Does antifouling paint select for antibiotic resistance?

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HIGHLIGHTS

• Antifouling paints often contain metals that may co-select for antibiotic resistance.
• Marine microbial biofilms established on painted surfaces were studied.
• The heavy-metal based paint co-selected for certain antibiotic resistant bacteria.
• The paint did not enrich known mobile antibiotic resistance genes in the communities.
• The paint selected for RND efflux systems and genes involved in mobilization of DNA.

ABSTRACT

There is concern that heavy metals and biocides contribute to the development of antibiotic resistance via co-selection. Most antifouling paints contain high amounts of such substances, which risks turning painted ship hulls into highly mobile refuges and breeding grounds for antibiotic-resistant bacteria. The objectives of this study were to start investigate if heavy-metal based antifouling paints can pose a risk for co-selection of antibiotic-resistant bacteria and, if so, identify the underlying genetic basis. Plastic panels with one side painted with copper and zinc-containing antifouling paint were submerged in a Swedish marina and biofilms from both sides of the panels were harvested after 2.5–4 weeks. DNA was isolated from the biofilms and subjected to metagenomic sequencing. Biofilm bacteria were cultured on marine agar supplemented with tetracycline, gentamicin, copper sulfate or zinc sulfate. Biofilm communities from painted surfaces displayed lower taxonomic diversity and enrichment of Gammaproteobacteria. Bacteria from these communities showed increased resistance to both heavy metals and tetracycline but not to gentamicin. Significantly higher abundance of metal and biocide resistance genes was observed, whereas mobile antibiotic resistance genes were not enriched in these communities. In contrast, we found an enrichment of chromosomal RND efflux system genes, including such with documented ability to confer decreased susceptibility to both antibiotics and biocides/heavy metals. This was paralleled by increased abundances of integron-associated integrase and ISCR transposase genes. The results show that the heavy metal-based antifouling paint exerts a strong selection pressure on marine bacterial communities and can co-select for certain antibiotic-resistant bacteria, likely by favoring species and strains carrying genes that provide cross-resistance. Although this does not indicate an immediate risk for promotion of such resistance, it highlights the potential for long-term impacts on marine ecosystems.

Keywords:
Marine bacteria
Metagenomics
Antibiotic resistance
Metal resistance
RND efflux pump
Integron
1. Introduction

Antibiotic resistance has become a growing global health problem. Our use and misuse of antibiotics has fueled this development during the last decades, leading to more and more antibiotic-resistant bacteria circulating in human and animal populations. However, development of antibiotic-resistant bacteria is not restricted to the microbial communities thriving on or inside human and animal bodies during antibiotic treatment. Antibiotic resistance genes (ARGs) were present in environmental bacterial communities long before the antibiotic era in human history (Bhullar et al., 2012; D’Costa et al., 2011). Several studies have also shown the presence of identical or almost identical ARGs in both environmental and pathogenic bacteria (D’Costa et al., 2011; Forberg et al., 2012; Poirel et al., 2002; Poirel et al., 2005). Accordingly, environmental bacterial communities have been postulated to be key players in the emergence of antibiotic resistance by providing a pool of ARGs that through horizontal gene transfer (HGT) events can eventually end up in pathogens (Finley et al., 2013; Gaze et al., 2013). In addition, environments, and especially aquatic environments, contaminated with feces have for long been recognized as a vector for dissemination of bacterial infectious agents, including antibiotic-resistant bacteria (Finley et al., 2013; Graham et al., 2014). Thus, the external environment is believed to play a critical role in both transmission and emergence of antibiotic-resistant bacteria, processes which are expected to be promoted in the presence of an adequate selection pressure.

There is growing evidence that not only antibiotics, but also heavy metals and biocides can exert selection pressures favoring antibiotic-resistant bacteria (SCENIHR, 2009). Co-selection by heavy metals/biocides can occur if resistance determinants against these and antibiotics are present in the same cell (co-resistance) or if a common resistance mechanism, such as an efflux pump, confer resistance to both types of toxicants (cross-resistance) (Baker-Austin et al., 2006; Chapman, 2003). However, for co-resistance to play a role in direct promotion of HGT, biocide/metal resistance genes (BMRGs) and ARGs need to be present on the same mobile genetic element.

