

A survey of crop-derived transgenes in activated and digester sludges in wastewater treatment plants in the United States

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ABSTRACT

The use of transgenic crops has become increasingly common in the United States over the last several decades. Increasing evidence suggests that DNA may be protected from enzymatic digestion and acid hydrolysis in the digestive tract, suggesting that crop-derived transgenes may enter into wastewater treatment plants (WWTPs) intact. Given the historical use of antibiotic resistance genes as selection markers in transgenic crop development, it is important to consider the fate of these transgenes. Herein we detected and quantified crop-derived transgenes in WWTPs. All viable US WWTP samples were found to contain multiple gene targets (*p35*, *nos*, *bla* and *nptII*) at significantly higher levels than control samples. Control wastewater samples obtained from France, where transgenic crops are not cultivated, contained significantly fewer copies of the *nptII* gene than US activated and digester sludges. No significant differences were measured for the *bla* antibiotic resistance gene (ARG). In addition, a nested PCR (polymerase chain reaction) assay was developed that targeted the *bla* ARG located in regions flanked by the *p35* promoter and *nos* terminator. Overall this work suggests that transgenic crops may have provided an environmental source of *nptII*; however, follow-up studies are needed to ascertain the viability of these genes as they exit WWTPs.

Key words | activated sludge, antibiotic resistance genes, digester sludge, transgenic crops, wastewater treatment

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INTRODUCTION

Transgenic crops have become increasingly popular relative to their conventional counterparts since their initial development in the 1980s (Clive 2012). As of 2012, 10 crops had been approved for cultivation and human consumption in the United States, including soybean, maize, cotton, canola, potatoes, alfalfa, papaya, sugar beets, squash and tomatoes (König *et al.* 2004). Many individual lines of each of these crops contain one or more genetically engineered traits (e.g., herbicide tolerance, pest resistance, drought tolerance, and virus resistance) conferred through genes of interest (GOI), amounting to hundreds of lines released for cultivation in the United States alone. The most popular engineered traits are pest resistance (Bt lines), herbicide tolerance (HT lines), or stacked pest and herbicide resistance. To date, more than 130 lines of transgenic crops have also been documented to contain one or more antibiotic resistance genes (ARGs), including β -lactamase (*bla*) and

neomycin (*nptII*) resistance genes (ISAAA 2015). ARGs were first used as selection markers during the plant cell transformation process, and were commonly incorporated into first- and second-generation transgenic crops beginning in 1990 (Horsch *et al.* 1985; Moss *et al.* 2006). While some ARGs such as *nptII* continue to be used as selection markers in genetic engineering, other ARGs have fallen out of favor in recent years due to the controversies surrounding their potential to contribute to the rise in global environmental antibiotic resistance (Conner *et al.* 2003; Bennett *et al.* 2004; Dunfield & Germida 2004; König *et al.* 2004). Instead, herbicide resistance genes such as *epsps* and *bar* have become popular alternative selection markers (D'Halluin *et al.* 1992).

In addition to being sold in supermarkets as the crop product itself, transgenic crops may be further processed into other forms of food. For transgenic maize, these products

