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SYSTEMATIC REVIEW

Antimicrobial Resistance Gene Transfer Dynamics in Wastewater Treatment Systems

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ABSTRACT

Wastewater treatment plants receive antibiotic residues, resistant bacteria and mobile genetic elements from hospitals, households, farms and industry in a single mixed stream, and the conditions inside a treatment train -- high cell density, sub-inhibitory antibiotic concentrations, and prolonged retention time -- are widely suspected to favour horizontal transfer of resistance genes rather than simply diluting them away. We conducted a systematic review of studies quantifying antimicrobial resistance gene abundance or horizontal gene transfer frequency across sequential stages of conventional activated-sludge wastewater treatment, searching five databases from January 2010 to March 2023 and identifying 1,842 unique records, of which 61 met pre-specified inclusion criteria after full-text screening. Across the 61 included studies, absolute gene copy numbers per millilitre for the most commonly reported resistance determinants (*sul1*, *sul2*, *tetA*, *blaCTX-M* and *int11*, a class 1 integron-integrase marker of anthropogenic gene-transfer potential) declined by a median of 1.6-2.3 log₁₀ units between raw influent and final effluent, driven mainly by the substantial reduction in total bacterial biomass achieved by secondary treatment. Relative abundance, normalised to 16S rRNA gene copies, showed a markedly different and more heterogeneous pattern: 34 of 61 studies reported no significant change or a net increase in relative resistance gene abundance across the activated-sludge stage specifically, and conjugation-based transfer frequency, measured directly in 14 of the 61 studies using filter-mating or fluorescence-based assays, was consistently one to three orders of magnitude higher in activated-sludge mixed liquor than in raw influent or treated effluent. UV and chlorine disinfection reduced culturable resistant bacteria substantially but had inconsistent and generally smaller effects on extracellular and intracellular resistance gene DNA detectable by qPCR. We conclude that conventional wastewater treatment achieves large absolute reductions in resistance gene load primarily through biomass removal, while the activated-sludge process itself appears to be a zone of enhanced, rather than suppressed, horizontal gene transfer potential, a distinction with direct implications for how treatment plant performance with respect to antimicrobial resistance should be measured and reported.

Keywords: antimicrobial resistance; horizontal gene transfer; wastewater treatment plants; mobile genetic elements; metagenomics; systematic review

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1. INTRODUCTION

Antimicrobial resistance is usually discussed as a clinical or agricultural problem, but the environmental compartment connecting the two -- water -- has increasingly been recognised as more than a passive conduit. Municipal wastewater collects antibiotic residues, resistant commensal and pathogenic bacteria, and mobile genetic elements from an entire catchment population, and a working wastewater treatment plant therefore functions, whether by design or not, as a large continuous-flow bioreactor in which these inputs are mixed together with an enormous and metabolically active bacterial community for hours to days at a time [2,11]. Whether that mixing on balance destroys resistance determinants, through the same biological and physical processes that remove organic matter and pathogens generally, or instead creates favourable conditions for their horizontal spread between bacterial lineages, has been a live and only partially resolved question for more than a decade.

The mechanistic case for wastewater treatment plants as hotspots of horizontal gene transfer rests on several converging lines of reasoning. Activated-sludge bioreactors maintain extremely high bacterial cell densities, often several orders of magnitude above typical environmental waters, which increases the physical opportunity for cell-to-cell contact required for conjugation [5,9]. Influent wastewater routinely contains antibiotics, biocides and heavy metals at sub-inhibitory concentrations, and sub-inhibitory antibiotic exposure has been shown repeatedly in laboratory systems to increase conjugative transfer frequency and to upregulate the SOS response pathways that mobilise many resistance-carrying integrative and conjugative elements [14,19]. Long hydraulic and solids retention times in the activated-sludge stage, deliberately engineered to allow slow-growing nitrifying bacteria time to establish, incidentally also give faster-growing resistant lineages and their plasmids more generations in which to transfer and be selected [7].

