Antibiotic resistance dissemination and sewage treatment plants

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Antibiotics are a class of naturally-occurring and synthetic chemical compounds with antimicrobial activity and are widely used in human and veterinary medicine. The increasing incidence of resistance to a wide range of antibiotic agents by a variety of organisms is a major concern facing modern medicine. There is widespread speculation that sewage treatment plants (STPs) and aquatic environments in general maybe a breeding ground for antibiotic resistant bacteria. In this mini-review, we examine and consider whether the presence of antibiotics in STPs facilitates the acquisition and proliferation of resistance characteristics amongst bacteria in that environment, or whether it just simply imparts a selective pressure for the propagation of clonal resistance. Literature reports relating to bacterial and resistance counts, upstream and downstream of hospital, pharmaceutical and town STPs in different geographical regions are appraised and inferences presented. The mini-review also appraises available literature on the acquisition of antibiotic resistance by previously non-resistant bacteria and the role of antibiotics in the transfer of resistant genes in STPs. Specific observations that would be required to confirm these phenomena are identified and experimental approaches, including culture-independent methods, are suggested.

Keywords antibiotics; bacteria; lateral gene transfer; resistance; sewage treatment plants (STPs)

1. Introduction

Antibiotics are a class of naturally-occurring, semi-synthetic and/or chemically synthesised compounds with antimicrobial activity. They are widely used in human and veterinary medicine to treat and prevent diseases and as growth promoters in animal intensive industries. The increasing incidence of resistance to a wide range of antibiotics by microorganisms is a major concern facing modern medicine. Clinical infections, disease and death caused by resistant bacteria are increasingly common. We know for a fact that antibiotic resistance can be established and propagated in human and animal digestive systems [1, 2]. The proliferation of resistant strains within the gastrointestinal track is facilitated by the co-occurrence of high concentrations of bacteria and sub-lethal doses of antibiotics. However, there is some conjecture and speculation about other environments, such as sewage treatment plants (STPs) [3, 4] and aquatic environments in general [5], which provide conducive conditions for the establishment and propagation of antibiotic resistant bacteria. As an example of the later, Muniesa and co-workers [6] reported bacteriophage isolated from sewage carrying various β-lactamase genes, a potentially simple route for disseminating antibiotic resistance amongst STP bacterial communities. Besides health fears, there is growing concern about the potential ecological impacts from both the presence of antibiotics and resistant bacteria in the environment.

Since the late 1990s, several classes of antibiotics have been reported in sewage and STPs including β-lactams, sulfonamides, trimethoprim, macrolides, fluoroquinolones and tetracyclines [7-9]. Some of these antibiotics are incompletely metabolized during therapeutic use and are excreted into sewage unchanged. Although variable, antibiotic concentrations in STP influents have been detected between 0.1 – 1.0 μg L^{-1}, whilst secondary effluent levels have been reported an order of magnitude lower.

Several authors have reported correlations between high concentrations of antibiotics in sewage and/or the environment and elevated levels of resistance to antibiotics by bacteria [5, 10]. However, such correlations are suggestive and do not confirm the existence of a cause-effect relationship. In many cases the correlation is to be expected given that both antibiotics and bacterial isolates are derived from a common source. That is, both are excreted by humans and discharged into the environment via sewage outfalls.

There are conflicting reports as to whether relative rates of antibiotic resistance are increased during STP processes. Some researchers have observed little difference in bacterial resistance levels between raw and treated sewage [11, 12]. Others have observed a decline in antibiotic resistance levels following sewage treatment [13], contradicting findings that resistance levels are proportionally elevated in treated effluent depending on the antibiotic, sewage treatment and bacterial genera under investigation. It should be noted that these observations are drawn from particular bacterial species and not reported as relative to the total number or diversity of bacteria.

The importance of and general interest in antibiotic resistance is supported by a large body of published reviews. Representative reviews encompassing this topic include: drug discovery and therapy [14, 15]; screening and epidemiology [16, 17], antibiotic resistance and its proliferation [18, 19], virulence and pathogenesis [20, 21]; genetics of antibiotic resistance [22, 23] and a host of others too numerous to list. The intension of this mini-review is not to revise this material but to provide the reader with an overview of antibiotic resistance, its acquisition and propagation

amongst bacterial community members in STP wastewaters and examine their relationship (causal or not). A cursory sample of some of the more innovative studies will also be discussed together with suggestions for future work.

2. Antibiotics and resistance mechanisms

Natural antibiotic producers are inherently resistant to the antibiotics they produce. Other bacteria survive by developing or acquiring antibiotic resistance mechanisms. Some of the prominent means of resistance include: altered permeability barriers across bacterial outer membranes, preventing uptake of the compound by inhibiting its corresponding transport carrier, modifying the target's binding sites so that it no longer recognizes the antibiotic(s), and the ability to chemically and/or enzymatically degrade the antibiotic (refer to fig. 1).

