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Antimicrobial resistance to 14 antimicrobials in marine coastal waters around Northern Ireland: Use of the novel *Relative Resistance Index* as a marker of ecological status

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ABSTRACT

Relatively little work has been published on the incidence of antibiotic resistance (ABR) in the marine microbiological environment, which is of importance to animal (fish, mammals, birds) health, zoonotic transmission, distribution of ABR bacteria with oceanic drift, and ultimately human health. A study was performed to determine the diversity of *total* ABR (intrinsic and acquired resistance) in marine bacteria in shallow coastal waters surrounding Northern Ireland through the use of a novel Relative Resistance Index (RRI) as a surrogate marker for ecological change, particularly in comparing marine water in commercial versus non-commercial sites. Total antibiotic resistance was observed to varying degrees in all marine water specimens and specific resistance levels were as follows, in order of diminishing antibacterial effectiveness: fluoroquinolones > rifampicin > polymyxin > tetracycline > sulphamethoxazole/trimethoprim > third generation cephalosporin and streptomycin > carbapenem > macrolide > clindamycin > vancomycin > fucidic acid > penicillin. None of the sampling sites contained endogenous bacteria that were resistant to ciprofloxacin, while nearly all (19 of 20 sites; 95%) contained bacteria that were resistant to penicillin. Commercial sites had a higher mean RRI score of 6.57 ± 3.58 than non-commercial sites (RRI $= 4.08 \pm 2.02$), which was statistically significant ($p = 0.037$), indicating that bacteria isolated from seawater in commercial coastal harbors had a higher frequency of antibiotic resistance than non-commercial sources. This novel RRI marker may be useful in assessing ecological change in marine water environments. In conclusion, this study demonstrated that there can be a high level of *total* ABR (intrinsic and acquired) in bacterial

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populations in marine water environments, which are multi- and pan-resistant to up to 11 major classes of antibiotics simultaneously. Ecological studies are urgently needed to help define the fate of ABR marine bacteria in their natural environment and their ability to act as reservoirs and donors of ABR to pathogenic bacteria, many of which transiently inhabit the natural environment.

1. Introduction

Antibiotic resistance (ABR) has now emerged as an important global threat to animal and human medicine. Although factors that promote emergence of antibiotic resistance in the clinical setting have been well documented, significantly less work has been performed relating to ABR organisms in the marine environment, their origins, persistence, fate, and contributions to human and animal health. Little is known about the fate of antibiotics which are excreted from animals and humans in a metabolically active form, their persistence in aquatic as well as marine environments, and their effect on their respective ecosystems. Important publications by Kümmerer (2009a, 2009b, 2009c) have highlighted the need for better risk assessment to be performed in order to identify the role of the environment for the dissemination of ABR organisms within human and animal ecosystems. Furthermore, a recent review by Kim and Aga (2007) has highlighted waste water treatment plants as an important source of antibiotics and ABR bacteria since they feed into other water sources, including water destined for drinking. Previously, surface water has been identified as being as source of ABR bacteria (Kümmerer, 2004). Given that rivers and other waterways may act as collection and delivery conduits for ABR bacteria into marine environments originating from (i) naturally intrinsic ABR in environmental organisms, (ii) acquired ABR in animal/zoonotic pathogens originating from farm/agricultural runoff, and (iii) acquired ABR organisms from human sewage entering through septic tank/sewage treatment works discharge, it was the aim of this study to examine generic ABR in bacterial populations within marine environments along the entire coastline of Northern Ireland, with particular emphasis on the comparison of marine waters from commercial and non-commercial sites. The study also wished to examine the concept of using a novel Relative Resistance Index (RRI) as a marker for ecological change in these two environments.