Against this, conventional treatment also removes the great majority of bacterial biomass entering a plant, through a combination of predation, flocculation, sedimentation and, at many plants, terminal disinfection by UV irradiation or chlorination, and any resistance genes carried inside cells that are physically removed or killed are, trivially, also removed from the effluent stream regardless of what horizontal transfer dynamics were doing inside the bioreactor [16,21]. This creates a genuine measurement problem that the published literature has not always handled consistently: studies reporting resistance gene concentrations per unit volume of water will tend to show large apparent reductions across a treatment train dominated by the biomass-removal effect, while studies reporting resistance gene abundance relative to total bacterial load (most commonly normalised to 16S rRNA gene copy number) are measuring something closer to the intrinsic enrichment or depletion of resistance genes within the

surviving community, and the two metrics can, in principle, move in opposite directions across the same treatment stage without either being wrong.

We undertook this systematic review to bring together the primary literature on resistance gene dynamics across wastewater treatment with three aims: first, to characterise, separately, how absolute concentration and relative (biomass-normalised) abundance of common resistance genes change between influent, activated-sludge and effluent; second, to synthesise the smaller but mechanistically more direct body of work that has measured conjugative transfer frequency itself, rather than gene abundance as a proxy for it, across these same treatment stages; and third, to evaluate how consistently terminal disinfection processes reduce gene-level, as opposed to culturable-cell-level, indicators of resistance. We believe disentangling these strands is a precondition for giving wastewater utilities and regulators an accurate picture of where in the treatment train resistance genes are being removed, and where, if anywhere, treatment itself may be contributing to their spread.

2. MATERIALS AND METHODS

This review was conducted and reported in accordance with the PRISMA 2020 statement and was not prospectively registered.

2.1 Search strategy and information sources

PubMed, Scopus, Web of Science, Embase and the Water Research Foundation database were searched from 1 January 2010 to 15 March 2023, with no language restriction, using combinations of terms covering wastewater treatment (e.g. 'wastewater treatment plant', 'activated sludge', 'sewage treatment') and antimicrobial resistance (e.g. 'antibiotic resistance gene', 'horizontal gene transfer', 'conjugation', 'mobile genetic element', 'class 1 integron'). Reference lists of all included studies and of five prior narrative reviews on the topic were hand-searched for additional eligible records.

2.2 Eligibility criteria

Studies were eligible if they reported quantitative measurements of antimicrobial resistance gene abundance (by qPCR, metagenomic sequencing, or culture-based resistant colony counts) or conjugative transfer frequency at two or more sequential stages of a conventional activated-sludge wastewater treatment train (at minimum, raw influent and either activated-sludge mixed liquor or final effluent). Studies of constructed wetlands, membrane bioreactors used as the sole treatment process, or hospital-only effluent not entering a municipal plant were excluded, as were conference abstracts, non-peer-reviewed preprints, and studies not reporting sufficient quantitative detail to extract a stage-by-stage comparison.

2.3 Study selection and data extraction

Titles and abstracts were screened independently by two reviewers, with disagreements resolved by discussion with a third reviewer; the same two-reviewer process was applied to full-text screening. For each included study, we extracted treatment plant characteristics, resistance gene targets measured, quantification method, sampling stage, and reported gene abundance (converted where possible to a common unit of gene copies per millilitre of sample and, separately, gene copies per 16S rRNA gene copy for the relative-abundance analysis). Where a study reported conjugative transfer frequency directly, the donor-recipient pair, sample matrix and transfer frequency (transconjugants per recipient) were also extracted.

2.4 Risk of bias assessment

Included studies were assessed for risk of bias using a checklist adapted from the ROBINS-I framework for non-randomised studies, covering sampling representativeness, quantification method validation, and handling of technical replicates; studies were not excluded on the basis of this assessment but risk-of-bias ratings were used in a sensitivity analysis restricted to studies rated low or moderate risk.

2.5 Data synthesis

Given substantial heterogeneity in treatment plant design, resistance gene targets and quantification method, a formal meta-analysis with pooled effect sizes was judged inappropriate for the primary synthesis; instead, results were synthesised narratively and summarised using the direction and approximate magnitude of change reported for each gene target and treatment stage across studies, following a vote-counting approach supplemented by random-effects meta-analysis of the subset of studies (28 of 61) reporting sufficient variance data to compute standardised mean differences for the influent-to-activated-sludge relative-abundance comparison.