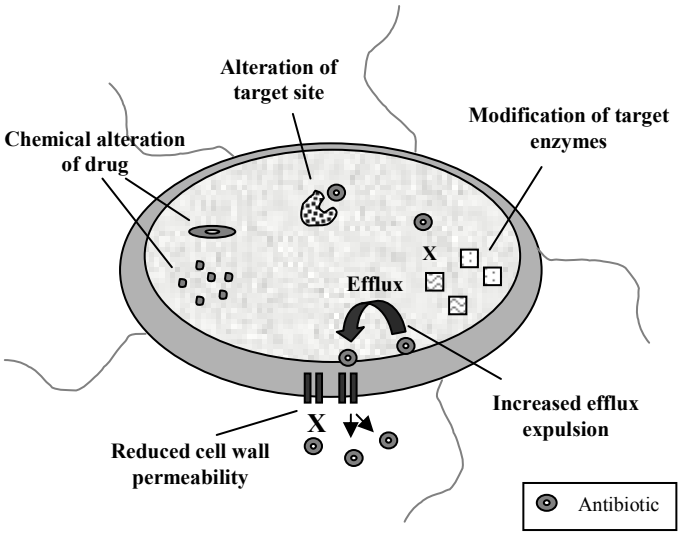

Fig. 1 Schematic diagram depicting the major antimicrobial resistance mechanisms

Antibiotics must enter the bacterial cell to access a target site in order to exert their bactericidal (cell death) or bacteriostatic (slow bacterial growth) action. Gram-negative bacteria are resistant to a greater number of antibiotics compared to gram-positives, largely because they possess an outer membrane. Migration of antibiotics between the external environment and a cell's periplasm occurs via 'porin channels'. Mutations within genes encoding for such porin channels could reduce the ability of the antibiotic to reach its target site as well as physical barriers such as extracellular gums and/or biofilms.

Efflux pumps are found in both gram-positive and negative bacteria and actively transport toxic substances from within the bacteria to its surrounding environment. DNA operons encoding for efflux genes are found either on chromosomes and are indicative of intrinsic resistance, or on plasmids which are suggestive of acquired resistance. There are five major efflux transporter families in prokaryotes, some selective, whilst others involved with expelling an array of compounds including antibiotics. The latter class of efflux exporters are cause for major concern since they can lead to multi-resistant bacteria. Mutations in the efflux repressor genes prompt over-expression of the structural genes which may lead to an increasing level of antibiotic tolerance [24]. Although over expression of efflux pump genes do not necessarily afford high level resistance; they guard against lower drug exposures and prolong survival until further possible mutations take place (such as within the antibiotics target site), potentially leading to highly resistant progeny.

2.1 Resistance to β-lactams

β-lactams such as penicillins and cephalosporins are narrow spectrum antibiotics, effective against the gram-positive genera *Streptococcus*, *Gonococcus* and *Staphylococcus* [25]. Prior to its introduction in the 1940s, almost all hospital acquired *Staphylococcus aureus* strains were sensitive to penicillin G, whilst today virtually all strains show resistance. Methicillin resistant *S. aureus* (MRSA) produce a low affinity penicillin binding protein PBP2a, encoded by the *mecA* gene, which provides resistance to virtually all β-lactams. *Neisseria* and *Streptococci* spp. also have reduced affinity for β-lactams due to altered penicillin binding proteins (PBPs). Another important resistance mechanism is the production of β-lactamase enzymes which inactivate the antibiotic molecule by hydrolysing the β-lactam ring [26]. The ability to produce β-lactamase enzymes is common amongst gram-negative bacteria, encoded on either plasmid or chromosomal DNA. Clinical introduction of cephalosporins initially halted the spread of plasmid encoded β-lactamases resistance, however, bacteria quickly acquired modifications to their β-lactamase genes (extended-spectrum β-lactamases) conferring resistance to penicillins and cephalosporins.

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2.2 Resistance to sulfonamides and trimethoprim

Sulfonamides and trimethoprim are synthetic competitive inhibitors of bacterial enzymes required for the synthesis of tetrahydrofolic acid (THF), which is necessary for the production of DNA and proteins [27]. Sulfonamides act as competitive inhibitors of dihydropteroate synthase (DHPS), while trimethoprim inhibits dihydrofolate reductase (DHFR). Resistance to sulfonamides and trimethoprim is almost exclusively associated with plasmid-encoded genes. To date, three sulfonamide resistance genes coding for different types of DHPS (insensitive to sulfonamides) have been identified: *Sul*1, *Sul*2 and the more recently described *Sul*3 gene [28]. Most multi-resistant gram-negative bacteria harbor Class 1 integrons which carry the *Sul*1 gene. The *Sul*2 gene is frequently associated with the small, multi-copy, non-conjugative IncQ plasmid group. Although less common, resistance can also be due to mutations within the chromosomally located dihydropteroate synthase gene (*folP*). Trimethoprim resistance is widespread amongst pathogenic bacteria, with up to 29 dihydrofolate reductase (*dfr*) resistance genes identified [29]. Most of these genes are associated with integrons and use elaborate transfer mechanisms to laterally spread and proliferate within the bacterial community.

2.3 Resistance to macrolides

Macrolide antibiotics, such as erythromycin, inhibit protein synthesis in most gram-positive bacteria by binding to the 50S ribosomal subunit [25]. Gram-negative bacteria are intrinsically resistant to macrolides, which cannot traverse their outer membrane. Gram positive bacteria may employ any one of three resistance mechanisms to negate the effects of these antibiotics; including alteration of the target ribosomal binding site, expulsion of the macrolides via efflux pumps, and direct inactivation by enzymes encoded on transmissible plasmids [30].

