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PERSPECTIVE

Using the class 1 integron-integrase gene as a proxy for anthropogenic pollution

Michael R Gillings[1], William H Gaze[2], Amy Pruden[3], Kornelia Smalla[4], James M Tiedje[5] and Yong-Guan Zhu[6]

[1]*Department of Biological Sciences, Genes to Geoscience Research Centre, Macquarie University, Sydney, New South Wales, Australia;* [2]*European Centre for Environment and Human Health, University of Exeter Medical School, Royal Cornwall Hospital, Truro, UK;* [3]*Via Department of Civil and Environmental Engineering, Virginia Tech, Blacksburg, VA, USA;* [4]*Institute for Epidemiology and Pathogen Diagnostics, Julius Kühn-Institut, Federal Research Centre for Cultivated Plants, Braunschweig, Germany;* [5]*Center for Microbial Ecology, Michigan State University, East Lansing, MI, USA and* [6]*Key Laboratory of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Sciences, Xiamen, China*

Around all human activity, there are zones of pollution with pesticides, heavy metals, pharmaceuticals, personal care products and the microorganisms associated with human waste streams and agriculture. This diversity of pollutants, whose concentration varies spatially and temporally, is a major challenge for monitoring. Here, we suggest that the relative abundance of the clinical class 1 integron-integrase gene, *intI1*, is a good proxy for pollution because: (1) *intI1* is linked to genes conferring resistance to antibiotics, disinfectants and heavy metals; (2) it is found in a wide variety of pathogenic and nonpathogenic bacteria; (3) its abundance can change rapidly because its host cells can have rapid generation times and it can move between bacteria by horizontal gene transfer; and (4) a single DNA sequence variant of *intI1* is now found on a wide diversity of xenogenetic elements, these being complex mosaic DNA elements fixed through the agency of human selection. Here we review the literature examining the relationship between anthropogenic impacts and the abundance of *intI1*, and outline an approach by which *intI1* could serve as a proxy for anthropogenic pollution.
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Introduction

Humans produce and use a diverse array of compounds in domestic, industrial and agricultural settings. These compounds can contaminate ecosystems, elevating local concentrations of pollutants such as heavy metals, synthetic organic compounds and radioactive isotopes. Together with microbiological contaminants, they create a zone of impact emanating from human activities. Managing impacts requires monitoring to assess the efficacy of preventative or remedial measures, by measuring the quantities and distribution of individual pollutants. However, because some 80 000 different compounds are now traded in the marketplace, testing for all pollutants is not feasible (Rockstrom *et al.,* 2009). Focussing on just one class of pollutant is also problematic, because the composition of pollutants varies both geographically and temporally. Furthermore, diverse classes of pollutants, such as antibiotics and endocrine disrupting compounds, have significant biological effects at extremely low concentrations (Diamanti-Kandarakis *et al.,* 2009; Gillings, 2013).

An alternative to direct detection is to use a proxy that exhibits rapid responses to diverse environmental pressures and could thus be a generic marker for anthropogenic pollutants. We propose that the class 1 integron-integrase gene, *intI1*, could serve as such a marker, because:

1. it is commonly linked to genes conferring resistance to antibiotics, disinfectants and heavy metals (Liebert *et al.,* 1999; Partridge *et al.,* 2001);
2. it has penetrated into diverse pathogenic and commensal bacteria of humans and their domestic animals (Goldstein *et al.,* 2001; Stokes and Gillings, 2011);
3. the abundance of *intI1* can rapidly change in response to environmental pressures, because the class 1 integron resides in diverse bacterial species that themselves have rapid generation times, and it is often located on mobile genetic elements that can readily transfer between bacteria; and

Correspondence: MR Gillings, Department of Biological Sciences, Macquarie University, Sydney, New South Wales 2109, Australia.
E-mail: michael.gillings@mq.edu.au
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4. the common 'clinical' forms of *intI1* are xenogenetic, that is, recently assembled under selection pressures imposed by human activities (Gillings *et al.,* 2008a).