Antifouling paints form a group of products where biocides and especially biocidal heavy metals are often present at high locally potent concentrations (Thomas and Brooks, 2010) although much effort have been spent on producing more eco-friendly paints (Ciriminna et al., 2012; Ruiz et al., 2002; Poirel et al., 2005). Accordingly, environmental bacterial communities established on surfaces painted with a copper and zinc-containing antifouling paint and unpainted control samples in the marine environment, and compared them by culturing bacteria and applying metagenomic DNA sequencing.

2. Materials and methods

2.1. Field site and antifouling paint

The field experiments were conducted during the fall of 2013 in the small marina of Långedrag close to the river mouth of Göta älvs in Gothenburg on the Swedish west coast (57°40’03.0″N 11°50’51.0″E). During the sampling period, surface water (0–2 m) temperature dropped from 17 °C to 10 °C. Salinity for the same period and depth ranged from 15% to 16%. Estimates were taken from measurements at nearby Skalkorgarna (57°40’03.0″N 11°46’10.0″E), and all data are available in the open data catalog of the Swedish Meteorological and Hydrological Institute (SMHI) (http://opendata-catalog.smhi.se/explore). A commercially available antifouling paint, Fabi CT Copper (Product Code YBB122, International Paint Ltd., Gateshead, United Kingdom), was used in this study. According to the manufacturer and the safety data sheet it is a copper (Cu2O)-based paint (2%–10%, w/w) that also contains significant amounts of zinc (ZnO) (10%–25%, w/w) but no booster biocides.

2.2. Chemical analyses

The antifouling paint was analyzed with regard to copper and zinc content. The paint (approximately 0.5 g) was digested using 5 mL of heated nitric acid (65% Suprapur, Merck, Darmstadt, Germany) for 2 h. The acid extract was diluted with MilliQ water and filtered through 0.45 μm syringe filters (Filtronop S, Sarstedt, Nürnberg, Germany). Analysis was done by inductively coupled plasma optical emission spectroscopy (Perkin Elmer Optima 2000 DV). For quality control, the paint was analyzed in duplicate alongside two blanks and a certified reference material (PACS-2, National Research Council, Canada).

2.3. Sampling

Plastic panels (styrene-acrylonitrile) measuring 300 × 300 mm were painted twice on one side using a roller while the other side was left unpainted as control. Biofilms were established on the painted and unpainted surfaces by submerging the panels in the Långedrag marina, positioned approximately 1 m below the water surface by using weights connected to the bottom part of the panels. Panels were left submerged until visible biofilms had been established. The sampling procedure was performed three times, and each time three panels were used (in total nine samples from unpainted surfaces and nine samples from painted surfaces were generated). Due to the declining ambient water temperature during fall, biofilms established within 2.5 weeks during the first...
experiment while the latter two experiments required three and four weeks of submersion respectively. Upon emersion the attached microbial communities were harvested using a plastic cell scraper. The samples were kept on ice and arrived to the lab within 1 h. Each sample was divided into two parts; one was immediately used for bacterial cultures whereas the other was frozen at −80 °C to be used for subsequent DNA isolation.

2.4. Culturing of bacteria

The biofilm samples were suspended in 2% NaCl and vortexed vigorously for 1 min. The cells of the biofilm were washed three times by centrifugation at 3000 × g for 10 min followed by resuspension in 2% NaCl. Serial dilutions of the samples were plated on marine agar (Scharlab, Barcelona, Spain) supplemented with cycloheximide (100 mg/L) (Sigma-Aldrich), St. Louis, MO, USA) and either tetracycline (20 mg/L, Sigma-Aldrich), gentamicin (10 mg/L, Sigma-Aldrich), copper sulfate (2.5 mM, Merck, Darmstadt, Germany) or zinc sulfate (2.5 mM, Sigma-Aldrich) as well as on control plates supplemented with cycloheximide only. Colony forming units (CFU) were counted after 5–6 days of incubation at room temperature. Bacterial growth on agar plates supplemented with antibiotics/metals was compared to the growth on control agar plates without supplementation, and expressed as % growth of control.