include grits, cornmeal, flour, cereals, and some fermented products. Genetically modified crops are also further broken down to obtain derivative products that are incorporated into other processed foods. Some of these products include corn starches (e.g., maltodextrin, dextrose, high fructose corn syrup, and sugar alcohols), soy lecithin, sugar (from sugar beets), and vegetable oils (Padgett *et al.* 1995). These byproducts can then be incorporated into other foodstuffs, including baked goods, breads, baby foods, and other snack foods. Approximately 75% of processed foods sold in US supermarkets contain one or more transgenic crop-derived ingredients leading to frequent consumption of transgenic crops and crop byproducts in the United States (Millstone & Lang 2008; Kamle & Ali 2013). Once ingested, DNA from foods can conceivably behave in three ways: (1) DNA can be degraded by acids or DNase I and II enzymes found within the digestive system, (2) DNA can be taken up by cells in the gut (host or bacterial), or (3) DNA can pass through the digestive system and be excreted in a broken-down form or wholly intact (Heritage 2004). Because transgenic crops contain genetic cassettes containing foreign DNA (e.g., pest resistance, herbicide tolerance and/or ARGs), controversy exists regarding the ability of transgenes to be transferred to surrounding gut microflora within the host (Uzogara 2000). All transgenic crop lines intended for human or animal consumption are subject to rigorous review for approval – including tests for allergenicity, toxicity, and other acute adverse health outcomes. However, these assessments are often only performed on animal models and employ classic toxicological endpoints (e.g., no observed adverse effect level (NOAEL) or lowest observed adverse effect level (LOAEL)) (Food & Drug Administration 1992; Paarlberg 2002), which should detect most significant acute health outcomes but may not observe subtle health effects or those associated with chronic ingestion of transgenic crops (König *et al.* 2004). It has been widely established that free DNA in any environment is highly susceptible to degradation by DNase I and II enzymes – this susceptibility would only be heightened in gastrointestinal systems with the added presence of digestive acids (Mazza *et al.* 2005). However, some free DNA contained within food matrices is protected from both enzymatic digestion and acid hydrolysis and can pass intact through a host's digestive tract (Hohlweg & Doerfler 2001). As a result, it is possible that transgenes derived from transgenic crops may be introduced into municipal wastewater treatment plant (WWTP) systems.

With approximately 16,000 in operation in the United States, municipal WWTPs are responsible for treating the wastewater produced by more than 75% of US households.

The treatment schematics of individual plants may vary, but generally consist of primary treatment, secondary treatment, and tertiary treatment (Metcalf & Eddy 1972). As both secondary treatment (e.g., activated sludge) and additional biosolids production (e.g., aerobic or anaerobic digestion) rely on mixed bacterial communities to remove unwanted organic compounds, any partially or wholly intact free DNA contained within human waste may be exposed to a range of microbes within those treatment processes (Kinney *et al.* 2006; Wu *et al.* 2010). Additionally, more than 50% of biosolids produced by WWTPs in the USA are used for downstream agriculture or land applications (Hale *et al.* 2001). This suggests that the crop transgenes entering into a WWTP may ultimately also be exposed to diverse soil microbial communities. While studies have been performed to estimate the fate of transgenic crop-derived ARGs in soil environments as well as the related risks to soil microbes (Nielsen *et al.* 1997; Gebhard & Smalla 1999; Gardner & Gunsch 2017), there remains a significant gap in understanding how WWTP bacteria are exposed to these transgenic constructs.

Given the high rates of transgenic crop cultivation and consumption in the USA as well as the previously noted possibility of crop transgenes being able to survive the digestive process, it is possible that partial or full-length transgenes derived from transgenic crops may be present in WWTPs. To investigate this question, in the present study, activated and digester sludge samples from WWTPs in the USA and France were screened for the presence of ARGs located between promoter and terminator gene sequences commonly used in transgenic crop development. Specifically, we targeted genetic sequences that contained the *p35* promoter sequence on the 5' end and the *nos* terminator gene on the 3' end and either the *bla* ARG or *nptII* ARG or both ARGs in between these two genetic elements. While not conclusively tying these ARGs to transgenic crops, the presence of ARGs in between the promoter and terminator sequences may be suggestive of transgenic crop origin as this expression cassette has been incorporated into a wide array of commercially available transgenic crop lines, including canola, wheat, tomato, maize, cotton, and soy (Hardegger *et al.* 1999). In addition, we quantified each transgene in the same samples.