Independent studies have already begun to note remarkable correlations between *intI1* and associated genetic elements with various measures of human impact (Gaze *et al.,* 2011; Pruden *et al.,* 2012; Jechalke *et al.,* 2013b). Here we review the recent evolutionary origins of the clinical class 1 integron, examine a series of case studies using *intI1* as an environmental marker of human pollution, and suggest methods for using this gene as a proxy for human impact.

The evolutionary history of the class 1 integron

Integrons are an ancient and common feature of bacterial genomes, where they usually reside on chromosomes (Gillings, 2014a). They have three core features: an integron-integrase gene (*intI*), a recombination site (*attI*) and a promoter (P_C). These features allow capture and expression of exogenous genes as part of gene cassettes that are recombined into the *attI* site using the integrase activity encoded by *intI* (Boucher *et al.,* 2007; Cambray *et al.,* 2010) and subsequently expressed from P_C (Collis and Hall, 1995) (Figure 1). This allows genes to be acquired and expressed with minimal disturbance to the existing genome. Integrons sample cassettes from an extraordinarily diverse pool that encodes functions of potential adaptive significance. Consequently, they are a hot spot of genomic diversity in a range of genera (Gillings *et al.,* 2005; Boucher *et al.,* 2011; Hall, 2012; Wu *et al.,* 2013).

Hundreds of integron classes have been described, defined on the basis of the relative homology of *intI* (Cambray *et al.,* 2010; Boucher *et al.,* 2011). Of these, the class 1 integrons, so named because they were first to be discovered, had properties that meant that they were well equipped to move by lateral DNA transfer into a wide range of commensal and pathogenic bacteria, and to accumulate diverse antibiotic resistance genes once humans tried to control bacteria with antimicrobial compounds. These fortuitous properties included: location on the chromosomes of *Betaproteobacteria* whose habitats intersect the human food chain; ability to move between chromosomal locations and between species (Gillings *et al.,* 2008a); carriage by 0.002% of cells in an unaffected soil (Gaze *et al.,* 2011) compared with as many as 5% of cells in affected soil, fresh water and biofilms (Gaze *et al.,* 2005; Hardwick *et al.,* 2008); ability to acquire a wide range of gene cassettes (Biskri *et al.,* 2005); and frequent association with *qac* genes that encode versatile efflux pumps (Gaze *et al.,* 2005; Gillings *et al.,* 2009a).

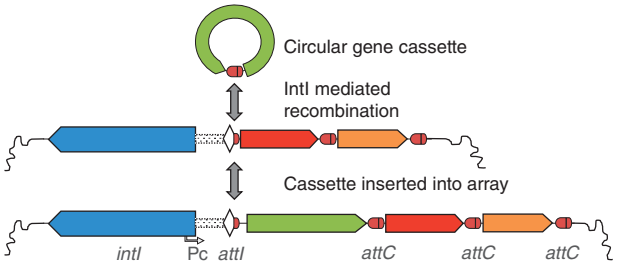

Figure 1 Integron structure and function. Integrons consist of a gene for an integron-integrase (*intI*) that catalyses recombination between the *attC* site of circular gene cassettes and the attendant integron recombination site, *attI*. This activity results in the sequential insertion of multiple, different cassettes to form a tandem cassette array that, in some cases, might contain hundreds of different genes. Inserted genes are expressed by an integron-encoded promoter, Pc.

When metagenomic DNA is examined from environmental sources, diverse genes belonging to *intI1* can be detected. In contrast, all examples of *intI1* recovered from clinical contexts have essentially identical DNA sequences, showing that there was a single common ancestor for the 'clinical' class 1 integron that has spread antibiotic resistance among Gram-negative pathogens (Gillings *et al.,* 2008b). Consequently, the class 1 integrons now circulating freely within human-dominated ecosystems have a conserved DNA sequence that, in the main, distinguishes them from the diverse class 1 integrons present in the more general environment.