2.5. DNA extraction

The biofilms were lysed and homogenized applying both enzymatic treatment and bead beating. The frozen samples were suspended in 180 μL Tris-HCl buffer, pH 8.0, supplemented with EDTA (2 mM), TritonX-100 (1.2%) and lysozyme (20 mg/mL, Sigma-Aldrich) and incubated at 37 °C for 2 h before subjected to two rounds of bead beating using a TissueLyzer (Qiagen, Hilden, Germany). In the first round, the samples were bead-milled with a single stainless bead (5 mm in diameter) and in the second round with 30 mg glass beads (150–212 μm in diameter), 30 s at 20 Hz for each round. After bead beating, the samples were again incubated at 37 °C (40 min) before total DNA was isolated from the homogenized samples using the DNeasy Blood and Tissue kit (Qiagen) including pretreatment with proteinase K and RNase according to the manufacturer’s protocol. The concentrations of eluted DNA were measured using a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Five samples from each group (two from the second sampling round and three from the third sampling round) were sent for sequencing at Science for Life Laboratories (Stockholm, Sweden), while the DNA extracted from the remaining samples unfortunately did not fulfill the quality criteria for DNA sequencing.

2.6. Metagenomic sequencing and bioinformatics analysis

The shotgun metagenomic sequencing libraries were prepared using the Rubicon ThruPLEX®-FD Prep Kit (Rubicon Genomics, Michigan, USA) and multiplexed on 8 lanes on the Illumina HiSeq2500 platform (Illumina Inc.). The flow cell was clustered using cBot and sequenced (HiSeq Control Software 2.0.12.0/RTA 1.17.21.13) with a paired-end 101 bp reads setup in High Output mode. The generated reads were de-multiplexed and the data in Bcl format was converted to Fastq using bcl2Fastq v1.8.3 from the CASAVA software suite 1.8. The raw sequence reads were quality-filtered and the Illumina sequencing adapters were removed using Trim Galore! (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) with a minimum quality score of 28 and a maximum error rate (errors per sequenced base) of 0.1 (parameters “–paired –phred33 –e 0.1 -q 28 –stringency 6”). After the quality filtering, reads shorter than 75 bp were discarded to avoid non-specific matches to resistance genes and taxa. The trimmed and quality-filtered reads were used as input for further analysis for resistance genes and taxonomic composition. The reads have been uploaded to MG-RAST, allowing for further investigation of the data.

To screen for resistance genes across all metagenomic datasets, we used the high-quality and manually curated Resqu version 1.1 (http://www.1928diagnostics.com/resdb) and BacMet predicted version 1.1 (http://bacmet.biomedicine.gu.se) databases (Pal et al., 2014). The Resqu database contains only ARGs reported to have been horizontally transferred between at least two different bacterial species and are therefore hereon referred to as mobile ARGs whereas BacMet contains genes conferring resistance to antibacterial biocides and/or metals. A set of markers for mobile genetic elements (MGEs), including integron-associated integrases and ISCR transposases, was also retrieved from the Resqu database. The resistance genes and markers of MGEs were identified in the metagenomes by mapping the DNA reads against the protein sequences retrieved from the Resqu and BacMet databases using Vmatch (http://vmatch.de/), requiring at least 90% identity over 20 amino acids (options “–showdesc 0 -dnasnprot 11 -I 20 -identity 90 -h 2”) (Bengtsson-Palme et al., 2016). Genes present in both BacMet database and CARD (https://card.mcmaster.ca/) (McArthur et al., 2013), a database containing both chromosomal and mobile ARGs, were regarded as resistance genes with cross-resistance potential.

