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ddPCR applied on archived Continuous Plankton Recorder samples reveals long-term occurrence of class 1 integrons and a sulphonamide resistance gene in marine plankton communities

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Summary

Antibiotic resistance is a rising threat for human health. Although in clinical settings and terrestrial environments the rise of antibiotic resistant bacteria is well documented, their dissemination and spread in the marine environment, covering almost two-thirds of the Earth's surface, is still poorly understood. In this study, the presence and abundance of sulphonamide resistance gene (*sul*2) and class 1 integron-integrase gene (*intI*1), used as markers for the occurrence and spread of antibiotic resistance genes since the beginning of the antibiotic era, were investigated. Twenty-nine archived formalin-fixed samples, collected by the Continuous Plankton Recorder (CPR) survey in the Atlantic Ocean and North Sea from 1970 to 2011, were analysed using Droplet Digital PCR (ddPCR) applied for the first time on CPR samples. The two marker genes were present in a large fraction of the samples (48% for *sul*2 and 76% for *intI*1). In contrast, results from Real-Time PCR performed on the same samples greatly underestimate their occurrence (21% for *sul*2 and 52% for *intI*1). Overall, besides providing successful use of ddPCR for the molecular analysis of CPR samples, this study reveals long-term occurrence and spread of *sul*2 gene and class 1 integrons in the plankton-associated bacterial communities in the ocean.

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Introduction

Antibiotic resistance is a global concern for human health (Marti *et al*., 2014). The massive use and misuse of antibiotics in human care settings and animal farming led to the release of high amounts of antibiotics in aquatic environments, causing the selection and spread of antibiotic resistant bacteria (Kümmerer, 2009). Antibiotic resistance genes (ARGs) have been found to be constitutively present within aquatic microbial communities, especially in coastal areas subjected to strong anthropogenic influence such as urban areas and aquaculture sites (Taylor *et al*., 2011; Di Cesare *et al*., 2012; 2015; Czekalski *et al*., 2015; Zhu *et al*., 2017). Antibiotic resistant bacteria (ARB) can be found both as free-living cells and associated with floating particles and plankton (Eckert *et al*., 2016; 2018); the latter was recently proposed as an important ecological driver for the evolution and spread of ARB in the aquatic ecosystem (Taylor *et al*., 2011). Zooplankton species may host abundant bacterial communities: copepods might even be regarded as microbial hot spots in the ocean (Tang, 2005). Eckert and colleagues (2016) hypothesized *Daphnia obtusa* as a refuge for ARB, supporting the idea that bacteria coming from wastewater treatment plants (WWTPs) could better survive when attached to the exoskeleton or gut of aquatic crustaceans (Hall-Stoodley *et al*., 2004). In addition, zooplankton species, with their diel circulation, are also responsible for vertical mobilization and consequent relocation of associated bacteria, which may be pathogenic (Grossart *et al*., 2010) and even antibiotic resistant. Similar to zooplankton, different cyanobacteria genera in the phytoplankton harbour their own microbial communities (Zhu *et al*., 2016; Callieri *et al*., 2017).

