**PROBING FOR CLINICAL INTEGRONS AND INTEGRON-ENCODED ANTIBIOTIC RESISTANCE GENES AS MOLECULAR INDICATORS OF WASTEWATER-RELATED DEGRADATION OF SUBTROPICAL COASTAL ENVIRONMENTS**

By

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## ABSTRACT

Wastewater discharges comprise the main source of fecal contamination across coastal environments in Puerto Rico. Enteric bacteria from hospital settings are frequently key hosts of integrons, a genetic system encoding multiple antibiotic resistance (ABR) genes. Nevertheless, it is unknown if bacterial hosts of integrons loaded with antibiotic resistance genes could become established across local coastal habitats. Using PCR and DNA sequencing analyses, prevalence patterns of integron integrases (classes 1, 2 and 3) and class 1-encoded ABR genes were detected in DNA samples recovered from wastewater-contaminated mangrove, beach and estuarine environments. Bacteriological analyses of mangrove ecosystems revealed that sediments and the rhizosphere are significant reservoirs of fecal bacteria (enterococci and fecal coliforms). Six different integron-encoded ABR genes were detected across impacted sites. These were related to determinants conferring resistance against four antimicrobial agents (aminoglycosides, trimethoprim, chloramphenicol and third generation cephalosporins) used for the treatment of infections of the urinary, respiratory and digestive tract in humans. Genes encoding resistance against disinfectants widely used in hospitals (quaternary ammonium compounds) were also detected. Genes encoding resistance against cephalosporins were specifically detected in wastewater-impacted mangrove and esturarine ecosystems. This study revealed that wastewater-contaminated coastal habitats are reservoirs of bacteria carrying ABR genes and that integrons may serve as indicators of fecal contamination and potential health risks at impacted sites.

## RESUMEN

Las descargas de aguas residuales constituyen la principal fuente de contaminación de origen fecal en los ambientes costeros de Puerto Rico. Las bacterias fecales aisladas en ambientes de hospitales frecuentemente son portadoras de integrones, un sistema genético que codifica múltiples mecanismos de resistencia a antibióticos (RAB). Sin embargo, se desconoce si bacterias con integrones cargados con genes de resistencia, pudieran establecerse en ambientes costeros expuestos a contaminación fecal. Utilizando la reacción en cadena de la polimerasa (PCR) y análisis de secuenciación de ADN, se detectaron patrones de prevalencia de integrasas de integrones (clases 1, 2 y 3) y genes de RAB codificados por integrones clase 1, en ADN de muestras ambientales tomadas en manglares, playas y estuarios contaminados por aguas residuales. Los análisis bacteriológicos de ecosistemas de mangle revelaron que los sedimentos y la rizósfera constituyen reservorios importantes de bacterias fecales tales como enterococos y coliformes fecales. Seis genes diferentes de RAB codificados por integrones clase 1 fueron detectados en ambientes impactados. Estos estaban relacionados a mecanismos que confieren resistencia contra cuatro agentes antimicrobianos (aminoglucósidos, trimetoprima, cloramfenicol, y cefalosporinas de tercera generación) usados para el tratamiento de infecciones del tracto respiratorio, urinario y digestivo en humanos. También se detectaron genes codificantes para resistencia a desinfectantes utilizados en hospitales (compuestos de amonio cuaternario). Los genes codificantes para resistencia en contra de cefalosporinas se detectaron en asociación con un manglar y un estuario impactado por aguas usadas. Este estudio reveló que hábitats costeros degradados por aguas usadas son reservorios de bacterias con genes de resistencia a antibióticos y que los integrones podrían servir como indicadores de contaminación fecal y riesgos a la salud en ambientes costeros.

## DEDICATION

I dedicate my thesis to my parents (Efrén Alvarez and Anaida Pérez) and my husband (Fernando Salas), for being there in the good and bad moments. To my parents for all their love, comprehension, support and encouragement, when I felt like "I was not walking". They are essential for me, to continue and to be who I am.

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# INTRODUCTION

Integrons are genetic systems described in bacteria which are responsible for the acquisition and expression of promotorless open reading frames that may encode antibiotic resistance traits. Although originally described among strains associated with hospital environments, integrons loaded with antibiotic resistance genes have been detected in natural environments, particularly in those exposed to fecal matter (Stokes et al., 2006; Cambray et al., 2010; Gu et al. 2008; Moura et al., 2007; Nandi et al., 2004; Rodríguez-Minguela et al., 2009). It has been recently demonstrated that nearly 90% of cultivable bacteria isolated from sewage-contaminated seawater are resistant to more than one antibiotic (Baquero et al., 2008). However, little is known about the prevalence of integrons across coastal habitats impacted by sewage-related pollution and potential risks to human health. We hypothesize that coastal environments exposed to sustained fecal pollution may act as reservoirs that promote the prevalence of integron-based antibiotic resistance, relative to undisturbed settings. Several studies conducted in coastal environments exposed to wastewater contamination demonstrate that beach sand constitutes a natural filter that accumulates fecal indicator bacteria (Bonilla et al., 2007; Wheeler Alm et al., 2003; de Oliveira and Pinhata, 2008). Fecal indicators are also commonly detected in estuarine environments impacted by human influence. Furthermore, recent investigations conducted in Vietnam revealed the presence of antibiotic residues and high densities of resistant bacteria in water and mud at mangrove habitats impacted by aquaculture activities (Cabral 2010; Chigbu et al., 2005; Jeng et al., 2005; Lipp et al., 2001; Ortega et al., 2009; Bachoon et al., 2010; Xuan et al., 2004; Xuan et al., 2005).

In Puerto Rico no studies have been conducted to evaluate the impact of fecal disturbances in coastal environments on the prevalence and dispersal of antibiotic resistance at a

genetic/molecular level. In this investigation culture-based and molecular techniques were used to determine the influence of fecal contamination on the prevalence of clinical integrons (classes 1-3) and integron-encoded antibiotic resistance genes (class 1) in costal environments. Special emphasis was given to mangrove, beach and estuarine environments. This was accomplished by focusing on the following objectives:

1. To evaluate the presence of clinical integrons as a molecular indicator of fecal contamination and potential exposure to antibiotic resistance genes by using PCR methods targeted at integrase genes (classes 1-3) and the variable region of class 1 integrons.

2. To quantify bacterial fecal indicators to determine possible relationships between the prevalence of resistance determinants encoded by class 1 variants and extant levels of fecal impact.

# LITERATURE REVIEW

## I. Antibiotic Resistance

Antibiotics are chemicals derived from naturally occurring organisms which are utilized for the treatment of bacterial infections in humans, as well as in animals and plants of economic importance. These drugs can be synthetically or semi-synthetically generated and are also widely used as growth promoting agents in animal farming facilities (Martinez 2009). Antibiotics exert their bactericidal or bacteriostatic effects by disrupting the integrity or function of key bacterial structures or by interfering with critical physiological processes (Kohanski et al., 2010). Their ecological role has been traditionally attributed to the inhibition of competitors (Martinez et al., 2009). Nevertheless, it has been reported that at low concentrations antibiotics may act as chemical messengers that control metabolic processes (Summers, 2002).

The extensive use of antimicrobial agents in agriculture, as well as in human and veterinary medicine is known to promote the rapid spread of resistance mechanisms (Isaacson and Torrence, 2001). Potential health hazards resulting from the creation of new environmental reservoirs bacteria carrying genetic systems implicated in the dispersal of resistance mechanisms are raising concerns surrounding the perpetuation of the above mentioned practices (Baquero et al., 2008; Converse et al., 2009; Martínez et al., 2009).

Resistance to antimicrobial agents is a natural consequence of bacterial cell adaptation. There are two major types of antibiotic resistance: intrinsic and acquired. Intrinsic or natural resistance is an innate property encoded by chromosomal genes, which is transmissible within a clonal linage but not laterally transferred between different bacteria. It may be shown as a reduced uptake of an antibiotic due to the presence of structures serving as barriers surrounding

the cell's surface such as the glycocalix or biofilms (White and McDermott 2001). Other determinants conferring intrinsic resistance include efflux proteins that fortuitously export antibiotics or modifying enzymes that degrade or inactive these chemicals (Alekshun and Levy, 2007; Wright and Sutherland, 2007; Martinez 2008). Intrinsic resistance can also occur as natural modifications or absence of antibiotic targets (Wright and Sutherland, 2007). In contrast, acquired resistance can be caused by the occurrence of rare mutations in housekeeping genes or other loci encoding cellular components targeted by antibiotics. However, acquired resistance is more commonly associated with the gain of foreign resistance genes via lateral gene transfer (White and McDermott 2001; Lambert, 2005, Martinez et al. 2009 and Alekshun and Levy, 2007). Lateral gene transfer involves the uptake and expression of genetic material that is not transmitted through a line of decent. Transformation, transduction, and conjugation are processes that mediate the dispersal of genes among unrelated organisms. Transformation involves the capture and expression of naked DNA released by dead cells in the environment. For transformation to occur a bacterium must have the genes encoding a functional state known as competence, which permits the capture, insertion, and subsequent assimilation of environmental DNA into the chromosome. Bacteria can also incorporate exogenous DNA dispersed by a bacteriophage through a process called transduction. During transduction a defective virus injects a DNA fragment from a previous host into a new bacterial cell. In contrast, during conjugation, cell to cell contact takes place while mobilizable genetic elements, such as plasmids or conjugative transposons are transferred (Caratolli, 2001).

## II. Integrons

Recently, it has been recognized that the widespread occurrence of antibiotic resistance is largely associated with genetic elements known as integrons, which are horizontally transferred by plasmid-associated transposons (Dubois et al., 2007). Integrons were first discovered in clinical settings and are known to incorporate promoterless open reading frames (genes cassettes) by a site specific recombination between *attI* and *att*C sequences, which are located within the integron and the gene cassettes, respectively (Figure 1). The integrative reaction is catalyzed by an integron encoded integrase (IntI) which belongs to the tyrosine recombinase family (Stokes et al., 2001; Roe and Pillai, 2003; Henriques et al., 2006; Demarre et al., 2007; Boucher et al., 2007). However, alternative recombination reactions (*attI* X *attC*, *attI* X *attI*, *attC* X *attC*) are known to be catalyzed by integrases encoded by different integron which facilitate the uptake and exchange of cassettes among variants of this genetic system (Frumerie et al., 2009).

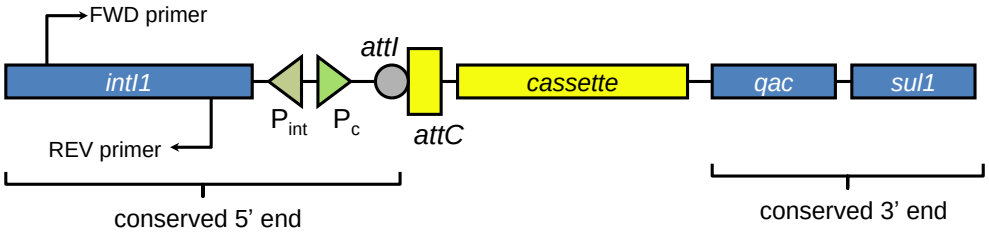



Figure 1. Schematic representation of a class 1 integron presenting the region common to all integrons (conserved 5'end) which generally consists of an integrase coding gene (*intI*) the site-specific recombination sequence *attI* and two promoters. Expression of the integrase relies on the $P_{int}$ promoter while $P_c$ drives the expression of inserted gene cassettes. The region designated as the conserved 3'end is characteristic of class 1 integrons detected in clinical strains and includes genes conferring resistance against quaternary ammonium compounds (*qacEΔ1*) and sulfonamide drugs (*sul1*).

The basic structure of all integrons also includes promoters for the independent expression of the integrase and the acquired cassettes (Figure 1; Mazel, 2006). It has been recently reported that integrase activity is up-regulated during the activation of the SOS response, since the RecA protease activity eliminates repressors blocking the expression of the integrase gene. This enhances the integron host's ability to capture new genetic material which could be useful to overcome adverse conditions, while retaining valuable traits by blocking integrase activity under favorable circumstances (Guerin et al., 2009 and Frumerie et al., 2009). Besides antibiotic resistance, gene cassettes are also known to encode a variety of determinants implicated in different functions. These include: virulence factors, toxin-antitoxin systems, polysaccharide biosynthesis, DNA modification, and degradation of industrial wastes (Koenig et al., 2008; Koenig et al., 2009). Although there are no current conventions with respect to their nomenclature, integrons are classified based on the identity percent of the nucleotide or predicted amino acid sequence of the integrase locus. So far, five classes of mobile integrons have been identified based on these criteria. Mobile integrons are commonly associated with transposons and laterally transferred via conjugative plasmids. Among these, classes 1, 2 and 3 have been described in association with clinical strains harboring multiple resistance mechanisms (Mazel, 2006; Koenig et al., 2008; Boucher et al., 2007).

Class 1 integrons are the most widely studied group and are associated with defective transposons (Tn*402*). They possess a 3' conserved region characterized by the presence of a *sulI* gene which mediates resistance to sulfonamide and a *qacEΔ1* gene that confers resistance to quaternary ammonium compounds (Figure 1). Additionally, a DNA segment of unknown function designated as *orf5* has been described in some class 1 integrons (Arduino et al., 2003). Although originally discovered in clinical strains, class 1 integrons have been also found in soil

and aquatic environments with different levels of anthropogenic disturbances (Hardwick et al., 2008; Moura et al., 2007 and Binh et al., 2009). Class 1 integrons are known to be widely distributed among the *Enterobacteriaceae* family. However, Gram-positive bacteria from fecal niches have been described as major reservoirs of this clinically important group (Nandi et al., 2004). Over 80 gene cassettes have been characterized among class 1 elements only. These include determinants encoding resistance against β-lactam antibiotics, aminoglycosides, chloramphenicol, trimethoprim, streptothricin, rifampin, erythromycin, fosfomycin, lincomycin and antiseptics of the quaternary-ammonium-compound family (Mazel 2006).

Class 2 integrons are the second most frequently detected integron type among clinical cultures. They are usually linked to Tn7 transposons and are also common carriers of *drfA1*, *sat* and *aadA1* genes, which mediate resistance to trimethoprim, streptothricin and spectinomycin, respectively (Moura et al., 2009). Additionally, a determinant encoding resistance against erythromycin *ere*(A) has been described in association with class 2 elements (Biskri and Mazel 2003). The 3' conserved segment of class 2 integrons have been reported to contain genes involved in transposition. Most of the class 2 elements described have a non functional integrase with a premature stop codon at the position 179 (Hansson et al., 2002). Nevertheless, fully functional class 2 integrases lacking this internal termination codon have been detected in cultivable strains (Barlow et al., 2004) and their partial sequences have been described in total environmental DNA (Rodríguez-Minguela et al., 2009). Moreover, IntI2 elements are also known to be prevalent in environments exposed to fecal impact relative to undisturbed settings (Rodríguez-Minguela et al., 2009). In contrast, class 3 integrons have been identified only in *Serratia marcescens* (Collis et al., 2002), *Klebsiella pneumoniae* (Correia et al., 2003) and *Delftia tsuruhatensis* as well as in association with genes encoding resistance against β-lactam

and aminoglycoside antibiotics (Xu et al., 2007). Furthermore, representatives from all these major classes have been found in clinical and environmental strains (Stokes et al., 2001; Moura et al., 2007; Hardwick et al., 2008).

Chromosomally-encoded variants of the integron system have been described in several bacterial groups (Boucher et al., 2007). These have been designated as chromosomal integrons (CI) or superintegrons. Nevertheless, a formal definition for the integron platform is still under debate (Hall and Stokes, 2004). Relative to their clinical counterparts (classes 1-3), CI are not associated with mobile elements and can carry more than 20 gene cassettes (Mazel, 2006). Most of the genes found in CI do not encode antibiotic resistance traits and many of them are of unknown function (Rowe-Magnus et al., 2001).

In Puerto Rico wastewater discharges from industrial, agricultural and domestic sources are a major cause of water pollution, particularly at coastal environments (Bonkosky et al., 2008). Due to the high concentration of organic matter, nutrients, bacteria and the prevalence of clinical integrons in fecal or intestinal environments, untreated wastewater effluents have great potential for promoting horizontal transfer of resistance genes from fecal strains to those naturally occurring in impacted coastal settings (Tennstedt et al., 2003; Nandi, et al., 2004; Moura et al., 2007; Binh et al., 2009; Rodríguez-Minguela et al., 2009). Coastal habitats are of great economical and ecological importance in Puerto Rico. Hence, their deterioration due to fecal pollution may also constitute potential risks to human health as the spread of resistant strains through the food chain (commercial and recreational fishing) or casual contact (bathing and water sport activities) could occur in contaminated environments. This could affect the course of infectious diseases as the effectiveness of treatments involving the use of antibiotics could be seriously limited by resistant strains (Martínez et al., 2009).

**III. Fecal Indicators**

Fecal coliforms and enterococci are conventional indicators of water quality in marine and fresh water environments (USAEPA, 2000; EQBPR, 2010). Fecal coliforms are facultative anaerobic, gram-negative rods that ferment lactose at 44.5°C. They comprise an abundant group of the intestinal flora of warmblooded-animals and humans. Hence, their presence in aquatic environments is assumed to be indicative of the potential presence of enteric pathogens. The production of β-D-glucuronidase activity at 44.5 °C is a trait associated with this group and very specific for *E. coli*. The activity of this enzyme is detected in mFC medium (membrane filtration procedure) as the presence of a blue or indigo color which forms after cleavage of the chromogen Indoxyl-β-D-glucuronide (USEPA 2003, Method 9222D).

Enterococci are gram-positive, ovoid bacteria present in fecal material of humans and other warm-blooded animals. Representatives from this group are routinely detected in a selective medium (mEI) using the membrane filter technique, based on their ability to produce the enzyme β-D-glucosidase which generates an indigo blue complex by reacting with the chromogen indoxyl-β-D-glucoside (USEPA 1997, Method 1600). *Enterococcus faecalis* and *E. faecium* are known to be dominant species in the human digestive tract. Epidemiological studies carried out by the USEPA have reported a correlation between the prevalence of enterococci and the incidence of swimming-related gastroenteritis in fresh and marine water habitats (USEPA, 2000). Moreover, recent reports have revealed that the number of enterococci isolated from nosocomial infections is on the rise (Sood et al., 2008, Cabral 2010, Domig et al., 2003).

## IV. Sampling site description

### A. Mangrove

Mangroves are natural ecosystems common in coastal tropical and subtropical regions. These habitats have ecological importance as they protect coastal areas from the impact of tidal cycles, serve as a shelter, as well as feeding and breeding areas for several marine species. Additionally, mangrove habitats are of economical importance for commercial fishing activities and constitute valuable sources of lumber, firewood and tanning agents (Al-Sayed et al., 2005). Despite their economical and ecological importance, these habitats are threatened by anthropogenic activities and wastewater discharges (Chen et al., 2009 and dos Santos et al., 2011). Microorganisms play an essential role in mangrove ecosystems with respect to nutrient transformation and the maintenance of the nitrogen and carbon flux in sediments and in the rhizosphere (Krauss et al., 2008; Chen et al., 2009; Ghosh et al., 2010). Moreover, photosynthetic, lithotrophic and organotrophic microorganisms provide an equilibrium state in mangroves to conserve different forms of life (Al-Sayed et al., 2005). Despite the importance of mangrove habitats, in Puerto Rico, no studies have been conducted in these environments with respect to relationships among fecal contamination, antibiotic resistance and potential health risks.

### B. Beaches and estuaries

In Puerto Rico beaches and estuaries are important sites for fishing, water sports and recreational activities. For years these areas have been in continuous deterioration due to overfishing, wastewater discharges, storm water runoff and habitat destruction (Levinton et al.,

2011; Lipp et al., 2001; Jeng et al., 2005). Estuaries can also receive urban sewage inputs and bring large quantities of pollutants to recreational beaches (Troussllier et al., 2004). Previous works have determined that after rain events fecal coliforms are prevalent in surface waters, thereafter they concentrate in the sediment (Cabral 2010; Chigbu et al. 2005). Fecal indicators in coastal areas have been traditionally monitored in the water column regardless of the possibility that solid materials may constitute a suitable reservoir for potentially harmful microorganisms. Previous investigations have demonstrated that beach sand can act as a filter, trapping nutrients and microorganisms (Bonilla et al. 2007 and Wheeler Alm et al. 2003). Whether these niches comprise reservoirs for antibiotic resistant bacteria in Puerto Rico is a subject that has not been investigated.