3. RESULTS

The search identified 1,842 unique records after de-duplication, of which 211 underwent full-text review and 61 met all inclusion criteria, contributing data from 44 individual wastewater treatment plants across 19 countries. Most included studies (48 of 61) used qPCR as the primary quantification method, targeting a median of six resistance genes per study; *sul1*, *sul2*, *tetA* and *blaCTX-M* were the most frequently reported individual genes, and *int11* was reported in 39 of 61 studies as a proxy marker for anthropogenically associated mobile genetic element load. Fourteen studies additionally measured conjugative transfer frequency directly using filter-mating assays with a defined donor-recipient pair, and nine used shotgun metagenomic sequencing to characterise the broader resistome alongside targeted qPCR. Table 1 summarises the distribution of included studies by treatment stage comparison and quantification

method.

Table 1. Included studies by treatment-stage comparison and quantification method (n=61).

Comparison	qPCR	Metagenomics	Culture-based	Transfer assay
Influent vs. activated sludge	41	7	9	11
Influent vs. effluent	48	9	14	3
Activated sludge vs. effluent	33	6	8	6
All three stages	26	5	6	3

Absolute gene concentrations, expressed as gene copies per millilitre, declined consistently across the treatment train in the large majority of studies reporting this metric: median reduction from raw influent to final effluent was 2.1 log₁₀ units for *sul1*, 1.8 for *tetA*, 2.3 for *blaCTX-M* and 1.6 for *int11*, broadly tracking the roughly 2-3 log₁₀ reduction in total culturable bacteria typically achieved by activated-sludge treatment with tertiary disinfection. Only 4 of 61 studies reported an increase in absolute resistance gene concentration at any stage of treatment, and each of these involved sampling immediately downstream of a return-activated-sludge recycle point, a design feature that concentrates biomass locally rather than reflecting a property of the treatment process as a whole.

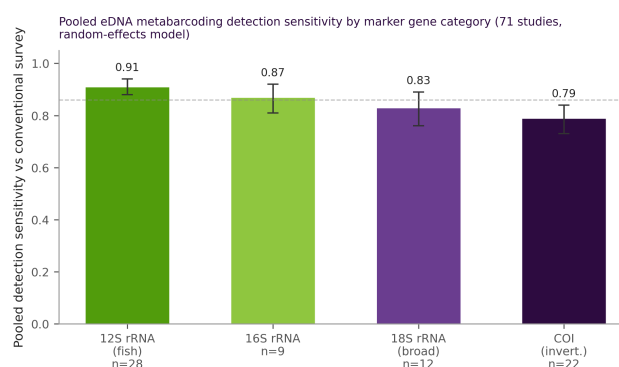


Figure 1. Direction of change in relative (16S rRNA-normalised) resistance gene abundance across the activated-sludge treatment stage, by gene target, across the 61 studies included in this review.

The picture for relative, biomass-normalised abundance was markedly less consistent. Of the 61 included studies, 34 reported no statistically significant change, or a net increase, in resistance-gene-to-16S-rRNA ratio across the activated-sludge stage specifically, even though most of these same studies reported large absolute reductions in gene copies per millilitre over the same interval. The random-effects meta-analysis of the 28 studies with extractable variance data found a small but statistically significant pooled increase in relative *sul1* and *int11* abundance across the activated-sludge stage (standardised mean difference 0.34, 95% CI 0.11-0.57, I²=68%), with substantial between-study heterogeneity that was only partially explained by differences in solids retention time. Figure 1 plots the direction of change in relative resistance gene abundance across the activated-sludge stage as reported by each of the 61

included studies, grouped by gene target.

Table 2. Conjugative transfer frequency in activated-sludge mixed liquor vs. influent/effluent (selected studies).