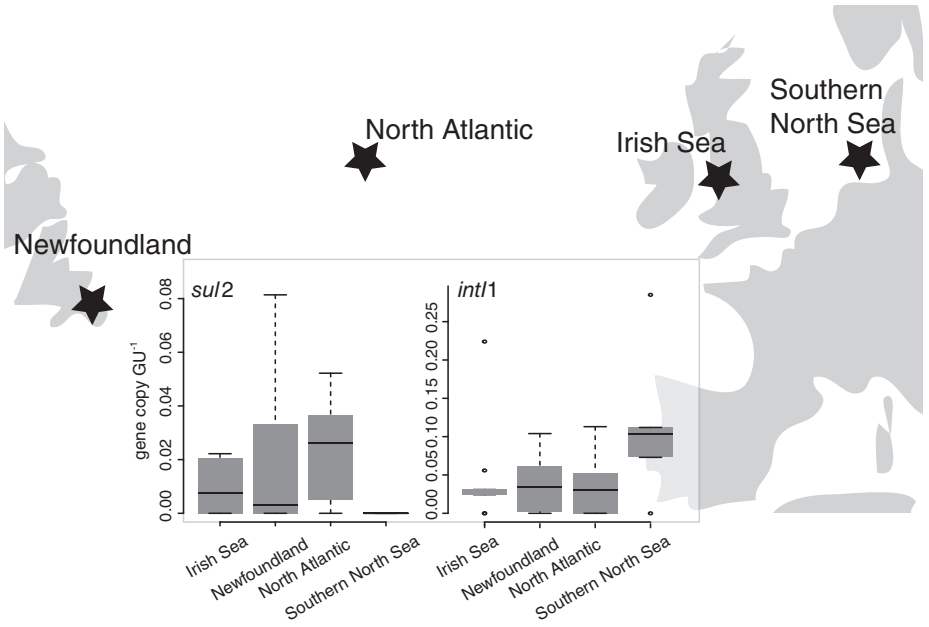

Fig. 1. Simplified map of the sampling area with the Atlantic Ocean in the centre. Sampling stations are indicated by a black star symbol. Boxplots represent the total gene copies GU^{-1} for *sul*2 and *intI*1 as measured in all the samples taken at a specific station by ddPCR. The black line in the box represents the median (2^{nd} quartile), the box edges mark the 1^{st} and 3^{rd} quartiles. Whiskers extend to the highest and lowest values in the ± 1.5 interquartile range from the box. Outliers, defined as values outside the range, are indicated as circles.

With the aim to investigate, for the first time, the long-term occurrence and spread of antibiotic resistance in the marine plankton community, we analysed by Droplet Digital PCR (ddPCR) the presence and abundance of *sul*2 and class 1 integron-integrase gene (*intI*1) in 29 archived formalin-fixed samples collected by the Continuous Plankton Recorder (CPR) survey in the Atlantic Ocean and North Sea from 1970 to 2011 (Fig. 1) (Vezzulli *et al*., 2016). The CPR is a high-speed plankton sampler designed to be towed from ships over long distances and the CPR archive, operating since 1931, is one of the longest and geographically most extensive collections of marine biological samples in the world (Reid *et al*., 2003). Therefore, archived CPR samples offer a unique opportunity to retrospectively investigate the marine plankton community over a multidecadal timescale.

Sulphonamides (SUL) are among the oldest used antibiotics, first introduced into medical practice in 1932 (Huovinen *et al*., 1995). They were extensively employed in human medicine until the 1970s and are nowadays only of limited use in clinic; however, they remain largely administered in veterinary medicine (summarized in Di Cesare *et al*., 2015). One of the plasmid SUL resistance gene, *sul*2, is widely distributed and permanently present within the aquatic microbial community (Czekalski *et al*., 2012; Di Cesare *et al*., 2015) and was suggested to be a good indicator of anthropogenic pollution (Gao *et al*., 2012; Pruden *et al*., 2012; Czekalski *et al*., 2014). Considering the early introduction of SUL antibiotic and the fact that a resistance gene can arise in clinical settings within few months or years from the first use of the antibiotic (Walsh, 2000), *sul*2 gene was selected as a suitable marker for ARGs circulating from the beginning of the antibiotic era.

In addition, class 1 integrons were selected as markers for the spread of ARGs in the marine environment (Stalder *et al*., 2014). Class 1 integrons are genetic capture elements often associated to ARGs, that play a recognized role in ARGs spread among the microbial communities (Gillings, 2014). Accordingly, *sul*2 has been found strongly associated to class 1 integrons (Di Cesare *et al*., 2016). It is worth noting that class 1 integrons are common in environmental bacteria including several marine bacterial species (e.g., all *Vibrio* species) (Gillings, 2014), and they were also recently proposed as proxies of anthropogenic pollution in aquatic ecosystems (Gillings *et al*., 2015).