In Puerto Rico beaches and estuaries are highly impacted by wastewater treatment plant discharges, agricultural runoff, storm water runoff, recreational users, fecal matter from domestic and wild animals, flawed septic system and illegal sewage discharges (Bonkosky et al 2008; Bachoon et al., 2010). The monitoring beaches program of the Environmental Quality Board of Puerto Rico (EQB) has reported high counts of fecal indicators (fecal coliform and enterococci) in monitored beaches of Puerto Rico (EQB, 2010) and estuaries (EQB and EPA Cycle 303, 2010) particularly after rain events, weekends and holidays.

## MATERIALS AND METHODS

**Sampling Sites.** To confirm our experimental hypothesis, different coastal habitats with varying exposure levels, to point and nonpoint sources of fecal contamination were analyzed and compared to reference environments exposed to severe and minimal anthropogenic impact (Table 1 and Appendices 7-11). The coastal environments analyzed included mangrove forests, beaches, and estuaries (Appendices 7-11). Mangrove habitats samples were retrieved from the water column surrounding the roots, sediments and the rhizosphere. In the case of beach environments, sand samples were collected from the wet zone below the high tide line and from the water column. Samples from estuaries were retrieved from the water column (Table 1). Samples from impacted, coastal settings were recovered from the Guanajibo River estuary (Mayagüez), Pico de Piedra (Aguada), Manatí River estuary (Manatí) and La Parguera (Lajas) (Table 1). Samples from wastewater treatment plants (input and outflow) and Sardinera (Isabela) were used as reference locations with severe fecal contamination whereas those from Culebra (mangrove), Peña Blanca beach, Cupeyes River, Fanduco Reef and forest reserves were reference locations with minimal anthropogenic impact and fecal contamination (Table 1). Altogether, references sites were used to elucidate patterns between the prevalence and distribution of integron-encoded antibiotic resistance genes and fecal impact with respect to disturbed coastal habitats. At each of the coastal habitat evaluated, four replicate samples were randomly collected during the dry and rainy seasons (Appendix 12) and analyzed via PCR for the presence of clinical integrases (classes 1-3) and integron-associated antibiotic resistance genes as well as for that of fecal indicators (fecal coliforms and enterococci).

**Quantification of Fecal Indicators and Prevalence of Resistance Integrons in Coastal Habitats**. To determine the relation between fecal contamination and the presence of resistance integrons (classes 1-3) in impacted coastal environments, molecular analyses were coupled to culture-based methods for the enumeration fecal coliforms and enterococci mFC and mEI agar plates, respectively (USEPA, 1997 and 2003). Four replicates were processed for each sampling location. Water samples (100 ml) and the supernatant of the washed sand (100ml), sediments (25ml) and mangrove rhizosphere (25ml) were filtered as described above and enumerated as specified in standard methods (USEPA, 1997 and 2003; EQBPR 2010). Chi-square test analyses were used to evaluate relationships between the prevalence of integron-encoded antibiotic resistance genes (class 1) and fecal contamination whereas ANOVA analysis was performed to test for significant differences among levels of anthropogenic impact and sampling season with respect to the abundance of fecal indicator bacteria.

**Extraction of Environmental DNA.** To collect microbial biomass from water, sand, sediment and mangrove rhizosphere, samples were homogenized to suspend cells and then processed using the membrane filter technique. Water samples were shaken manually (25 times) following the filtration of a volume of 500 ml in 47 mm, 0.45 µm (pore size) cellulose nitrate membrane filters (Whatman, Germany). Sand samples (300 g) were mixed with 300 ml of dilution buffered water (USEPA, 1997) and homogenized in a shaker (200 rpm) for 30 min at room temperature, then left to settle for 20 min. Mangrove rhizosphere and sediment samples were processed similarly except that 50 g of the corresponding material were diluted in buffered water (USEPA, 1997). The supernatant (100ml) of the washed sand (100ml), sediments (25ml) and mangrove rhizosphere (25ml) were filtered as described above.

Total DNA, from soil and filter-captured biomass was extracted using the FastDNA SPIN Kit for Soil (Qbiogene Inc., Carlsbad, CA) according to the manufacturer's manual except for the following modifications. Cell lysis by bead beating was carried out in a vortex using a Mo-Bio adapter (Mo Bio Inc., Carlsbad, CA) for 5 minutes at maximum speed followed by centrifugation at 10,000 rpm for 10 minutes. Samples were centrifuged at 14,000 rpm for 15 min after the addition of the PPS reagent. The lysates were mixed with the DNA Binding Matrix Suspension for 10 minutes, followed by two washes with the SEW-S solution. Finally the DNA was eluted in 150 µl of DES, electrophoresed in a 1% agarose gel and quantified (optical density at 260 nm) using a nanodrop ND-1000 instrument (Thermo Scientific, Wilmington, DE).

**PCR Amplification of *intI* loci of Clinical Integrons and Gene Cassettes Associated with Class 1 Elements.** Small sub-unit 16S rRNA genes were amplified to test the quality of the extracted DNA. Amplification reaction mixtures consisted of 12.5 µM dNTPs; 0.025 U/µl of *Taq* polymerase (Promega, Madison, WI); 0.5X Buffer, 3.5 mM $MgCl_2$, 100 µg of BSA (New England® Biolabs, Ontario, Canada), 6.25 p/moles of each primer (827 FWD and 1392 REV, Table 2) and 22 ng of template DNA, in a final volume of 25 µl. Cycling conditions were initial denaturation at 95ºC for 3 minutes, followed by 25 cycles of melting at 94ºC for 45 sec, 45 sec of annealing at 57ºC, 1 min 30 sec of extension at 72ºC and a final extension of 72ºC for 7 min. Four replicate PCR reactions were prepared for each of the four replicate DNA samples and genomic DNA from *E. coli* strain R388 (Dillon et al., 2005) was used as template in positive controls.

Integrase genes (classes 1-3) and the variable region of class 1 integrons were independently amplified using the above mentioned PCR cocktail adjusted for a 12.5 µl reaction volume and ~30 ng of template DNA. The appropriate primers (Table 2) were added at a final concentration of 6.25 p/moles. Cycling conditions for integrase genes were initial denaturation at 94ºC for 2 minutes, followed by 35 cycles of melting at 94ºC for 30 sec, 1 min of annealing at 59 ºC, 30 sec of extension at 72ºC and a final extension of 72ºC for 7 min. Four replicate PCR reactions were prepared for each of the four replicate DNA samples while genomic DNA from *E. coli* strains harboring class 1, class 2 and class 3 integrons were used as positive controls (Avila and de la Cruz, 1988; Dillon et al., 2005 and Arakawa et al., 1995). For each PCR product a total of five clones were sequenced to corroborate the identity of the amplified fragment.

Gene cassettes carried by class 1 integrons were amplified using previously described primers (Lévesque et al., 1995 and Arduino et al., 2003) (Table 2) and 30 ng of environmental DNA as template, in a final volume of 25 µl. Four replicate PCR reactions were prepared for each of the four replicate DNA samples. Cycling parameters consisted of initial denaturation at 94ºC for 2 minutes, followed by 30 cycles of melting at 94ºC for 30 sec, 1 min of annealing temperature at 62ºC, 5 min 30 sec at 72ºC of extension and a final extension of 72ºC for 7 min. All PCR reactions were carried out in a Robocycler Gradient 96 instrument (Stratagene, La Jolla, CA).

The amplified gene cassettes were electrophoresed in a 1.5% agarose gel to determine the product size and extracted using the IBI Gel/PCR DNA Fragments Extraction Kit (IBI Scientific, Peosta, IA). The gel-purified amplicons were ligated into the pCR4-TOPO plasmid vector for subsequent transformation of *E. coli* cells using the TOPO TA cloning for sequencing kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, except for the following

modifications: the ligation reaction were carried out for 3 hours and the entire transformation mix was spread on LB plates (~75 µl per plate) containing 50 µg/ml of kanamycin. For each PCR product a total of five clones were sequenced to corroborate the identity of the amplified fragment. The cloned inserts were identified relative to matching sequences in GeneBank using BLAST. Multiple sequence alignments were constructed with ClustalX in order to conduct phylogenetic analyses using the MEGA 4 software package as previously described (Rodríguez-Minguela et al., 2009).

Table 1. Description of sampling sites.

| Site Code | Description | Habitat | Levels of fecal impact | Sample type |
|---|---|---|---|---|
| UWP | Urban secondary wastewater treatment plant (output and input) | NA | Severe | Liquid phase |
| RWP | Rural secondary wastewater treatment plant (output and input) | NA | Severe | Liquid phase |
| GRM | Guanajibo River estuary, Mayagüez | Estuarine | Severe | Water column[1] |
| PPA | Pico de Piedra Beach, Aguada | Beach | Severe | Water column[1] and beach sand[2] |
| LPM | Mangrove habitat, La Parguera, Lajas | Mangrove | Severe | Water column[1], sediment[3] and mangrove |
| SBPO SBPA | Pipeline outfall, Sardinera Beach, Isabela | Beach | Severe | Water from **o**utfall and **a**djacent effluent |
| MRB | Manatí River estuary, Barceloneta | Estuary | Severe | Water |
| PBA | Peña Blanca, Aguadilla | Beach | Minimal | Water column[1] and beach sand[2] |
| EDC | Ensenada Dakity, Culebra | Mangrove | Minimal | Water column[1], sediment[3] and mangrove |

Table 1. Continued.

| Site Code | Description | Habitat | Levels of fecal impact | Sample type |
|---|---|---|---|---|
| GF | Undisturbed Forest soil (Palma Manaca Plantation), Guajataca Reserve | Forest | Minimal | Soil[4] |
| UUM | Urban Region, University of Puerto Rico at Mayagüez | Forest | Minimal | Soil[4] |
| CRU | Cupeyes river upstream, Susua Reserve | Estuary | Minimal | Water |
| CRD | Cupeyes river downstream, Susua Reserve | Estuary | Minimal | Water |
| FR | Fanduco Reef, Offshore region, Puerto Real, Cabo Rojo | Beach | Minimal | Water |

[1] Retrieved from a depth of 20 cm below the surface.

[2] Retrieved from a depth of 15 cm between the low and high tide lines

[3] Retrieved from a depth of 15 cm below the water column.

[4] Retrieved from a depth of 15 cm below the surface.

Table 2. Primer sets used for the amplification of integron integrases (classes 1-3) and the variable region of class 1 integrons.

| Primer | Target | Sequence (5'-3') | Position | Product Size (pb) | Annealing Temperature °C | References |
|---|---|---|---|---|---|---|
| Int1 F | *IntI1* | CAG TGG ACA TAA GCC TGT TC | 2734–2751 | 160 | 59 | Koeleman et al., 2001 |
| Int1 R | | CCC GAG GCA TAG ACT GTA | 2874–2891 | | | |
| Int1 F | *IntI1* | TCTCGGGTAACATCAAGG | 468-485 | 300 | 56 | Leverstein-van et al., 2002 |
| Int1 R | | AGGAGATCCGAAGACCTC | 693-710 | | | |
| Int1 F | *IntI1* | AAA ACC GCC ACT GCG CCG TTA | 4–24 | 1,200 | 56 | Bezanson et al., 2008 |
| Int1 R | | GAA GAC GGC TGC ACT GAA CG | 584-603 | | | |
| Int2 R | *IntI2* | GTA GCA AAC GAG TGA CGA AAT G | 11524–11545 | 788 | 59 | Mazel et al., 2000 |
| Int2 F | | CAC GGA TAT GCG ACA AAA AGG T | 12291–12312 | | | |
| Int3 R | *IntI3* | ACG GAT CTG CCA AAC CTG ACT | 1697–1717 | 979 | 59 | Mazel et al., 2000 |
| Int3 F | | GCC TCC GGC AGC GAC TTT CAG | 738–758 | | | |
| 5'-CS F | *IntI* | GGC ATC CAA GCA GCA AG | 24-43 | Variable | 62 | Lévesque et al., 1995 |
| 3'-CS R | *ΔqacE/sul1* | AGC CCC ATA CCT ACA AAG CC | 2160–2181 | | | Arduino et al., 2003 |

# RESULTS

***Overall fecal indicator counts in coastal habitats.*** Enterococci and fecal coliform bacteria were enumerated during the dry and rainy seasons in samples from disturbed beaches, mangrove ecosystems and estuarine environments (Table 1) with respect to reference samples from relatively undisturbed sites. According to water quality standards for beaches enviroments, permissible levels for enterococci and fecal coliforms have been established as 35 cfu's and 200 cfu's per100 ml, respectively (EQBPR, 2010). Analysis of variance (ANOVA) was used to test for significant differences in levels of anthropogenic impact, abundance of fecal indicators and sampling season. In general, during the rainy season coliforms counts (143 CFU/100 ml) were significantly greater across polluted environments (pvalue<0.05, $F_{1,70}$= 16.22) whereas those for enterococci were above permissible levels (53-108 CFU/100 ml) regardless of the sampling season. No significant differences (pvalue>0.05, $F_{1,70}$= 1.09) were observed for enterococci counts in association with the seasonal factor. Virtually all samples from sites with minimal impact showed indicator counts within permissible parameters and no significant differences for coliforms (pvalue>0.05, $F_{1,46}$ = 3.12) and enterococci (pvalue>0.05, $F_{1,46}$ = 1.16) counts were observed with respect to sampling seasons.


Figure 2: Validation of mFC agar plates with respect to sterility (a) and detection of *E. coli* as a representative fecal coliform (b).

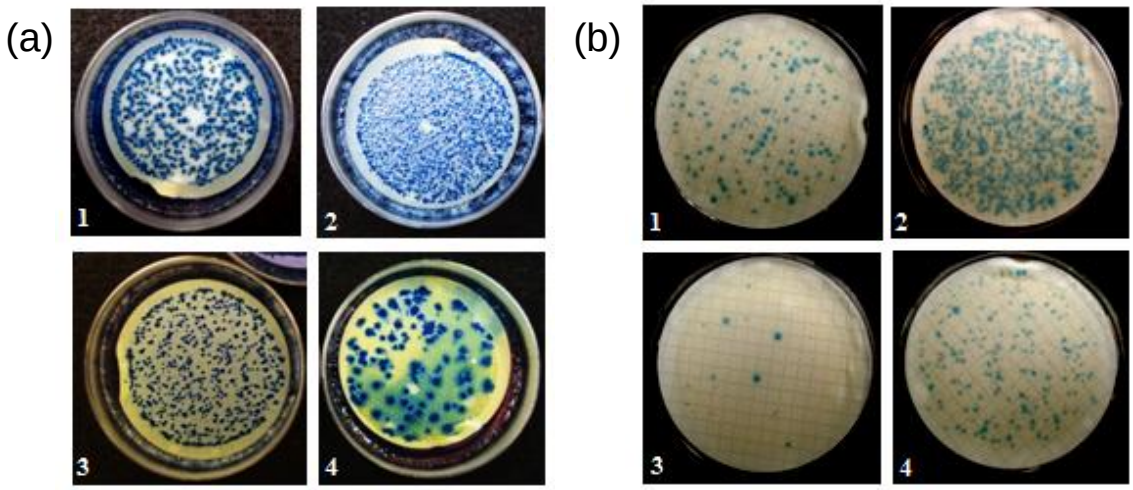



Figure 3.Representative results for the enumeration of fecal indicators in water samples from Pico de Piedra beach, Aguada, PR. (a) = total coliforms (USEPA, Method 9222). (b) = total enterococci (USEPA, Method 1600).

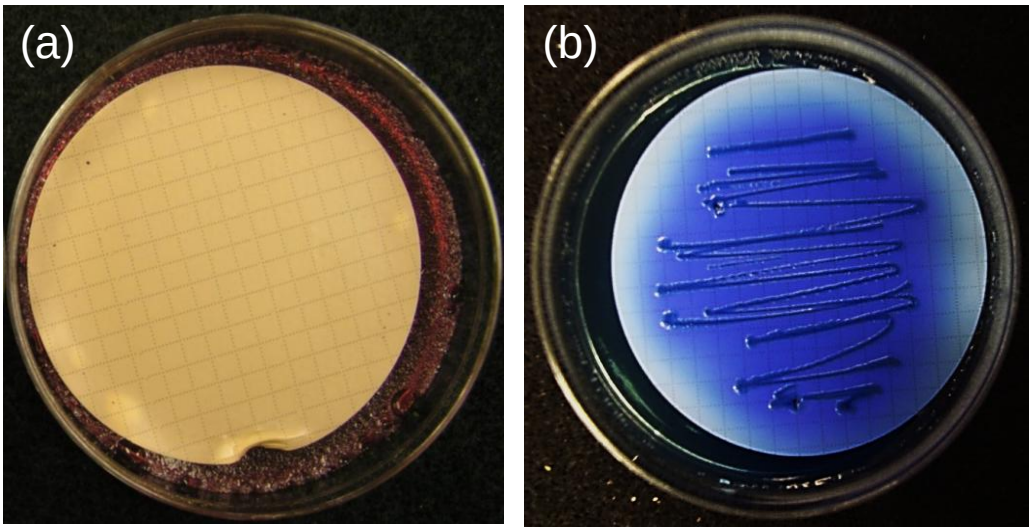



Figure 4. Validation of mEI agar plates with respect to sterility and detection of *E. faecalis* as a representative enterococci.

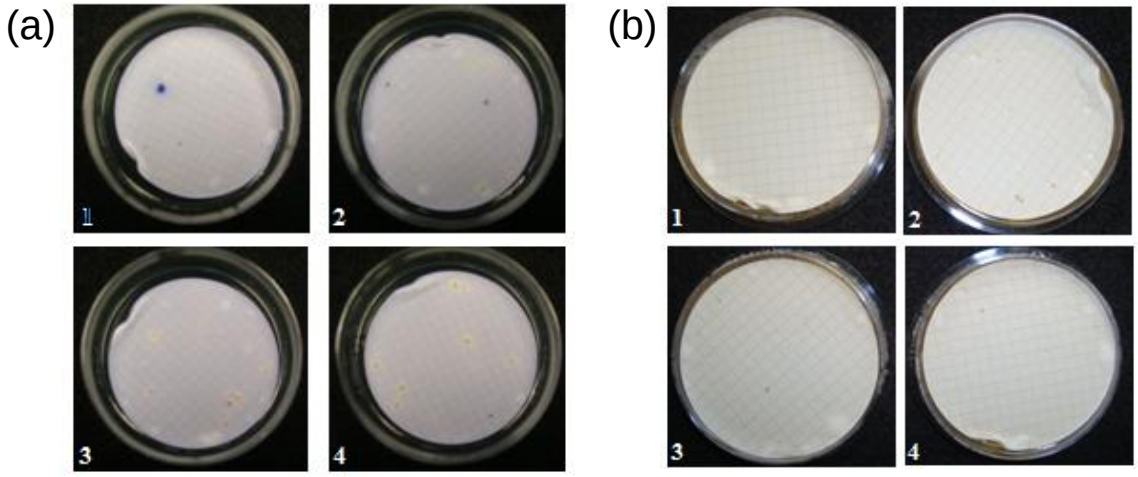



Figure 5. Representative results for the enumeration of fecal indicators in water samples from Peña Blanca beach, Aguadilla, PR. (a) = total coliforms (USEPA, Method 9222). (b) = total enterococci (USEPA, Method 1600).

***Fecal indicator counts in mangrove habitats.*** Fecal coliforms and enteroccocci were enumerated in water, sediment and rhizosphere samples from impacted (La Parguera, Lajas) and relatively undisturbed (Ensenada Dakity, Culebra) mangrove ecosystems (Table 1). Counts for coliforms were within permissible levels in water samples at both sites. However, coliforms numbers were significantly greater in water samples from La Parguera ($F_{1,15}$= 23.33, pvalue<0.05) with respect to those from Culebra. At the later site, both indicators were undetected or found within acceptable levels. (Table 3). No significant differences were observed with respect to the sampling season for coliform counts ($F_{1,15}$= 0.68, pvalue>0.05).

However, in water samples from La Parguera counts for enterococci revealed significant differences in association with sampling seasons ($F_{1,15}$= 7.60, pvalue<0.05). During the rainy season, enterococci counts were at permissible levels at both sites. Nevertheless, during the dry season enterococci numbers were significantly higher in water samples from La Parguera, whereas these were undetected at Culebra.