METHODS

Wastewater sludge collection and DNA isolation

Activated sludge samples were obtained from 26 WWTPs located in North Carolina, Ohio, Oregon, Pennsylvania,

Washington, West Virginia, Missouri, Kansas, and Iowa in the USA. Control samples were obtained from a WWTP in France where transgenic crop cultivation is banned. These WWTPs were selected based on geographic regions and the broad range of treatment capacities (0.3–155 million gallons per day (MGD)) (Table S1, Supplementary Information, available with the online version of this paper). Approximately 50 mL were collected in triplicate from each WWTP and samples were shipped overnight on dry ice and were stored at -80°C upon receipt until DNA could be extracted. DNA was isolated from triplicate WWTP samples using the DNeasy PowerWater Kit (QIAGEN Carlsbad, CA, USA) according to kit manufacturer instructions. Eluted DNA was quantified using a QuBit 2.0 (ThermoFisher Scientific, Waltham, MA, USA) and stored at -20°C for downstream analysis. Extracted DNA from three WWTPs appeared highly degraded and these samples were excluded from further analysis.

In addition, of the 26 WWTPs mentioned above, 10 facilities from North Carolina, Missouri, Kansas, Iowa, and Oregon also provided samples of digester sludge. Control digester samples were also obtained from France. For each WWTP, approximately 50 mL were collected in triplicate and samples were shipped overnight on dry ice and were stored at -80°C upon receipt until DNA could be extracted. Aliquots of 5 mL were centrifuged in triplicate to obtain biosolids for DNA extraction using the DNeasy PowerLyzer PowerSoil kit (QIAGEN Carlsbad, CA) according to manufacturer instructions. Eluted DNA was quantified using a QuBit 2.0 (Life Technologies) and stored at -20°C for downstream analysis.

End point PCR and qPCR assay conditions

First, extracted DNA was screened to detect the presence of *p35*, *nos*, *bla*, and *nptII* genes with end point polymerase chain reaction (PCR) using the primers shown in Table 1. All end point PCR reactions were performed using a Bio-Rad T100 Thermal Cycler (Hercules, CA, USA) under the following conditions: initial denaturation at 95°C for 3 min; 35 cycles of 95°C for 30 s, 60°C for 60 s, and 72°C for 30 s; and 72°C for 5 min, before storing at 4°C .

In addition, we used end point PCR to identify larger fragments originating from potential transformation plasmids containing ARGs used in transgenic crop development. As *bla* and *nptII* genes found in WWTPs originating from transgenic crops are likely to be located in gene sections flanked by *p35* promoter and *nos* terminator genes, we first performed end point PCR with *p35F/nosR* primers to amplify that large DNA section and followed up with a nested PCR targeting the ARGs. The nested PCR primers used in this study are outlined in Table 1. Finally, extracted DNA was also screened for the presence of the cauliflower mosaic virus (CaMV) ORF (open reading frame) VII region to determine if the *p35* genes detected in WWTPs were viral in origin. Negative and positive controls were incorporated into each end point PCR run.

All samples yielding positive results by end point PCR were then further analyzed by quantitative real-time PCR (qPCR) using the Stratagene Mx3000P thermocycler (Agilent Technologies, Santa Clara, CA, USA). For each transgene assay, all triplicate WWTP samples were run in

Table 1 | Primer and TaqMan™ probe sequences for qPCR assays

Target gene	Primer sequence (5'-3')	TaqMan probe (5'-3')	Amplicon length (bp)	Source
<i>p35</i>	F: GCCTCTGCCGACAGTGGT R: AAGACGTGGTTGGAACGTC TTC	FAM-CAAAGATGGACCCCCAC CCACG-TAMRA	82	Waiblinger et al. (2008)
<i>nos</i>	F: GTCTTGCGATGATTATCAT ATAATTTCTG R: CGCTATAATTTGTTTTCTATC GCGT	FAM- AGATGGGTTTTTATGAT TAGAGTCCC GCAA-TAMRA	120	Mano et al. (2008)
<i>bla</i>	F: TGC GCGGAACCCCTATTTGT R: GCGGCGACCGAG TTGCTCTT	Not applicable	382	Badosa et al. (2004)
<i>nptII</i>	F: AGGAAGCGGTCAGCCCAT R: GCGTTGGCTACCCGTGATAT	FAM-CGCCGCAAGCTCTTCA GCAA- TAMRA	60	Mason et al. (2002)
16S	F: TCCTACGGGAGGCAGCAG R: GGACTACCAGGGTATCTAA TCCTGTT	FAM- CGTATTACCGCGGCTGC TGGCAC-TAMRA	466	Nadkarni et al. (2002)
CaMV ORF VII	F: AGCGGTCAAATATTGCTTA R: AACTTACCGTATGCTAGATTACCT	Not applicable	141	Love et al. (2005)