Fluroquinolones on the other hand, are effective against both gram negative and positive bacteria. Unlike other antibiotics, fluoroquinolones selectively inhibit nucleic acid synthesis by inhibiting bacterial DNA topoisomerase II and thus prevent bacterial growth. They enter gram-negative cells via porin channels and gram-positives by lipophilicity (ability to diffuse through the lipid bilayer of the cell membrane). Resistance may be conferred by reduced internal drug build up owing to diminished cell wall permeability and/or increased efflux expulsion [30]. The main mechanism of resistance is believed to involve the modification of fluroquinolone's target, chiefly DNA gyrase and topoisomerase IV. These enzymes consist of two subunits: GyrA and GyrB for DNA gyrase and ParC and ParE for topoisomerase IV, all of which are encoded by the genes *gyrA*, *gyrB* and *parC, parE* respectively. Resistance occurs in response to chromosomal mutation of these genes. In clinical isolates, quinolone resistance genes (*qnrA)* are either chromosomally located or plasmid borne as in *qnrB*, *qnrS* and *qnrS2* . The *qnrA* gene is located within an integron and is associated with the *sul1* gene and confers resistance to nalidixic acid but not to fluoroquinolones. The gene product essentially binds to DNA gyrase subunits and minimises quinolone action. Plasmid mediated resistance has been found shown to enhance pre-existing quinolone resistant mechanisms such as the efflux system [30]. Consequently, bacteria resistant to fluoroquinolones are often multi-resistant.

2.4 Resistance to tetracyclines

Tetracycline antibiotics act by blocking the binding of aminoacyl tRNA to the ribosome thereby inhibiting protein synthesis [25]. There are 38 known tetracycline (*tet*) and oxytetracycline (*otr*) resistance genes [31]. Of these, 23 encode for efflux pumps, 11 encode for ribosomal proteins, 3 for an inactivation enzyme and 1 for an unknown resistance mechanism. Efflux genes from gram-positive bacteria are associated with small plasmids, whilst those from gram negatives are often linked to large conjugative plasmids. Given that any of the latter harbor resistance determinants for several antibiotic drugs and heavy metals, selection for resistance to tetracycline will generally render the recipient multi-resistant [2]. Genes encoding for ribosomal protection proteins are usually located within conjugative transposons, which accounts for their large host range. Other resistance mechanisms, conferred by the *tet*(X) genes, are responsible for enzymatic alteration of the drug [31]. Point mutations within the 16S rRNA gene and mutations which alter the permeability of the porin channels also increase tetracycline resistance [2].

2.5 Resistance to nitroimidazoles

Nitroimidazoles such as metronidazole are microbiocidal drugs active against most anaerobic bacterial species [32] and a range of pathogenic anaerobic protozoa causing infections such as *giardiasis, amoebiasis* and trichomoniasis. They bind to macromolecules including DNA and inhibit its synthesis. Metronidazole is administered in an inactive form and enters the cell by diffusion. Its activation is subject to a reduction of the molecule's nitro group by the ferredoxin mediated electron transport system, thereby creating toxic free radicals which kill sensitive strains [33]. This reductive mechanism appears to be unique to the anaerobes. Resistance is associated with the *nim* genes encoding 5-nitroimidazole reductase enzymes located on the chromosome (*nimB*) or on low copy self-transmissible plasmids. Nitroimidazole reductase acts by removing an electron from the intermediary ferredoxin compound, thus eliminating the drug's trigger mechanism. Some organisms may impart resistance by their reduced intracellular concentrations of ferredoxin, leading to reduced activation of the drug.

2.6 Resistance to glycopeptides

Vancomycin is a bacteriocidal glycopeptide antibiotic which until recently was used as the final safeguard against multi-resistant gram-positive bacterial infections such as MRSA and multi-resistant enterococci [34]. Vancomycin inhibits cell wall formation of gram-positive bacteria by binding to its target site within the peptidoglycan assembly preventing cross-linking. Vancomycin resistance has been associated with seven *van* genes, which code for the promotion of an abnormal target site with lower affinity for the drug [35]. The *van*A genotype also confers a high level of resistance to the glycopeptide teicoplanin. Vancomycin resistant enterococci (VRE) are resistant to vancomycin and teicoplanin due to a gene cluster which encodes for the synthesis of a novel cell envelope with a 1000-fold reduced affinity for glycopeptide binding. Thickening of the cell wall or slowing cell growth is also implicated with vancomycin resistance, especially in *Lactobacillus casei, Pediococcus pentosaceus* and *Leuconostoc mesenteroides* which are intrinsically resistant to the glycopeptide.

The glycopeptide avoparcin has been used as a growth promoter in animals, thereby giving rise to a large *van* resistance gene pool [36]. Banning avoparcin use in Denmark did not lead to reduced levels of glycopeptide-resistant enterrococci (GRE) in pigs and it wasn't until all macrolide-based growth promoters were also barred that GRE levels substantially dropped. This apparent co-selection of resistance genes has been explained by a genetic linkage between the glycopeptide resistance gene *vanA* and the macrolide resistance gene *ermB* originating from pigs [37].

Aminoglycosides such as kanamycin, gentamycin and streptomycin bind to bacterial ribosomes and prevent the initiation of protein synthesis. Bacterial resistance is generally due to chemical alteration of the drug thus preventing it from binding to its ribosomal target site [38]. Resistance genes are commonly found on self-transmissible plasmids and transposons but may also reside in the chromosome [35]. Mutations associated with ribosomal genes and efflux systems may also be linked to aminoglycoside resistance.