2. Materials and methods

a. Collection of environmental marine samples

The bacteriological quality of marine coastal waters around Northern Ireland was examined, as detailed in Table 1 and Figure 1. This sampling plan included the systematic sampling/collection of marine waters at 20 consecutive locations along the 650 km coastline of Northern Ireland, commencing in northwest Northern Ireland (GPS reference: 55.044228° −7.091761°), in a clockwise manner and finishing in southeast Northern Ireland (GPS reference: 54.092472° − 6.191043). Sampling locations were assigned into two categories, either (i) commercial (C), supporting an active fishing (trawler) fleet and/or heavy usage of harbor facilities, or (ii) non-commercial (NC), indicating open sea or a harbor with

Table 1. Antibiotic susceptibility profiles of marine sampling locations along Northern Ireland coastline. Antibiotics tested were β-lactam (penicillin; BL_{pen}), β-lactam (3rd generation cephalosporin; BL_{3rdGen}), carbapenem (CARB), fluoroquinolone (FQ), glycopeptide (G), macrolide (M), polymyxin (P), rifampicin (R), streptomycin (S), sulphamethoxazole/trimethoprim (ST), and tetracycline (T).

Sample number	Sampling location	Location category	Microbial count (colony forming units/ml)	GPS Co-ordinates	Antibiotic susceptibility profile	Relative Resistance Index (RRI)
1	Greysteel, Limavady	NC	50	55.044228° −7.091761°	Resistant:CARB,FA,G,M,BL^{*}_{pen}, BL^{**}_{3rdG},S,T	9
2	Magilligan Point, Limavady	NC	10	55.191124° −6.966029°	Resistant:BL_{pen},BL_{3rdG},FA,G,M Sensitive:CARB,FQ,P,R,S,ST,T	5
3	Portrush Harbour	NC	250	55.206940° −6.658444°	Resistant:BL_{pen} Sensitive:BL_{3rdG},CARB,FA,FQ,G,M,P,R,S,ST,T	1
4	Ballycastle Beach	NC	850	55.206333° −6.238319°	Resistant:BL_{pen},BL_{3rdG},FA,G,M,S T Sensitive: CARB,FQ,P,R,S,T	6
5	Ballycastle Harbour	C	100	55.206326° −6.238743°	Resistant:BL_{pen} Sensitive: BL_{3rdG},CARB,FA,FQ,G,M,P,R,S,ST,T	1
6	Cushendall Lifeboat Station	NC	570	55.075695° −6.052453°	Resistant:BL_{pen},FA,G Sensitive:BL_{3rdG},CARB,FQ,M,P,R,S,ST,T	3
7	Waterfoot Coast Road	NC	530	55.055611° −6.033983°	Resistant: BL_{pen},FA,G Sensitive: BL_{pen},BL_{3rdG},CARB,FQ,M,P,R,S,ST,T	3
8	Garron Coast Road	NC	100	55.043873° −5.962744°	Resistant:FA Sensitive:BL_{3rdG},CARB,FA,FQ,G,M,P,R,S,ST,T	1
9	Carnlough Harbour	NC	240	54.993275° −5.987999°	Resistant:BL_{pen},CARB,FA,G Sensitive:BL_{3rdG},CARB,FA,FQ,G,M,P,R,S,ST,T	4
10	Glenarm Beach	NC	500	54.969452° −5.954880°	Resistant:BL_{pen},CARB,FA,G,M Sensitive:BL_{3rdG},FQ,P,R,S,ST,T	5

(Continued)

Table 1. (Continued)

Sample number	Sampling location	Location category	Microbial count (colony forming units/ml)	GPS Co-ordinates	Antibiotic susceptibility profile	Relative Resistance Index (RRI)
11	Glenarm Bridge	NC	1,350	54.968563° −5.953399°	Resistant:BL_{pen},CARB,FA,G Sensitive:BL_{3rdG},FQ,M,P,R,S,ST,T	4
12	Carnfunnock Pier	NC	300	54.891521° −5.840494°	Resistant:BL_{pen},CARB,FA,G Sensitive:BL_{3rdG},FQ,M,P,R,S,ST,T	4
13	Bangor Harbour	C	1,510	54.664429° −5.669353°	Resistant:BL_{pen},BL_{3rdG},CARB,FA,G,M,R,S,T Sensitive:FQ,P,ST	9
14	Ballyhalbert Harbour	C	1, 080	54.490475° −5.439997°	Resistant:BL_{pen},BL_{3rdG},FA,G,M,T Sensitive:CARB,FQ,P,R,S,ST	6
15	Portavogie Harbour	C	1,720	54.456551° −5.438737°	Resistant:BL_{pen},BL_{3rdG},FA,G,M,S,T Sensitive:CARB,FQ,P,R,ST	7
16	Portaferry Harbour	C	770	54.380718° −5.550327°	Resistant:BL_{pen},FA Sensitive:BL_{3rdG},CARB,FQ,G,M,P,R,S,ST,T	2
17	Newcastle Harbour	NC	1,220	54.198067° −5.884391°	Resistant:BL_{pen},FA,ST Sensitive:BL_{3rdG},CARB,FQ,G,M,P,R,S,T	3
18	Annalong Harbour	C	3,050	54.108093° −5.895603°	Resistant:BL_{pen},BL_{3rdG},CARB,FA,G,M,P,S,ST,T Sensitive:FQ,R	10
19	Kilkeel Harbour	C	3,010	54.061008° −5.994764°	Resistant:BL_{pen},BL_{3rdG},CARB,FA,G,M,P,R,S,ST,T Sensitive:FQ	11
20	Carlingford Lough	NC	1,930	54.092472° −6.191043°	Resistant:BL_{pen},FA,G,P,S Sensitive:BL_{3rdG},CARB,FQ,M,R,ST,T	5