In this study, *sul*2 and *intI*1 genes were investigated in historical CPR samples as markers for the occurrence and spread of antibiotic resistance genes in the plankton associated bacterial community of the ocean. The correlation between presence and abundances of the tested genes, biotic (i.e., copepods, phytoplankton and *Vibrio* spp., this latter used as proxy of abundance for indigenous human pathogenic bacteria), and abiotic (i.e., sea surface temperature [SST]) factors was also tested.

Results and discussion

In recent studies, molecular analyses applied on CPR samples have been successfully employed to investigate

the plankton associated microbial community in the North Sea and North Atlantic over the past half century (Vezzulli *et al*., 2012; 2016). The performance of molecular quantification methods such as quantitative Real-Time PCR (qPCR) applied on CPR samples could however be problematic because of the low quality of extracted DNA (largely dependent upon sample age and storage in formalin, which might precludes the detection and quantification of low-abundance molecular targets) (Vezzulli *et al*., 2012). To overcome this problem, ddPCR was applied for the first time on CPR samples to detect and quantify *sul*2 and *intI*1 genes. ddPCR is a relatively new PCR technology which improves classical PCR and qPCR, giving the possibility to quantify a target sequence of DNA in an absolute way without external references and with a proven resistance to the variability of PCR efficiency (Hindson *et al*., 2013). In recent years, it has been used in environmental microbiology for different purposes, from the quantification of faecal indicator bacteria in water (Wang *et al*., 2016), to the quantification of ARGs in soil, manure, or urban waste (Cavé *et al*., 2016).

Results from the analyses were surprising as 14 out of 29 analysed samples collected in different marine areas from 1970 to 2011 scored positive for *sul*2 gene. Its concentration ranged between 3.22×10^{-3} and 8.14×10^{-2} copies genomic unit^{-1} (GU, previously quantified by qPCR and expressed as the ratio between 16SrDNA gene copy number in tested samples and average 16SrDNA copy number in Proteobacteria, Vezzulli *et al*., 2016) (Fig. 2). The *intI*1 marker was also found by ddPCR analysis in 22 out of 29 samples, with a concentration ranging between 3.22×10^{-3} and 2.85×10^{-1} copies GU^{-1} (Fig. 2). Interestingly, both genes were found in aged CPR samples, the oldest of which date back to August 1970. In contrast, results from qPCR performed on the same samples greatly underestimate the occurrence of *sul*2 and *intI*1 genes (6 positive samples for *sul*2 and 15 for *intI*1), whose abundances were below the limits of quantification (LOQ, Supporting Information Table S1). This is related to the higher sensitivity of ddPCR compared with qPCR protocols, as shown by sensitivity assays (Supporting Information Fig. S1). The measured *sul*2 copies GU^{-1} did not vary significantly over time (Linear Model: $F_{6,10} = 0.5$, $p = 0.760$, Supporting Information Table S1, Supporting Information Text S1), while the relative abundance of *intI*1 was shown to be significantly different between quinquennia ($F_{6,10} = 7.7$, $p = 0.003$), with 1990–1994 being higher than 2000–2005 (Tukey: $p = 0.011$) (Supporting Information Table S2). Previous studies based on the analysis of the abundances of ARGs from long-term series of soil and sediment samples showed, with some exceptions, a general increase in abundance of ARGs over time (Knapp *et al*., 2010; Madueno *et al*., 2018). Although care must be taken due to the limited sampling size, such a trend was not observed in the present study (Supporting Information Text S2). As a speculation, this might be related to the different nature of the environmental matrices analysed: soils and sediments are static matrices, accumulating ARGs over long period of time, while zoo- and phyto-plankton are motile organisms, more directly influenced by water quality conditions (e.g., improved water quality due to improved wastewater treatment practices over time may have had a detectable influence on ARGs presence in the pelagic and planktonic communities).