3. Disseminating antibiotic resistance

There are two routes for acquired resistance, vertical evolution via mutation and selection or horizontal evolution via exchange of genes between similar and different species. Vertical evolution is determined by natural selection whereby a spontaneous mutation in the bacterial chromosome bestows resistance to a bacterium and its progeny within the population.

Horizontal evolution (or lateral gene transfer) generally occurs via three routes; transformation (DNA uptake), conjugation (direct contact transfer of mobile plasmids) or transduction (uptake of naked DNA). Lateral gene transfer is believed to be the major route for widespread global dissemination of antibiotic resistance and is responsible for transfers of plasmids carrying antibiotic resistance genes (R plasmids) in 60-90% of gram-negative bacteria [39]. Lawrence and Ochman [40] deduced that 17.6% of *E. coli* genes have been acquired by lateral gene transfer.

Regardless of their physical location, i.e. chromosome, plasmid or integrons within transposons, antibiotic resistance genes can undergo lateral gene transfer. Transposons, are the most conducive means of transferring antibiotic resistance genes amongst bacterial populations. They typically carry a selectable phenotype (antibiotic resistance) bordered by two insertion sequences, and are unique in their ability to 'jump' from one genetic locus into another, irrespective of taxanomic class. Transposons often contain integrons, genetic elements which harbor a range of antibiotic resistance genes, a promoter site, a recombination site downstream of the resistant genes and an integrase coding gene. They are transferred between bacteria, integrating into bacterial genomes and/or plasmids. Multi-resistance is achieved when several antibiotic resistance cassettes are inserted into the integron.

There are five major classes of integrons [41]. Class 1 integrons are derived from transposon Tn402 that can insert into the large Tn21 transposon; Class 2 is exclusively derived from the Tn7 transposon which is highly adept at integrating into the chromosome of *E. coli* and other *Proteobacteria* thus disseminating its resistance genes throughout a large community of bacteria; Class 3 is probably transposon associated; and Classes 4 and 5 are linked to trimethoprim resistance in *Vibrio* species. Integrons are thought to play a major role in the spread of bacterial antibiotic resistance. Some resistance genes reside within highly efficient transfer elements. For example *dfr1,* the most common trimethoprim resistance gene, is located on both Class1 and Class 2 integrons. Class 1 integrons have been identified in 40-70% of gram-negative bacteria isolated from humans and animals.

A well studied group of highly promiscuous plasmids are the IncP-1 plasmids. In addition to self-transfer, they are capable of coordinating the movement of non-mobilisable plasmids, which in some cases enables genetic material to be transferred across taxonomic barriers. This was verified by Schluter et al [42] who compared the entire DNA sequence of nineteen IncP-1 plasmids isolated from STPs, environmental and clinical isolates. These plasmids were found to contain mobile genetic elements (MGEs) carrying resistance to most antibiotics, heavy metals such as mercury and chromate, and quaternary ammonium compounds. They found genes responsible for replication, conjugation, mating pair formation, plasmid stability and control present on all of the plasmids. They also found similarities within the IncP-1α and IncP-1β subsets which were not common to both. Interestingly, their comparative study showed that the backbone sequences of the IncP-1β plasmids were highly similar (in one instance 100%) to an IncP-1 degradative plasmid. As a general rule, similar incompatibility plasmid groups are unable to co-exist within a bacterium at any one

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time. It follows that IncP-1β could transfer antibiotic resistance or degradative genes to other IncP-1 plasmids provided appropriate selective pressures are maintained on the host. The study also found significant identities within genomes of bacteria from human, animal and plant origins, suggesting that bacteria from a wide range of environments had access to a common gene pool at some point in their evolution.

Multi-drug resistance is often achieved by the acquisition of a single mobile genetic cassette harboring several different resistance mechanisms. In addition to the selective pressure exerted by antibiotic drugs themselves, other antibiotics and/or agents such as disinfectants and heavy metals may also contribute to the maintenance of antibiotic resistance [42]. Consequently, bacteria can retain resistance to drugs such as streptomycin and sulphonamides which are rarely used today, simply because their resistance genes are closely associated with contemporary antibiotics or heavy metal resistance mechanisms.

Resistance gene transfer rates are affected by factors both internal and external to the bacterium. External influences include those which facilitate DNA transferability such as temperature, pH, detergents and organic solvents. Internal influences include the 'SOS' response to DNA damage which appears to increase the frequency of transfer of certain resistance traits. An SOS response regulates transcription in reply to external stresses such as UV radiation and certain antibiotics (ciprofloxacin, trimethoprim and β-lactams), thus causing metabolic changes and mutations facilitating survival and resistance [43].

4. Bacterial resistance to antibiotics in STPs

Biological reactors such as activated sludge tanks, trickling filters and membrane bioreactors concentrate bacteria in conditions designed to encourage their proliferation and activity. For example, 10^{12} - 10^{13} bacteria per gram of volatile suspended solids (VSS) have been reported in activated sludge, of which an estimated 80% were viable [44]. Most of these bacteria, however, represent different groups from those originally in faecal matter or even the sewer, due to the different selective pressures in sewage treatment.