The best explanation for the origin of the clinical class 1 integron is that a chromosomal class 1 integron from an environmental betaproteobacterium was captured by a transposon of the Tn*402* family (Figure 2). This integron carried a gene cassette encoding resistance to disinfectants (*qacE*), and subsequently captured a gene for sulphonamide resistance (*sul1*), deleting the terminus of the *qacE* cassette (Kholodii *et al.,* 1995; Gillings *et al.,* 2008a; Gillings, 2014a).

The Tn*402* transposon has the unusual property of targeting the *res* sites of plasmids (Minakhina *et al.,* 1999) and, consequently, the Tn*402*-class 1 integron hybrid was able to transpose into a wide variety of plasmids (Figure 2) that then enabled lateral transfer into an equally wide variety of bacterial species. One of the most successful of these insertion events associated the Tn*402*-integron with a mercury resistance operon (*mer*) to spawn the Tn*21* element that itself went on to generate a series of complex derivatives (Liebert *et al.,* 1999; Partridge *et al.,* 2001). The Tn*402*-integron has also subsequently generated extensive internal variation by deletion of parts of *qacE*, *sul1* and/or the Tn*402* transposition machinery (Hall *et al.,* 1994; Brown *et al.,* 1996; Partridge *et al.,* 2001). Variation in the cassette array has been generated by the collective acquisition of over 130 different antibiotic resistance gene cassettes (Figure 2) (Partridge *et al.,* 2009), conferring resistance to the majority of antibiotics used to control Gram-negative pathogens (Mazel,

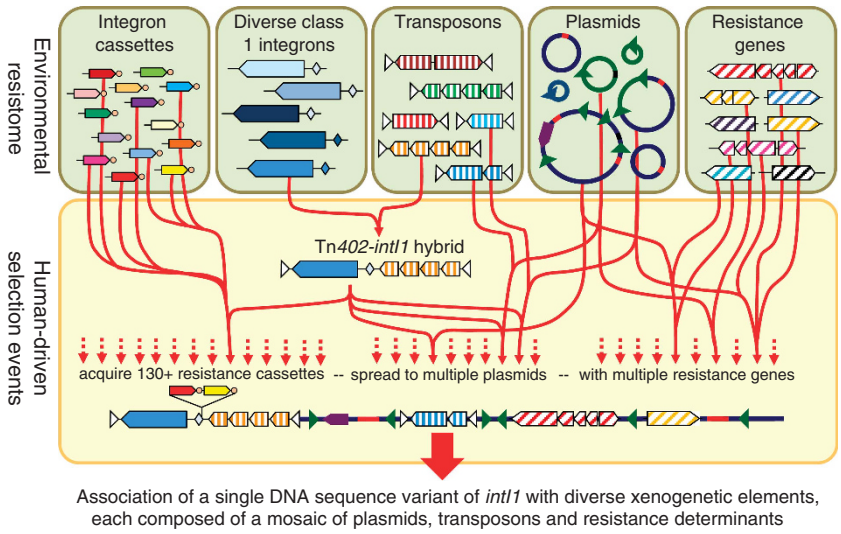

Figure 2 The recent evolutionary origin of the clinical class 1 integron and its incorporation into diverse xenogenetic elements. The raw materials for the assembly of the complex mosaic DNA elements that now carry the clinical *intI1* were all present in the environmental resistome. A single sequence variant from the diverse pool of class 1 integrons in natural environments was captured by a Tn*402* transposon, thus forming a Tn*402–intI1* hybrid, and giving the integron greater mobility. This hybrid integron, in total, has captured at least 130 different gene cassettes encoding resistance to diverse antibiotics. At the same time, the Tn*402* portion of the hybrid element targeted the *res* sites of plasmids, transposing the whole hybrid molecule into a diverse collection of plasmids. This, in turn, promoted movement of clinical *intI1* between different bacterial species by conjugation of those plasmids. Human selection events have also independently fixed the acquisition of diverse resistance genes onto the collection of plasmids invaded by the hybrid integron. These independent acquisitions resulted in the accumulation of genes for resistance to metals, antibiotics, disinfectants and other compounds, along with other genetic elements such as insertion sequences and transposons. As a result, a single molecular species (the clinical *intI1* sequence variant) has become associated with an ever expanding and diverse set of plasmids, transposons and resistance genes. These mosaic elements can be thought of as xenogenetic, in the sense that they owe their current structures and abundance to human activity.