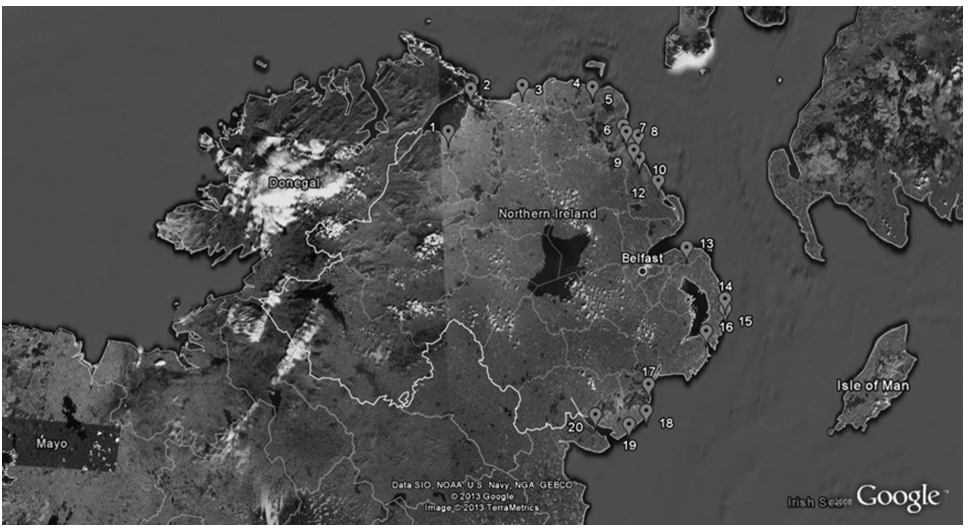

Figure 1. Map details of marine sampling locations along Northern Ireland coastline. Map and satellite data from SIO, NOAA, US Navy, NGA, and GEBCO, © 2013 Google; image © 2013 TerraMetrics.

non-commercial activity and/or low usage of harbour facilities. All sampling was completed during a two week period in August 2011. Sampling at each site was performed by aseptically obtaining 100 ml marine water in a sterile disposable plastic universal container (Sterilin Ltd., UK). All samples were stored at 4 °C until processed and were processed microbiologically within 6 hours of sampling.

b. Quantitative bacteriological examination of water

Total culturable bacterial numbers were estimated in each water sample by employing quantitative enumerative methods. Briefly, serial dilutions (to 10^{-2} dilution) were prepared in 0.1% (w/v) peptone saline (Oxoid CM0733; Oxoid Ltd., Basingstoke, UK) solution. Enumeration studies were performed employing the Spiral Plater (Don Whitley Scientific Ltd., Yorkshire, England) and a Protocol Digital Colony Counter (Don Whitley Scientific Ltd., Yorkshire, England) using Plate Count Agar (PCA) (Oxoid CM325) (30 °C/48h) in accordance with the manufacturer's instructions. Water from each sampling site was counted in duplicate.