Biological sewage treatment processes reduce viable concentrations of faecal bacteria such as coliforms and enterococci down to 1-4 log units, depending on the plant efficiency [12]. However, relatively high numbers of bacteria remain in STP effluents, up to 10^3 cfu.mL^{-1} enterococci have been reported. Some of these bacteria carry antibiotic resistance genes, especially in the influent, which reportedly contain up to 10^3 mL^{-1} antibiotic resistant coliforms. In fact, Reinthaler and co-workers [45] found that 40% of the cultivable *E. coli* strains isolated from sewage were resistant to one or more antibiotics and 9.8% were resistant to more than three antibiotics. Relative levels of cultivable bacterial antibiotic resistance in sewers and STPs reported in the literature are summarised in Table 1.

Based on the presence of cultivable faecal bacteria, hospital wastewaters appear to contain high levels of antibiotic resistant bacteria compared to other sewage sources. Typically, 2-5% of coliforms isolated from the latter contain resistance (R) plasmids compared to over 50% of coliforms isolated from hospital sewage [46]. The diversity and concentration of antibiotics in hospital sewage presumably accounts for the high incidence in resistance. These high levels of resistance may otherwise reflect a relatively high excretion rate of resistant bacteria and genes from the source populations. A study by Novais et al [47] implicated hospital sewage as a source of vancomycin resistance genes because they found zero VRE upstream of a hospital but 79% of samples tested downstream contained VRE. Some 29% of individual enterococci isolates taken from samples downstream displayed vancomycin resistance. Likewise, STPs in larger cities have been reported to contain significantly greater numbers of resistant enterococci, compared to sewage derived from smaller towns [12]. Interestingly, faecal bacteria isolated from a STP receiving municipal sewage incorporating a nursing home also reported higher rates of resistance to vancomycin compared to two other STPs investigated [48].

As a general rule, conventional sewage treatment processes appear to have little (if any) effect on the relative levels of resistance of select cultivable bacteria isolated from sewage (Table1). However, there are some exceptions to this observation. For example, enterococci resistance to ciprofloxacin has been reported to increase during sewage treatment [49]. Conversely, municipal STPs have been reported to reduce the relative proportion of penicillin resistant pseudomonads by about half [48]. Indeed, certain species have been identified which show variable degrees of antibiotic resistance compared to other members of the genus. For example, high levels of ampicillin resistance among thermotolerant coliforms displayed significant correlations with increased levels of *Klebsiella* [50].

Acinetobacter spp. isolated from sewers receiving wastewaters from a pharmaceutical manufacturing plant have been reported to exhibit significant increases in resistance to a number of antibiotics manufactured at the plant [55]. Sulfonamide resistance increased from non-detectable levels amongst isolates collected upstream of the plant to 51% downstream. Moreover, the number of *Acinetobacter* spp. carrying resistance to more than three antibiotics increased from undetected levels upstream to 36% downstream. These results are highly suggestive that the pharmaceutical waste had either been a source of resistant bacteria, or had exerted a selective pressure for resistant bacteria proliferation in sewage. In the same study, *Acinetobacter* resistance levels downstream of the hospital were found to be slightly elevated, especially with regards to tetracycline. MAR bacteria were reported to have increased from 0% upstream of the hospital to 1.3% downstream.

Table 1 Percentage of cultivable bacteria from various sewage sources exhibiting resistance to antibiotic classes.

Bacteria	Sources	% bacteria resistant to each antibiotic								Ref
		β-lact	Ceph	Tet	Macro	Amino	Glyco	Quin	Sul	
Total coliforms	City sewage	10-30	10-26	1-15		1-30			20-55	[51]
	Hospital sewage	31-52	10-21	16-74		21-93			60-83	[51]
	STP Influent	8.3[a]		4.4[a]		5.8[a]				[52]
	STP Effluent	11.5[a]		3.4[a]		5.2[a]				[52]
Faecal coliforms	STP untreated domestic sewage	35		17		6			20	[50]
	STP treated domestic sewage	20		10		1.5			4	[50]
	STP influent	24-33	28	18	0.4-4	0.2-28			26	[53]
	STP effluent	17-19	23	14	0.3-2	0-17			19	[53]
	short ret. (5 days) lagoon influent	10-19	39	14	0-0.7	0-21			16	[53]
	short ret. (5 days) lagoon effluent	28-35	10	15	0-3	0-13			5	[53]
	long ret. (90 days) lagoon influent	30-56	48	26	10-13	0.6-8			12	[53]
	long ret. (90 days) lagoon effluent	59-65	51	33	35-50	19-26			25	[53]
	STP Influent	10-87[b]		0-5[b]	0-25[b]	0-15[b]	35-95[b]	0[b]	0-25[b]	[48]
	STP Effluent	0-100[b]		0-26[b]	0-41[b]	0[b]	7-100[b]	0[b]	0-40[b]	[48]
E. coli	STP influent	0-18[b]	0-31[b]	6-29[b]				0-10[b]	2-4[b]	[54]
	Activated sludge tank	0-6[b]	0-28[b]	7-28[b]				0-6[b]	0-2[b]	[54]
	STP effluent	0-16[b]	0-35[b]	16-35[b]				0-4[b]	0-10[b]	[54]
	STP sludge	2-16[b]	0-28[b]	21- 57[b]				0-15[b]	6-13[b]	[54]
Enterococcus spp.	Sewage upstream of hospital	11		41	59			27		[47]
	Sewage downstream of hospital	45		37	69			75		[47]
	STP influent	63-100[b]		0-4[b]	0-8[b]			0[b]		[48]
	STP effluent	67-100[b]		0[b]	0-7[b]			0[b]		[48]
	District capitals STPs (influent + effluent + sludge)	2-17		25-53	18-43	0-9	0-3	8-28		[12]
	Smaller towns STPs (influent + effluent + sludge)	0-6		12-50	15-29	0-5	0	6-20		[12]
	STP influent	0		31	33			9	0	[49]
	STP effluent	3		33	23			25	1	[49]
Acinetobacter spp.	Pharmaceutical plant sewer	26		49				0	51	[55]
	Hospital sewer	3		37				3	3	[55]
	STP influent	0-3[b]	11-28[b]	6-9[b]				0-11[b]	2-5[b]	[56]
	STP effluent	0-1[b]	5-22[b]	7-13[b]				0-10[b]	0[b]	[56]
	Digested STP sludge[b]	0[b]	5-32[b]	4-13[b]				1-10[b]	0-1[b]	[56]
Pseudomonas spp.	STP influent	61-93[b]		0[b]	17-32[b]			0[b]	21-96[b]	[48]
	STP effluent	34-100[b]		0-3[b]	13-34[b]			0[b]	20-83[b]	[48]
Native heterotrophic bacteria	STP effluent (trickling filter)	66-83		1				4		[57]
	STP effluent (aerated lagoon)	66-84		3				9		[57]
	STP effluent (activated sludge)	78-96		5				12		[57]