Coliform counts in sediment samples were higher at La Parguera (Table 3) and revealed significant differences associated with the sampling site ($F_{1,15}$= 14.38, pvalue<0.05) and the sampling season ($F_{1,15}$= 5.30, pvalue<0.05). At Culebra, coliforms were undetected during the dry season although during the rainy season these were present within acceptable levels. Similarly, counts for enterococci were higher at La Parguera and demonstrated significant differences in connection with sampling sites ($F_{1,15}$= 483.90, p<0.05) although no significant difference was found with respect to sampling seasons ($F_{1,15}$= 2.49, p>0.05). Enterococci were undetected in samples from Culebra during both seasons. (Table 3).

As observed in sediments, coliforms counts in rhizosphere samples were higher at La Parguera during both seasons. These showed significant differences corresponding to human influence ($F_{1,15}$= 13.53, $p<0.05$) and sampling seasons ($F_{1,15}$= 11.73, $p<0.05$). Coliform indicators were undetected at Culebra during the dry season. Nevertheless, they were present within permissible parameters during the rainy season (Table 3). Enterococci indicators were also significantly higher at La Parguera ($F_{1,15}$=9.22, $p<0.05$) but showed no significant difference with respect to sampling seasons ($F_{1,15}$=0.26, $p>0.05$). In contrast, these were undetected during the dry season and found above permissible levels during the rainy season at Culebra.

Table 3. A fecal indicator counts for mangrove habitats.

| Season | Site | Sample | Fecal Coliform[1] | Enterococci[1] | Geometric Mean[2] Fecal Coliform | Enterococci |
|---|---|---|---|---|---|---|
| Rainy | Parguera | Water[3] | 7 | 9 | 5.96 | 7.52 |
| | | Sediment[4] | 48 | 101 | 39.75 | 94.14 |
| | | Rhizosphere[4] | 517 | 7 | 315.03 | 5.62 |
| | Culebra | Water[3] | 6 | 2 | 5.56 | 2.28 |
| | | Sediment[4] | 6 | 0 | 6 | 0 |
| | | Rhizosphere[4] | 47 | 95 | 19.48 | 24.15 |
| Dry | Paguera | Water[3] | 38 | 146 | 24.8 | 115.09 |
| | | Sediment[4] | 58 | 230 | 16.62 | 191.06 |
| | | Rhizosphere[4] | 53 | 340 | 24.07 | 307.51 |
| | Culebra | Water[3] | 0 | 0 | 0 | 0 |
| | | Sediment[4] | 0 | 0 | 0 | 0 |
| | | Rhizosphere[4] | 0 | 0 | 0 | 0 |

[1]Average of four replicates (CFU/100 ml).
[2]Geometric mean according to EPA standard method and EQBPR 2010.
[3]Volume filtered 100 ml.
[4]Supernatant volume filtered of 15-20 ml.

***Fecal indicator counts in beach habitats.*** Indicator bacteria were quantified in water and sand samples from impacted (Pico de Piedra Beach, Aguada) and relatively undisturbed (Peña Blanca Beach, Aguadilla) beach ecosystems. During the rainy season, significantly high levels of fecal coliforms ($F_{1,15}$= 192.31, $p<0.05$) and enterococci ($F_{1,15}$= 39.48, $p<0.05$) were detected in water samples from Pico de Piedra Beach relative to those from Peña Blanca Beach (Table 4). However, no significant differences with respect to sampling seasons were detected in association with fecal coliforms ($F_{1,15}$= 0.48, $p>0.05$) whereas the opposite was observed for enterococci indicators ($F_{1,15}$= 28.61, $p<0.05$) as these were detected above permissible levels during the rainy season (Table 4).

Sand samples from Aguada presented acceptable counts for fecal coliforms during the rainy season which were significantly higher than those from Aguadilla ($F_{1,15}$= 51.56, $p<0.05$). During both seasons while enterococci were detected within acceptable levels at Aguadilla. Nevertheless, no significant relationships were found relative to sampling seasons (fecal coliforms: $F_{1,15}$= 1.09, $p>0.05$; enterococci: $F_{1,15}$= 1.50, $p>0.05$) in counts reported for sand samples (Table 4).

Table 4: Fecal indicator counts for beach habitats.

| Season | Site | Sample[1] | Fecal Coliform[2] | Enterococci[2] | Geometric Mean[3] Fecal Coliform | Enterococci |
|---|---|---|---|---|---|---|
| Rainy | Aguada | Water | 52 | 118 | 44.83 | 72.3 |
| | | Sand | 185 | 8 | 96.11 | 3.85 |
| | Aguadilla | Water | 1 | 0 | 1.19 | 0 |
| | | Sand | 1 | 2 | 1.41 | 1.56 |
| Dry | Aguada | Water | 55 | 1 | 48.92 | 1 |
| | | Sand | 50 | 19 | 39.5 | 18.82 |
| | Aguadilla | Water | 0 | 0 | 0 | 0 |
| | | Sand | 0 | 1 | 0 | 1 |

[1]Volume filtered 100 ml.
[2]Average of four replicates (CFU/100 ml)
[3]Geometric mean according to EPA standard method and EQBPR 2010.

***Fecal indicator counts in estuarine habitats.*** In order to evaluate the input of fecal pollution from river–associated nonpoint sources into coastal habitats, the abundance of indicator bacteria was evaluated in water samples from impacted estuarine settings (Guanajibo River estuary, Mayagüez and Manatí River estuary, Barceloneta) with respect to a relatively undisturbed river ecosystem (Cupeyes River, Sabana Grande) (Table 1). Fecal coliform counts ($F_{1,23}$= 110.04, $p<0.05$) and enterococci indicators ($F_{1,23}$= 65.41, $p<0.05$) were significantly higher in samples from Guanajibo River relative to La Boca estuary and Cupeyes River (Table 5). Coliform indicators were above permissible parameters at the Guanajibo River estuary whereas these were within allowable levels and undetected at La Boca site and Cupeyes River, respectively. During the rainy season enterococci counts were above permissible limits at the Guanajibo and La Boca estuaries but were undetected at the Cupeyes River. During the dry season

enterococci counts were above acceptable values at Guanajibo River estuary while these were within permissible limits at Manatí River estuary, and at Cupeyes River (Table 5). However, no significant differences were detected between the abundance of fecal indicators with respect to the sampling seasons (fecal coliforms: $F_{1,23}$=2.18, p>0.05; enterococci: $F_{1,23}$= 5.6, p>0.05).

Table 5: Fecal indicator counts for estuarine and river-related sites.

| Season | Site | Sample[1] | Fecal Coliform[2] | Enterococci[2] | Geometric Mean[3] Fecal Coliform | Enterococci |
|---|---|---|---|---|---|---|
| Rainy | Guanajibo River, Mayaguez | Water | 150 | 151 | 150 | 150.6 |
| | Manati River, Barceloneta | Water | 19 | 54 | 15.95 | 35.55 |
| | Cupeyes, S. Grande[4] | Water | 0 | 0 | 0 | 0 |
| Dry | Guanajibo River, Mayaguez | Water | 150 | 167 | 150 | 166.81 |
| | Manati River, Barceloneta | Water | 1 | 2 | 1.41 | 2.06 |
| | Cupeyes, S. Grande[4] | Water | 33 | 13 | 28.42 | 10.85 |

[1]Volume filtered 100 ml.
[2]Average of four replicates (CFU/100 ml)
[3]Geometric mean according to EPA standard method and EQBPR 2010.
[4] Standard quality for fecal coliform was <2000 CFU/100 ml (EQBPR 2010).
[*]Fecal coliform count was above 150 CFU/100 ml.

***DNA extraction of total environmental DNA.*** The yield of total DNA recovered from membrane filters and reference soil samples ranged from 20-250 ng/µL (Figure 6). Small sub-unit 16S rRNA genes were consistently amplified from all the processed samples demonstrating that the extracted DNA was suitable for subsequent integron–targeted PCR assays (Figure 7).

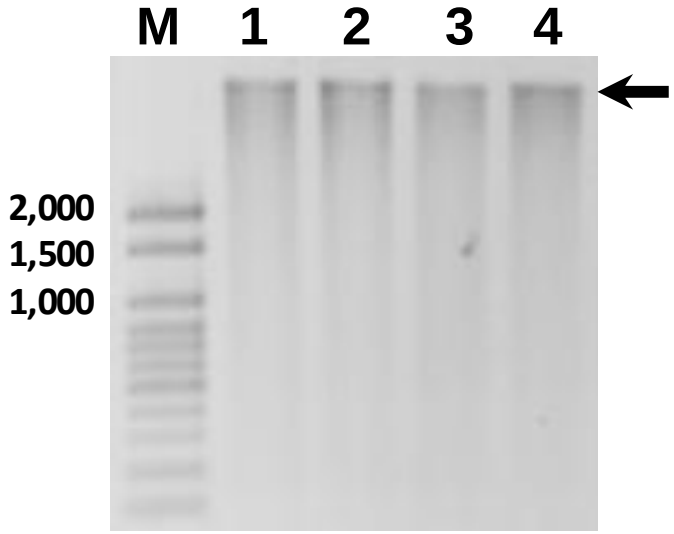



Figure 6. Agarose gel electrophoresis showing representative results of total DNA extractions from biomass associated with water samples from the Guanajibo River estuary, Mayagüez (lanes1-4). M= 100 bp DNA Marker (Applied Biological Materials Inc., Richmond, BC, Canada).

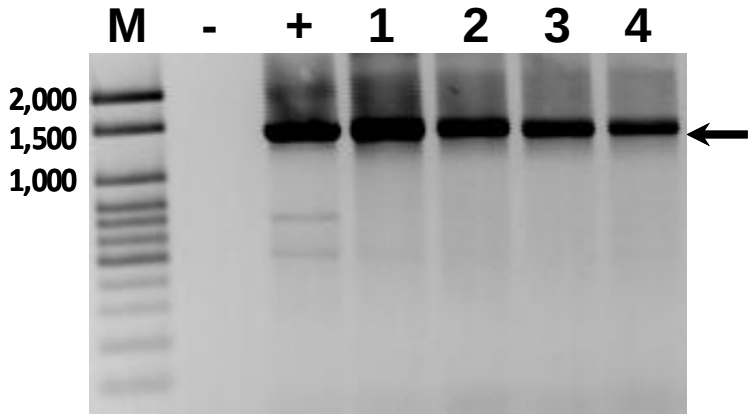



Figure 7. Representative results for the amplification of small subunit 16S rRNA genes corresponding to biomass present in four replicate water samples recovered from the Guanajibo Estuary, Mayagüez. (-) = Negative control, no template DNA. (+) = Positive control (Genomic DNA from *E. coli* R388), samples from Guanajibo River estuary, Mayagüez (lanes 1-4).

***Validation of PCR assays targeting integron integrases (classes 1-3).*** A PCR protocol targeting conserved regions among integrases encoded by clinical integrons (intl genes, Figure 8) was implemented as an initial screening method to evaluate potential dispersal of these elements in the environment through wastewater-related and anthropogenic disturbances. To this end, integrase genes were PCR-amplified, cloned and sequenced from reference samples which included influents and effluents from anonymous wastewater treatment plants (UWP, RWP, and impacted terrestrial (UUM) environments (Table 1). Samples from coastal (sand, PBA) and terrestrial reference (FUM) sites with moderate or minimal human influence were also analyzed (Table 1).

Results from these tests using equal amounts of total community DNA (11ng) from each environmental sample revealed different distribution and prevalence trends among integrases (Figure 8).

Class 1 and class 2 integrases were detected among impacted and relatively undisturbed sites. However their detection signals were stronger in DNA from sites exposed to a high fecal or anthropogenic impact (sewage and urban soil) with respect to those from less disturbed environments (forest soil and wet sand from a secluded beach, Peña Blanca, Aguadilla). This trend was further evaluated for *intI2*-like genes through an experiment conducted with soil DNA from an isolated sector of the Guajataca Forest. Amplification experiments revealed that *intI2* elements were not readily detected when probing a site with a history of minimal human influence (Guajataca Forest), unless the template was spiked with DNA from a bacterial control strain harboring a class 2 integron (Figure 9, Panel B and Panel C). Class 3-like integrases were detected only in the outflow of the rural, secondary, wastewater treatment plant (Figure 8, Panel C).

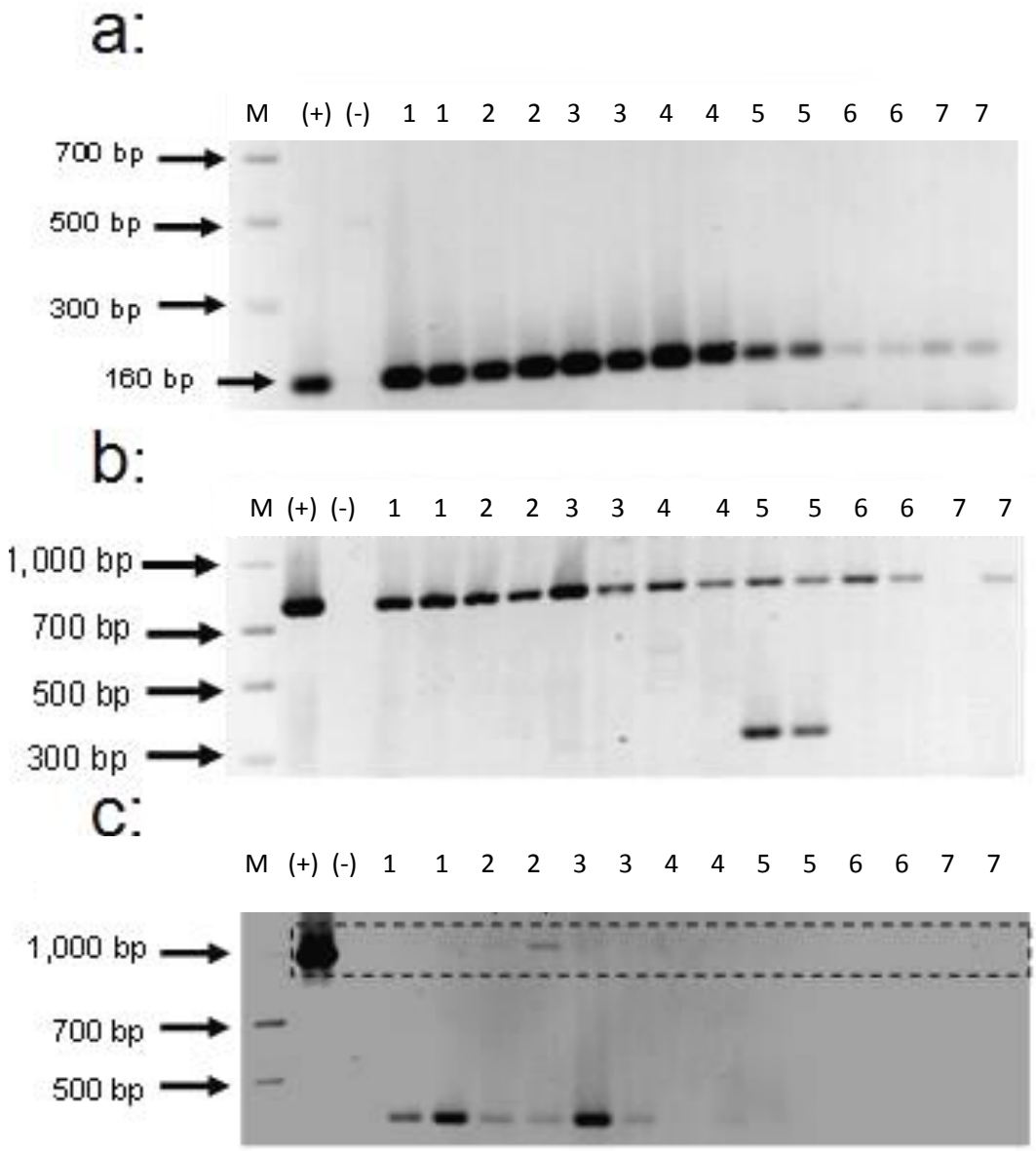



Figure 8. Agarose gel electrophoresis showing representative results of the amplification of integrase genes from class 1 (Koeleman et la., 2001) (Panel A) class 2 (Mazel et al., 2000) (Panel B) and class 3 (Mazel et al., 2000) (Panel C) integrons from total community DNA recovered from environments with different levels of fecal impact. M = 24kb Max DNA Ladder (Fisher BioReagents, Pittsburgh, PA); (+) = positive control, template DNA containing the target intI sequence, (-) = negative control, no template DNA added. 1=RWPI (rural wastewater treatment plant influent), 2=RWPO (rural treatment plant outflow), 3=UWPI (urban treatment plant influent), 4=UWPO (urban treatment plant outflow), 5=UUM (urban region, University of Puerto Rico at Mayagüez), 6=FSM (forest soil adjacent to urban soil, Mayagüez), 7=PBA (beach sand with moderate human influence (Peña Blanca site, Aguadilla, PR).

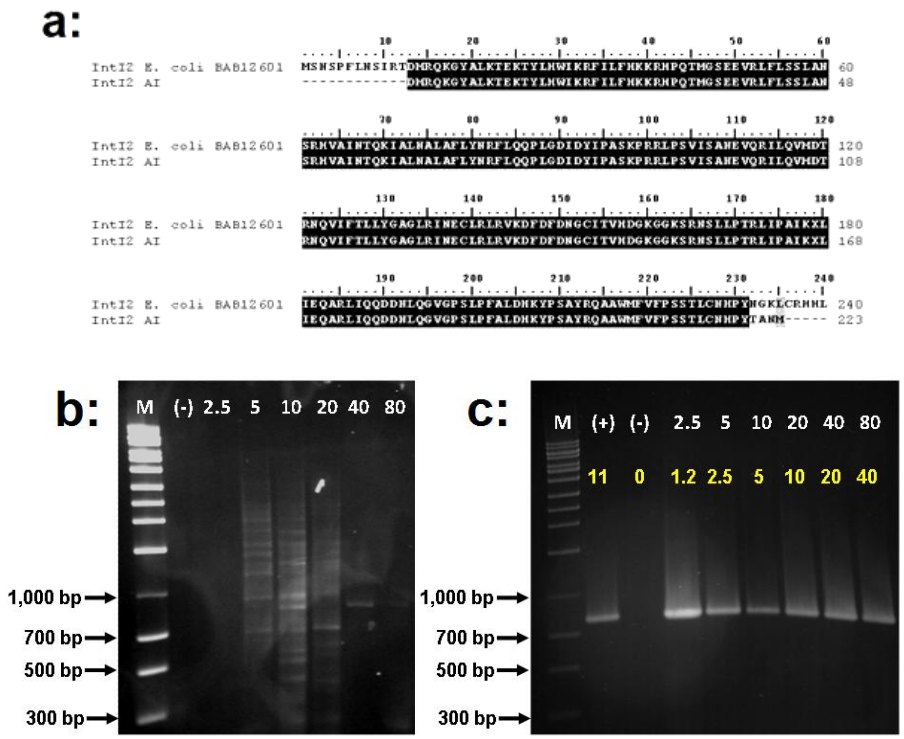



Figure 9. Panel (a): Alignment of the predicted amino acid sequence of the PCR product recovered from Peña Blanca beach (Aguadilla) using primers targeted at intl2 elements against that of a class 2 integrase described in *Escherichia coli*. The Genbank accession number follows the designation of the reference Intl2 sequence. Panel (b): PCR amplification of intl2 genes from total soil DNA (undisturbed area Guajataca Forest). Panel (c): PCR amplification of intl2 genes from total soil DNA (undisturbed area Guajataca Forest) spiked with increasing amounts of intl2 template DNA. Numbers in white and yellow describe the respective mass (ng) of soil DNA and intl2 template used for each reaction. All experimental and control reactions were run in the same batch.

***Optimization and validation of a cassette-targeted PCR assay for the amplification of the variable region of class 1 integrons.*** Since class 1 and class 2 integrons appeared to be widespread and class 3 integrons showed a more restricted distribution, an additional PCR assay was implemented for the detection of class 1 integrons loaded with antibiotic resistance gene cassettes. The purpose of this cassette-targeted PCR method was to evaluate any pattern on the prevalence of antibiotic resistance mechanisms and human influence under the hypothesis of dominance of class 1 integrons carrying resistance mechanisms in impacted habitats. To tests this hypothesis total DNA from influents retrieved from an urban wastewater plant was analyzed and an amplicon within the 1,000-1500 base was detected (Figure 10).