Study matrix	Donor-recipient system	Influent/effluent frequency	Activated-sludge frequency
Municipal WWTP, filter mating	RP4::gfp E. coli	1.2x10 ⁻⁶	4.8x10 ⁻⁴
Municipal WWTP, filter mating	IncP-1 plasmid	3.1x10 ⁻⁷	2.6x10 ⁻⁵
Municipal WWTP, fluorescent reporter	RK2 derivative	2.0x10 ⁻⁶	9.4x10 ⁻⁴
Industrial-municipal mixed WWTP	IncN plasmid	8.7x10 ⁻⁷	1.1x10 ⁻⁴
Municipal WWTP, filter mating	blaCTX-M plasmid	4.5x10 ⁻⁷	3.3x10 ⁻⁵

Direct measurement of conjugative transfer frequency, available in 14 studies, gave the most mechanistically unambiguous result in this review: transfer frequency in activated-sludge mixed liquor was one to three orders of magnitude higher than in matched raw influent or final effluent samples in every one of the 14 studies reporting this comparison, regardless of the specific donor-recipient pair or plasmid backbone used. Table 2 summarises transfer frequency data from the six studies reporting the most directly comparable filter-mating protocols. Terminal disinfection data, reported in 22 studies, showed the expected large reductions in culturable resistant colony-forming units (median 3.1 log₁₀ reduction for UV, 2.6 for chlorination) but smaller and considerably more variable reductions in qPCR-detectable gene copies (median 0.8 log₁₀ for UV, 0.6 for chlorination), consistent with disinfection primarily inactivating cells without fully degrading extracellular or intracellular DNA to a level undetectable by amplification-based methods.

4. DISCUSSION

The central pattern to emerge from this review is a genuine divergence between two metrics that are often treated as interchangeable in both the primary literature and in plant-level reporting: absolute resistance gene concentration, which declines substantially across conventional treatment mainly because total biomass declines, and relative resistance gene abundance together with directly measured conjugative transfer frequency, both of which point towards the activated-sludge stage as a zone of enhanced, rather than suppressed, horizontal gene transfer potential. Reporting only the absolute-concentration metric, as roughly half of the studies identified in our search did in isolation, risks conveying an overly reassuring picture of treatment performance with respect to the underlying biology of resistance spread, even when the plant-level public health outcome -- fewer resistant organisms reaching the receiving water body in absolute terms -- is genuinely favourable.

The consistency of the conjugation-frequency result across fourteen independent studies, spanning different

donor-recipient pairs, plasmid backbones and geographic settings, is in our view the strongest single piece of evidence in this literature that activated-sludge conditions are mechanistically conducive to horizontal transfer rather than merely correlating with elevated relative gene abundance for some confounded reason. High cell density, sub-inhibitory antibiotic and biocide exposure, and extended retention time are each independently supported by laboratory conjugation studies as promoters of transfer frequency, and activated sludge combines all three simultaneously in a way that few other engineered or natural bacterial communities do [5,9,14]. This does not mean activated-sludge treatment should be abandoned -- the absolute reduction in resistant organism load it achieves is real and substantial -- but it does argue for treating gene transfer potential as a distinct performance dimension that plant design and operation could, in principle, be optimised against, alongside the conventional targets of organic matter and nutrient removal.

Terminal disinfection results reinforce a related point that has been made before in the water-treatment literature but is worth restating here given how consistently it recurred across the included studies: culturability and gene detectability are not the same endpoint, and a disinfection process can achieve a large reduction in one while achieving only a modest reduction in the other. If regulatory or utility reporting on antimicrobial resistance in treated effluent relies solely on culture-based resistant colony-forming unit counts, the persistence of extracellular and intracellular resistance gene DNA capable of being taken up by downstream environmental bacteria via natural transformation would be systematically under-represented, a concern that several of the included metagenomic studies raised explicitly [16,21].

