intI*1

280   gene to below detection limits (p < 0.0001), with log removals ranging from 2.14 for the *tet*(L)

281   gene to 4.62 for the *sul*1 gene. Similar to the results from pyrolysis temperature experiments, the

282   ARG with the highest observed removal rate was the *sul*1 gene. In the current study, the

283   abundance of the s*ul*1 gene in the biosolids influent samples was 2.56 x 10^8 copies/g dry weight

284   on average. The results observed in this study are congruent with previous studies that have

285   reported *sul*1 as one of the most prevalent ARGs detected in municipal WRRFs.[18,25,63]

286   ARGs such as sulfonamide resistance genes can proliferate in biological processes at

287   WRRFs and previous studies have reported *sul*1 concentrations of up to 10^{11} copies/g dry weight

288   in dewatered sludge.[25] The *sul*1 gene is generally harbored in class 1 integrons containing

289   multiple resistance genes, and encodes dihydropteroate synthase that is not inhibited by

290 sulfonamides.[64] The *intI*1 gene was also detected at elevated levels in municipal biosolids in the

291 current study, with an observed abundance of 6.66 x 10^7 copies/g dry weight on average. This is

292 congruent with previous research that observed a positive correlation between *sul*1 and *inti*1 in

293 the feed and effluent of anaerobic and aerobic digesters.[28] The enrichment of class 1 integrons

294 and ARGs such as *sul*1 in biological treatment processes at WRRFs underscores the need for

295 rigorous biosolids treatment technologies that can significantly reduce levels of ARGs in

296 biosolids prior to land application. The results of the current study demonstrate that pyrolysis can

297 effectively reduce the levels of total bacterial biomass, ARGs, and class 1 integrons in municipal

298 biosolids and could provide WRRFs with a means of mitigating the discharge of ARGs to the

299 environment.

300 **Comparison of Pyrolysis to Other Biosolids Handling Processes for Removal of ARGs**

301 There is a growing body of literature regarding existing biosolids handling processes and

302 their influence on ARG removal from residual biosolids. Biosolids handling processes are

303 currently designed to reduce pathogenic microorganisms, water content, and organic carbon

304 content.[8] Commonly used biosolids handling processes such as air-drying, aerobic digestion, and

305 anaerobic digestion can significantly reduce the abundance of various ARGs and class 1

306 integrons in wastewater sludge and residual biosolids (Table 2). However, Table 2 illustrates the

307 fact that removal of ARGs through typical biosolids handling processes is highly variable, and

308 multiple studies have observed enrichment of ARGs (e.g. *erm*(B), *erm*(F), *tet*(O)) during

309 anaerobic digestion of municipal biosolids.[27,28]

310 Previous studies have demonstrated that temperature plays an important role in ARG

311 removal, and removal of ARGs is typically greater under thermophilic conditions compared to

312 mesophilic conditions.[27,65] Treatment technologies such as thermophilic anaerobic digestion and

14

313   thermal hydrolysis pretreatment (THP) have demonstrated increased removal of ARGs compared

314   to traditional methods (e.g. mesophilic anaerobic digestion, aerobic digestion).[8,28,66] The high

315   temperature (130-170°C) and pressure of THP sterilizes sludge, destroys cell walls, and releases

316   readily degradable components.[28] Ma et al. (2011) performed thermal hydrolysis of sewage

317   sludge and documented over 2-log removal of *intI*1, *sul*1, and *tet*(O) genes. Comparatively,

318   biosolids pyrolysis achieved greater than 3.5-log removal for *inti*1, *sul*1, and *tet*(O) genes in the

319   current study. It should also be noted that most ARGs (excluding the *sul*1 and *tet*(G) genes) were

320   observed to rebound during subsequent anaerobic and aerobic digestion following THP

321   pretreatment.[28]

322   Biosolids handling processes such as sludge bio-drying and sludge composting have also

323   been investigated for ARG removal. Sludge bio-drying of municipal biosolids effectively

324   reduced levels of the 16S rRNA, *intI*1, *sul*1, and *erm*(B) genes by 0.3 to 0.99-log.[67] Similarly,

325   composting of sewage sludge (20 - 60°C) substantially reduced the *erm*(B) genes levels by 1.55-

326   log, but failed to reduce levels of the *sul*1 and *intI*1 genes.[68] Previous studies have suggested that

327   more rigorous technologies such as biosolids incineration are zero-risk solutions for the

328   reduction of ARGs, although there are trade-offs with air quality and the loss of value-added soil

329   amendment products.[62] Therefore, processes with operating temperatures exceeding those

330   typically used for biosolids handling, such as pyrolysis and incineration, could potentially

331   provide additional removal of ARGs compared to existing biosolids treatment technologies. In

332   the current study, pyrolysis of municipal biosolids at operating temperatures ≥300°C

333   significantly reduced the abundance of total bacteria (i.e., 16S rRNA), ARGs, and class 1

334   integrons by greater than 99%.

335 It should be noted that the influent feed to many of the processes referenced in Table 2

336 was undigested sludge which is different than the digested, heat-dried biosolids used for feed in

337 this study. It is possible that the production of Milorganite® could also reduce ARGs.

338 Nevertheless, ARGs were present in in the pyrolysis experiments and these experiments

339 demonstrated that pyrolysis could reduce ARGs to below detection limits.

340 **Conclusions**

341 Biosolids pyrolysis has potential to contribute to future sustainability plans of WRRFs

342 because it produces valuable products (py-gas, py-oil, and biochar). The current study

343 demonstrated that pyrolysis of dried municipal biosolids at operating temperatures of ≥300°C

344 resulted in ARG and the *intI*1 gene levels that were below the detection limit of the qPCR assays

345 (i.e., similar to negative controls). The significant reduction in all genetic biomarkers quantified

346 in this study likely corresponded with the destruction of prokaryotic genetic material and ARGs.

347 This research makes a vital contribution to new knowledge by identifying a potentially

348 sustainable approach to mitigating the spread of antibiotic resistance. In the U.S., over 8 million

349 tons of biosolids are produced annually,[1] and this study identified an approach to significantly

350 reduce the levels of total bacteria (i.e., 16S rRNA), ARGs, and class 1 integrons in municipal

351 biosolids prior to land application. Additionally, the resultant biochar from biosolids pyrolysis

352 represents a valuable source of organic carbon, nutrients (N, P), and energy that can be recovered

353 from the pyrolysis process itself to help offset operating costs and power requirements.[30]

354 The investigation of ARG removal from wastewater biosolids is an important issue in

355 controlling the dissemination of antibiotic resistance in the natural environment. Traditional

356 biological treatment methods may result in the selective increase of antibiotic resistant bacteria

357 and ARGs due to conditions present in WRRFs that appear to foster HGT and the development

358 of multidrug-resistant bacteria.[62,69] As a result, advanced biosolids treatment technologies, such

359 as pyrolysis, could provide WRRFs with a method of further decreasing ARG levels in

360 municipal biosolids prior to land application.

361 **Conflicts of Interest**

362 There are no conflicts of interest to declare.

363 **Acknowledgments**

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