[a] mean value of 5 sampling dates; [b] Combined range from different STPs
β-lact: β-lactams; Ceph: Cephalosporins**;** Tet: tetracyclines; Macro: macrolides; Amino: aminoglycosides; Glyco: glycopeptides; Quin: quinolones; Sul: sulphonamides.

Several multi-resistant plasmids harbouring transposons, each carrying up to 10 different antibiotic resistance genes, have been isolated from STPs [58]. In a study by Gallert [48], 96% of the pseudomonads isolated from effluent incorporating a nursing home were multi-resistant (>3 antibiotics) with some isolates carrying up to seven different resistant traits. An increased proportion of coliforms with resistance genes to different types of antibiotics were reported for wastewater treatment in long-term retention lagoons [53]. However, it is not clear from these studies whether resistant strains simply were more environmentally persistent, actively selected for, or if lateral gene transfer had occurred.

5. Concluding Remarks

A key factor for the acquisition and proliferation of antibiotic resistance amongst bacteria appears to be the co-occurrence of high concentrations of faecal bacteria and sub-lethal antibiotic levels. The presence of low concentrations of antibiotics in STP wastewaters may serve as a selective pressure for the proliferation of resistant organisms. A

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potential scenario depicting a bacterial floc in a conventional STP during primary or secondary treatment stages is illustrated in Fig. 2. As a consequence of high bacterial density, lateral gene transfer is assumed to occur via conjugation, phage-mediated transduction, or free DNA take-up by transformation. External and internal influences (previously mentioned) play a part in evolving the complex resistance mechanisms.

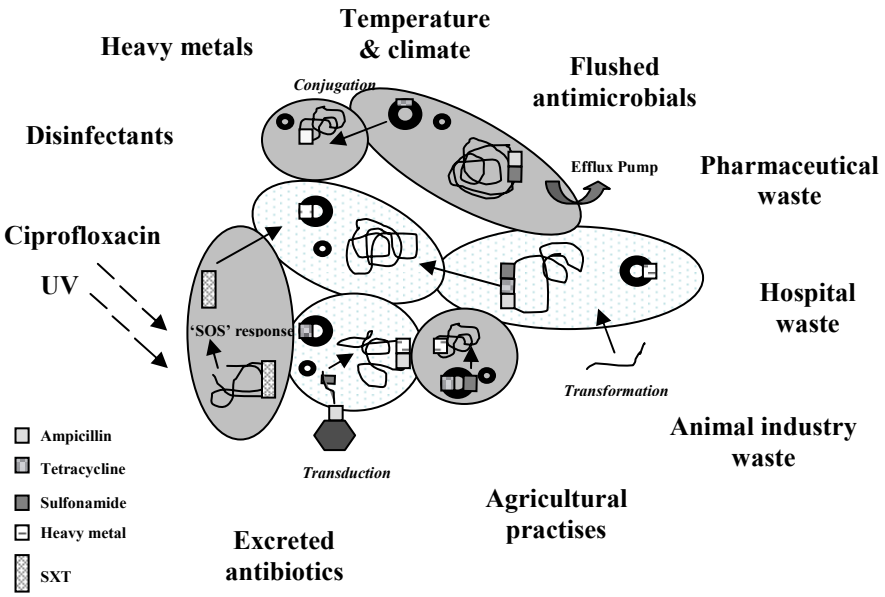

Fig.2. Illustration showing bacterial floc in STP, potential genetic events (italics) and influences (bold) leading to antibiotic resistance amongst environmental bacteria.

While such influences and mechanisms summarised in fig.2 appear plausible, it's difficult to ascertain whether or not such resistance-responses actually occur in STPs. Hence, conclusions based on comparative analysis of published microbial studies must be viewed with caution because factors such as sewage source, species composition, type of STP and operational conditions, significantly influence the outcomes. Similarly, biases associated with analytical methodology such as sampling regimes, culture vs non-cultured techniques, antibiotic type, number and concentration also affect and shape inferences. Accordingly, care has been taken to avoid over-interpreting apparent differences in observations reported from diverse studies.