2006; Cambray *et al.,* 2010; Stokes and Gillings, 2011).

Consequently, the 'clinical' *intI1* variant is now found on a range of different mobile elements that are freely transmissible between diverse commensal and pathogenic bacteria associated with humans and domestic animals (Nandi *et al.,* 2004; Bailey *et al.,* 2010; Djordjevic *et al.,* 2013; Liu *et al.,* 2013). This 'clinical' *intI1* is also closely linked to various genes that confer phenotypes of environmental significance, such as antibiotic, disinfectant and heavy metal resistances (Figure 2) (Liebert *et al.,* 1999; Norman *et al.,* 2009; Gillings *et al.,* 2009b; Moura *et al.,* 2010; Heuer *et al.,* 2012; Domingues *et al.,* 2013). Finally, 'clinical' *intI1* comprises a single molecular species with essentially identical DNA sequences, regardless of the diverse genetic and cellular landscapes they now inhabit (Figure 2).

Antibiotics and antibiotic resistance genes as pollutants

Between 30% and 90% of ingested antibiotic is excreted unchanged by both animals and humans (Sarmah *et al.,* 2006). Antibiotics are only partly removed by wastewater treatment (Giger *et al.,* 2003; Watkinson *et al.,* 2007) and, depending on the antibiotic, can still be found at levels between 10 and 1000 ng l^{-1} in secondary effluent (Le-Minh *et al.,* 2010). Antibiotics can enter soils via animal manure used for fertilization (Chee-Sanford *et al.,* 2009), whereas other antibiotics are excreted preferentially in urine (Subbiah *et al.,* 2012). As much as 80% of the antibiotics used in aquaculture flow into the environment (Cabello *et al.,* 2013). Consequently, there is a zone around human activities that is enriched with antibiotics.

The use of antibiotics has vastly increased the abundance of 'clinical' class 1 integrons, such that they are now present in up to 80% of enterobacteria in humans and farm animals (Tenaillon *et al.,* 2010; Marchant *et al.,* 2012; Liu *et al.,* 2013). Consequently, large numbers of bacteria containing integrons are released into the environment, with one estimate suggesting that disposal of sewage sludge in the United Kingdom adds 10^{19} integron-containing bacteria to waste streams each year (Gaze *et al.,* 2011). Wastewater treatment is not designed to remove DNAs, and the abundance of *intI1* often increases during the water treatment process (LaPara *et al.,* 2011; Ma *et al.,* 2011b, 2013; Chen and Zhang, 2013; Cheng *et al.,* 2013; Du *et al.,* 2014). This might be a consequence of selection driven by the antibiotics, disinfectants and heavy metals that are also inefficiently removed during water treatment (Baker-Austin *et al.,* 2006; Selin, 2009; Hegstad *et al.,* 2010; Rosewarne *et al.,* 2010). As a result, any bacteria that carry class 1 integrons associated with resistance determinants, or that are able to acquire them by lateral gene transfer, would increase in abundance during various stages of water treatment.

Resistance genes and DNA vectors are increasingly being recognized as environmental contaminants (Pruden *et al.,* 2006; Stalder *et al.,* 2014), and their abundance in natural environments and wild animals has been increasing since the first human use of antibiotics (Knapp *et al.,* 2009; Gillings, 2013). The complex DNA molecules that now bear class 1 integrons often also carry genes for resistance to diverse antibiotics, disinfectants and other environmental contaminants, all embedded in a mosaic of mobile elements. These individual components often have a separate phylogenetic origin, each having been acquired in a separate event, and then fixed by human selection. Because human activities have had a direct role in the selection of sequential gene acquisitions, these complex mosaics of resistance elements can be thought of as xenogenetic. Such xenogenetic molecules have properties of both pollutants and invasive species, as they are pollutants that can replicate (Storteboom *et al.,* 2010; Gillings and Stokes, 2012; Pruden *et al.,* 2012). Methods to control pollution by antibiotics and their respective resistance genes have been suggested, including limiting the use of antibiotics in agriculture, and improving treatment of urban, industrial and hospital waste water (Pruden *et al.,* 2013; Berglund *et al.,* 2014).