Partial sequencing analysis of the aforementioned fragment confirmed the occurrence of genes associated with class 1 integrons encoding a putative dehydrofolate reductase (Dhfr) (Figure 11) that was ~98.7% identical to that previously detected in pathogenic strains of *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Enterobacter faecalis*, *Salmonella enterica* and *Escherichia coli* (GenBank accession numbers: ABC50093, ABJ15828, BAD77819, AAT45231 and ACD02027, respectively). Furthermore, a gene encoding an enzyme capable of inactivating the antibiotic chloramphenicol (a protein synthesis inhibitor) and a determinant coding for an efflux pump (qacΔE1) which protects against disinfectants widely used in hospitals (quaternary ammonium compounds [qac]) were also recovered (Figures 12 and 13). The former showed an amino acid identity of 97% with respect to a chloramphenicol modifying enzyme reported in *Klebsiella pneumoniae* (GenBank accession number:

YP_001965795) while the later was 100% identical to a qac pump detected in *Klebsiella pneumoniae* (GenBank accession number: ACF06163).

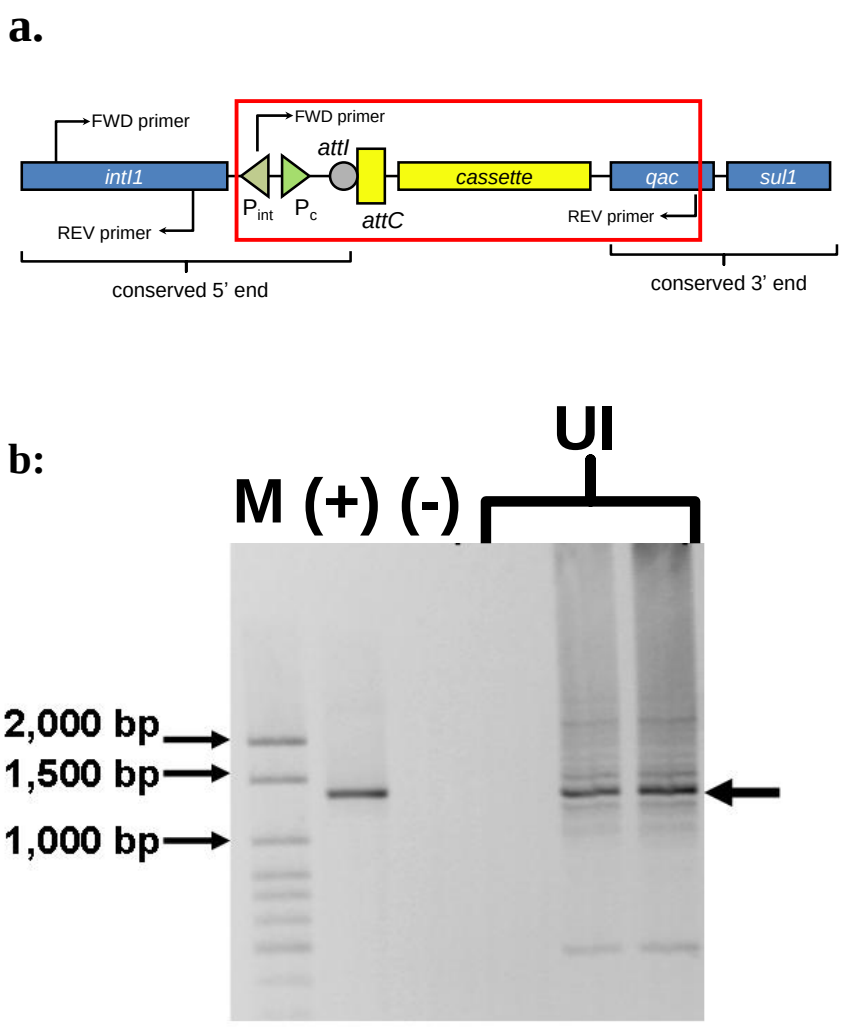



Figure 10. Panel (a): Diagram depicting the structure of a class 1 integron and the relative position of primers targeted at conserved regions flanking the gene cassettes insertion site. Panel (b): Agarose gel electrophoresis showing the amplification (three replicate reactions) of gene cassettes (~1.3 Kb long) associated with class 1 integrons detected in DNA recovered from the influents of an urban wastewater treatment plant. M=100bp ladder DNA marker (Applied Biological Materials Inc., Richmond, BC, Canada); (+) = positive control; (-) = negative control.

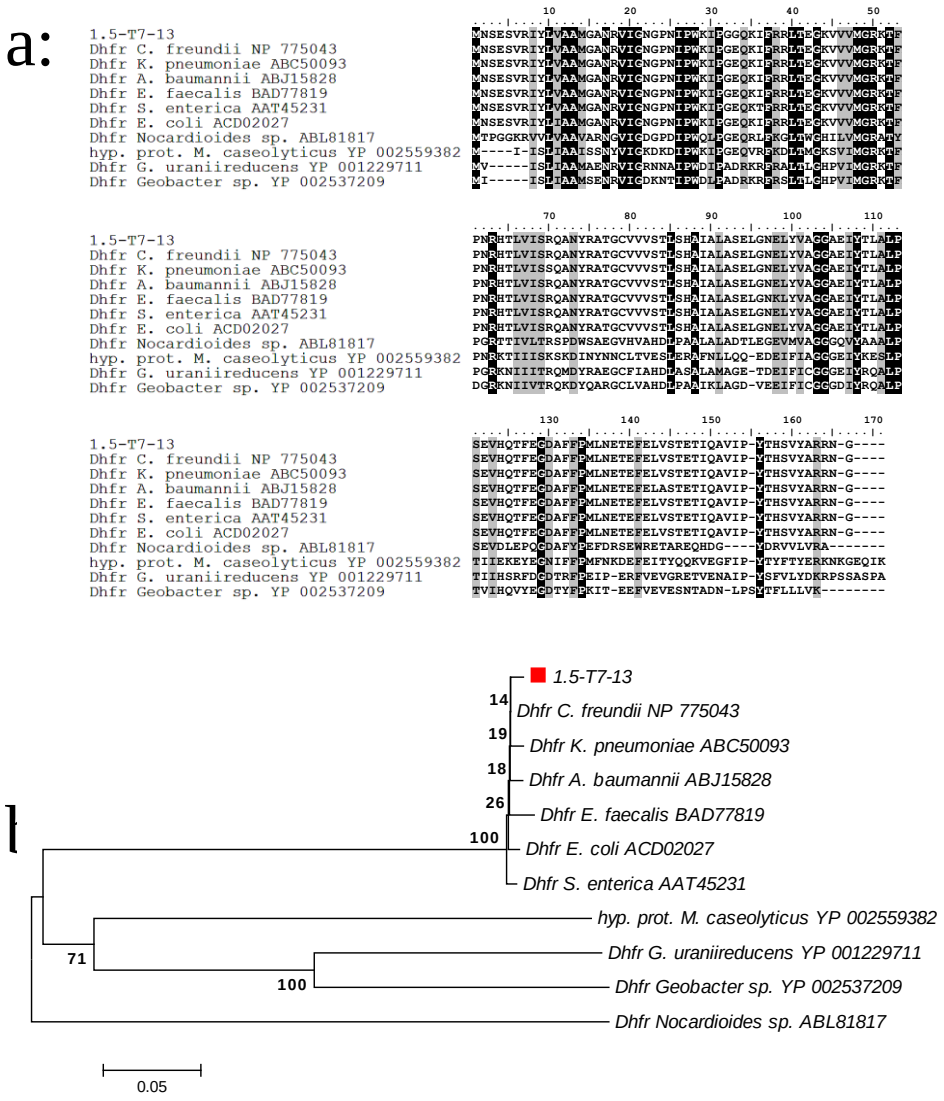
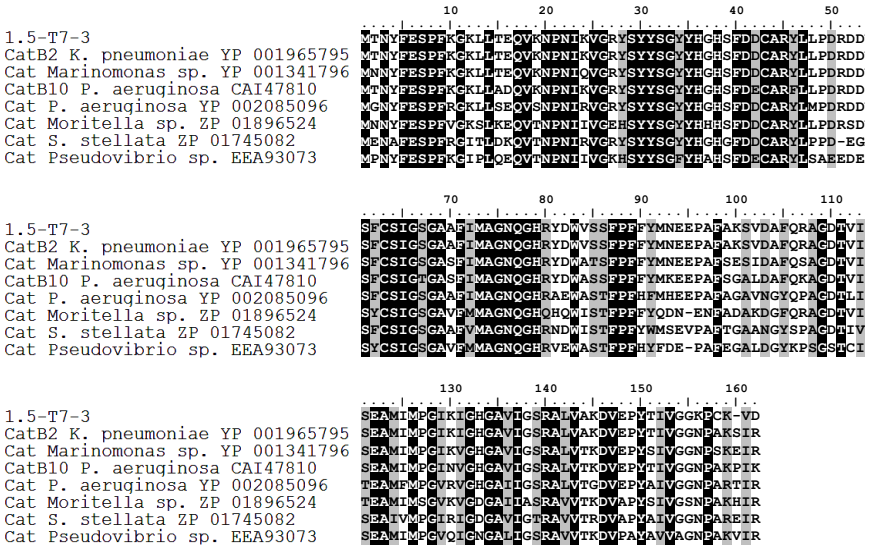



Figure 11. Panel (a): Diagram depicting the structure of a class 1 integron and the relative position of primers targeted at conserved regions flanking the gene cassettes insertion site. Panel (b): Agarose gel electrophoresis showing the amplification (three replicate reactions) of gene cassettes (~1.3 Kb long) associated with class 1 integrons detected in DNA recovered from the influents of an urban wastewater treatment plant. M=100bp ladder DNA marker (Applied Biological Materials Inc., Richmond, BC, Canada); (+) = positive control; (-) = negative control.

a:




b:

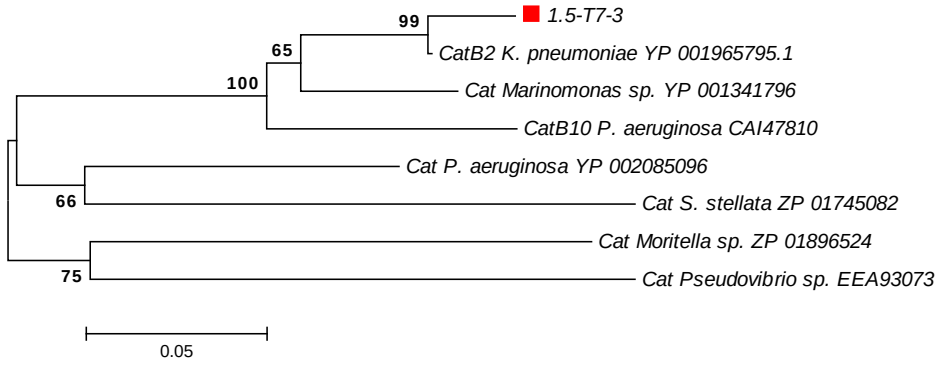
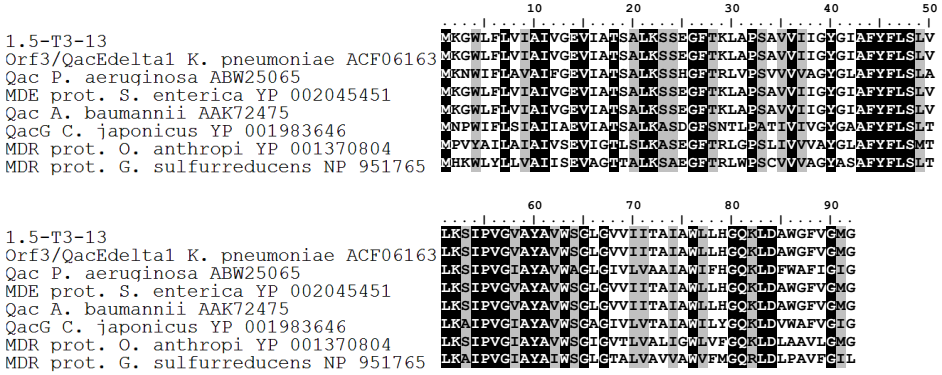



Figure 12. Multiple sequence alignment (Panel a) and neighbor joining tree (Panel b) depicting the relationships between database matches and the partial amino acid sequence of a class 1 integron gene cassette encoding a chloramphenicol adenyltransferase (clone 1.5-T7-3) detected in the influents of an urban wastewater treatment plant. The GenBank accession number follows the designation of reference sequences.

a:




b:

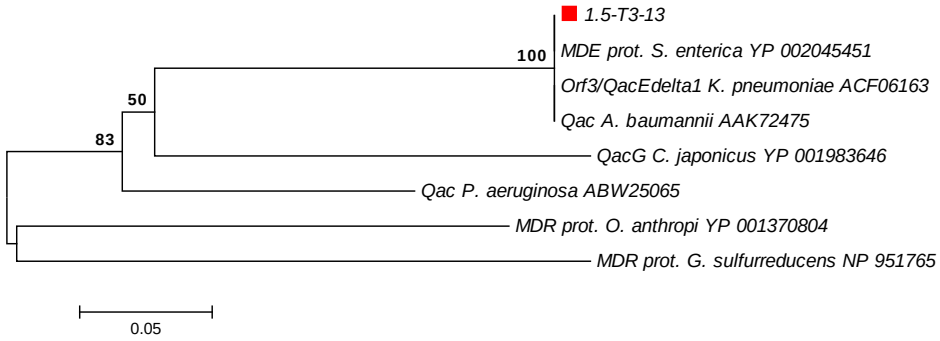



Figure 13. Multiple sequence alignment (Panel a) and neighbor joining tree (Panel b) depicting the relationships between database matches and the deduced, partial amino acid sequence of the region corresponding to the qac gene associated with the conserved 3' end of class 1 integrons (clone 1.5-T3-13). The GenBank accession number follows the designation of the reference sequences.

An additional preliminary experiment was conducted to further validate the specificity of the cassette-targeted PCR. This consisted of sequencing multiple bands amplified in three replicate PCR reactions using total DNA isolated from water samples of the Guanajibo estuary, Mayagüez. A site extensively impacted by fecal pollution (Appendix 9). Except for a single ~800 bp amplicon, sequencing analysis of PCR products corresponding to fragments of approximately 2Kb, 1.3Kb, 1Kb, and 400bp revealed the presence of a diverse pool of class 1 integrons loaded with gene cassettes encoding different resistance determinants (Figure 14 and Table 6). Moreover, the 1.5 Kb amplicon detected in *E. coli R388* (positive control) was also found in beach water receiving the sewage discharges from a pipeline outfall (Sardinera Beach, Isabela) and undetected in water samples from the Fanduco Reef, a remote site (10 km offshore the Cabo Rojo municipality coast) with minimal human impact, further reinforcing the link between fecal contamination and the prevalence of Intl1-encoded antibiotic resistance (Figure 15).

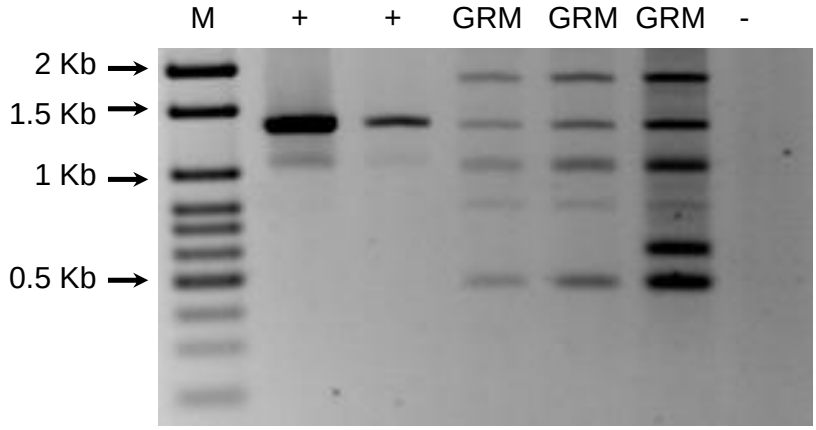



Figure 14. PCR-Amplification of gene cassettes associated with multiple class 1 integrons detected in three replicate reactions. M= 100 bp DNA Marker (Applied Biological Materials Inc., Richmond, BC, Canada), (+)= positive control, GRM= Guanajibo River estuary, Mayagüez, (-)= negative control.

Table 6. Characterization of amino acid sequences derived from IntI1-associated amplicons detected in water samples from Guanajibo River estuary, Mayagüez.

| Clone | Amplicon Size | GeneBank Database Match | Function of matching protein | Identity % | GeneBank Accesion Number |
|---|---|---|---|---|---|
| GRM: 1.7 | ~2,000 bp | *Yersinia pestis biovar Orientalis str. IP275* | Aminoglycoside adenylyltransferase | 99% | ABO42050 |
| GRM: 2.3 | ~1,300 bp | *Yersinia pestis biovar Orientalis str. IP275* | Streptomycin 3"-adenylyltransferase | 98% | ZP_02226678 |
| GRM: 3.3 | ~1,000 bp | *Klebsiella pneumoniae* | Dihydrofolate reductase | 100% | YP_003517586 |
| GRM: 4.4 | ~800 bp | *Polaromonas naphthalenivorans CJ2* | Tryptophanyl-tRNA synthetase | 82% | YP_983497 |
| GRM: 5.5 | ~400 bp | *Klebsiella pneumoniae* | Orf3/QacEdelta1 fusion protein | 100% | ACF06163 |

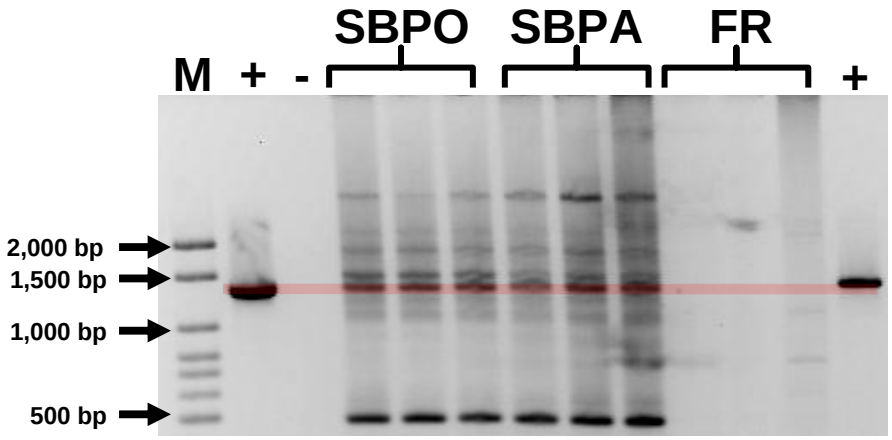



Figure 15. Agarose gel electrophoresis showing representative results for the amplification of gene cassettes associated with class 1 integrons. The red line highlights the detection of a ~1,300 bp amplicon encoding antibiotic resistance genes in the Isabela samples (wastewater pipeline outfall (SBPO) and receiving sea water (SBPA) near Sardinera Beach). M= 100 bp DNA Marker (Applied Biological Materials Inc., Richmond, BC, Canada), (+)= positive control, (-)= negative control.

Since the detection of class 1 integrons carrying antibiotic resistance genes in sewage and polluted estuarine waters was feasible, this cassette-targeted assay was used to probe for integron encoded resistance in impacted coastal ecosystems and reference sites with minimal human influence. Four replicate samples from these sites were collected during the rainy and dry seasons. Four replicates PCR reactions were prepared for each individual sample. Only bands having approximately the same size of the amplicon generated by the positive control (~1.3Kb) were purified for subsequent cloning and sequencing analyses. For each PCR product a total of five clones were sequenced to corroborate the sequencing analyses.

***Detection frequency of integrase genes and intl1-encoded gene cassettes in mangrove habitats.*** A PCR-based assay was used to evaluate the distribution and dominance of clinical integrases (classes 1-3) and class 1 integrons loaded with gene cassettes in impacted (La Parguera, Lajas) and secluded (Ensenada Dakity, Culebra) mangrove habitats (Appendices 1-2). During the rainy season, *intl1*, *intl2* and *intl3* integrases were found in water samples from La Parguera a at a detection frequency (DF) ranging from 31.1 to 37.5% (Appendix 1). In contrast, gene cassettes encoded by class 1 integrons showed a detection frequency of 75%. *Intl1* (43.8% DF) and intl2 elements (12.5% DF) were the most prevalent integrases in water samples from Ensenada Dakity while class 3 sequences were undetected. However, *intl1* gene cassettes were found at a lower detection frequency in water samples from Ensenada Dakity (31.1%) with respect to those from La Parguera (75%).