5.1 Evidence of lateral transfer of antibiotic resistance in sewage treatment plants

The assumption that antibiotic sensitive bacteria can acquire resistance in STPs as a consequence of lateral resistance gene transfer is plausible, given that several transmissible plasmid harboring resistance genes have been isolated from sewage plants [58, 59]. However, demonstrating transfer of such genes between bacteria in STPs remains challenging. Most studies have been performed under laboratory conditions. Soda et al [60] recently reported the *in vitro* transfer of the multi-resistant plasmid RP4 (IncP-1) from an *E. coli* donor to activated sludge bacterial recipients. Such experiments give an indication of feasible events but do not truly mirror conditions found in STPs.

Several research groups have independently conducted *in situ* experiments to determine whether lateral gene transfer does in fact take place in a STP. Marcinek and co-workers established a sterile recirculating membrane diffusion chamber (a microcosm) within an activated sludge compartment of a German STP [3]. Dialysis tubing was used to facilitate mating experiments between *E. faecalis* strains harboring various natural plasmids whilst allowing diffusion of soluble sewage compounds and preventing contaminating phage and bacteria from entering into the sterile chamber. Although the transfer rate between strains was at least 10, 100 and 10^5-fold lower for Tn*916*, pIP501 and the sex pheromone plasmids, respectively, under environmental conditions compared to the lab; the study demonstrated that gene transfer could occur in municipal STPs.

Likewise, Mach and Grimes [61] used membrane diffusion chambers (non-circulating) placed in primary and secondary clarifier tanks of a STP to observe *in situ* transfer of antibiotic resistance gene between environmental and clinical bacterial isolates. Sterile sewage was added to the chamber and allowed to equilibrate in the tanks prior to addition of the recipient and donor strains. They used naturally occurring strains rather than lab strains. They reported low transfer frequencies (10^{-5}/donor) within the STP compared to *in vitro* (10^{-3}/donor) observations, and concluded that the density and concentration of the influent bacteria within a STP were high enough to facilitate the transfer of resistant genes.

Others such as De Gelder et al [62] used marker genes (red fluorescent protein- *rfp*) to track the movement of the multi-resistant plasmid pB10 amongst a microbial community from an activated-sludge municipal STP. Besides demonstrating lateral gene transfer, De Gelder and co-workers found that plasmid distribution throughout the bacterial community was not solely due to the plasmid, but was in part influenced by the host it resides in.

5.2 Are antibiotics in STP responsible for transfer of resistance genes?

Antibiotic resistance acquisition and/or proliferation may conceivably occur in sewage regardless of the presence of small concentrations of antibiotic drugs. However, most bacteria isolated prior to the antibiotic drug era were sensitive to antibiotics and many contained conjugative plasmids free of resistance genes [63]. The widespread evidence of subsequent increase in antibiotic resistance is almost universally presumed to be related to the use (and thus presence) of antibiotic drugs. Consistent with this, it seems reasonable to assume that the presence of antibiotics in sewage may also contribute to the evident rise in resistant bacteria isolated from municipal STPs.

Hospital sewage typically carries increased numbers of antibiotic resistant bacteria compared to other municipal sewage [51, 64, 65]. Since hospital sewage also contain elevated concentrations of antibiotics [66], the increased resistance may reflect a selective pressure imparted by the elevated levels of these compounds. However, the correlation itself does not provide evidence for such an influence since it does not distinguish the situation from an increased rate of excretion of antibiotic resistant bacteria by hospital patients.

For some classes of antibiotics, such as the β-lactams, a link between the presence of antibiotics and a selective pressure for proliferation in sewage seems unlikely. One reason is that many of the early generation β-lactams are intrinsically unstable in aqueous solutions [26]. The 4-membered β-lactam ring, a feature common to all, is a strained and cyclic amide which is highly susceptible to hydrolysis. The degradation of penicillin takes place under alkaline or acidic conditions, or by reaction with weak nucleophiles such as water or metal ions. Besides, penicillin is enzymatically hydrolysed by β-lacatamase enzymes via the same pathway as acid hydrolysis. β-lacatamase or 'penicillinase' are produced by many bacteria. Accordingly, penicillins have rarely been observed at measurable concentrations in STPs. Nonetheless, β-lactam resistance traits carried on multiple-gene cassettes could conceivably be maintained due to co-selection i.e. selective pressure targeting a neighboring gene residing on the on same cassette.

While cephalosporin β-lactam drugs such as cephalexin appear to be somewhat more persistent than penicillins during sewage treatment, the means by which some organisms impart resistance may prevent the realization of a selective pressure. That is many organisms rely on deactivating and/or degrading the antibiotic enzymatically outside of the confines of the cell. In these circumstances, all community members would receive equal benefit from the enzymatic degradation, regardless of which community member(s) were responsible for producing the enzyme. In some circumstances, low concentrations of antibiotics may act as a selective pressure for resistant organisms without eliciting the expression of degradative enzymes. Instead, the action leading to resistance imparts a benefit directly to and solely for the organism responsible. Examples of such traits include reduced cell wall permeability, biochemical modification of the antibiotic's target and/or the acquisition of effective efflux pumps.