To estimate the abundance of resistance genes and MGEs, reads mapped to resistance genes and markers of MGEs were counted and subsequently normalized to the length of the respective genes to avoid introducing bias due to length variations of genes present in the databases (Bengtsson-Palme et al., 2014; Pal et al., 2016). The length-normalized numbers were further normalized by the number of 16S rRNA sequences detected in each metagenome for deriving values of gene abundance per bacterial 16S rRNA, accounting for the average length of the 16S rRNA gene (Bengtsson-Palme et al., 2014; Pal et al., 2016).

Taxonomic assignment of extracted small-subunit (SSU) rRNA reads was performed with Meta-taxa2 (version 2.0.1) using default parameters (Bengtsson-Palme et al., 2015). Relative abundances of each taxon from each taxonomic level were calculated by normalizing 16S rRNA sequence counts for each taxon against the total sequence counts in that metagenome. All metagenomes were down-sampled to 35 million reads before alpha-diversity of the bacterial communities was assessed by determining Shannon’s index, genus richness, Chao1 index and Pielou’s evenness using the ‘vegan’ statistical package (Oksanen et al., 2016) in R version 3.2.2 (R Development Core Team, 2016).

2.7. Statistical analysis

Differences in bacterial growth and alpha-diversity measurements between unpainted and painted surfaces were tested using a two-way Analysis of Variance (ANOVA) with time and treatment as independent variables. The normality assumption was assessed using quantile-quantile plots of the residuals and data with a non-normal distribution was log-transformed. Tests with p-values < 0.05 were considered significant. Differentially abundant resistance genes were tested with an overdispersed Poisson generalized linear model in an ANOVA-like design including covariates for time and treatment (Kristiansson et al., 2009). This model has been shown to have a high performance for metagenomic gene count data generated by Illumina sequencing (Jonsson et al., 2016). The p-values were adjusted for multiple testing using Benjamini-Hochberg false discovery rate (FDR) (Benjamini and Hochberg, 1995). An FDR < 0.05 was considered statistically significant. Tests to evaluate differences in total relative abundance for groups of genes were performed under the same Poisson model including all genes in the group and assuming a gene-specific base-line. All analysis was performed in the statistical language R version 3.3.1 (R Development Core Team, 2016).
2.8. Accession numbers

The raw metagenomic sequencing data of marine biofilm samples has been deposited in the MG-RAST under the project ID20345.

3. Results

3.1. Chemical analysis of the paint

Chemical analyses of the antifouling paint showed copper and zinc concentrations of 110 g/kg (SD 18.6) and 250 g/kg (SD 34.2), respectively. The measured concentrations are comparable to the upper boundaries provided in the safety data sheet from the manufacturer.

3.2. Elevated phenotypic heavy metal and antibiotic resistance in antifouling paint-selected bacterial communities

Culturing on selective media showed significantly higher proportions of tetracycline, copper and zinc resistant bacteria in harvested biofilms from painted surfaces compared to unpainted surfaces (Fig. 1). The proportions of tetracycline, copper and zinc resistant bacteria increased nine-fold, 53-fold and four-fold, respectively. Elevated resistance against gentamicin was also observed among bacteria collected from painted surfaces, but this increase was not regarded statistically significant (p = 0.09) (Fig. 1).

3.3. Antifouling paint altered the taxonomic composition of the marine biofilms

The taxonomic compositions of the collected biofilms were analyzed based on SSU rRNA reads extracted from the metagenomic datasets. The numbers of bacterial SSU reads were in the ranges 8843–13310 and 40134–45146 in samples from unpainted surfaces and painted surfaces, respectively, whereas the average sequencing depth was fairly similar for the two groups (53 and 61 million filtered reads, respectively). The lower relative abundance of bacterial SSU reads in samples from unpainted surfaces could largely be explained by the significantly higher proportion of non-bacterial SSU reads in these samples compared to those from painted surfaces (64.5% vs 14.0%). This is most likely a result of the antifouling effect of the paint, which limits the attachment and growth of many eukaryotic organisms (Table S1). In accordance, the bacterial communities established on painted surfaces displayed a lowered diversity compared to bacterial communities from unpainted surfaces manifested by decreased Shannon’s index, genus richness, Pielou’s evenness and Chao1 index, although the latter decrease was not statistically significant (Fig. 2).