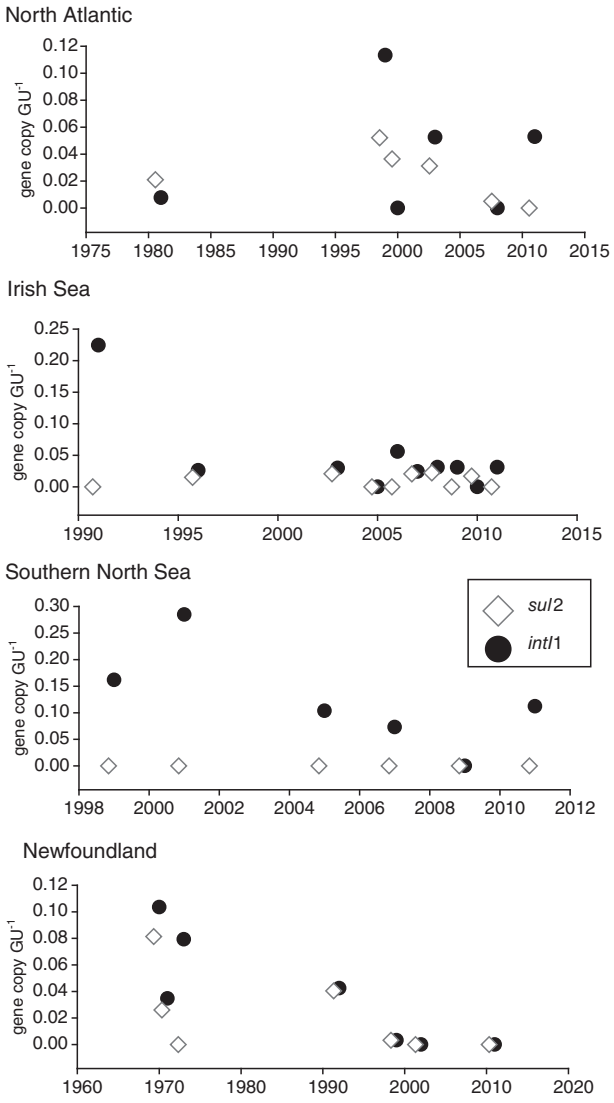

Fig. 2. Temporal changes for *sul*2 (diamond) and *intI*1 (circle) gene copies GU^{-1} as measured in the North Atlantic, Irish Sea, Sothern North Sea and Newfoundland sampling stations by ddPCR. Note that the span of the X- and Y-axis differ for each of the plots.

The abundance of *sul*2 gene was also not significantly different between geographic areas ($F_{3,10} = 2.2$, $p = 0.150$, Fig. 1, Supporting Information Table S2). In contrast, the abundance of *intI*1gene was significantly higher in the Southern North Sea than in the other

sampling sites (Tukey tests with p values between 0.007 and 0.011, Fig. 1, Supporting Information Table S3). This might be related to the high level of anthropogenic pollution in the area (Gillings *et al*., 2015). Indeed, several rivers, for example, Scheldt, Meuse, Rhine, Ems, Weser, Elbe and Thames, receive high amount of nutrients, heavy metals, and organic compounds when crossing different towns of Western Europe (Moulder and International Association on Water Pollution Research and Control, 1986).

*intI*1 and *sul*2 abundances did not correlate with any of the measured environmental variables, that is, SST, zoo- and phyto-plankton abundances, and *Vibrio* spp. concentrations. This suggests that they are not likely to play a major role in determining long-term occurrence of these genes in the investigated areas (Supporting Information Table S4). Anthropogenic influence (e.g., ARGs discharge through human pollution) and/or environmental factors other than those investigated in this study likely play a more significant role in this framework (Di Cesare *et al*., 2015).

Furthermore, co-occurrence between *intI*1 and *sul*2 genes in the analysed samples was not observed (Pearson's $r = 0.07$, $p = 0.734$), despite it recently being reported for freshwater WWTPs microbial communities (Di Cesare *et al*., 2016). These findings suggest that different factors drive the dynamics of these two genes within the ocean microbial community.

Taken together, these results support the effective use of ddPCR for the analysis of CPR samples, which also allows for the quantitative analysis of target genes that are otherwise not quantifiable by qPCR. In addition, ddPCR applied on historical CPR samples revealed long-term occurrence and spread of *sul*2 gene and class 1 integrons carrying bacteria in the ocean. Zoo- and phyto-plankton are relevant components of the aquatic food web, and they could influence the dynamics of ARGs carrying bacteria within the whole microbial community of the aquatic system.