IntI1 as a potential marker of anthropogenic pollution

The 'clinical' *intI1* gene has key advantages as a generic marker of anthropogenic influence. These include: universal presence and high abundance in the commensal bacteria of humans and domestic animals, a consequently high representation in waste streams, low abundance in less affected environments and a uniform and highly conserved DNA sequence. Based on these properties, a number of research groups have used quantitative analysis of *intI1* to track human influence (Table 1).

Examining the relationship between pollutants, antibiotic resistance and class 1 integrons reveals a number of general trends (Table 1). *IntI1* is poorly removed during water treatment, and its abundance often increases downstream from water treatment plants and human habitation. The *intI1*-carrying bacteria are abundant in manure, in digestates from biogas plants and in pesticide biopurification systems (Dunon *et al.,* 2013; Jechalke *et al.,* 2013a). Mesocosms designed to test land application of wastewater solids show that *intI1* has a slow decay rate (Burch *et al.,* 2014). In this regard, *intI1*-carrying bacterial populations are similar to other persistent pollutants, such as metals, antibiotics and disinfectants.

The co-occurrence of integrons, resistance genes and pollutants is probably causal, as co-selection of antibiotic resistance genes and integrons occurs in environments polluted with heavy metals and disinfectants (Baker-Austin *et al.,* 2006; Hegstad *et al.,* 2010; Seiler and Berendonk, 2012). This co-selection is most likely caused by the physical location of class 1 integrons on a range of transposons and plasmids that also carry genes for resistance to antibiotics, heavy metals and disinfectants (Table 1), and consequently, class 1 integrons can be selected via simple linkage. Similarly, *intI1* abundance has been associated with pesticide pollution, via the co-occurrence of integrons and genes for degradative pathways on IncP-1 plasmids (Dealtry *et al.,* 2014b).

Although the class 1 integron integrase gene does not directly confer resistance to any particular pollutant, its linkage to a diverse suite of antibiotic, metal and disinfectant resistance genes means that it is an excellent *de facto* measure of the general level of resistance determinants. For instance, there is a strong correlation between the abundance of *intI1* in reclaimed water and the abundance of antibiotic resistance genes such as *sul1* and *tetG* (Wang *et al.,* 2014). Similarly, at the scale of whole watersheds, there is a strong correlation between *sul1*, which is commonly linked to *intI1*, and the upstream capacities of wastewater treatment and animal feeding operations (Pruden *et al.,* 2012). Because resistance determinants confer selective advantages on those bacterial cells that carry them, *intI1* abundance should then reflect the general response of the bacterial community to selection imposed by anthropogenic pollution. Consequently, *intI1* abundance should be a good measure of general selective pressure. In contrast, targeting specific resistance determinants such as *tet* or *sul* is not a generic measure, as abundance of these genes is dependent on both their presence in a waste source and the presence of specific antibiotics to which they confer resistance.

Towards practical application of *intI1* as a marker

Resistance genes and their vectors originate from environmental sources, where they form part of the resistome (D'Costa *et al*., 2006; Wright, 2010). This is also the case for *intI1*, which occurs naturally in environmental samples (Figure 2). The use of generic *intI1* PCR primer pairs (Stokes *et al.,* 2006) effectively amplifies both clinical and environmental variants of *intI1*, potentially contributing noise to quantification of *intI1* shed from human sources. In environmental samples, *intI1* exhibits considerable sequence diversity (Gillings *et al.,* 2008b), whereas the clinical *intI1* has a uniform, conserved sequence. For example, the Fungene database (http://fungene.cme.msu.edu/index.spr) (Fish *et al.,* 2013) has over 500 sequences with $>99\%$ identity to *intI1*. These are mostly from clinical isolates, although a few are from environmental strains.