The bacterial communities from painted surfaces were dominated by the class of Gammaproteobacteria, which showed a three-fold increase in relative abundance compared to communities from unpainted surfaces (Fig. 3 and Table S2). No other classes of bacteria showed an increased relative abundance in samples from painted surfaces. The majority of the increase in Gammaproteobacteria could be ascribed the order of Alteromonadales, which in turn received its major contributions from the families of Alteromonadaceae, Pseudoalteromonadaceae and Shewanellaceae (Tables S3 and S4). These families were dominated by the genera Alteromonas, Pseudoalteromonas and Shewanella, respectively (Table S5). However, the family showing the highest relative increase was Piscirickettsiaceae with the major contribution coming from Cycloclasticus, which likewise belong to Gammaproteobacteria but not to the order Alteromonadales (Tables S4 and S5). Alteromonas and Cycloclasticus were also the two most abundant genera identified in samples from painted surfaces (Table S5).

3.4. Enrichment of known BMRGs but not known mobile ARGs in antifouling paint-selected bacterial communities

The abundances of different genes were determined in relation to bacterial SSU rRNA genes. Resistance genes that showed significantly differential abundance (FDR < 0.05) between samples from painted and unpainted surfaces are shown in Tables S6 (BMRGs) and S7 (mobile ARGs). There was an overall 46% increase in annotated BMRGs in samples from painted surfaces, which to a large extent could be attributed to genes involved in metal resistance (Fig. 4A). In line with this, there were also significantly higher relative abundances of genes known to confer resistance to copper (136% increase) and zinc (22% increase) in communities from painted surfaces (Fig. 4A). When metal resistance genes described to be situated on plasmids were analyzed separately, the enrichments of copper and zinc resistance genes in samples from painted surfaces were even more pronounced, showing a four-fold and five-fold increase, respectively (Fig. 4B).

In comparison to BMRGs, annotated mobile ARGs were much less abundant in all samples. Total relative abundance of such ARGs was on average 260 times lower than observed for BMRGs (Fig. 4A). Also in contrast to the BMRGs, there was no overall elevation in relative abundance of mobile ARGs in bacterial communities from painted surfaces. Rather there was slightly higher relative abundance of mobile ARGs in samples from unpainted surfaces (Fig. 4C). When the detected ARGs were assigned into nine different antibiotic classes, only macrolide-lincosamide-streptogramin (MLS) and trimethoprim resistance genes were enriched in antifouling paint-selected communities of which the latter was not statistically significant. Instead six of the ARG classes showed significantly higher relative abundance in bacterial communities from unpainted surfaces (Fig. 4C). Noteworthy is that seven of twelve individual mobile ARGs that showed significantly lower relative abundance in communities from painted surfaces were tetracycline resistance genes (Table S7).

3.5. Enrichment of RND efflux genes including those with cross-resistance potential in antifouling paint-selected bacterial communities

Almost half (48%) of all detected BMRGs encode proteins involved in efflux systems, of which the great majority (68%) could be connected to RND-mediated efflux. In samples from painted surfaces there was a four-fold higher relative abundance of genes encoding components of tripartite RND efflux systems (Fig. 5). Many of these systems have the ability to also pump antibiotics out from the cell and thus confer
cross-resistance between biocides/metals and antibiotics to the host (Anes et al., 2015; Li et al., 2015). A seven-fold higher relative abundance of such genes with cross-resistance potential was observed in samples from painted surfaces (Fig. 5). The individual RND efflux genes detected at higher relative levels in samples from the painted surfaces represented efflux systems whose documented substrate profiles comprise a wide diversity of antibiotic classes including aminoglycosides, amphenicols, beta-lactams, fluoroquinolones, macrolides, rifamycins sulphonamides, tetracyclines and trimethoprim.