Guardabassi et al [55] suggested that wastewaters from both hospital and pharmaceutical manufacturing sources led to an increase in multi-antibiotic resistance of *Acinetobacter* spp. Although they observed little difference between antibiotic sensitivity amongst isolates from either side of the hospital's outlet, they found that the number of completely sensitive isolates downstream of the pharmaceutical plant had decreased from 68.4% (upstream) to 8.8%. This study demonstrated that pharmaceutical plant wastewaters were responsible for selecting antibiotic resistant bacteria. Moreover, they showed that the antibiotic resistance patterns of MAR bacteria isolates were in fact phylogentically distinct *Acinetobacter* strains and not just clonal variants.

Similarly, a recent study was undertaken by Knapp etal [67] to assess whether low levels of antibiotics released into waterways could promote bacterial antibiotic resistance. The research was carried out in mecocosm tanks filled with surface water, sediment, macrophytes and varying levels of oxytetracycline (0-250 ugL^{-1}) over a period of 56 days. Using quantitative PCR techniques, the authors demonstrated that resistant gene levels remained relatively constant irrespective of prevailing antibiotic concentration. However, they found that the ratio of tetracycline resistance gene to 16S rRNA gene levels did increase, suggesting that the microbial community had shifted in favor of maintaining oxytetracycline resistant bacteria, thus eliminating sensitive bacteria.

5.3 Future work: validating interaction between antibiotics and acquisition of resistance in STPs

Most studies concentrate on a few specific bacterial genera as indicators of overall antibiotic resistance. However, this is a biased view because less than 15% of wastewater bacteria can be cultivated under laboratory conditions [68]. It does not represent the true community population dynamics, which remains unknown. Determination of antibiotic phenotypic behavior of culturable bacteria will not provide a complete picture of the resistance levels within sewage. Although sewage bacteria have been shown to laterally transfer genetic determinants (R plasmids, integrons, transposons) *in situ* [3], circumstances for these events (i.e. presence of antibiotics or other selective pressures) remain unknown. Molecular based approaches would undoubtedly provide additional insight into such studies.

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Metagenomic methodologies could be adapted to the study of uncultivable bacterial communities and potentially yield important information about environmental and management impacts on bacterial community dynamics. For example, Szczepanowski and co-workers [69] devised an approach to delve into the plasmid metagenome of cultured, antibiotic resistant bacteria isolated from a German activated sludge sample. Using 454-pyrosequencing technology, over 36 Mbase sequence data (corresponding to 346,427 reads) was generated, analysed and functionally annotated by the Sequence Analysis and Management System (SAMS). Most plasmid genes were identified and associated with plasmid replication, stability, mobility and transposition. Gene tags potentially encoding for proteins with environmental function (EGT) were assigned a cellular function following comparison to the Pfam database [70]. The study confirmed a high degree of plasmid diversity residing in STP bacteria and several accessory plasmid modules which encoded for MGE's such as transposons, insertion sequences, integrons, resistance and virulence determinants. Resistant genes to all antibiotic classes were found including those newly identified in clinical isolates. Although this particular study was focused on cultured bacteria, EGT analysis proved to be a powerful tool and undoubtedly, its use and application in the study of the entire STP metagenome would greatly broaden our knowledge basis.

In an attempt to estimate MGE's and thus potential lateral gene transfer, quantification of MGE's, especially the Class 1 integron integrase gene (intL1), have recently been reported for bacterial communities subjected to selective pressures stemming from industrial metal contamination. This study was undertaken using quantitative PCR techniques and targeted the intL1 gene. Wright and co-workers reported that bacterial communities in freshwater, estuarine and microcosm samples had increased levels of Class1 integrons when exposed to industrial metal contaminants [71].

Controlled pilot scale biological reactors would further facilitate our understanding of the impact of antibiotics and the movement of resistance determinants within the bacterial population of a STP. Instead of employing submerged sterile microcosms to investigate DNA transfer events, one could literally visualize antibiotic resistance transference and/or proliferation by tagging resistant bacteria and the genes responsible with photoactive fluorescent proteins (PAFP). For example De Gelder et al [62] used a PAFP to track the multi-resistance plasmid pB10 in activated sludge. Two different strategies could be used; the first approach would involve inserting PAFP gene(s) into a host specific plasmid-borne antibiotic resistant integron cassette and reintroducing the construct into a native bacterial host. The second method would entail injecting naked DNA (harboring PAFP and AB^R genes) directly into the reactor or pockets where biofilms tend to arise. Through either means, it should be possible to quickly and easily trace antibiotic resistant gene movements amongst STP reactor populations under natural conditions.

Bacterial community profiling based on 16S rRNA gene using techniques such as length heterogeneity polymerase chain reaction (LH-PCR), denatured gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP) [72] may also be employed to delineate bacterial population dynamics in relation to reactor manipulations and more importantly to reveal the extent of bacterial community disparity between influent and effluent. Correlations between bacterial community profiling, real time-PCRs and other chemical/physical measurements may also be used to assess the prevalence and distribution of the antibiotic resistance genes within the bacterial population, and ultimately gauge the impacts of operating conditions and STP designs.

With bacterial pathogens fast approaching multi-drug resistant status and available defences diminishing at comparable speeds, we may soon find ourselves resorting to drastic measures to combat them. Controlling possible point-of-source and incumbent resistance gene transfer events maybe our only hope in halting and preventing the spread of antibiotic resistance.

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