Experimental procedures

Selection of CPR samples

CPR samples retain zooplankton and phytoplankton (Vezzulli *et al*., 2012). We selected 29 CPR samples coming from different locations: 6 from the Southern North Sea, 10 from the Irish Sea, 7 from Newfoundland (coastal sites) and 6 from the North Atlantic (pelagic site) (Fig. 1) collected in August from 1970 to 2011. SST, phytoplankton (expressed as Phytoplankton Colour Index [PCI], an index of total phytoplankton biomass) and the total number of copepods per CPR sample (a detailed description of how CPR plankton data are generated is provided by Richardson *et al*., 2006) were previously measured for 28 out of 29 samples (Supporting Information Table S1) (Vezzulli *et al*., 2016). The sample with which we could not recover such data has been excluded from the statistical analyses. DNA was previously extracted and analysed for the abundance of the total bacteria (using the primers, 967F 5′-CAACGCGAAGAACCTTACC-3′ and 1046R 5′-CGACAGCCATGCANCACCT-3′) (Sogin *et al*., 2006) expressed as GU and the abundance of *Vibrio* spp. expressed as *Vibrio* index (VAI) by qPCR (Supporting Information Table S1) (Vezzulli *et al*., 2016).

qPCR and ddPCR analyses

All the DNA samples were tenfold diluted and analysed in duplicate for *sul*2 and *intI*1 gene abundances by both qPCR and ddPCR. The sequences of primers used to amplify the targeted genes in qPCR and ddPCR were sul2F (5′-TCCGGTGGAGGCCGGTATCTGG-3′) and sul2R (5′-CGGGAATGCCATCTGCCTTGAG-3′) for *sul*2 gene (Pei *et al*., 2006) and intI1LC5 (5′-GATCGGTCGAATGCGTGT-3′) and intI1LC1 (5′-GCCTTGATGTTACCCGAGAG-3′) for *intI*1gene (Barraud *et al*., 2010). The qPCR assays were carried out in 20 μl:5 μl of DNA, 0.5 μM of each primer, 10 μl of SsoAdvanced universal SYBR Green Supermix (Bio-Rad), and filtered and autoclaved MilliQ water (Millipore) to the final volume, using a CFX96 Real-Time System (Bio-Rad). The qPCR program was 95°C for 2 min, 35 cycles of 95°C for 15 s, 60°C for 30 s and 72°C for 15 s. Melt curve analysis was performed from 60°C to 95°C with increments of 0.5°C/5 s. Standard curves were carried out as described in Di Cesare *et al*. (2013). The specificity of reaction was verified by melting profile analysis on Bio-Rad CFX Manager IDE v2.2 software (Bio-Rad) and by electrophoresis run (1% agarose gel, 80 V, 40 min) of positive samples. The efficiencies of reaction were 94% and 100% for *sul*2 and *intI*1 genes, respectively, with R^2 values always >0.99. The LOQ were determined according to Bustin *et al*., (2009) and were 9 and 3 copy μl^{-1} for *sul*2 and for *intI*1 genes respectively. The inhibition test of qPCR was carried out as described in Di Cesare *et al*. (2013) and was shown to be always less than 1.5 threshold cycle. The ddPCR assays were performed on QX200 Droplet Digital PCR System (Bio-Rad). The reaction mix was prepared assembling 11 μl of 2× QX200 ddPCR EvaGreen Supermix with specific forward and reverse primers (final concentration 150 nM), 5 μl of DNA and nuclease free grade water to a final volume of 22 μl for each sample. Aliquots of 20 μl for each reaction were thoroughly mixed, centrifuged and loaded into a sample well of a DG8 Cartridge for QX200 Droplet Generator (Bio-Rad) followed by 70 μl of QX200 Droplet Generation Oil as per manufacturer's