3.6. Enrichment of genetic elements involved in mobilization of genes in antifouling paint-selected bacterial communities

Finally, we also searched the metagenomic datasets for genes encoding integron-associated integrases (intI) and ISCR transposases since such genetic elements are known to be involved in mobilization of genes, not least ARGs (Gillings, 2014; Toleman et al., 2006; Toleman and Walsh, 2011). There was an overall higher relative abundance of both intI (two-fold) and ISCR transposase genes (nine-fold) in communities from painted surfaces compared to those from unpainted surfaces (Fig. 6). Out of the eight detected intI genes, five showed significantly higher relative abundance in samples from painted surfaces, whereas only one showed significantly lower compared to samples from unpainted surfaces. The most abundant intI genes in communities from painted surfaces were intI9 and intI10. The difference in relative abundance of ISCR transposase genes between the two sample groups was clearly dominated by the elevated level of ISCR2 in communities from painted surfaces. Nevertheless, six of the nine detected ISCR transposase genes were significantly more abundant in communities from painted surfaces, whereas only one was significantly decreased.

4. Discussion

To the best of our knowledge the present study is the first to describe how antifouling paint shapes bacterial communities with regard to antibiotic resistance. Although the antifouling paint exerted a strong pressure on marine bacterial communities and selected for both heavy metal and antibiotic-resistant bacteria, the process was not accompanied by an enrichment of mobile ARGs. Rather the antifouling paint seems to have potential to select for antibiotic resistance primarily via chromosomally encoded efflux systems and cross-resistance mechanisms. The genetic profiles of the selected bacterial communities also suggest increased capacity for integron- and ISCR-mediated mobilization of genes that might include yet unknown resistance genes.

Given the high content of copper and zinc in the antifouling paint used, bacteria from painted surfaces were expected to display higher tolerance against those heavy metals. The observed increase in copper and zinc resistance served rather to confirm that the antifouling paint exerted a selection pressure on the bacterial communities established on painted surfaces under the study conditions. The increase in phenotypic tetracycline resistance among bacteria from the painted surfaces is, however, a result of co-selection. Whether this co-selection only is a result of promotion of tolerant species, or if it also involves the selection of resistant strains within species cannot be concluded. Nevertheless, both copper and zinc exposure of complex microbial communities have previously been linked to higher level of tetracycline resistance. Increased community tolerance to tetracycline have been detected in copper-contaminated soils (Berg et al., 2010; Fernández-Calviño and Bååth, 2013), whereas increased tet(A) abundance has been observed in the intestinal content of weaned pigs after dietary zinc supplementation (Vahjen et al., 2015). Even though our experimental design does not allow us to disentangle the effects of the individual components present in the antifouling paint, it is thus highly plausible that the selection/co-selection of phenotypic resistance observed in our study to a large extent is a heavy metal-mediated effect. It should also be noted that there are many antifouling paints on the market containing significantly higher levels of copper than the paint included in this study, suggesting that even more pronounced effects would be observed if these were used.

Even though we could clearly detect increased phenotypic tetracycline resistance in communities from painted surfaces, metagenomic sequencing showed a reduction of tet genes. This was in consistency with the overall decrease of known mobile ARGs, which also indicates lack of general co-selection of such genes. In a recent screening for known BMRGs and mobile ARGs in bacterial isolates from twelve different types of environments, aquatic environments stood out as one of those harboring genomes and plasmids with the lowest rates of BMRG
and ARG co-occurrence (Pal et al., 2015). If transferable to the marine environment studied here, it should together with the overall low detection of mobile ARGs in our samples provide a limited number of opportunities for co-selection due to co-resistance involving known mobile ARGs. However, a recent functional metagenomic screening of DNA isolated from marine environments revealed that the great majority of genes conferring resistance to four different antibiotics, including tetracycline, were not previously classified as ARGs (Hatosy and Martiny, 2015). From this perspective it is intriguing that Alteromonadales, comprising the genus Shewanella, was one of the most highly enriched orders in biofilms from painted surfaces. Shewanella species have been suggested to carry the progenitors of several ARGs that today circulate among clinical bacteria, to which they have reached via HGT (Poirel et al., 2004; Poirel et al., 2005; Potron et al., 2011). In addition, our analyses of MRGs suggest selection of plasmid-borne resistance in biofilms from painted surfaces.