c. Direct antimicrobial susceptibility testing of water

For bacteriological examination, Columbia Blood Agar (Oxoid CM0331), supplemented with 5% (v/v) defibrinated horse blood, was employed. Individual 100-ml water samples were inoculated and spread uniformly onto the surface of the agar with the aid of a L-shaped spreader and the plates were incubated at 30 °C for 48 hours prior to reading. Following

this, each plate was flooded with 9 ml 0.1% (w/v) peptone saline (Oxoid CM0733) solution and all colonies were emulsified with the aid of a L-shaped spreader prior to harvesting in a sterile glass universal. The Direct Antimicrobial Susceptibility Testing (DAST) assay was employed in this study, as originally described by Zebouh and colleagues (2008). This assay allows for the determination of *total* levels of antibiotic resistance from a mixed population of organisms, thereby detecting the most resist phenotypes present. Briefly, a cotton swab was charged with inoculum of the total bacterial population and was inoculated onto the surface of Mueller-Hinton agar (Oxoid CM0337). On drying, a standard disk diffusion assay was performed, where antibiotic disks of the following 14 antibiotic agents were placed onto the surface with a semi-automated stamper, which dispensed six different antibiotics (□g) to a single plate: Cefoxitin (30), Ciprofloxacin (5), Clindamycin (2), Colistin (25), Erythromycin (5), Fusidic Acid (10), Meropenem (10), Penicillin (2), Piperacillin/Tazobactam (110), Rifampicin (5), Streptomycin (10), Sulphamethoxazole/Trimethoprim, Tetracycline (30), and Vancomycin (30).

Plates were incubated at 20°C for 24 hours prior to reading. Resistance was recorded as any bacterial morphology growing to the edge of the antibiotic disk, whereas sensitivity was defined as a complete clear zone of inhibition for all bacterial taxa represented on the susceptibility agar.

d. Relative Resistance Index

RRI values were calculated for each sampling location, by assigning a value of 1 for resistance and 0 for sensitivity for each antibiotic tested, where an RRI score = 14 indicates total resistance and an RRI score = 0, indicates total sensitivity. Statistical significance was calculated by employment of the Student's t-test, where a probability value of less than 5% ($p < 0.05$), indicated a significant difference.

3. Results

Total numbers of culturable bacteria at each sampling point along the Northern Ireland coastline are shown in Table 1, which ranged from 10 to 3,050 colony forming units per milliliter of seawater (cfu ml^{-1}), with a mean culturable number of 957 ± 891 cfu ml^{-1}. There was a statistical difference between cultural counts at commercial and non-commercial sampling points ($p = 0.0076$).

Total antibiotic resistance was observed to varying degrees in all marine water specimens, as detailed in Table 1, and specific resistance levels were as follows in order of diminishing antibacterial effectiveness: fluoroquinolones > rifampicin > polymyxin > tetracycline > sulphamethoxazole/trimethoprim > third generation cephalosporin and streptomycin > carbapenem > macrolide > clindamycin > vancomycin > fucidic acid > penicillin. Specific resistance levels in all water samples examined, as determined through a direct antibiotic susceptibility assay, are shown in Figure 2. None of the sampling sites contained endogenous bacteria that were resistant to ciprofloxacin, while nearly all (19 of 20 sites; 95%) contained

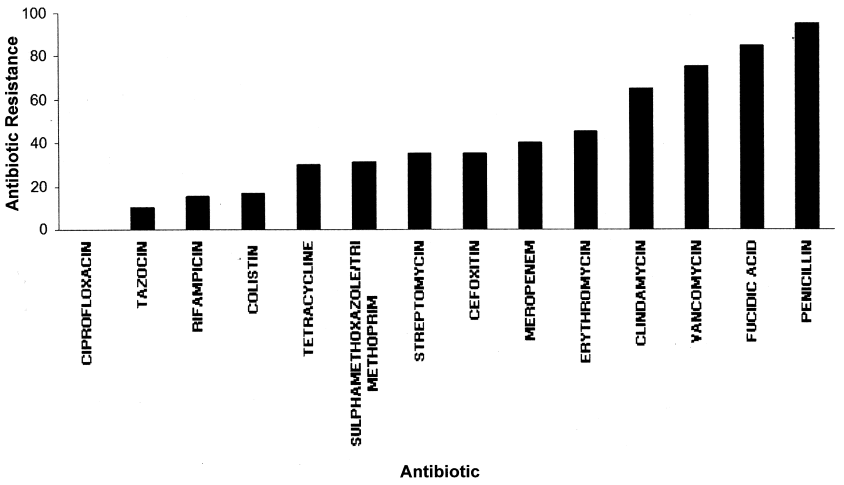

Figure 2. Direct antibiotic susceptibility testing (DAST) of a marine water specimen against 14 antibiotics.