Class 2 (43.8% DF) and class 1 (25% DF) elements were the only integrases detected in sediment samples from La Parguera and Ensenada Dakity, respectively. Nevertheless, the highest detection frequencies for intl1 gene cassettes were observed in sediment habitats at both mangrove ecosystems (La Parguera, 87.5% DF; Ensenada Dakity 100% DF) relative to the corresponding samples of water and rhizosphere material (50% DF). Rhizosphere samples from La Parguera only showed the presence of class 3 integrases at a low detection frequency (6.3%) whereas those from Ensenada Dakity only revealed the presence of class 2 (87.5% DF) and class 1 ( 25% DF) integrases.

Class 1 integrases dominated in water (100% DF), sediment (50% DF) and rhizosphere (43.8% DF) samples from La Parguera during the dry season (Appendix 2). However, despite their prevalence in water, *intl1*-encoded gene cassettes were only detected in sediment (100% DF) and rhizosphere (75% DF) material. Class 2 integrases were more prevalent in sediments (43.8% DF) relative to water (37.5% DF) and rhizosphere (37.8% DF) samples, whereas class 3 integrases were undetected in sediments but were more abundant in rhizosphere (37.5% DF) with respect to water samples (6.3% DF).

During the dry season integron-related sequences were poorly represented at Ensenada Dakity with respect to La Parguera site (Appendix 2). These were undetected in water samples, whereas only class 1 (68.8% DF) and class 2 integrases (37.5% DF) were present in sediments. Class 2 elements were the only integrases detected in rhizosphere material (37.5% DF). *Intl1* gene cassettes were undetected in water, sediment and rhizosphere habitats.

***Detection frequency of integrase genes and intl1-encoded gene cassettes in beach habitats.*** The prevalence of integron encoded sequences was evaluated in water and sand samples retrieved from an impacted (Pico de Piedra, Aguada) and a secluded beach (Peña Blanca, Aguadilla) habitat (Appendices 3-4). During the rainy season, class 2 integrases were the most common intl elements present (81.3% DF) in water samples from Pico de Piedra Beach (Appendix 3). Class 1 integrases and intl1-encoded gene cassettes were also present at detection frequencies of 31.3 and 37.5%, respectively, whereas class 3 integrases were undetected. Relative to water samples, all three integrase classes were detected in sand samples. Moreover, *intl1* gene cassettes were present in sand at a detection frequency of 100%. In contrast with results from Pico de Piedra, *intl1* gene cassettes were undetected in water and sand samples from Peña Blanca. Although class 1 and class 2 integrases were detected in water and sand samples, their detection frequencies were lower with respect to the corresponding samples from Pico de Piedra (Appendix 3). Furthermore, class 3 integrases were undetected.

During the dry season, *int1*-encoded gene cassettes were present in water and sand samples at both locations at detection frequencies ranging from 62.5 to 100% (Appendix 4). Class 1 integrases were more abundant in water and sand samples from Pico de Piedra (93.8 and 81.3% DF, respectively) relative to those from Peña Blanca (31.3 and 62.5% DF, respectively). Likewise, class 2 integrases were more abundant in sand samples from Pico de Piedra (31.3% DF) relative to those from Peña Blanca (18.8% DF). However, *intl2* sequences dominated in water samples from Peña Blanca (81.3% DF) with respect to those from Pico de Piedra (31.3% DF). Class 3 integrases were undetected at both sites.

***Detection frequency of integrase genes and intl1-encoded gene cassettes in estuarine and river-related habitats.*** In order to evaluate the contribution of polluted stream water as a vehicle for the dispersal of integrons into coastal environments, impacted estuarine settings (Guanajibo River, Mayagüez and Manatí River, Barceloneta) were analyzed with respect to downstream and upstream regions of a secluded river (Cupeyes River, Sabana Grande)(Appendix 5-6). During the rainy season, class 1 integrases were detected at the three sites, but these were more abundant in water samples from the Manatí (68.8% DF) and Guanajibo (56.3% DF) River estuaries, relative to those from the downstream site of the Cupeyes River (43.8% DF) (Appendix 5). Similarly, class 2 integrases were present in all three locations, however, these were found at higher detection frequencies at Cupeyes (downstream, 37.5%) and Manatí (37.5%) relative to Guanajibo (12.5%). Class 3 integrases were detected only at the Manatí estuary (43.8% DF) whereas *intl1* gene cassettes were amplified from all sites with detection frequencies ranging from 37.5 to 62.5%.

During the dry season, the only integron-associated sequences detected upstream Cupeyes River were those of class 1 integrases (Appendix 6). However, these were present at a relatively low detection frequency (12.5%). In contrast, all clinical integrases and *intl1* gene cassettes were detected in Guanajibo and Manatí. Class 1 integrases dominated in samples from Manatí (87.5% DF) followed by class 2 (25% DF). The opposite was observed among samples from Guanajibo (class 1, 62.5% DF; class 2, 81.3% DF). Class 3 integrases were not detected at neither estuary. Class 1-encoded gene cassettes were present at detection frequencies of 56.3% and 43.8% at Manatí and Guanajibo estuaries, respectively.

***Molecular characterization of intl-like sequences.*** PCR amplification of *intl1* genes resulted in the detection of a 1,200 Kb amplicon (Figure 16). Alignments of predicted amino acids sequences (Figure 17) demonstrated that the cloned fragments were 99-100% identical to class 1 integrases described among Gram-negative pathogens from clinical settings, *Vibrio cholera* and aquatic strains of *Aeromonas caviae*, an emerging pathogen associated with diahereal disease in children (Lee et al., 2008; Strateva and Yordanov 2009; Beatson et al., 2011; Roy Chowdhury et al., 2011; Morinaga et al., 2011; Bravo et al., 2011, Mishra et al., 2011).

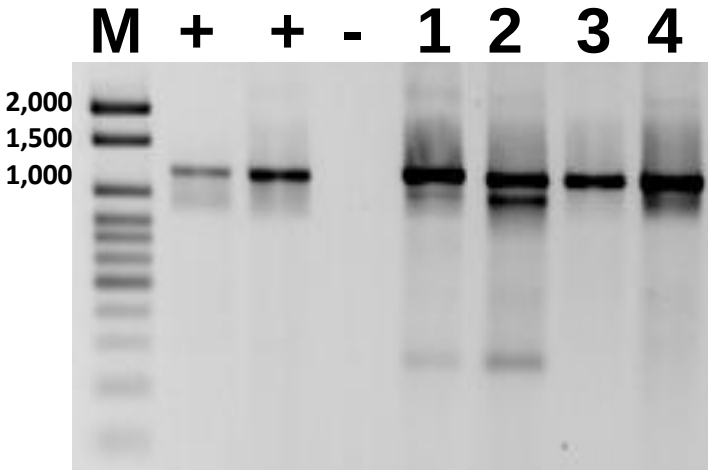



Figure 16. Representative results for the amplification of integrase class 1 genes of four replicates extractions of total DNA recovered from biomass present in water samples from the Pipeline outfall, Sardinera beach, Isabela (lanes 1-4). (-) = Negative control, no template DNA. (+) = Positive control (Genomic DNA from *E. coli* R388).

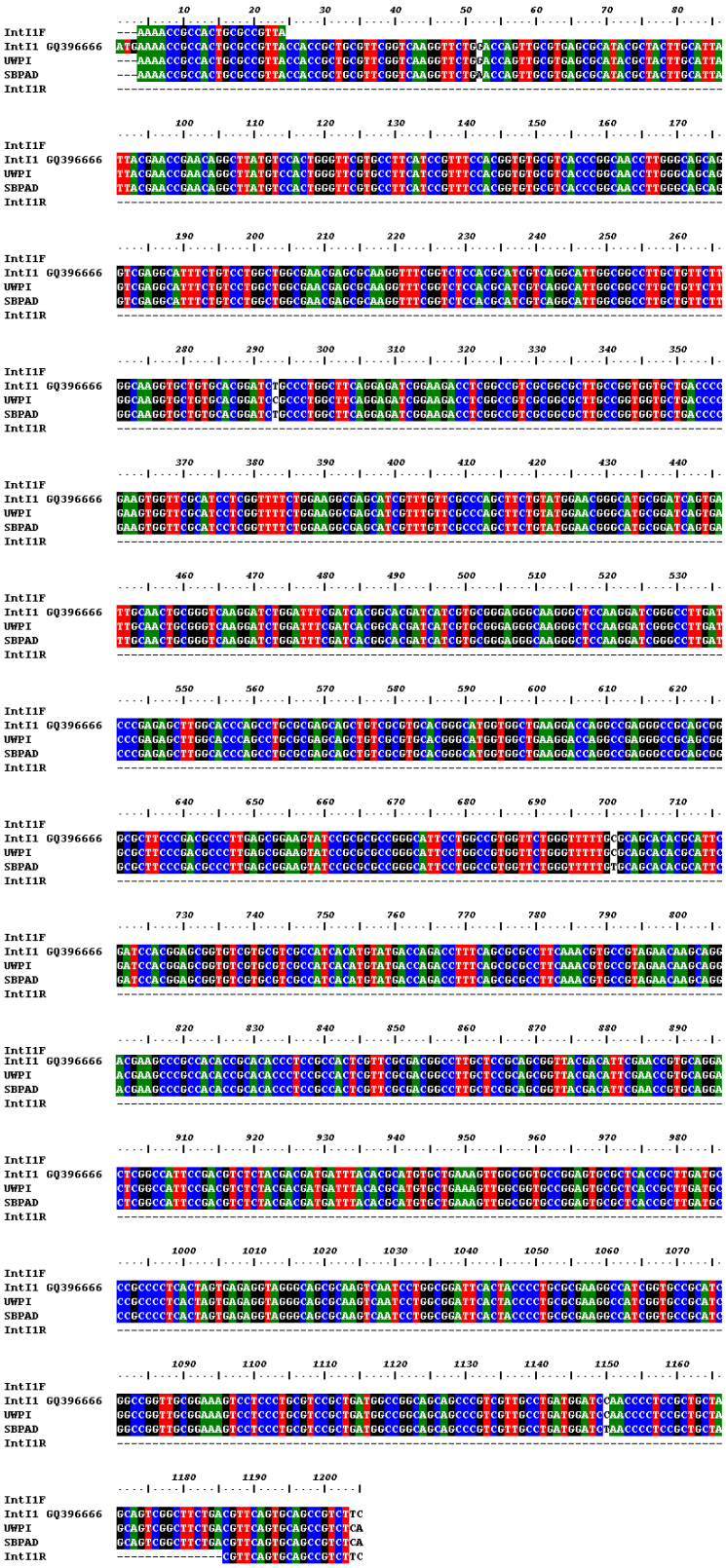



Figure17. Full length alignment of representative *intlI1* amplicons (1.2 Kb) retrieved from influents from an urban wastewater treatment (UWPI) plant and sea water receiving sewage influents (SBPAD). GenBank accession no follow the designation of a reference *intI1* sequence. The sequences of the forward and reverse primers used are presented at the top and the bottom of the alignment, respectively.

Amplification of class 2 integrases showed a product size of 788 bp (Figure 18). Deduced amino acid sequences from clones were 98-100% identical to class 2 integrases described in *Shigella sonnei* (GenBank accession no. AAT72891), *Klebsiella pneumoniae* (GenBank accession no. ADH82143), *Escherichia coli* (GenBank accession no. BAB12601) and *Providencia stuartii* (GenBank accession no. ABG21674) which have been associated with serious infectious diseases (Barlow and Gobius 2006, Jafari et al., 2009; Pourakbari et al., 2010; Quirante et al., 2011; Abreu et al., 2011, Flannery et al., 2009). Putative functional IntI2 sequences which lacked the internal stop codon at position 179 were detected in water samples at Guanajibo (Mayagüez), and in those from off shore sewage discharges and impacted seawater (Isabela) (Figure 19). Furthermore, Intl2 genotypes lacking the 179 ochre codon were retrieved from rhizosphere and sediment samples at La Parguera (Lajas) and sewage-contaminated seawater (Isabela).

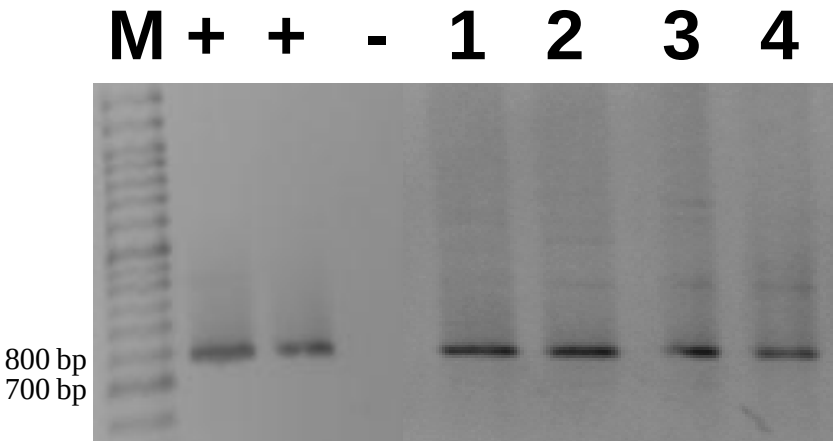



Figure 18: Agarose gel electrophoresis showing representative results of the amplification of integrase genes from class 2 integrons from total community DNA recovered from Guanajibo River estuary, Mayagüez (GRM, lanes 1-4) during the rainy season. (+)= positive control containing the target sequence; (-)= negative control, no template DNA added.

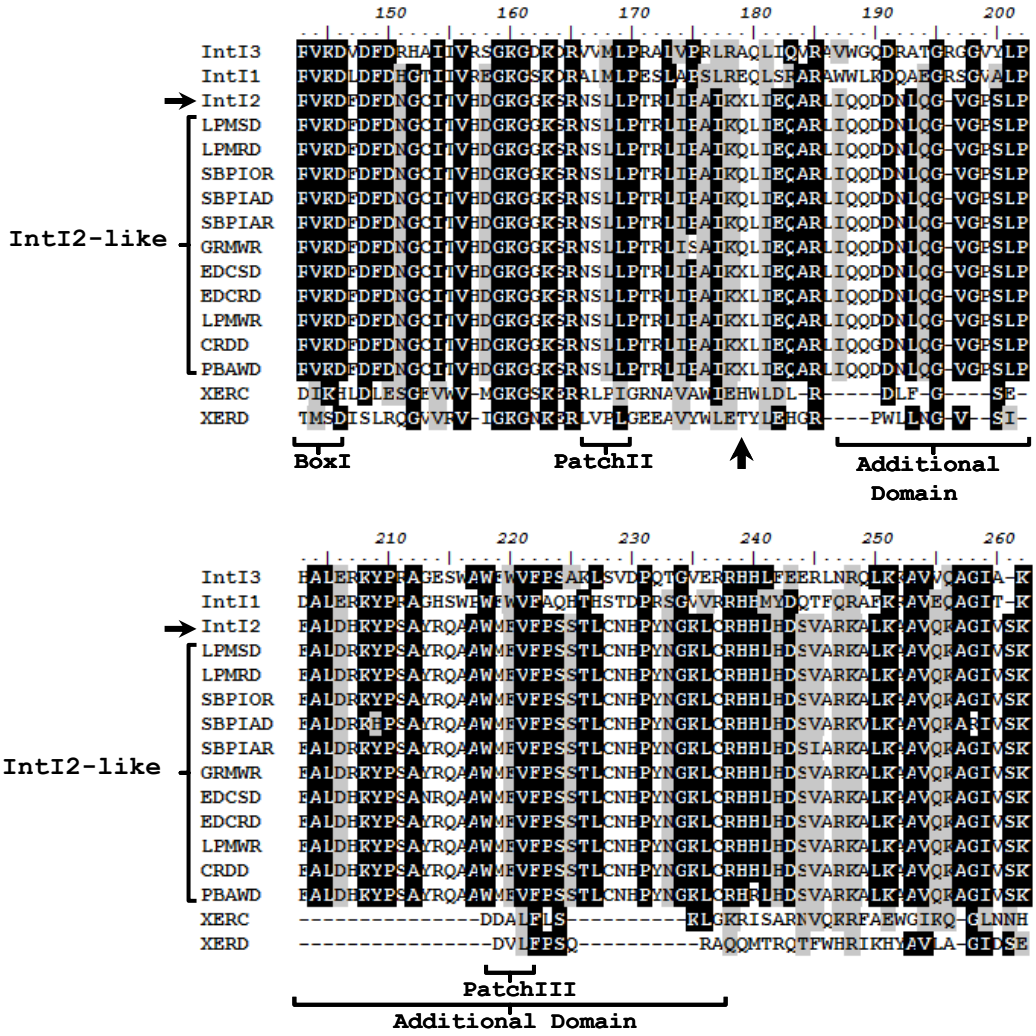



Figure 19. Partial alignment of predicted amino acid sequences derived from environmental clones of IntI2-like elements with respect to IntI1, IntI2 and IntI3 elements (GenBank accession nos. AAQ16665, AAT72891and AAO32355, respectively) and tyrosine recombinases (XerC and XerD, GenBank accession nos. P0A8P6, and P0A8P8, respectively). The arrow at the bottom indicates the presence of a glutamine residue at position 179 among 6 potentially functional IntI2 sequences. This residue corresponds to the position of the ochre codon present in conventional class 2 integrases. The degree of conservation across the alignment is indicated relative to XerC and XerD sequences as follows: black (80% identical), gray (80% similar). Gaps are indicated by "-" characters. The numbering is based on the amino acid sequence of a class 2 integrase (GenBank accession no. AAT72891).

The amplification of class 3 integrases revealed the presence of a fragment with a length of ~979 bp (Figure 20). Predicted amino acid sequences of representative clones of putative *intl3*-like sequences were 98-99% identical to class 3 integrases found in a clinical isolate of *Serratia marcescens* (GenBank accession no. BAA08929) and in an environmental strain of *Delftia tsuruhatensi* (GenBank accession no. ABR28408) originally recovered from activated sludge from a wastewater treatment plant (GenBank accession no. ABR28408) (Figure 21).

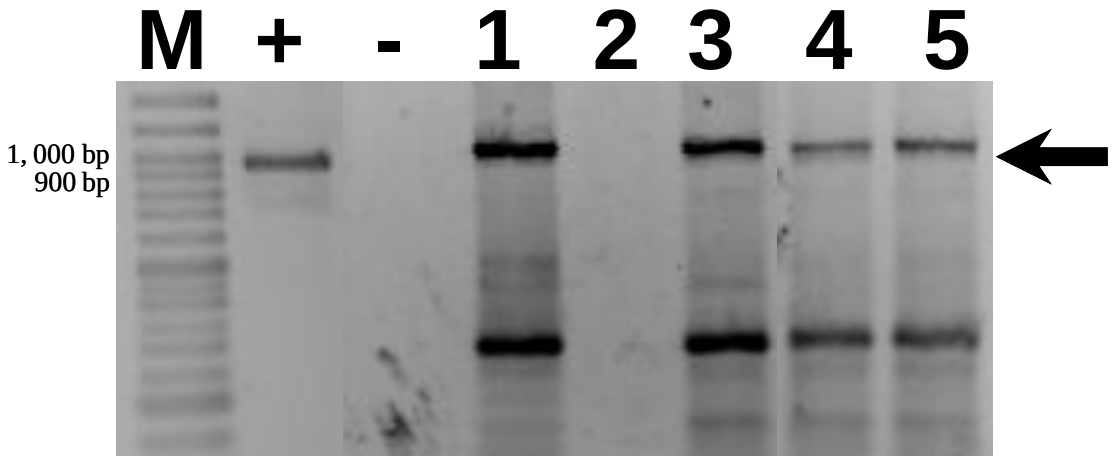



Figure 20. Representative results for the amplification of the integrase genes from class 3 integron corresponding to biomass present in replicates water samples recovered from the La Parguera mangrove, Lajas during the rainy season (lanes 1-5). (+) = positive control containing DNA template with the target sequence; (-) = negative control, no template DNA added.