Although our generated profiles of known mobile ARGs could not support antifouling paint-induced co-selection of antibiotic resistance, the metagenomic DNA sequencing showed increased relative abundance of RND systems involved in efflux of biocides/metal ions as well as antibiotics, which thus provide good conditions for cross-resistance in biofilms from painted surfaces. RND efflux systems are widely distributed among Gram negative bacteria and show specificity for broad and varying ranges of compounds (Anes et al., 2015; Li et al., 2015). Since many bacterial species can carry several different RND efflux systems with varying substrate profiles in their genomes there are also possibilities for increased RND-mediated co-resistance in the communities exposed to the antifouling paint. In addition, although most often reported to be chromosome-borne, RND efflux systems have been detected on conjugative plasmids (Flach et al., 2015; Norberg et al., 2014). Such localizations on mobile genetic elements suggest that RND-mediated co-selection can promote horizontal transfer of antibiotic resistance in certain cases.

It should be noted that the detected efflux systems most likely have much broader substrate ranges than indicated by their annotations. The contribution of a given efflux system to tolerance against different toxicants can also vary depending on in which species or genetic context it is expressed as shown in different E. coli and S. enterica serovar Typhimurium strains (Conroy et al., 2010; Nishino et al., 2006). Not least, this was shown for GesAB, which was one of the most abundant and enriched RND systems in samples from painted surfaces in the present study. Taken together, it is likely that there are many yet unrecognized co-selection opportunities mediated by genes detected to be enriched in the antifouling paint-exposed communities.

Among the intI genes showing increased relative abundance in biofilms from painted surfaces it is intI2 that has previously been associated with ARGs and is frequently detected in clinical settings (Gillings, 2014; Ramirez et al., 2010). However, earlier observations have shown that integron gene cassettes from various environments provide functions related to prevailing physical and chemical conditions (Koenig et al., 2009; Nemer gut et al., 2004). Conceivably, integrons are thought to be important for bacteria’s adaptation to changing conditions. Thus, it is plausible that yet unknown mobile resistance genes are embedded in integrons found in bacteria living in hostile/polluted environments. Tentatively, such integrons could include those associated with intI9 and intI10, which were highly enriched in the antifouling paint-selected communities in the present study. Indeed integrons have been pointed out as hotspots of diversity in bacterial genomes and a
A large proportion of identified gene cassettes have unknown functions (Gillings, 2014; Hall, 2012; Oliveira-Pinto et al., 2016). Beside the great diversity of gene cassettes associated with integrons, there is also a variety with regard to the associated intI genes in different environments including the marine (Abella et al., 2015). Due to this diversity of integron-associated integrases it is expected that not all such gene types are detected when metagenomic datasets are screened against the intI sequences present in the database applied here. Nevertheless, genera representing the most enriched families after antifouling paint-induced selection, including the two most abundant genera (Alteromonas and Cycloclasticus) in the biofilms from painted surfaces, have been described to carry integron-associated integrases (Abella et al., 2015). Altogether this indicates importance of integrons in the paint-induced selection process. In addition, the enrichment of ISCR transposase genes, which are the hallmarks of another class of genetic elements involved in mobilization of DNA and often associated with ARGs (Toleman et al., 2006; Toleman and Walsh, 2011), further reinforce an increased adaptive capacity of the bacterial communities from painted surfaces.

5. Conclusions

To conclude, the present study shows co-selection of antibiotic-resistant bacteria by a heavy metal-based antifouling paint in the marine environment although no such selection was observed for known mobile ARGs. However, the antifouling paint enriched for bacteria with genetic profiles suggesting an increased capacity for extrusion of antibiotics via RND efflux systems and good adaptive capacity due to capture and mobilization of genes via integrons and ISCRs. Given the bacterial density in biofilms, the presence of genetic elements promoting mobilization of DNA, and the prevailing selection pressure, surfaces painted with antifouling paint such as ship hulls might be hotspots for emergence and dissemination of resistant bacterial strains in the marine environment.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2017.01.213.
References