pseudo-triplicate ($n = 9$). qPCR amplification to detect the *bla* gene was performed using SYBR Green chemistry under the following conditions: initial denaturation at 95 °C for 3 min, followed by 40 cycles of 95 °C for 45 s, 55 °C for 60 s, and 72 °C for 30 s. Fluorescent measurements were taken during elongation. The dissociation curve was assessed at the end of the run under the following conditions: 95 °C for 60 s, 55 °C for 30 s, and 95 °C for 30 s. qPCR amplification using TaqMan™ Probe chemistry was performed for *p35*, *nos*, and *nptII* assays using the following conditions: initial denaturation at 95 °C for 60 s, followed by 40 cycles of 95 °C for 30 s and 60 °C for 30 s. The following conditions were used for *16S*: initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s and 60 °C for 60 s. Fluorescence measurements for each assay were taken during annealing steps for all qPCR assays.

Statistical analysis

The concentration of each transgene was determined by normalizing gene copy numbers to the volume of wastewater used to extract DNA. Gene copies for each target were also normalized to the gene copies of *16S* (Supplementary Information, Figures S5–S8, available with the online version of this paper) to compare the relative abundance of each transgene to the total bacteria in each sample (Ma *et al.* 2011). Multiple linear regression analyses and correlation analyses were performed to investigate the relationships between gene concentration and WWTP characteristics. Averages and standard deviations of all data were calculated using Microsoft Excel 2007. All other statistical tests including t-tests and analyses of variance (ANOVA) were conducted in GraphPad PRISM (version 7). Strong statistical significance was defined as $p < 0.05$ and marginal statistical significance was defined as $p < 0.10$.

RESULTS AND DISCUSSION

End point PCR screenings

Activated sludge obtained from 26 WWTPs and digester sludge from 10 WWTPs were screened for the presence of crop transgenes using end point PCR. In the first step, we screened for individual transgenes (i.e., *p35*, *nos*, *nptII*, and *bla*) and all were detected during this preliminary screening step in both WWTP activated sludge and digester sludge (Tables S2 and S3, Supplementary Information, available with the online version of this paper). All but three samples

contained at least one transgene, and 12 samples contained all four transgenes. In follow-up analyses, we found that the extracted DNA from the three WWTPs with no detected transgenes appeared highly degraded when run out on an agarose gel, both pre- and post-PCR. In addition, qualitative PCR screenings for *16S* genes failed to amplify from these samples suggesting that the DNA within the sludge may have become damaged in transit and therefore we cannot conclusively state whether the transgenes were present or absent in these samples. These samples were excluded from further analysis.

The *p35* promoter gene found in many transgenic crops is derived from the CaMV. To verify that the *p35* promoter regions detected in this study originated from crop transgenes as opposed to CaMV, DNA extracted from WWTP activated sludge and digester sludge was also screened for CaMV ORF VII genes. This end point PCR screening was negative for all samples, suggesting that the detected *p35* genes were not viral in origin. Rather, these data suggest that the *p35* gene regions detected herein existed either as extracellular DNA or within the intracellular DNA of WWTP bacteria. However, the exact source was not identified herein.

Finally, we wanted to ascertain as to whether the fragments spanned by the *p35* promoter region and *nos* terminator regions contained the *bla* ARG. This was accomplished by nested PCR using the forward *p35* primer (*p35F*) and reverse *nos* primer (*nosR*) described in Table 1 (Supplementary Information, Figure S1, available online). PCR products obtained from the *p35F/nosR* screening step were then subjected to an additional PCR amplification using the *bla* primers detailed in Table 1. *P35F/nosR* primer screenings resulted in the amplification of multiple large gene fragments in all WWTP samples (Supplementary Information, Figure S2, available online). Some of these bands were found to contain the *bla* ARG within each WWTP sample. Others have shown that multiple copies of promoters, terminators, GOI, and selection markers are often incorporated into a single transformation plasmid (Taylor *et al.* 1999), and could therefore generate multiple bands using a single PCR primer set.