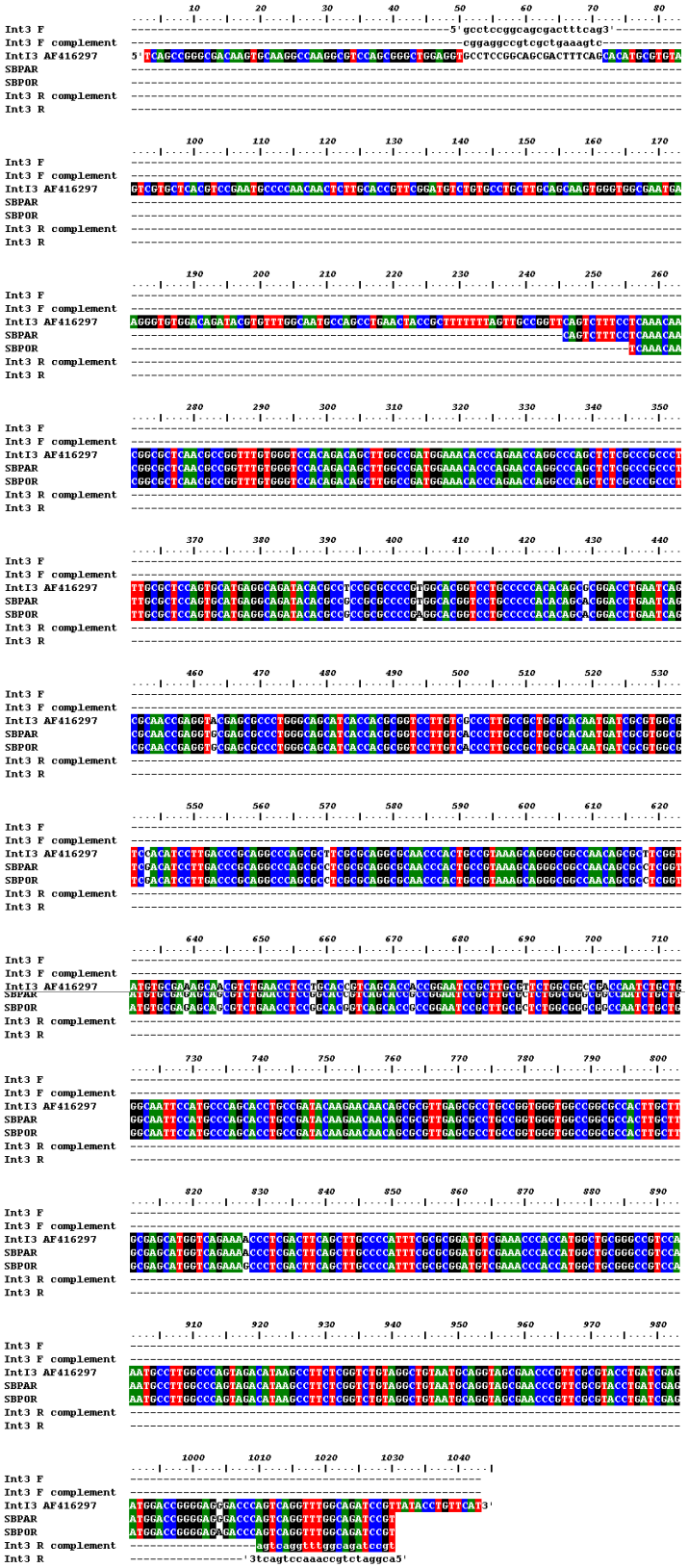
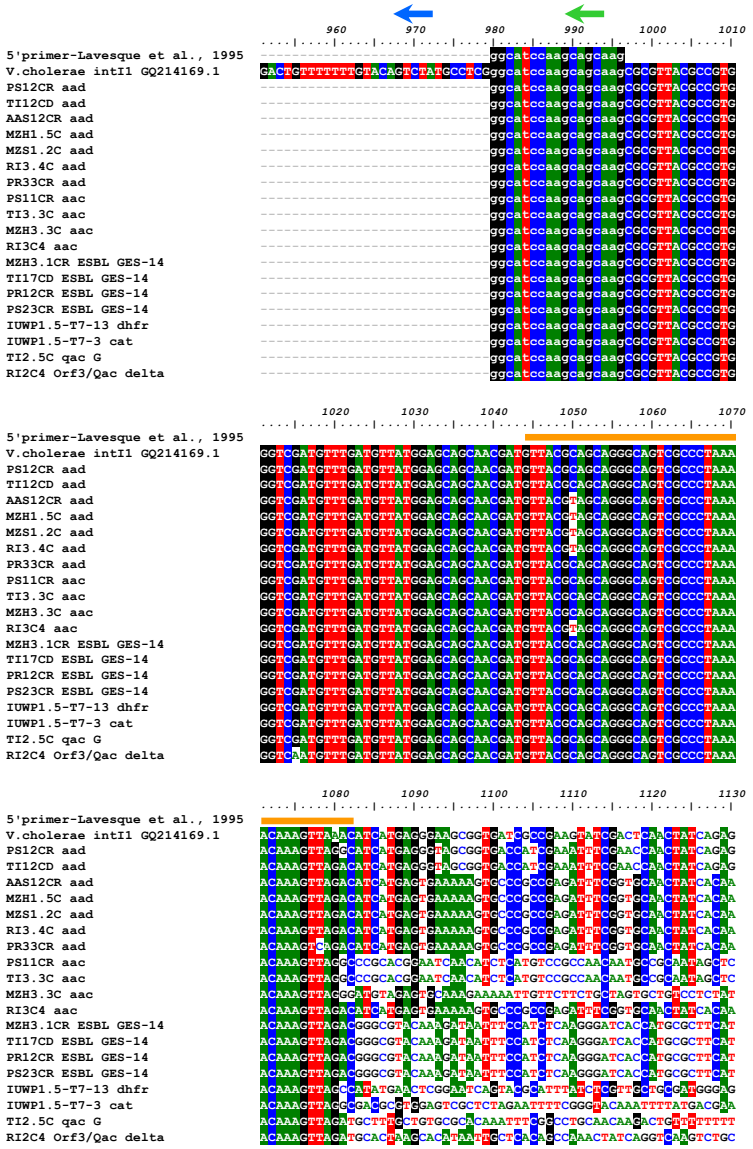



Figure 21. Alignment of representative, partially sequenced *intlI3* amplicons (979 bp) from off shore sewage discharges (SBPOR) and impacted seawater (SBPAR). GenBank accession no. follow the designation of a reference *intI3* sequence. The sequences of the forward and reverse primers used are presented at the top and the bottom of the alignment, respectively.

***Molecular characterization of intl1-encoded gene cassettes.*** Representative clones of the variable region of class 1 integrons (1.3Kb amplicon) were submitted to comparative alignment analysis. Functional and structural features of class 1 integrons were identified in all of them, confirming class 1 elements as the source of the recovered fragments (Figures 22 and 23). A total of seven different antibiotic resistance genes were detected across the tested sites. These included three variants of amino glycoside modifying enzymes, a dehydrofolate reductase, an extended spectrum beta-lactamases, and two variants of quaternary ammonium compounds efflux pumps (Figure 24 and Table 7). All of them were closely related 98-100% amino acid identity) to resistance determinants described in known human pathogens harboring class 1 integrons (Cloeckaert et al., 2006; Chen et al., 2007; Chen et al., 2009; Nawaz et al., 2009; Bogaerts et al., 2010; Shi and Yamasaki, unpublished, GenBank accession no. BAE66663).

a:




b:

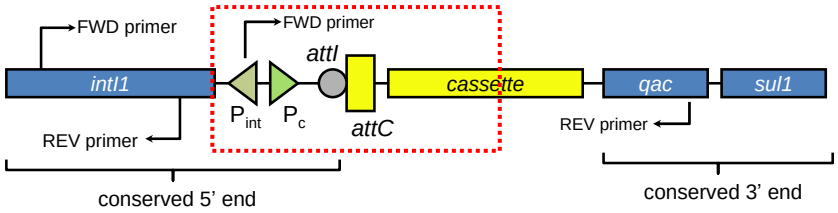
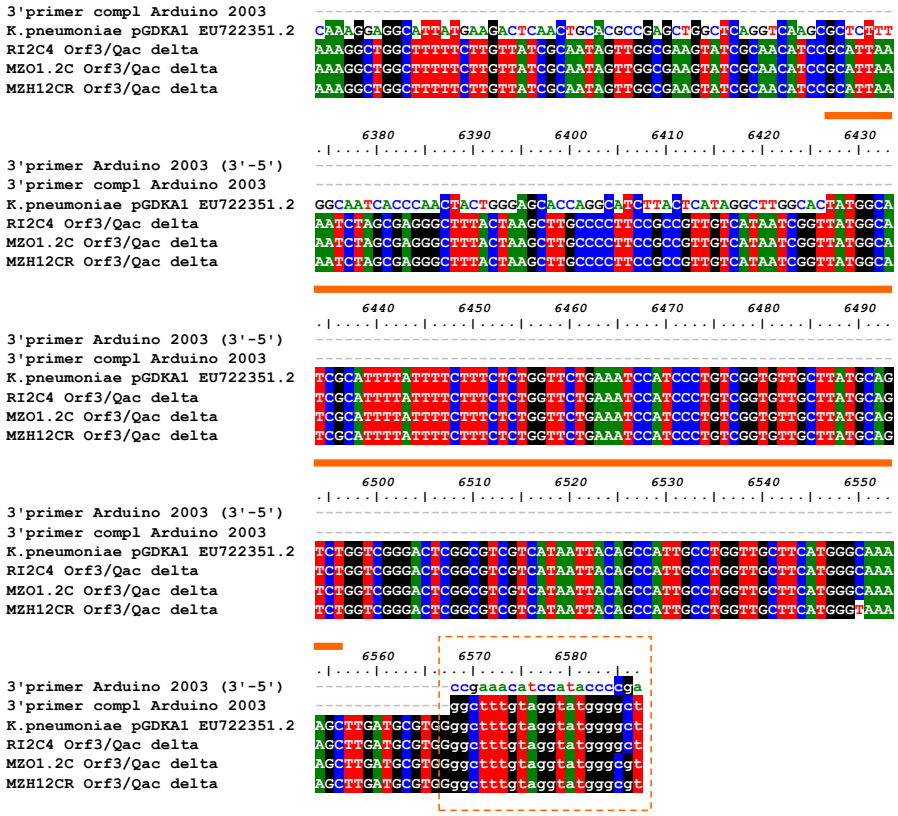



Figure 22. Panel (a): Partial alignment of representative, PCR-amplified nucleotide sequences corresponding to the conserved 5'end of class 1 integrons (Panel [b] red doted lines) detected in environmental samples. The blue and green arrow depict the positions of the -10 and -35 signals of the intl1 promoter, respectively, The orange bar shows the position of the attI1 site. Numbering is based on the *V. cholerae intI1* (GenBank accession no. GQ214169 ).

a:




b:

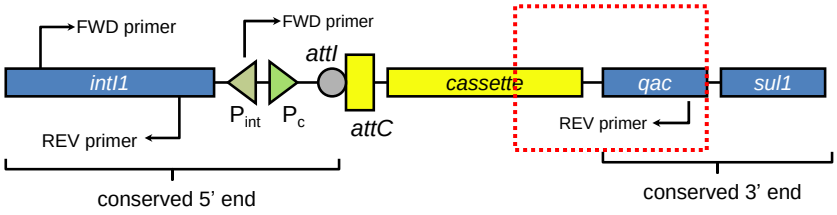
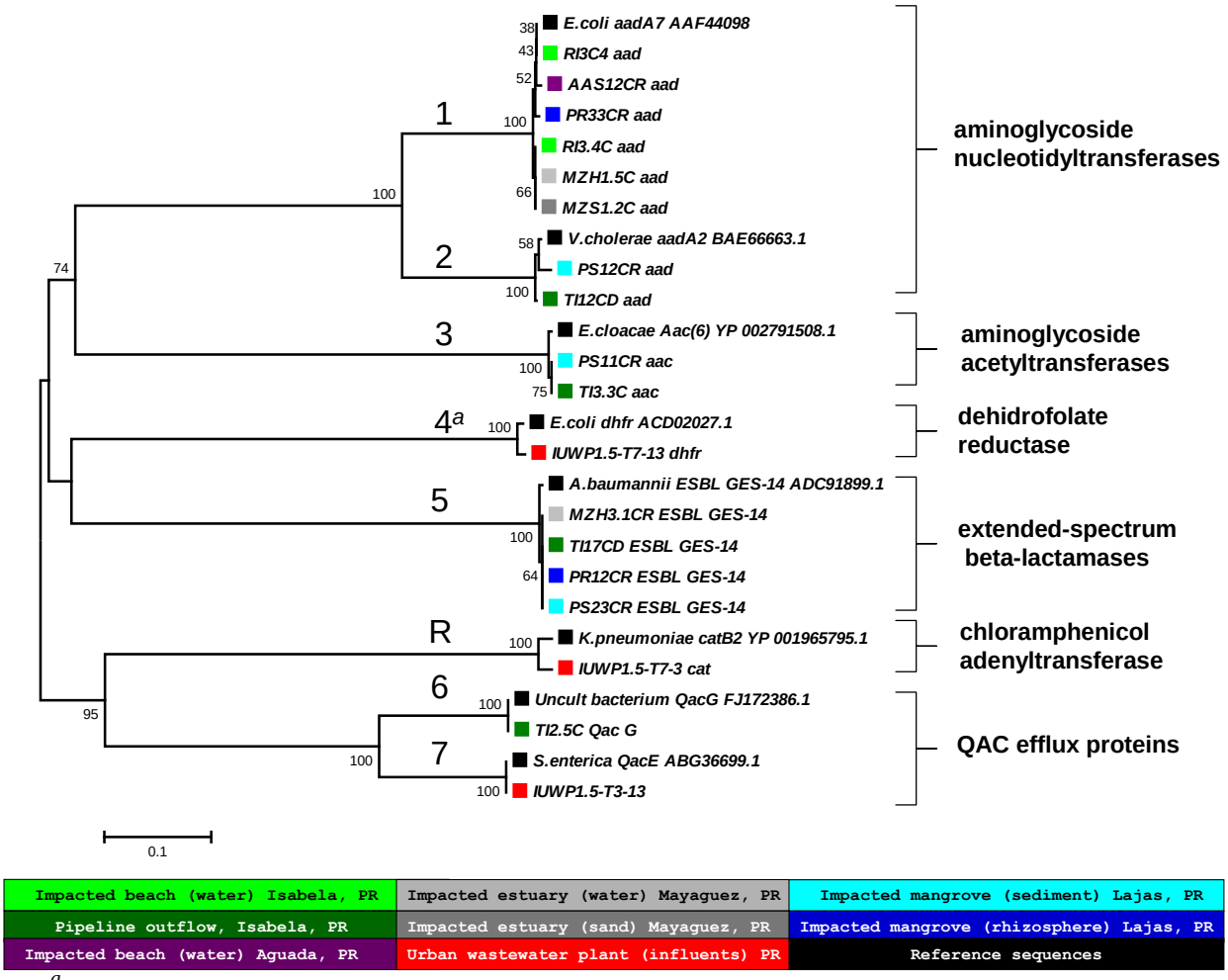



Figure 23 Panel (a). Partial alignment of representative, PCR-amplified nucleotide sequences corresponding to the conserved 3'end of class 1 integrons (Panel [b]) detected in environmental samples. The orange bar shows the position of the qacEΔ1 gene. Numbering is based on the *K. pneumoniae intI1* (pGDKA1) (GenBank accession no. EU722351).




[a] Detected in water samples from an impacted estuary (Guanajibo River, Mayagüez) besides reference wastewater treatment plant.

Figure 24. Unrooted neighbor-joining tree illustrating the relationship of deduced amino acid sequences of representative IntI1 antibiotic resistance gene cassettes recovered from a variety of impacted coastal habitats (1-7). Values represent the percentage of 2,000 bootstrap replications that supported the branching order. GenBank accession numbers follow the designation of the reference sequences. R= detected only in influents from an urban wastewater treatment plant used as reference site.

Table 7. Frequency and distribution of IntI1-encoded gene cassettes.

| Site Code | Site Description | Level of human influence | No. of successfully sequenced clones | No. of clones encoding aminoglycoside nucleotidyl transferases | No. of clones encoding aminoglycoside acetyltransferases | No. of clones encoding dehidrofolate reductases | No. of clones encoding a extended spectrum of betalactamases | No. of clones encoding QAC efflux proteins | No. of clones encoding putative ORF's | No. of clones encoding no ABR function |
|---|---|---|---|---|---|---|---|---|---|---|
| MRBR | Water | Severe | 5 | 4 | 0 | 0 | 0 | 0 | 0 | 1 |
| MRBD | Water | Severe | 4 | 1 | 0 | 0 | 0 | 0 | 1 | 2 |
| GRMWR | Water | Severe | 11 | 5 | 0 | 0 | 1 | 2 | 0 | 3 |
| GRMWD | Water | Severe | 9 | 4 | 2 | 1 | 1 | 1 | 0 | 0 |
| PBAWD | Water | Moderate | 3 | 1 | 0 | 0 | 0 | 0 | 1 | 1 |
| PBASD | Sand | Moderate | 2 | 0 | 0 | 0 | 0 | 0 | 1 | 1 |
| PPASR | Sand | Severe | 9 | 2 | 0 | 0 | 0 | 1 | 0 | 6 |
| LPMMR | Rhizosphere | Severe | 9 | 3 | 0 | 0 | 1 | 0 | 0 | 5 |
| LPMWR | Water | Severe | 6 | 0 | 0 | 0 | 0 | 0 | 0 | 6 |
| LPMSR | Sediment | Severe | 12 | 5 | 2 | 0 | 1 | 0 | 0 | 4 |
| LPMMD | Rhizosphere | Severe | 7 | 0 | 4 | 0 | 0 | 2 | 1 | 0 |
| EDCWR | Water | Moderate | 5 | 2 | 0 | 0 | 0 | 1 | 0 | 2 |
| EDCMR | Rhizosphere | Moderate | 2 | 1 | 0 | 0 | 0 | 1 | 0 | 0 |
| EDCSR | Sediment | Moderate | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| SBPO | Water | Severe | 7 | 2 | 1 | 0 | 1 | 2 | 0 | 2 |
| SBPA | Water | Severe | 9 | 1 | 0 | 0 | 0 | 5 | 0 | 3 |

***Overall prevalence trends of intl-encoded sequences in coastal settings with respect to human influence and sampling season.*** Our results indicate that class 1 elements are the most widely distributed among the tested integrases followed by class 2 sequences, regardless the levels of human influence. In contrast, class 3 elements were poorly represented and mainly associated with high levels of fecal impact. The Chisquare analysis ($X^2$= 22.50, $p<0.05$) demonstrate significant differences among the detection of antibiotic resistance genes encoded by class 1 integrons and the levels of fecal impact. Chi-square tests revealed that detection frequency of antibiotic resistance genes encoded by class 1 integrons was significantly higher (0.83) in disturbed environments relative to those less exposed (0.42) to human influence.

# DISCUSSION

***Fecal indicator counts***. In general, fecal indicator counts were significantly higher in coastal environments with a history of fecal pollution with respect to those relatively undisturbed. Among the different niches analyzed in mangrove habitats, sediment and rhizosphere material showed significantly higher, indicator loads relative to water samples. Traditionally, potential risks to human health associated with microbial pollutants in coastal habitats have been assessed by the exclusive analysis of the water column (Cabral 2010; Bachoon et al., 2010). Nevertheless, it has been increasingly documented that sediments may harbor fecal indicator bacteria, including strains with enhanced ability to cause disease (González-Fernández et al., 2010 and Luna et al., 2010). Various factors have been reported to promote the association and survival of microbial pollutants in sediments and dissolved organic matter. These include the inherent load of organic matter in sediments, the availability of nutrients due to interactions among physiologically diverse microbial populations (Dai and Sun, 2007), as well as protection against viral infections, protozoan grazing, UV radiation, and toxic heavy metals (Davies et al., 1995). Moreover, as previously documented for terrestrial plants, it has been recently reported that the mangrove rhizosphere niche may promote the stable settlement of rhizo-competent bacteria, including representatives from foreign environments (Singh et al., 2004 and Gomes et al., 2010). Although several studies have documented the colonization of internal tissues and root-related environments of plants of agricultural importance by enteric bacteria (Dong et al., 2003; Tyler and Triplett, 2008) knowledge on the potential of enterics for becoming established within the rhizosphere setting of vegetation from coastal areas is scarce.

Similar to what was observed in mangrove settings, water and sand samples from the relatively undisturbed beach site, showed indicator counts within permissible levels. Although, water samples from the impacted beach site presented fecal coliform counts within acceptable parameters, these indicators were in elevated numbers in suspensions derived from sand samples. As described in sediments, beach sand from lakes and marine environments have been reported to act as a reservoir of fecal indicator bacteria (Whitman and Nevers, 2003; Ishii et al., 2007; Hartz et al., 2008). Various studies have indicated that sand grains may acts as a filter that concentrates waterborne, fecal indicators which are able to survive and reproduce within this niche (Hartz et al., 2008; Heaney et al., 2009; Bonilla et al. 2007; Wheeler Alm et al. 2003). It has been also argued that sand particles provide a solid phase that promotes the formation of biofilms and the accumulation of nutrients which favors conditions for enhanced survival and reproduction (Hartz et al., 2008). Moreover, fissures in sand particles can also serve as isolated micro-habitats that protect against protozoan predation and UV radiation (Hartz et al., 2008; Bonilla et al. 2007; Wheeler Alm et al. 2003).