bacteria which were resistant to penicillin (Figure 2). Commercial sampling points had a higher mean RRI score of 6.57 ± 3.58 than non-commercial sites (RRI $= 4.08 \pm 2.02$), which was statistically significant ($p = 0.037$).

4. Discussion

The island of Ireland consists of approximately 5,631 km (3,500 miles) of coastline (OSi, n.d.), of which 650 km belongs to Northern Ireland (DOE NIEA, 2014). This 650 km of coastline is used for diverse purposes, such as tourism, recreation and leisure (sailing, wind surfing, and surfing), agriculture, aquaculture, inshore fisheries, industry, commercial harbors and quays, as well as in waste disposal, aggregate mining, and power generation. This range of activities may thus have consequences for local marine biodiversity and, coupled with extensive environmental protection, is an interesting area for further research.

Changes in water ecosystems have been previously assessed by employment of several ecological indicators, including diversity, biomass and abundance of macroinvertebrates, focal populations, river bed stability, contaminants in fish, and river chemistry (including determination of inorganic suspended sediment, pH, soluble reactive P, NH_4, and NO_3). We now wish to examine the potential for examining the presence of antibiotic resistant bacteria as a potential indicator of ecological change of the marine environment, through the introduction of the novel RRI marker.

The presence of ABR bacteria in the marine environment is becoming increasingly important, given the changing ecology associated with such environments, particularly where they interface with changing human social and demographic factors at the coast. There have been

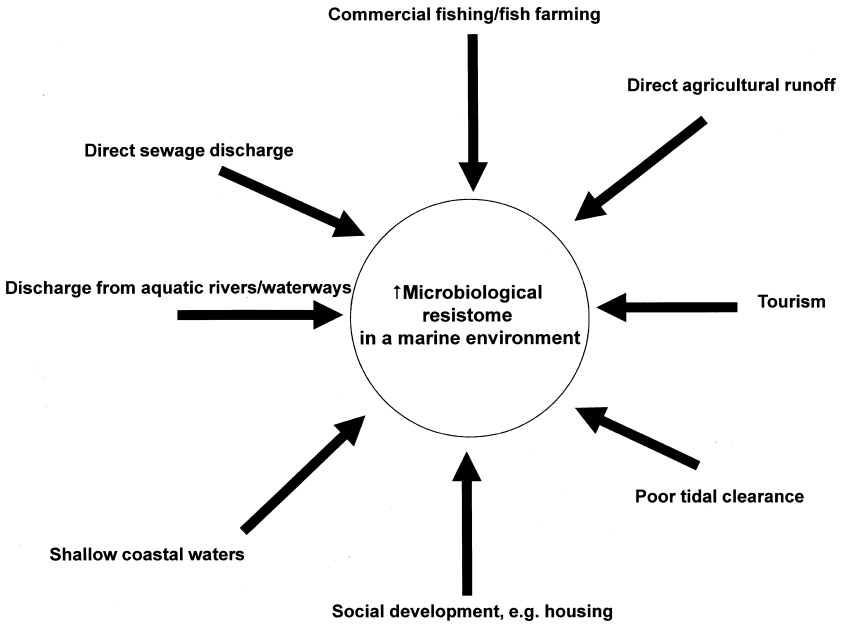

Figure 3. Contributors to antibiotic resistance in bacteria in the marine environment.

several reports of antibiotic resistant bacteria being isolated from the marine environment (Di Cesare et al., 2012; Moscot et al., 2012; Voolaid et al., 2012). In most of these previous reports, specific factors such as fish farming have usually prompted such work to be undertaken. Our interest in this work arises due to the high level of intensive terrestrial farming in Northern Ireland.