Table 1 Environmental and laboratory studies examining the relationship between diverse pollutants, antibiotic resistance genes and class 1 integrons

System	*Location*	*Sample, method*	*Comments*	*Reference*
Hospital effluent	France	Water treatment, qPCR	*IntI1* abundance increases because of effluent	Stalder *et al.* (2014)
Medical center effluent	France	*E. coli* isolation, PCR	*IntI1* abundance increases because of effluent	Oberle'X' *et al.* (2012)
Sewage treatment	USA	Aerobic digester, qPCR	*IntI1* has longest half-life of genes tested	Burch *et al.* (2013)
Sewage treatment	China	Activated sludge, qPCR	*IntI1* abundance increases	Ma *et al.* (2013)
Sewage treatment	China	Isolation, water, qPCR	*IntI1* abundance increases in effluent	Ma *et al.* (2011a)
Sewage mesocosms	USA	Sludge, effluent, qPCR	Efficiency of *intI1* removal dependent on treatment system	Ma *et al.* (2011b)
Wastewater treatment	China	Water, qPCR	Efficiency of *intI1* removal dependent on treatment system	Du *et al.* (2014)
Wastewater treatment	China	Water, qPCR	Efficiency of *intI1* removal dependent on treatment system	Chen and Zhang (2013)
Wastewater treatment	China	Water, sediment, qPCR	*intI1* increases in abundance downstream from city	Zhang *et al.* (2009)
Wastewater treatment	USA	Water, sediment, qPCR	*IntI1* abundance significantly increases in effluent	LaPara *et al.* (2011)
Wastewater treatment	UK	Bacterial isolation, PCR	Shows co-selection of *intI1* and disinfectant resistance	Gaze *et al.* (2005)
Freshwater microcosm	USA	Bacterial isolation	Shows co-selection of antibiotic and metal resistance	Stepanauskas *et al.* (2006)
Waste streams	UK	Sludge, manure, qPCR	Shows selection of *intI1* by waste antibiotics/ disinfectants	Gaze *et al.* (2011)
River catchment	Cuba	Sediment, water, qPCR	Ab resistance correlates with degree of pollution	Graham *et al.* (2011)
River catchment	Pakistan	Water, qPCR	*IntI1 and* other gene abundance increases with human impact	Khan *et al.* (2013)
River catchment	USA	Sediment, water, qPCR	*IntI1* abundance increases with industrial pollution	McArthur *et al.* (2011)
Stream catchment	Australia	Sediment, qPCR	*IntI1* abundance increases with human impact	Hardwick *et al.* (2008)
Freshwater habitats	Canada	Water, floc, microarray	*IntI1* cassette abundance increases with human impact	Drudge *et al.* (2012)
Estuary, catchment	France	*E. coli* isolation, qPCR	*IntI1* and Ab resistance correlates with degree of pollution	Laroche *et al.* (2009)
Estuary	USA	Sediment, water, qPCR	*IntI2* abundance increases with human impact	Uyaguari *et al.* (2013)
Estuary	Canada	Sludge, PCR	*IntI1* and diverse cassettes associated with industrial waste	Koenig *et al.* (2009)
Various	Worldwide	PCR, cloning	*IntI2* abundance increases with human impact	Rodríguez-Minguela *et al.* (2009)
Environ. gradient	USA	Sediment, qPCR	*IntI1* abundance increases with metal/anti-biotic pollution	Wright *et al.* (2008)
Environ. gradient	China	Sediment, sequencing	Integron and plasmid abundance increases with impact	Chen *et al.* (2013)
Environ. gradient	Argentina	Bacterial isolation, PCR	Trend for *intI1* to increase in abundance with urbanization	Nardelli *et al.* (2012)
Environ. gradient	Australia	Sediment, qPCR	*IntI1* abundance increases with heavy metal pollution	Rosewarne *et al.* (2010)
Environ. gradient	Worldwide	Soil, sediment, PCR	IncP plasmid abundance increases with pesticide impact	Dealtry *et al.* (2014a)
Swine production	Not stated	Soil and water, qPCR	*IntI1* and other genes increase in abundance	Hong *et al.* (2013)
Slaughterhouse water	Portugal	Bacterial isolation, PCR	*IntI1* increased in abundance during treatment	Moura *et al.* (2007)
Farm manuring	Germany	Soil, rhizosphere, qPCR	*IntI1* and other genes increase in abundance	Jechalke *et al.* (2014)
Farm manuring	UK	Soil, qPCR	*IntI1* increased in abundance	Byrne-Bailey *et al.* (2011)
Farm manuring	Germany	Soil, manure, PCR	*IntI1* and other genes increase in abundance	Binh *et al.* (2009)
Manure, wastewater	China	Water, manure, qPCR	*IntI1* and other genes increase in abundance	Cheng *et al.* (2013)
Manure treatment	China	Manure, qPCR array	Transposons and resistance genes increase in abundance	Zhu *et al.* (2013)
Animal microbiota	Various	*E. coli* isolation, PCR	*IntI1* increases in frequency with increased human contact	Skurnik *et al.* (2006)
Archived soils	Scotland	Soil, qPCR	Correlation of resistance genes with copper pollution	Knapp *et al.* (2011)
Diverse	Various	Review	Shows co-selection of antibiotic and heavy metal resistance	Baker-Austin *et al.* (2006)
Diverse	Various	Review	Shows co-selection of antibiotic and heavy metal resistance	Seiler and Berendonk (2012)
Diverse	Various	Review	Shows co-selection of antibiotic and disin-fectant resistance	Hegstad *et al.* (2010)