As opposed to fecal coliforms, enterococci indicators were present above permissible levels in water samples from the impacted beach but within permissible limits in sand. Relative to fecal coliforms, enterococci are known to be better adapted to salt concentrations approximately two times higher (6.5%) to those commonly found in sea water (3.5%) (Cabral 2010). Additionally enterococci are well known to form biofilms (Cook et al., 2011) which may have interfered with the detachment of the cells during the resuspension procedure. However, this later point should be further evaluated relative to other fecal indicators or reference strains and verified with further sampling.

The analysis of water samples from impacted, river-related environments revealed increasing levels of fecal indicators according to the intensity of anthropogenic impact, suggesting that bacterial populations associated with polluted beach sand, sediments and mangrove rhizosphere ecosystems may become reservoirs of clinical integrons and integron-encoded antibiotic resistance genes, as these genetic elements have been associated with fecal bacteria (Nandi et al., 2004; Li et al., 2010; Yang et al., 2009). Relative to the degradation of coastal settings, the above mentioned ecosystems have been traditionally overlooked in terms of potential hazards to human health and could serve as complementary, reference sampling sites for risk assessment.

***Validation of PCR assays targeting integron integrases (classes 1-3)***.

Optimization experiments of integrase-targeted PCR assays revealed that sequences from class 1 and class 2 integrases were the most frequently detected with respect to those from class 3 variants across environments with different histories of human impact. The detection signals for *intl1* and *intl2* sequences were stronger in reference sites which consisted of influents and effluents from rural and urban secondary wastewater treatment plants, relative to soil under strong human influence and soil and beach sand from areas remote from evident sources of anthropogenic disturbances.

Class 1 integrons are known to be associated with Tn21 replicative transposons which in turn are embedded into self-replicative plasmids widely disseminated among clinical isolates of enteric bacteria (Liebert et al., 1999; Fluit and Schmitz 1999). Moreover, Gram-positive bacteria from avian fecal material have been identified as a major source of class 1 integrons. (Nandi et al., 2004). It is thought that genetic linkage of class 1 integrons with highly mobile or laterally transferred elements accounts for their

widespread distribution across clinical and environmental settings exposed and unexposed to wastewater pollution (Boucher et al., 2007; Gillings et al., 2008). Similarly, class 2 integrons are linked to non-replicative Tn7 transposons (Hansson et al., 2002). Hence, since these transposons have a more limited mobility relative to Tn21 elements the detection of IntI2 variants is less common with respect to the class 1 group. Nevertheless, Intl2 genotypes are known to be transferred by conjugative plasmids among Gram-negative bacteria and to harbor several antibiotic resistance genes (Marquez et al., 2008; Byrne-Bailey et al., 2011; Hansson et al., 2002; Barlow et al., 2006). Moreover, IntI2 elements are being increasingly reported outside the clinical environments (Rodríguez-Minguela et al., 2009; Xu et al., 2011).

During the optimization stage, class 3 integrases were only detected in effluents from a rural wastewater treatment plant which is consistent with the description of a chromosomally-encoded version of this integron class in *Delftia acidovorans and Delftia tsuruhatensis,* both recovered from a wastewater treatment plant in Canada (Xu et al., 2007). The gene cassettes identified within this class 3 integron did not encode any antibiotic resistance function (Xu et al., 2007). However, D. *tsuruhatensi* has been described as an emerging opportunistic pathogen associated with the use of a central venous catheter (Preiswerk et al., 2011). Despite being the less frequently detected integron variant, class 3 integrons loaded with antibiotic resistance genes have been identified in clinical isolates of *Serratia marcescens*, *Klebsiella peumoniae*, and *Pseudomonas putida*. (Arakawa et al., 1995; Correia et al., 2003; Shibata et al., 2003). However, knowledge on the genetic elements implicated in the dispersal of class 3 integrons is still scarce, although it is presumed that they might be horizontally

transferred by conjugative plasmids (Xu et al., 2007). The presence of class 3 integrons in wastewater may enhance the disease causing potential of environmental strains as wastewater environments are known to contain high numbers of diverse bacteria, which promotes the transfer and exchange of genetic material. These results comprise the first evidence for the presence of class 3 integrons in the Caribbean region as indicated by the detection of *intI*3 elements. In general, results from the optimization experiments of the *intl*-targeted PCR assay, demonstrated a link between fecal pollution, clinical integrons and the existence of environmental reservoirs of these genetic elements whose magnitude appeared to be amplified by human activity.

***Optimization and validation of a PCR assay targeted at the variable region of class 1 integrons***. The amplification of the variable region of class 1 integrons from reference samples retrieved from an urban wastewater treatment plant resulted in the detection of a PCR product within the 1-1.5 Kb size range, similar to that present in a control strain (*E. coli* R388) known to carry a class 1 integron. Cloning and sequencing analysis of this environmental amplicon revealed the existence of at least three class 1 integrons each loaded with a single cassette within the 1-1.5 Kb range but encoding different antibiotic resistance mechanisms, respectively. As many as 8 different gene cassettes encoding antibiotic resistance traits have been reported within a single class 1 integron (Nass et al., 2001). The identified gene cassettes encoded a dehydrofolate reductase, an acetyltransferase and an efflux pump which confer resistance against trimethoprim, chloramphenicol and quaternary ammonium compounds, respectively.

Class 1 integrons are known to encode an alternative dehydrofolate reductase which is insensitive to inhibition by the antibiotic trimethoprim (Cambray et al., 2010),

the most widely prescribed antibiotic for the treatment of urinary tract infections (Lawrenson and Logie, 2001). Production of the resistant dehydrofolate reductase variant, allows the integron host to produce precursors needed for the synthesis of nucleotides in the presence of the drug. Resistance mechanisms against chloramphenicol, a wide spectrum protein synthesis inhibitor usually reserved for serious bacterial infections, have been described in class 1 integrons (Feder et al., 1981; Guo et al., 2011). Integron encoded chloramphenicol resistance consists of the production of acetyl transferases that chemically modify the antibiotic by the addition of acetyl groups, which prevents binding to the large subunit of the bacterial ribosome (Hansen et al., 2003; Guo et al., 2011). Another resistance determinant commonly associated with class 1 integrons are those encoding efflux pumps (Qac) for quaternary ammonium compounds, a group of chemicals widely used as disinfectants in food and hospital-related settings (Gillings et al., 2009). Sequences encoding Qac pumps are typically located at the conserved 3'end of class 1 integrons and also as incorporated gene cassettes (Gillings et al., 2009).

Amplification of the variable region of class 1 integrons from environmental DNA from polluted sites not always resulted in the exclusive presence of the 1.3 Kb fragment detected in the control IntI1 host (*E. coli R388*). In a second optimization experiment, PCR analyses of water samples from a polluted estuary (Guanajibo River estuary, Mayagüez) revealed the presence of five dominant bands, including the reference 1.3 Kb fragment, which indicated the existence of several class 1 integrons carrying different gene cassettes. With only one exception, sequencing analyses demonstrated that the detected gene cassettes were 98-100% identical to antibiotic resistance determinants present in human pathogens. These included a dehydrofolate reductase, a Qac efflux

pump, and two variants of adenyltransfereases. The later are enzymes known to chemically inactivate aminoglycoside antibiotics (Vakulenko and Mobashery, 2003). Aminoglycoside antibiotics are protein synthesis inhibitors of broad spectrum activity that comprise treatment options against tuberculosis (Vakulenko and Mobashery, 2003).

Finally, to determine if naturally occurring class 1 integrons could also contribute to the detection signal of antibiotic resistance genes in polluted coastal environments, water samples from secluded, marine (Fanduco Reef) and river (Cupeyes upstream site) environments were analyze with respect to beach water contaminated by direct sewage discharges (Isabela). As observed in the contaminated estuary, multiple bands were also detected in water samples from the Isabela site along with the reference 1.3 Kb amplicon indicating the detection of multiple class 1 integrons with varying cassette loads. The 1.3 Kb fragment detected at Isabela encoded beta-lactamses enzymes, aminoglycoside modifying enzymes and Qac efflux pumps. However, the 1.3 Kb amplicon was undetected in remote reference sites. The amplification patterns of the class 1 variable region along with overall sequencing results obtained from these validation experiments suggest that bacterial populations from relatively undisturbed marine and river environment are not major sources of IntI1-encoded antibiotic resistance. These findings indicate that the prevalence of class 1 integron loaded with resistance determinants is mainly associated with sewage related contamination. Additionally, these results suggest that several class1 variants loaded with different resistance mechanisms can be potentially associated with different hosts. This could have important implications as class 1 integrons are laterally transferred and known to exist beyond the hospital setting, including variants lacking gene cassettes encoding antibiotic resistance traits (Hardwick

et al., 2008). Hence, these environmental variants could ultimately become stable receptors of intl1-encoded resistance genes from introduced microbial pollutants.

***Frequency and distribution of IntI-encoded sequences in coastal environments.***

As observed in optimization experiments, class 1 and class 2 integrases dominated in impacted coastal settings relative to those exposed to minimal impact. This is consistent with the association of class 1 and class 2 integrons with replicative and non-replicative transposons, respectively (Liebert et al., 1999; Hansson et al., 2002). However, class 3 integrases were only detected in water and rhizosphere samples from impacted mangrove (La Parguera) as well as in water samples from contaminated estuaries (Guanajibo and Manatí River). The mangrove forest sampled at La Parguera is adjacent to a secondary wastewater treatment plant. Class 3 elements have been previously described among representatives of the genus *Delftia* (formerly *Commomonas*) recovered within wastewater treatment facilities (Xu et al., 2007), which suggests wastewater impact from the treatment system on this mangrove habitat. In a recent study *intl3* genes were not detected among a collection of 654 *E.coli* isolates retrieved from contaminated estuaries (Laroche et al., 2009). This is in contrast with the detection of *intl3* sequences in water samples from the Guanajibo and Manatí River estuaries, which demonstrated the usefulness of a molecular metagenomic approach as a superior screening method with respect to culture based assessments.

The implementation of a molecular method in this study also facilitated the detection of potentially functional class 2 integrases which lacked the signature ochre

codon at position 179 (Barlow et al., 2006 and Márquez et al., 2008). These class 2 variants were distributed across rhizosphere and sediment samples from the impacted mangrove site at La Parguera, the outflow of a direct sewage discharge and receiving sea water at Isabela, and water samples from the Guanajibo River estuary. Integrons carrying functional class 2 integrases are not as common as those having the ochre 179 codon.

The former have been described in isolates of *Providencia stuartii* and *E.coli* associated with beef cattle and human urinary tract infections, respectively (Barlow et al., 2006; Márquez et al., 2008). Additionally, partial sequences of potentially functional class 2 variants have been detected in metagenomic DNA extracted from biosolids and cropland fertilized with swine manure in temperate regions (Rodríguez-Minguela et al., 2009). These findings constitute the first report of potentially functional class 2 integrases in the Caribbean area. This study also uncovered coastal habitats as reservoirs of potentially functional Class 2 IntlI genotypes, expanding previous distribution estimates of these Intl2 variants, which so far have been reported only in terrestrial habitats.

Overall, gene cassettes inserted in the variable region of class 1 integrons were more frequently detected in impacted settings than class 1 integrases. However, the presence of class 1 integrases was not always correlated with the presence of Intl1 gene cassettes. Only in the case of sediment and rhizosphere samples from La Parguera (rainy season) class 1 integrases were not detected whereas intl1-encoded gene cassettes showed detection frequencies ranging from 50-87%. Inconsistent results for the independent screening of these two intl1 markers could be due to the presence of class 1 integrons lacking DNA sequences important for primer attachment sites in the integrase gene which

are located upstream the binding sites for primers targeted at the variable region. In a study conducted with 32 strains of *Xanthomonas* most of the integron integrases found were not functional due to the presence of premature stop codons, frameshift mutations and substantial deletions (Gillings et al., 2005). In this study it was hypothesized that loss of integrase activity can prevent the loss of captured genes that could provide and advantage to the host (Gillings et al., 2005). Furthermore, the analysis of genomic data has also revealed the prevalence of *intl*-pseudogenes (Nemergut et al., 2008).

In contrast, intl1-encoded gene cassettes were undetected and intl1 genes were present in water samples from La Parguera, (dry season) whereas both markers were detected in water samples during the rainy season, suggesting that class 1 hosts lacking gene cassettes (Rosser and Young 1999) or deleted priming sites for the variable region (Laroche et al., 2009) were dominant in the water column during the dry season while class 1 variants lacking the above mentioned defects could have been prevalent due to sewage dispersal by rain events. The analysis of samples from the isolated mangrove site (Ensenada Dakity, Culebra) during the dry season revealed the presence of the *intl1* marker only in sediment samples while Intl1 gene cassettes were undetected in water, sediments and rhizosphere material. This site is considerably far from any obvious source of fecal contamination and it has been argued that in the absence of sustained antibiotic selective pressures, integrons persist devoid of antibiotic resistance gene cassettes (Rosser and Young 1999). Nevertheless, both markers were detected during the rainy season which appeared to be associated with the presence of sailing boats and migratory waterfowl near the sampling area. Illegal wastewater discharges from recreational boats is known to contribute to the degradation of coastal habitats (Leon and

Warnken, 2008) whereas avian fecal matter has been described as a major reservoir of antibiotic resistant bacteria and integrons (Tsubokura et al., 1995; Nandi et al., 2004; Garmyn et al., 2011).

During the rainy season all *intl*-associated targets were detected in the impacted beach (Pico de Piedra) except for Intl3 sequences in water samples. At this site the bathing area is flanked by a polluted stream constantly carrying high loads of sediments and by a channel that funnels stormwater runoff. Except for the detection of *intl3* sequences in water and sand, the detection trends for integron-related sequences were similar at Pico de Piedra during the dry season, which suggested a sustained pollution impact. In contrast, during the rainy season, Intl1 gene cassettes were undetected in the secluded beach site (Peña Blanca) whereas *intI1* and *intl2* sequences were the only markers found, which is in agreement with the existence of background levels of these elements and lower levels of human influence. However, except for *intl3* elements, all integron-associated markers were detected in water and sand samples at Peña Blanca during the dry season, contrary to what would be expected since rain events are commonly associated with the dispersal of fecal pollution (Cho et al., 2010). Additionally, no obvious pollution sources were identified at this site. It appears that in addition to meteorological factors, increased human activity during the dry season in conjunction with tidal cycles may promote the mobilization and resuspension of bacteria in the beach environment as it has been argued that bacteria present in beach sand could be dispersed an average distance of 1.6 m in 4 h by human transit (Bonilla et al., 2006; Hartz et al., 2008).

***Molecular characterization and prevalence trends of IntI-encoded gene cassettes in coastal environments***. The existence of different class 1 integrons having different gene cassette contents was observed in various instances. However, in order to simplify the identification of an integron signature marker indicative of sewage-related pollution, sequencing analyses were limited to the IntI1 5'region of 12 clones per site, that contained those amplicons found in coastal settings that had a size similar to that yielded by the control host of a class 1 integron (~1.3 Kb, *E. coli R388*). Sequencing analyses revealed gene cassettes encoding putative protein sequences or functions unrelated to antibiotic resistance. Nevertheless, a total of seven different antibiotic resistance mechanisms were detected including those found in validation experiments: an aminoglycoside nucloetidyltransferease (aadA7), a dehydrofolate reductase and a Qac E efflux pump. In addition to these, a second amino glycoside nucloetidyltransferease (aaA2), a Qac G efflux pump, an Aac6 amino glycosideacetyltransferase and an extended spectrum beta-lactamase (GES-14) were detected.

Except for gene cassettes cloned from water samples collected at La Parguera, a considerable fraction (25-100%, 61% average, $n$ =1 to 12) of the analyzed clones revealed the presence of IntI-encoded antibiotic resistance genes. Gene cassettes encoding aminoglycoside nucloetidyltransfereases were the most abundant ($n$ = 32) and widely distributed across the tested habitats, followed by those encoding Qac efflux pumps ($n$ = 15), extended spectrum beta lactamases ($n$ = 9), aminoglycoside acetyltransferases ($n$ = 5) and dehydrofolate reductases ($n$ = 1).

The gene cassettes encoding extended spectrum beta-lactamases (ESBL's) had predicted amino acid sequences 99% identical to that of a novel representative of the

GES (Guiana Extended Spectrum ) group (GES 14) (Bonnin et al., 2011). GES enzymes were first reported in a rectal isolate of *Klebsiella pneumoniae* carrying a class 1 integron (Poirel et al., 2000). ESBL's are known to confer resistance against penicillin antibiotics, as well as against first, second and third generation cephalosporins, which were produced as a counter measure against increasing resistance to commonly used beta-lactam antibiotics (Paterson and Bonomo, 2005). They also confer resistance against last resort antibiotics such as imepenem (Queenan and Bush, 2007). Relative to other beta-lactamases, those of the GES group are considered to have a limited occurrence among clinical isolates (Queenan and Bush, 2007). However, this study uncovered the presence of GES 14 variants in sediments and rhizosphere samples from a mangrove ecosystem in close proximity to a wastewater treatment plant (La Parguera) and in water samples from a polluted estuary (Guanajibo River), demonstrating the dispersal of this highly versatile resistance determinant beyond the clinical setting. This may constitute a future threat to human health.

Similar to aminoglycoside nucloetidyltransfereases, Aac6 acetyltransferases are broad-spectrum enzymes capable of chemically inactivating the aminoglycosides antibiotics most widely used for the treatment of bacterial infections. Acetyltransferase variants mediate the addition of acetyl groups to aminoglycosides, which prevents binding of the drug to the 30S subunit of the bacterial ribosome. Genes encoding Aac6 acetyltransferases have been previously detected in transposons or integrons (Wright 1999). The substrate specificity of this enzyme appears to be low, suggesting alternative functions unrelated to aminoglycoside resistance such as the acetylation of peptides

which may promote their prevalence in the absence of antibiotic selective pressure (Wright 1999).

The cassette-targeted PCR assay appeared to be particularly sensitive in detecting resistance determinants in environments with moderate human influence. At the isolated mangrove site (Ensenada Dakity, Culebra), IntI1-encoded antibiotic resistance was undetected by PCR during the dry season. However, resistance determinants against aminoglycoside drugs and quaternary ammonium compounds were identified by sequencing a limited number of randomly chosen clones ($n$ = 1 to 2). Integrons having these gene cassettes are common among clinical isolates (Díaz-Mejía et al., 2008; Fonseca et al., 2005; Gu et al., 2008; Chang et al., 2007; Ploy et al., 1998). These were found in water, sediment and rhizosphere material at the sampling point corresponding to the rainy season, during which wastewater discharges from sailing boats and fecal matter from migratory waterfowl could have contaminated the site. During the rainy season, PCR failed to detect Intl1-encoded genes in water and sand from the secluded beach site (Peña Blanca, Aguadilla) whereas these were detected in the water column during the dry season in two of three randomly chosen clones. The presence of these resistance determinants could have been influenced by higher human transit during dry weather conditions.

The prevalence and distribution patterns of IntI1-encoded antibiotic resistance genes revealed in this study identified potential molecular markers that may serve as alternative indicators of fecal contamination in coastal settings. Penicillin and related antibiotics are the most widely used drugs for treating bacterial infections in humans and Intl1-encoded resistance determinants against these chemicals were detected in

environments subjected to sustained wastewater pollution and human influence. Hence, molecular methods targeted at the conserved 5'end of class 1 elements and GES genes could be developed and further evaluated as tools for detecting wastewater contamination from human sources. A similar approach could be used that also includes gene cassettes that provide resistance against aminoglycoside antibiotics as these are frequently detected in clinical strains and fecal bacteria harboring class 1 integrons (Gu et al., 2008; Heir et al., 2004), while Intl1 elements from natural environments commonly lack antibiotic resistance functions and the conserved 3' region used as a primer binding site in this study (Rosser and Young 1999). Antibiotic resistant bacteria are known to endure wastewater treatment in oxidation ponds receiving discharges from animal husbandry operations (Mispagel and Gray 2005). Moreover, resistant bacteria, conjugative resistance plasmids and integrons can also persist after chlorination in wastewater treatment facilities (Figueira et al., 2011;Tennstedt et al., 2003). Hence, a comprehensive knowledge of sources and environmental reservoirs of resistance determinants would be beneficial in predicting potential health hazards and developing measures that may attenuate the degradation of coastal habitats.