Our first aim was to identify various inputs and outputs of ABR bacteria in the marine environment (Fig. 3). The antibiotic resistance profile of bacteria in seawater should be considered in a state of continuous flux. In shallow coastal water, ABR bacteria may be composed of two types of bacteria, namely (i) ABR bacteria that have been displaced from terrestrial or aquatic sources and have entered the marine environment via waterways and rivers or directly from agricultural runoff from land adjacent to the sea and (ii) endogenous marine bacteria. Little is known about the resistome of true marine bacteria. The survival of the former type of bacteria in the marine environment is poorly described, however recent studies from our group have demonstrated the survival of the environmental Gram-negative pathogen, *Pseudomonas aeruginosa*, for greater than one year in seawater (Moore et al., 2007).

The presence of ABR bacteria in marine waters is entirely natural, in that certain bacteria have a natural resistance to certain antibiotics, which is known as intrinsic resistance. For example, Gram-negative bacteria have a natural resistance to the oxazolidone class of antibiotics, such as linezolid, whereas Gram-positive organisms are usually susceptible to

this class of antibiotics. Furthermore, ABR may be present due entirely to acquired resistance. In such resistance, the organism was originally susceptible to the presence of the antibiotic agent, but has evolved a mechanism of resistance to the antibiotic agent.

In these experiments, we employed Plate Count Agar (PCA) for enumeration purposes, as well as Mueller-Hinton Agar (MHA) for antibiotic sensitivity studies. These are standard media that we employ in our microbiological work with water and waste water. Moderate halophilic organisms may require NaCl concentrations ranging between 5%–10% (w/v), whereas the extreme halophile requires salt concentrations ranging from 15%–30%. Because PCA and MHA are not supplemented with sodium chloride, we anticipated that halophilic organisms might not grow under such conditions. However, these same halophilic organisms may not be as clinically significant due to their likely inability to grow and survive in human hosts with relatively high temperatures and low salt concentrations, therefore we restricted our studies to those organisms which were culturable under standard laboratory conditions.

The origins of ABR bacteria in the marine waters tested may be several-fold. First, we may be detecting a high degree of intrinsic resistance from populations of environmental bacteria, which have zero or virtually zero exposure to antibiotic agents. Second, we may have been detecting acquired-ABR bacteria which originated in humans or animals and which were deposited in the sea via watercourses through waste water discharge operations, agricultural run-off, or discharge from septic tanks. Or third, the presence of ABR may have originated in environmental bacteria which developed acquired resistance mechanisms due the chronic presence of sub-lethal concentrations of antibiotic agents from agricultural run-off or discharge from human sewage waste treatment works, the effects of which may have been magnified in shallow water. Regardless of which origin the ABR bacteria detected in this study evolved, their presence in marine environments creates a mass balance of finite and tangible ABR determinants that may have downstream consequences for environmental management, as well as for human health and the well-being of sea life.

The introduction of the RRI marker in this study is an interesting development, in its potential as a surrogate marker for helping to quantify ecological shift in marine environments. Our inital hypothesis was that stable, unpolluted marine environments would have a stable and relatively low RRI score, whereas as marine environments exposed to higher levels of fecal pollution, would have a higher RRI score. Our data demonstrate that commercial marine sources have a statistically higher RRI score, indicating increased resistance levels associated with commercialization of the marine environment.

In conclusion, this study demonstrated that there can be a high level of *total* ABR (intrinsic and acquired) in bacterial populations in marine water environments that are multi- and pan-resistant of up to 11 major classes of antibiotics simultaneously. This depends largely on whether there is any commercial involvement with the immediate marine environment, as demonstrated by significant changes in the RRI score between commercial harbors and non-commercial sites. The potential for marine environmental organisms to be promiscuous and exchange genetic determinants for resistance with each other and more importantly,

with transient pathogens entering the human ecosystems, is a cause for concern. Ecological studies are urgently needed to help define the fate of ABR marine bacteria in their natural environment and their ability to act as reservoirs and donors of ABR to pathogenic bacteria, many of which transiently inhabit the natural environment. Furthermore, a comprehensive risk assessment is urgently required to help define the ecological significance of ABR in the marine environment for animal and human health, as well as to assess the fate of natural marine environmental organisms and their persistence within the marine ecosystem.

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