instructions. Droplets were carefully transferred into a clean 96-well plate for the amplification step on a Bio-Rad C1000 Touch Thermal Cycler. Amplification conditions were optimized for both the target genes using a gradient of annealing temperatures (from 56.2°C to 61°C for both *sul*2 and *intI*1 genes), testing standards used in qPCR. The final program, according to manufacturer's instructions, was 5 min at 95°C for enzyme activation, followed by 40 cycles of a two-step denaturation (95°C for 30 s) and annealing/extension (60.2°C for 1 min for both genes) with a reduced ramp rate of 2°C s^{-1}. Signal was stabilized with subsequent steps at 4°C for 5 min and 90°C for 5 min, followed by holding at 4°C. The plates were then transferred in the QX200Droplet Reader (Bio-Rad) to analyse the fluorescence signal and to acquire concentration data. Two no template controls (NTC) and two positive controls (standards from qPCR) were included together with samples in each run. Only reactions with more than 10 000 droplets were analysed (droplets were $1.54 \times 10^4 \pm 1.87 \times 10^3$ for *sul*2 and $1.64 \times 10^4 \pm 1.60 \times 10^3$ for *intI*1). Thresholds to discriminate between positive and negative droplets were manually set up and only samples with ≥ 3 positive droplets were considered as positive. An example of result output is given in Supporting Information Fig. S2. Quantification data were analysed by QuantaSoft Analysis Pro software v1.0.596 (Bio-Rad, California) and expressed as gene copy μl^{-1}. In order to evaluate the sensitivity of ddPCR protocols the standard curves were carried out as above and the LOQ were 2.1×10^{-1} and 2.3×10^{-1} for *sul*2 and *intI*1 genes respectively. The inhibition test was performed as for qPCR and no inhibition was observed. Finally, concentrations of the tested genes were expressed in a relative manner as gene copy GU^{-1}.

Statistical analysis

The first question to be addressed was whether abundances of *sul*2 and *intI*1 GU^{-1} were different between different stations and different time periods. Four stations and seven quinquennia (from 1970–1974 to 2010–2014) were considered to address the first question. Because of the scarce temporal resolution of the data set, with 29 samples from four areas across more than 40 years, single years could not be used, but time periods were identified in groups of 5 years. Analysis of variance as linear model (LM) was used to test the effect of differences between stations and quinquennia and of the interaction between stations and quinquennia. In case of significant differences, Tukey Honestly Significant Difference (HSD) test was used to identify which stations or quinquennia were different from the others. Given the inherent potential bias introduced by grouping years in quinquennia, with some stations having a wider temporal span than others, we provide additional support on temporal trends by directly performing Mann–Kendall trend tests for each station independently (Supporting Information Text S1).

Then, after assessing whether spatial (stations) and temporal (quinquennia) patterns were present, the drivers of such differences were inferred from four environmental variables. The statistical models asked whether SST, phytoplankton, copepods and VAI could influence the relative abundances of *sul*2 and *intI*1 genes. Linear models were used with the four environmental variables and no interactions as explanatory variables. Given the potential pseudoreplication problems of variables being inherently more similar between samples within the same station or the same quinquennium, linear mixed effect models (LMEMs) were used by including as random effects the stations, which came out as significant in several analyses. Before assessing the effect of environmental variables, the correlation between them was tested using Pearson multiple correlation tests. Also, a multivariate analysis of variance (MANOVA) was used to assess whether the environmental variables were influenced by the spatial (four stations) and temporal (seven quinquennia) groups, mirroring the same structure of the analyses performed for *sul*2 and *intI*1 genes (Supporting Information Text S2). All analyses were performed in R 3.3.3 (R Core Team, 2017). Model fit for linear models was visually checked by assessing normal distribution of model residuals, absence of deviations in QQ-plots, and small values of Cook's distances (Crawley, 2013). For the analyses including random effects, we used linear mixed effect models (LMEMs) with R package nlme v 3.1–131 (Pinheiro *et al*., 2017). Mann–Kendall trend tests were performed with R package trends v1.0.1 (Pohlert, 2017).

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1: Supporting information.