# CONCLUSIONS

1. The analysis of mangrove habitats uncovered rhizosphere and sediment niches as key reservoirs of indicators bacteria. Higher numbers of fecal indicators were observed in sediments and rhizosphere with respect to water samples. In some instances, these indicators counts were above permissible levels relative to water samples. Hence, decisions made based solely on the analysis of the water column may be false safety indicators as exposure to potentially harmful bacteria residing in close proximity to the water column are being ignored.

2. Variance analysis indicated that levels of fecal indicators were significantly higher in impacted settings relative to less disturbed sites, suggesting the presence of clinical integron hosts, as these are commonly reported in enteric bacteria.

3. The partial amplification of clinical integrases demonstrated that class 3 elements are restricted to settings severely impacted by wastewater, whereas class 1 and class 2 variants are more widely distributed in the environment. Moreover, the intensity of the amplification signal of class 1 and class 2 integrases was associated with high levels of human influence particularly in sites exposed to wastewater contamination, indicating the dispersal of clinical integrons outside the hospital environment.

4. Chi-square tests indicated a higher detection frequency in impacted environments of gene cassettes associated with the variable region of IntI1 variants commonly found in pathogens (conserved 5’ and 3’ ends).

5. Amplification and sequencing analyses of the variable region of class 1 integrons revealed the presence in polluted coastal environments of distinct class 1 integrons carrying various gene cassettes, many of them encoding different resistance determinants. The analysis of a limited number ($n$ = <12) of randomly chosen clones showed to be highly consistent for the detection of resistance determinants across impacted environmental niches relative to undisturbed sites. This reflects the high specificity of the assay for the intended target.

6. Sequencing of the variable region of class 1 integron revealed six different antimicrobial resistance determinants. Those encoding resistance against amino glycoside antibiotics followed by those encoding Qac efflux pumps were the most commonly detected. A gene cassette encoding a protein highly related to a novel extended spectrum beta lactamase (GES 14) was detected in environment directly impacted by sewage discharges or in close proximity to wastewater treatment facilities. This trend is suggestive of sewage contamination from human sources. Hence, genes encoding amino glycoside modifying enzymes, Qac pumps and beta lactamases could be further evaluated as signature sequences indicative of fecal pollution and source tracking. However, further investigation is needed to evaluate the full potential of this molecular technique for the above mentioned purposes and for indicating relationships with respect to recent, late and sustained contamination events.

7. This research showed the need to establish measures for the appropriate use of antimicrobial agents and controlling the sources of fecal pollution in coastal settings as the genetic pool represented by the integron platform appeared to be shifted in favor of those carrying resistance determinants at impacted sites. This could promote lateral gene transfer between introduced and indigenous integron hosts, perpetuating the prevalence of resistance traits.

# RECOMMENDATIONS AND FUTURE DIRECTIONS

1. It is recommended to use sequences covering the junction between 5' and 3' ends of the variable region on class 1 integrons of the detected antibiotic resistance gene cassettes, for the development of quantitative PCR assays. Absolute molecular quantification of these target sequences relative to levels of fecal pollution may facilitate more accurate risk assessments with respect to health hazards since the most abundant resistance determinants can be identified. This information can also be used to develop alternative indicators for the degradation of coastal settings and elucidate contamination sources.

2. Screening of fecal indicator bacteria for the presence of integron–encoded antibiotic resistance may serve as an additional warning with respect to the dispersal of traits that threatens the efficacy of treatment options. The molecular characterization of integrons circulating in local environments can reveal additional molecular markers that could be evaluated as potential tracers of sewage contamination. Moreover, surveying in coastal settings the presence of known and emerging pathogens implicated in nosocomial outbreaks and their subsequent screening for the presence of clinical integrons can provide new insights on the extent of risks to human health outside the clinical environment as well as sequence data for the development of additional molecular markers.

3. To concentrate isolation and sequencing efforts on the characterization of class 2 and class 3 integrons in order to identify suitable regions for the design of primers that

may allow the amplification of gene cassettes present in the variable region of these understudied variants.

4. To conduct future studies with additional sampling sites and increased sampling frequency with supporting statistical analysis to further evaluate the trends detected in this work.

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# APPENDICES

Appendix 1. Detection frequency of integrase genes (classes 1-3) and intl1-encoded gene cassettes independently amplified (4 replicates) from four samples of total DNA retrieved from mangrove ecosystems during the rainy season.

| Site code and description | Sample type | Target sequence | DNA samples 1 | 2 | 3 | 4 | Detection frequency %* |
|---|---|---|---|---|---|---|---|
| LPM: | Water | *intl1* | 0 | 0 | 2 | 3 | 31.3 |
| Mangrove habitat, | column | *intl2* | 0 | 2 | 2 | 2 | 37.5 |
| La Parguera, Lajas | | *intl3* | 0 | 1 | 2 | 2 | 31.3 |
| | | *intl1* cassettes | 3 | 2 | 4 | 3 | 75 |
| | Sediment | *intl1* | 0 | 0 | 0 | 0 | 0 |
| | | *intl2* | 2 | 3 | 2 | 0 | 43.8 |
| | | *intl3* | 0 | 0 | 0 | 0 | 0 |
| | | *intl1* cassettes | 4 | 4 | 4 | 2 | 87.5 |
| | Rhizosphere | *intl1* | 0 | 0 | 0 | 0 | 0 |
| | | *intl2* | 0 | 0 | 0 | 0 | 0 |
| | | *intl3* | 0 | 1 | 0 | 0 | 6.3 |
| | | *intl1* cassettes | 0 | 2 | 2 | 4 | 50 |
| EDC: | Water | *intl1* | 1 | 2 | 2 | 2 | 43.8 |
| Mangrove habitat, | column | *intl2* | 2 | 0 | 0 | 0 | 12.5 |
| Ensenada Dakity, | | *intl3* | 0 | 0 | 0 | 0 | 0 |
| Culebra | | *intl1* cassettes | 0 | 1 | 2 | 2 | 31.3 |
| | Sediment | *intl1* | 0 | 2 | 2 | 0 | 25 |
| | | *intl2* | 0 | 0 | 0 | 0 | 0 |
| | | *intl3* | 0 | 0 | 0 | 0 | 0 |
| | | *intl1* cassettes | 4 | 4 | 4 | 4 | 100 |
| | Rhizosphere | *intl1* | 0 | 2 | 2 | 0 | 25 |
| | | *intl2* | 3 | 4 | 4 | 3 | 87.5 |
| | | *intl3* | 0 | 0 | 0 | 0 | 0 |
| | | *intl1* cassettes | 0 | 3 | 2 | 3 | 50 |

* Based on a total of 16 replicate PCR reactions (four per environmental DNA sample).

Appendix 2. Detection frequency of integrase genes (classes 1-3) and intl1-encoded gene cassettes independently amplified (4 replicates) from four samples of total DNA retrieved from mangrove ecosystems during the dry season.

| Site code and description | Sample type | Target sequence | DNA samples 1 | 2 | 3 | 4 | Detection frequency %* |
|---|---|---|---|---|---|---|---|
| LPM: | Water | *intl1* | 4 | 4 | 4 | 4 | 100 |
| Mangrove habitat, | column | *intl2* | 0 | 2 | 2 | 2 | 37.5 |
| La Parguera, Lajas | | *intl3* | 0 | 0 | 0 | 1 | 6.3 |
| | | *intl1* cassettes | 0 | 0 | 0 | 0 | 0 |
| | Sediment | *intl1* | 0 | 0 | 4 | 4 | 50 |
| | | *intl2* | 0 | 4 | 3 | 0 | 43.8 |
| | | *intl3* | 0 | 0 | 0 | 0 | 0 |
| | | *intl1* cassettes | 4 | 4 | 4 | 4 | 100 |
| | Rhizosphere | *intl1* | 0 | 3 | 2 | 2 | 43.8 |
| | | *intl2* | 0 | 0 | 2 | 4 | 37.5 |
| | | *intl3* | 1 | 0 | 3 | 2 | 37.5 |
| | | *intl1* cassettes | 0 | 4 | 4 | 4 | 75 |
| EDC: | Water | *intl1* | 0 | 0 | 0 | 0 | 0 |
| Mangrove habitat, | column | *intl2* | 0 | 0 | 0 | 0 | 0 |
| Ensenada Dakity, | | *intl3* | 0 | 0 | 0 | 0 | 0 |
| Culebra | | *intl1* cassettes | 0 | 0 | 0 | 0 | 0 |
| | Sediment | *intl1* | 2 | 3 | 2 | 4 | 68.8 |
| | | *intl2* | 2 | 4 | 0 | 0 | 37.5 |
| | | *intl3* | 0 | 0 | 0 | 0 | 0 |
| | | *intl1* cassettes | 0 | 0 | 0 | 0 | 0 |
| | Rhizosphere | *intl1* | 0 | 0 | 0 | 0 | 0 |
| | | *intl2* | 3 | 3 | 0 | 0 | 37.5 |
| | | *intl3* | 0 | 0 | 0 | 0 | 0 |
| | | *intl1* cassettes | 0 | 0 | 0 | 0 | 0 |

* Based on a total of 16 replicate PCR reactions (four per environmental DNA sample).

Appendix 3. Detection frequency of integrase genes (classes 1-3) and intl1-encoded gene cassettes independently amplified (4 replicates) from four samples of total DNA retrieved from beach ecosystems during the rainy season.

| Site code and description | Sample type | Target sequence | DNA samples 1 | 2 | 3 | 4 | Detection frequency %* |
|---|---|---|---|---|---|---|---|
| PPA: | Water | *intl1* | 0 | 0 | 3 | 2 | 31.3 |
| Pico de Piedra beach, | column | *intl2* | 3 | 2 | 4 | 4 | 81.3 |
| Aguada | | *intl3* | 0 | 0 | 0 | 0 | 0 |
| | | *intl1* cassettes | 1 | 0 | 2 | 3 | 37.5 |
| | Sand | *intl1* | 4 | 1 | 4 | 3 | 75.0 |
| | | *intl2* | 2 | 4 | 3 | 4 | 81.3 |
| | | *intl3* | 0 | 0 | 2 | 0 | 12.5 |
| | | *intl1* cassettes | 4 | 4 | 4 | 4 | 100.0 |
| PBA: | Water | *intl1* | 0 | 3 | 3 | 3 | 56.3 |
| Peña Blanca beach, | column | *intl2* | 2 | 2 | 2 | 2 | 50.0 |
| Aguadilla | | *intl3* | 0 | 0 | 0 | 0 | 0 |
| | | *intl1* cassettes | 0 | 0 | 0 | 0 | 0.0 |
| | Sand | *intl1* | 4 | 4 | 0 | 2 | 62.5 |
| | | *intl2* | 2 | 4 | 4 | 2 | 75.0 |
| | | *intl3* | 0 | 0 | 0 | 0 | 0 |
| | | *intl1* cassettes | 0 | 0 | 0 | 0 | 0 |

* Based on a total of 16 replicate PCR reactions (four per environmental DNA sample).

Appendix 4. Detection frequency of integrase genes (classes 1-3) and intl1-encoded gene cassettes independently amplified (4 replicates) from four samples of total DNA retrieved from beach ecosystems during the dry season.

| Site code and description | Sample type | Target sequence | DNA samples 1 | 2 | 3 | 4 | Detection frequency %* |
|---|---|---|---|---|---|---|---|
| PPA: | Water | *intl1* | 4 | 3 | 4 | 4 | 93.8 |
| Pico de Piedra beach, | column | *intl2* | 1 | 0 | 0 | 4 | 31.3 |
| Aguada | | *intl3* | 0 | 0 | 0 | 0 | 0 |
| | | *intl1* cassettes | 4 | 4 | 4 | 4 | 100 |
| | Sand | *intl1* | 4 | 3 | 4 | 2 | 81.3 |
| | | *intl2* | 2 | 0 | 0 | 3 | 31.3 |
| | | *intl3* | 0 | 0 | 0 | 0 | 0 |
| | | *intl1* cassettes | 3 | 4 | 4 | 4 | 93.8 |
| PBA: | Water | *intl1* | 2 | 1 | 1 | 1 | 31.3 |
| Peña Blanca beach, | column | *intl2* | 3 | 4 | 4 | 2 | 81.3 |
| Aguadilla | | *intl3* | 0 | 0 | 0 | 0 | 0 |
| | | *intl1* cassettes | 4 | 2 | 2 | 2 | 62.5 |
| | Sand | *intl1* | 0 | 4 | 2 | 4 | 62.5 |
| | | *intl2* | 0 | 0 | 3 | 0 | 18.8 |
| | | *intl3* | 0 | 0 | 0 | 0 | 0 |
| | | *intl1* cassettes | 4 | 4 | 4 | 4 | 100 |

* Based on a total of 16 replicate PCR reactions (four per environmental DNA sample).

Appendix 5 Detection frequency of integrase genes (classes 1-3) and intl1-encoded gene cassettes independently amplified (4 replicates) from four samples of total DNA retrieved from estuarine and river-related ecosystems during the rainy season.

| Site code and description | Sample type | Target sequence | DNA samples 1 | 2 | 3 | 4 | Detection frequency %* |
|---|---|---|---|---|---|---|---|
| GRM: | Water | *intl1* | 2 | 4 | 3 | 0 | 56.3 |
| Guanajibo River | column | *intl2* | 0 | 0 | 0 | 2 | 12.5 |
| estuary, Mayagüez | | *intl3* | 0 | 0 | 0 | 0 | 0 |
| | | *intl1* cassettes | 4 | 3 | 0 | 3 | 62.5 |
| MRB: | Water | *intl1* | 4 | 4 | 2 | 1 | 68.8 |
| Manatí River | column | *intl2* | 1 | 2 | 1 | 2 | 37.5 |
| estuary, Barceloneta | | *intl3* | 0 | 3 | 4 | 0 | 43.8 |
| | | *intl1* cassettes | 4 | 3 | 0 | 0 | 43.8 |
| CRU: | Water | *intl1* | 1 | 3 | 0 | 3 | 43.8 |
| Cupeyes River | column | *intl2* | 0 | 3 | 3 | 0 | 37.5 |
| downstream site, | | *intl3* | 0 | 0 | 0 | 0 | 0 |
| Sabana Grande | | *intl1* cassettes | 2 | 4 | 1 | 3 | 62.5 |

* Based on a total of 16 replicate PCR reactions (four per environmental DNA sample).

Appendix 6. Detection frequency of integrase genes (classes 1-3) and intl1-encoded gene cassettes independently amplified (4 replicates) from four samples of total DNA retrieved from estuarine and river-related ecosystems during the dry season.

| Site code and description | Sample type | Target sequence | DNA samples 1 | 2 | 3 | 4 | Detection frequency %* |
|---|---|---|---|---|---|---|---|
| GRM: | Water | *intl1* | 3 | 2 | 4 | 1 | 62.5 |
| Guanajibo River | column | *intl2* | 4 | 4 | 4 | 1 | 81.3 |
| estuary, Mayagüez | | *intl3* | 0 | 1 | 0 | 0 | 6.3 |
| | | *intl1* cassettes | 2 | 0 | 3 | 2 | 43.8 |
| MRB: | Water | *intl1* | 4 | 4 | 3 | 3 | 87.5 |
| Manatí River | column | *intl2* | 2 | 2 | 0 | 0 | 25 |
| estuary, Barceloneta | | *intl3* | 0 | 2 | 0 | 0 | 12.5 |
| | | *intl1* cassettes | 4 | 3 | 2 | 0 | 56.3 |
| CRU: | Water | *intl1* | 0 | 2 | 0 | 0 | 12.5 |
| Cupeyes River | column | *intl2* | 0 | 0 | 0 | 0 | 0 |
| upstream site, | | *intl3* | 0 | 0 | 0 | 0 | 0 |
| Sabana Grande | | *intl1* cassettes | 0 | 0 | 0 | 0 | 0 |

* Based on a total of 16 replicate PCR reactions (four per environmental DNA sample).

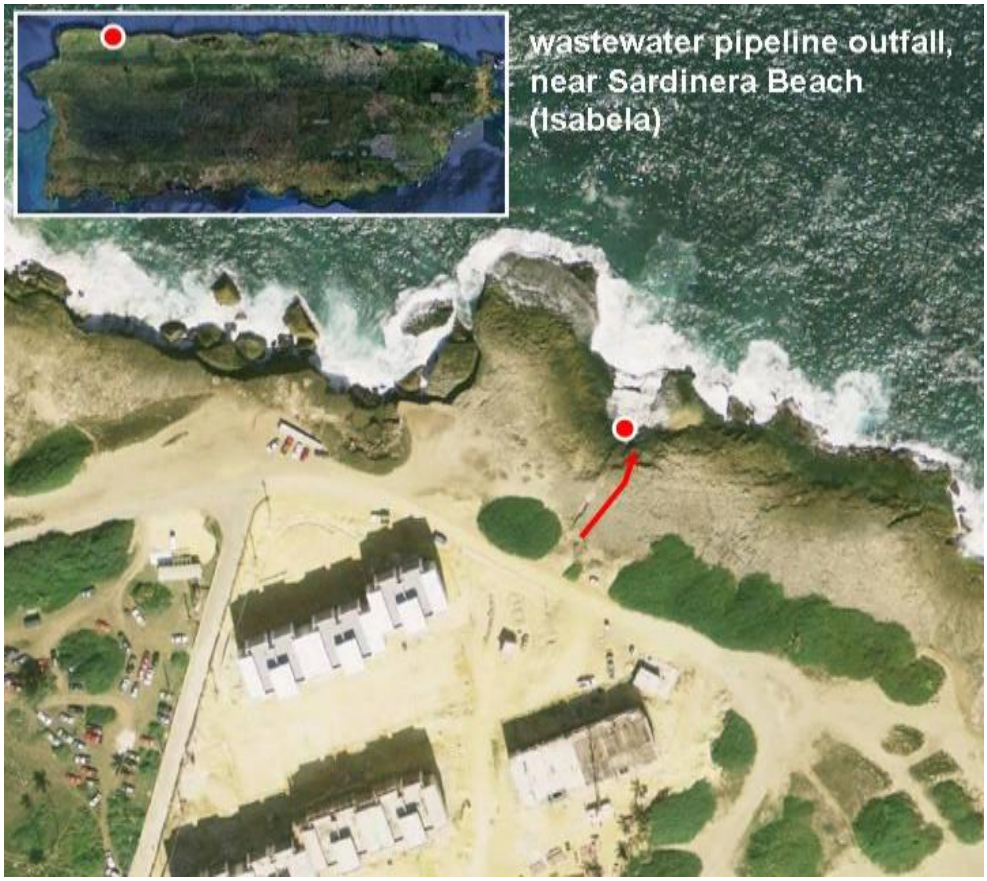
Appendix 7. Locations of impacted (La Parguera, Lajas) and relatively undisturbed (Ensenada Dakity, Culebra) mangrove ecossytems.


Appendix 8. . Locations of impacted (Pico de Piedra, Aguada) and relatively undisturbed (Peña Blanca, Aguadilla) beach ecosytems.

Appendix 9. Location of impacted estuarine environments Manati River estuary, Barceloneta (left) and Guanajibo River estuary, Mayagüez (right).


Appendix 10. Location of reference undisturbed river (Cupeyes, Sabana Grande).

Appendix 11. Location of reference beach habitats impacted by direct sewage discharge (Sardinera Beach, Isabela).



Appendix 12. Table depicting the sampling timing of the analyzed coastal environments.

| Site Code | Description | Impact Level | Location | September | November | December | January | Febraury | April | June | August |
|---|---|---|---|---|---|---|---|---|---|---|---|
| GRM | Estuarine | Severe | Mayagüez | X | | | | X | | | |
| MRB | Estuarine | Severe | Barceloneta | | X | | X | | | | |
| CRU | Estuarine | Minimal | Sabana Grande | X | | | | | | | |
| CRD | Estuarine | Minimal | Sabana Grande | | | | X | | | | |
| PPA | Beach | Severe | Aguada | | | | X | | | X | |
| PBA | Beach | Minimal | Aguadilla | | | | X | | | | X |
| LPM | Mangrove | Severe | Lajas | X | | | | | X | | |
| EDC | Mangrove | Minimal | Culebra | | | X[a] | | | X | | |

[a] Samples collected during a three days of rainy events.