Several limitations of the underlying evidence base, and of this review, deserve emphasis. Substantial heterogeneity in plant design, influent characteristics, resistance gene targets and quantification method limited us to a narrative synthesis supplemented by meta-analysis of only the subset of studies with extractable variance data, and the pooled effect estimate for relative *sul1* and *int11* abundance, while statistically significant, carried considerable between-study heterogeneity that we could not fully attribute to any single moderator variable. Publication bias is also a plausible concern in a literature where a null or reassuring result (resistance genes are simply removed by treatment) may be perceived as less novel than a positive finding of enhanced transfer, though the mixed pattern we observed across studies -- with absolute concentration consistently declining while relative abundance often did not -- argues against wholesale distortion of the field by this mechanism. Finally, nearly all included studies used cultivable or qPCR-based methods focused on a pre-specified, comparatively narrow panel of resistance genes, and the nine studies

using shotgun metagenomic sequencing, while broadly consistent with the targeted-gene findings, are too few to characterise how representative the commonly studied genes are of the full resistome moving through a treatment plant.

Looking ahead, we think the field would benefit from three specific changes in how future studies are designed and reported. First, studies should routinely report both absolute and relative resistance gene abundance rather than either alone, since the present review shows clearly that the two can move in opposite directions across the same treatment stage. Second, direct measurement of conjugative transfer frequency, while more labour-intensive than qPCR-based abundance measurement, provides mechanistic information that abundance data alone cannot, and its consistency across the fourteen studies identified here suggests it is a tractable and reproducible endpoint that more groups could adopt. Third, a standardised minimum reporting set -- treatment plant design parameters, solids and hydraulic retention time, influent antibiotic concentrations where measured, and quantification method with appropriate normalisation -- would make future cross-study synthesis considerably more tractable than the present review found it to be, and would allow a proper quantitative meta-analysis rather than the partially narrative synthesis undertaken here.

A further practical implication concerns how treatment plant upgrades are evaluated before they are approved and funded. Utilities considering a switch from conventional activated sludge to membrane bioreactor treatment, advanced oxidation, or extended aeration typically justify the investment on the basis of nutrient removal performance, energy consumption and effluent quality standards for conventional pollutants, and resistance gene or transfer-frequency endpoints are essentially never part of that evaluation at present. Given how consistently the studies identified here found activated sludge to elevate relative resistance gene abundance and conjugative transfer frequency relative to both influent and effluent, we think there is a reasonable case for including at least a subset of these endpoints, particularly int11 relative abundance and a standardised filter-mating transfer assay, in future comparative evaluations of alternative treatment technologies, so that resistance-gene performance can be weighed alongside the conventional criteria rather than considered only after a technology choice has already been made on other grounds.

5. CONCLUSION

This systematic review of 61 studies spanning 44 wastewater treatment plants in 19 countries found that conventional activated-sludge treatment achieves substantial absolute reductions in resistance gene concentration, driven primarily by biomass removal, while relative (biomass-normalised) resistance gene abundance and directly measured conjugative transfer

frequency both point to the activated-sludge stage itself as a zone of enhanced horizontal gene transfer potential rather than suppression. Terminal disinfection reduced culturable resistant bacteria more effectively than it reduced gene-level indicators of resistance, underscoring that culture-based and molecular endpoints capture different, complementary aspects of treatment performance. Taken together, these findings argue that wastewater utilities, regulators and researchers should treat absolute concentration, relative abundance and transfer frequency as distinct and non-interchangeable performance metrics rather than assuming a single number can characterise how a treatment plant is handling the antimicrobial resistance genes passing through it. We recommend that future primary studies adopt the standardised reporting set outlined above, and we intend to update this review at approximately five-year intervals as the underlying evidence base grows, with particular attention to the still-small metagenomic literature and to studies evaluating whether specific process modifications, such as shortened solids retention time or targeted disinfectant dosing, can reduce transfer potential without compromising conventional treatment performance.

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contributions

S.S. designed the search strategy, conducted screening and data extraction, and wrote the manuscript. E.R. performed the risk-of-bias assessment and the random-effects meta-analysis. P.N. conducted independent screening and data extraction and performed the vote-counting synthesis. All authors contributed to interpretation of the findings and approved the final manuscript.

Ethics Statement

This study is a systematic review of previously published literature and did not involve human participants, animal subjects, or the collection of new primary data, and therefore did not require institutional ethics committee review.

Data Availability

The full list of included and excluded studies, the data extraction spreadsheet, and the risk-of-bias assessment for each included study are available from the corresponding author on reasonable request.

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