qPCR screenings

Samples that tested positive for potential transgenes during the end point PCR screening were then further analyzed using qPCR to determine the abundance of each transgene in each sample. Standard curves and efficiencies for each qPCR assay are shown in Supplementary Information (Table S4, Figures S3 and S4, available online). Copy numbers of the *p35* promoter gene in US wastewater samples ranged from 2.92×10^2 to 3.61×10^4 copies per mL of wastewater,

with an average of 1.12×10^4 copies/mL (Figure 1(a)). The abundance of *p35* in activated sludge was not significantly different among wastewater treatment facilities sampled in North Carolina, Ohio, Pennsylvania, and West Virginia (WWTPs 1–14). The abundance of *p35* detected in activated sludge from facilities sampled in Missouri, Iowa, and Kansas was more variable (WWTPs 15–21); *p35* levels were significantly lower in WWTPs 15 and 18 ($p < 0.05$). Overall copy numbers among digester sludge were lower than those measured in activated sludge, but the same trends were observed. The abundance of *p35* genes ranged from 2.53×10^2 to 2.28×10^5 copies per mL of sludge, with an average of 1.13×10^3 copies per mL. The levels of *p35* were significantly higher in activated sludge compared to digester sludge obtained from US WWTPs ($p < 0.05$). In contrast, the concentration of *p35* genes in control activated sludge obtained from France (WWTP 1) was significantly lower compared to that of US wastewaters ($p < 0.05$).

Similar trends were observed in the detected copy numbers of the *nos* terminator genes in US wastewater samples (Figure 1(b)). *Nos* terminator copy numbers ranged from 2.26×10^2 to 4.78×10^4 copies per mL of wastewater, with an average of 1.15×10^4 copies per mL. Similar to the *p35* promoter genes, the abundance of *nos* in activated sludge was not significantly different among wastewater treatment facilities sampled in North Carolina, Ohio, Pennsylvania, and West Virginia (WWTPs 1–14). The abundance of *nos* detected in activated sludge from facilities sampled in Missouri, Iowa, and Kansas was more variable (WWTPs 15–21); *nos* gene concentrations were significantly lower in WWTPs 15 and 18 ($p < 0.05$). Detected *nos* values in WWTP 20 were only marginally significantly lower than in other WWTPs ($p = 0.093$). Lower *nos* copy numbers were observed in digester sludge samples than in activated sludge samples. In these samples, *nos* abundance ranged from 4.19×10^1 to 7.95×10^3 copies per mL of wastewater,