Abbreviations: Ab, antibiotic; *intI2*, class 2 integron-integrase gene; qPCR, quantitative PCR.

Sequences for environmental variants of *intI1* are still in the minority in databases, and the region of *intI1* for which most data are available is that amplified by primers HS464/HS463a (Gillings *et al.,* 2008b). Examination of the sequence data (Supplementary Table S1) reveals a number of nucleotide positions where the clinical *intI1* can be distinguished from most reported environmental variants. A primer pair targeting *intI1* nucleotide positions 165–184 and 456–476 (intI1F165 5′-CGA ACGAGTGGCGGAGGGTG-3′ and intI1R476 5′-TAC CCGAGAGCTTGGCACCCA-3′) is one possibility for specifically amplifying the clinical version of *intI1*. Because these primers target the clinical *intI1* sequence, but not the diverse *intI1* variants known to be present in environmental bacteria, they should allow a more precise quantitative analysis. As more complete sequences from environmental variants of *intI1* become available, better regions for discrimination could be identified.

Sample collection and processing

Environmental monitoring of human impact and the efficacy of remediation could be conducted using quantitative analysis of *intI1* abundance. Careful consideration should be given to sampling strategies and data generation. Samples of sediment, soil or water should be taken in a uniform, reproducible manner. The likelihood of temporal variation should be taken into account. For instance, sewage treatment water can vary considerably over a 24-h period, and composite or flow proportionate samples should be considered. Ideally, each sampling time or point should be represented by at least triplicate samples, to be treated as triplicates in all subsequent steps such as DNA extraction and quantitative PCR (qPCR). Each sampling point for soil or sediment can be laid out in a grid to capture microvariation. At the minimum, samples should be identified by date, GPS coordinates and land use. The GSC (Genomic Standards Consortium) provides a guide to collection of environmental data under its MIMARKS environmental packages (Yilmaz *et al.,* 2011), conveniently implemented by RDP according to habitat type with prepopulated googlesheets (http://rdp.cme.msu.edu/wiki/index.php/RDP_MIMARKS_GoogleSheets). For a detailed description of one multipurpose soil sampling procedure, see the BASE website (http://www.bioplatforms.com.au/special-initiatives/environment/soil-biodiversity/sample-collection-procedure). Soil can be stored at 4 °C, or snap-frozen immediately upon collection, and maintained frozen during transport to minimize changes to microbial populations.

IntI1 monitoring could be used for analysis of water samples, such as wastewater effluents, feedlot runoff and affected streams, rivers, lakes and oceans. Water samples can be collected by bulk grab techniques, using methods described for coliform monitoring. Water samples contain a particulate fraction, and many microbes, including microbes carrying *intI1*, attach to particulate matter suspended in the water. Most methods employ filtration, with 0.22 μm cutoff capturing the majority of bacteria and other particulates. The filter is then directly subject to DNA extraction. However, extracellular DNA may also be of interest, and this will pass through filters under some conditions. Recent studies have introduced techniques for analysing extracellular forms of antibiotic resistance genes (Mao *et al.,* 2013). Further assessment and standardization of filter membrane composition and pore size employed for analysis of *intI1* in water samples for different purposes (extracellular versus intracellular) would be of interest.

Sufficient sample should be taken for multiple analyses, and for archival storage. To ensure representative subsampling, the cone and quarter method can be used (Ferrari *et al.,* 2008). The DNA extraction method employed should be suitable for diverse cell types and for removal of inhibitors present in soil, sediment, manure, sludge and other intractable substrates (Yeates and Gillings, 1998; Gillings, 2014b). The integrity of extracted DNA should be assessed using agarose electrophoresis and the concentration estimated photometrically. Alternatively, double-stranded DNA could be quantified using fluorometric methods (Singer *et al.,* 1997).

Concentrations of *intI1* can then be determined using real-time qPCR, correcting by the total bacterial abundance as measured by 16S rRNA gene PCR performed on the same sample. Ideally, three independent environmental samples should be processed in parallel to control for variation introduced during processing. PCR inhibition caused by co-extracted compounds can be overcome using bovine serum albumin (Gaze *et al*., 2011), an environmental master mix or template dilution. Primers for amplification of 16S rRNA genes should be specific for bacteria (Nadkarni *et al.,* 2002). Primer sets need to be optimized across a range of concentrations and annealing temperatures. Standard curves for each target gene need to be determined, and positive control standards of known copy number prepared by PCR (Hardwick *et al.,* 2008; McKinney and Pruden, 2012). The qPCR results could also be normalized by the total DNA in a sample, which would generate an idea of the relative abundance of *intI1* in relation to the entire metagenome. If a housekeeping or other gene is used to normalize *intI1* abundance, it should be established that it has a similar amplification efficiency to that of *intI1*.

Rapidly advancing molecular technologies will add new capabilities for understanding human impact. Highly parallel qPCR equipment from several vendors allows analysis of multiple primer sets and samples, such that hundreds of antibiotic resistance genes, mobile elements and their variants

can be analysed (Looft *et al.,* 2012; Zhu *et al.,* 2013), allowing more comprehensive spatial and temporal studies. Furthermore, amplicons can be sequenced, providing diagnostic-level insight into the probable origin of these genes. A recent example of the latter shows clusters of *intI1* and antibiotic resistance gene identities at a country scale and at an intercontinental scale for *intI1* (Johnson *et al.,* 2014).

As DNA sequencing becomes more efficient and cheaper, direct sequencing of metagenomic samples may replace qPCR approaches. In such an analysis, clinical *intI1* sequences could be extracted from the sequence data and normalized to a single copy housekeeping gene. Already, such approaches are being used, based on high-throughput next-generation sequencing methods.

Conclusion

The clinical version of the *intI1* gene has some unique advantages as a universal marker of selective pressures imposed by anthropogenic pollution. Its recent emergence into human-dominated ecosystems means that it has a homogenous and conserved DNA sequence, simplifying detection. It has seen a rapid increase in abundance and geographic distribution, fuelled by the extensive use of antibiotics and its insertion into diverse mobile elements, coupled with its penetration into a wide range of bacterial species associated with human-dominated ecosystems. During this expansion, it has become closely linked with genes that confer resistance to disinfectants and heavy metals, as well as the wide range of antibiotic resistance determinants for which it is well known. Consequently, versions of the clinical *intI1* gene are capable of conferring diverse advantages to those cells that carry them, and these advantageous phenotypes correspond with the selective agents that are most likely to be present in human waste streams.

Conflict of Interest

The authors declare no conflict of interest.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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