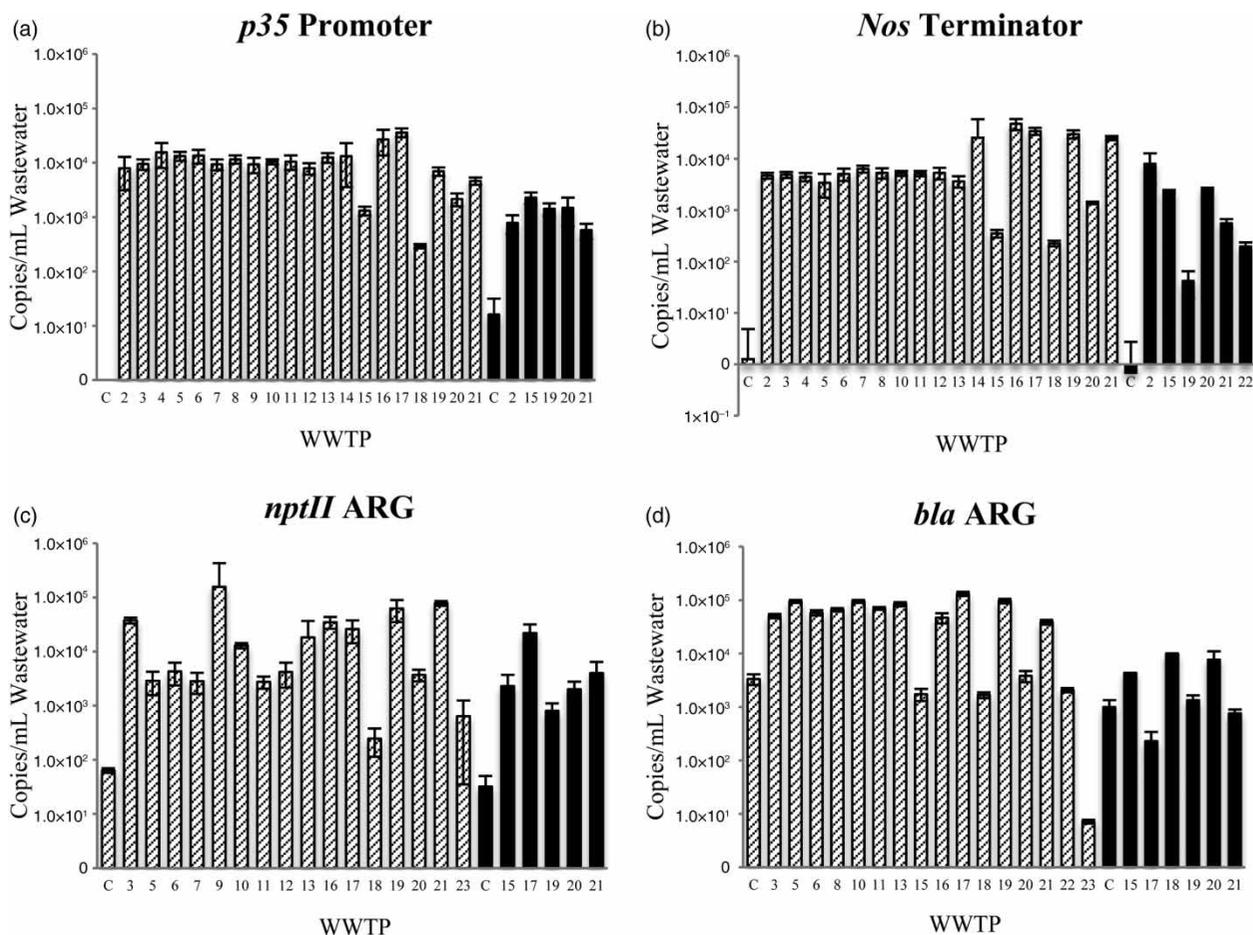


Figure 1 | Gene copies of the (a) *p35* promoter gene, (b) *nos* terminator gene, (c) *nptII* ARG, and (d) *bla* ARG in WWTP activated sludge and digester sludge. Bars representing activated sludge samples are denoted by black hatch patterns and digester sludge samples are represented by solid black. WWTPs have been reported anonymously, with control samples denoted by 'C'. Error is reported as standard deviation ($n = 9$).

with an average of 2.77×10^3 copies per mL. *Nos* gene copy numbers were highest in WWTPs 2, 15, and 20, but these levels were not significantly different from other digesters ($p = 0.052$). *Nos* copy numbers were also not significantly different between activated and digester sludge ($p = 0.058$). Again, the concentration of *nos* genes in control activated and digester sludge samples was significantly lower compared with those from US wastewaters ($p < 0.05$).

Copy numbers of *nptII* ARGs in US activated sludge wastewater varied widely among sampling locations (Figure 1(c)). *NptII* concentrations ranged from 2.46×10^2 to 1.58×10^5 copies per mL of wastewater, with an average of 2.80×10^4 copies per mL. Unlike *p35* and *nos* genes, *nptII* ARG abundance in activated sludge did not appear to be linked to the location of WWTPs ($p = 0.091$). The highest concentrations of *nptII* among activated sludge samples were found in WWTPs 3, 9, 19, and 21, and were significantly different from other WWTPs ($p < 0.05$). Overall copy numbers among wastewater digesters were similar to those observed in activated sludge samples ($p = 0.086$). *NptII* abundance in US digesters ranged from 8.04×10^2 to 2.20×10^4 copies per mL of wastewater, with an average of 5.29×10^3 copies per mL. The concentration of *nptII* ARGs in control activated and digester sludge (WWTP 1) was 6.30×10^1 and 3.20×10^1 per mL, respectively, and again was found to be significantly lower compared to that of US wastewaters ($p < 0.05$).

The detected copy numbers of the *bla* ARGs in US wastewater samples were similar to those of *nptII* in that they varied greatly among WWTPs (Figure 1(d)). In activated sludge, *bla* ARGs copy numbers ranged from 7.18 to 1.31×10^5 copies per mL of wastewater, with an average of 5.25×10^4 copies per mL. The lowest concentrations of *bla* among activated sludge samples were found in WWTPs 15, 18, 20, 22, and 23 and were significantly different from other WWTPs ($p < 0.05$). Overall copy numbers among digester sludge samples were also highly variable. Among these samples *bla* ARG abundance ranged from 2.29×10^2 to 9.71×10^3 copies per mL of wastewater, with an average of 3.67×10^3 copies per mL. The concentration of *bla* genes in control activated sludge was not significantly different from that of US wastewaters ($p = 0.24$).

Correlation analyses

Overall, gene copy numbers of the *p35* promoter region and *nos* terminator region as well as *nptII* and *bla* ARGs varied widely among the sampled WWTPs. *P35* promoter and *nos* terminator genes were less abundant than *nptII* and *bla*

ARGs on average. This is not surprising given that *p35* promoter and *nos* terminator genes are not known to exist in natural environments at high concentrations and may only be found in WWTPs as a result of ingestion and excretion of transgenic foods (Badosa *et al.* 2004). By contrast, ARGs have been measured at high concentrations in environmental bacterial populations (Ma *et al.* 2011; Laht *et al.* 2014; Mao *et al.* 2015). Additional statistical analyses suggest that some transgenes were positively associated with WWTP location and size. The concentrations of all potential transgenes (*p35*, *nos*, *nptII*, and *bla*) among individual WWTPs were found to be correlated to WWTP state locations ($p < 0.05$), determined by an ANOVA and multiple linear regression. *P35* promoter gene copy numbers were most strongly correlated with WWTP location ($p < 0.05$), while *nptII* and *bla* copy numbers were marginally correlated with WWTP location ($p = 0.051$ and $p = 0.099$, respectively). *Nos* terminator and *16S* gene copy numbers were not significantly correlated with WWTP location ($p = 0.38$, $p = 0.27$ respectively). Associations with WWTP locations were linked only to individual states and not entire geographic regions. For example, transgene abundance was linked to the US states in which they were located, as noted above. However, the correlations between entire geographic regions were not significant (e.g., Midwestern regions vs. Northeastern regions). This appears counterintuitive, given the large quantities of transgenic crops cultivated in Midwestern states. One explanation is that the consumption of transgenic crops may be a more important factor in assessing the prevalence and quantity of transgenes in WWTP sludge.

Notably, the abundance of the *p35*, *nos*, and *nptII* genes was markedly lower in control sludge samples as compared to US sludge samples. The significant differences in *p35*, *nos*, and *nptII* concentrations in French WWTP systems relative to US WWTP systems are probably due to the fact that France does not cultivate transgenic crops and human consumption is limited to imported processed foods containing transgenic crops (Kuntz 2014). The lower quantities of these transgenes in control samples further supports the idea that transgene presence in WWTPs is linked to human consumption and excretion of transgenic crops or their byproducts. The levels of *p35*, *nos*, and *nptII* genes in these control wastewater samples may be a reflection of the small amounts of foods consumed that contain ingredients derived from transgenic crops. Although France does not allow for the cultivation or human consumption of transgenic crops, France still imports processed foods containing transgenic byproducts from other countries, which

may explain the baseline levels measured (Gruere 2006). Finally, as the abundance of the *bla* ARG was not found to differ significantly between control and US wastewater samples, these data may suggest that the *bla* genes detected in US wastewater are not derived from transgenic crops, but may instead be a reflection of naturally occurring antibiotic resistance. In fact, *bla* genes have been widely detected in WWTP systems across multiple countries in both biofilms and suspended bacterial communities (Marti *et al.* 2013).

The statistical association between WWTP size, in terms of typical daily flow rates in MGD, and transgene prevalence was also investigated. Daily flow rates in sampled WWTPs varied greatly, from 1.8 to 155 MGD (Table S1, Supplementary Information). *P35* gene copy numbers were strongly correlated with WWTP size ($p < 0.05$). *Nos* gene concentrations as well as *bla* and *nptII* ARGs were not significantly correlated with WWTP size ($p = 0.14, 0.33$ and 0.67 , respectively). The prevalence of *16S* genes was most strongly correlated with WWTP size ($p < 0.05$). This is to be expected, as influent rate and sludge retention time are known to be strongly correlated with the carrying capacities, and therefore bacterial abundance, of WWTP sludges (Wéry *et al.* 2008; Xia *et al.* 2012).

CONCLUSIONS

In conclusion, all four selected transgenes (i.e., *p35*, *nos*, *bla*, and *nptII*) were detected in all viable samples obtained from WWTPs in the United States. Our data suggest that some of the transgenes detected in WWTPs are most likely derived from digested transgenic crops or foods containing their byproducts within human waste. The *p35* promoter gene was detected at high concentrations in all US wastewater samples. Although this gene is naturally found within the CaMV the authors were unable to detect additional CaMV genes (e.g., ORF VII) in any WWTP sample, suggesting the *p35* genes were derived from transgenic crop material rather than CaMV. The *nos* terminator gene was also detected at high copy numbers within sampled WWTPs. While this *nos* gene is derived from bacteria, it is specific to *Agrobacterium tumefaciens*, which is commonly found in soils, but has not been noted in the literature to reside in WWTP systems. Finally, *bla* ARG primers used by this study are crossborder primers, with the forward primer targeting a region 109 bp upstream of the *bla* gene and the reverse primer targeting a region 273 bp into the *bla* gene on the transgenic transformation plasmids. Utilizing crossborder primers, which include additional elements of the

host plasmid, lends supports to the idea that the detected *bla* construct is derived from the transformation plasmid contained from transgenic crops. This is also supported by the strong differences in gene abundance between US and French control WWTPs, where the rates of transgenic crop cultivation and human consumption are significantly lower (Kuntz 2014). It is important to note that while our study suggests this association, additional in-depth metagenomic analysis is needed to confirm this association. Furthermore, additional studies are needed to determine if the DNA fragments amplified in this study originated from broken-down gene templates or full-length templates which may be transferred via horizontal gene transfer (HGT). HGT has been documented to occur within WWTPs at elevated rates, possibly as a function of the high concentration and mobility of aquatic bacteria as well as the elevated availability of dissolved Ca^{2+} and Mg^{2+} ions (Moura *et al.* 2007; Zhang *et al.* 2009a, 2009b; Caucci & Berendonk 2014). The potential risk of ARG transfer to bacteria may be heightened by the presence of promoter genes and terminator genes incorporated into transformation plasmids (e.g., *p35* and *nos*, respectively); however, the integrity and viability of these larger fragments should be further verified. Transgenes detected within WWTP anaerobic sludges are of particular interest as more than 50% of these sludges are further utilized as agricultural fertilizers (Holzem *et al.* 2016). Upon land application of biosolids fertilizers, these transgenes may be able to adsorb to the surfaces of soil and clay particles, which are able to shield this DNA from physical and enzymatic degradation in additional environments (Gardner & Gunsch 2017). Therefore, the present study highlights the importance of verifying the sources of potential transgenes, as documented herein, with future metagenomic studies of WWTPs and evaluating their ability to be taken up by environmental bacteria and persist across multiple environments.

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CONFLICT OF INTEREST STATEMENT

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