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Does antifouling paint select for antibiotic resistance?

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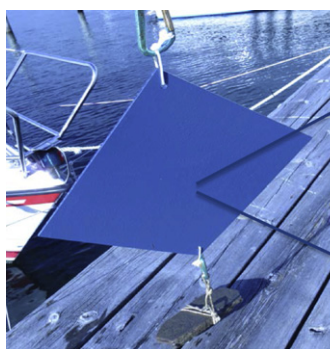
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HIGHLIGHTS

- 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.

GRAPHICAL ABSTRACT



Metal resistance ↑
 Tetracycline resistance ↑
 RND efflux systems ↑
 Integron integrases ↑
 ISCRs ↑
 Mobile ARGs ↔

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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

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mobile antibiotic resistance, the clear increase of genes involved in mobilizing DNA provides a foundation for increased opportunities for gene transfer in such communities, which might also involve yet unknown resistance mechanisms.

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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; Forsberg et al., 2012; Poirel et al., 2002; Poirel et al., 2005). Accordingly, environmental bacteria 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 often are present at highly potent concentrations (Thomas and Brooks, 2010) although much effort have been spent on producing more eco-friendly paints (Ciriminna et al., 2015; Lejars et al., 2012). These products are used extensively within the commercial shipping and recreational boating sectors to reduce drag and thus retain speed and fuel efficiency. Since the ban of tributyltin (TBT), copper has been the leading active ingredient used in antifouling paints (Yebra et al., 2004). Copper is often accompanied by another heavy metal, zinc, which is added to many formulations as a pigment and/or to control polishing rates.

The marine environment, which via hulls of ships and boats as well as other underwater constructions becomes exposed to antifouling paints, is known to harbor various bacterial species carrying ARGs and has also been pointed out as a reservoir of ARGs (Hatosy and Martiny, 2015). Not least *Shewanella* species have been suggested to carry the progenitors of several ARGs frequently found in human pathogens today (Poirel et al., 2004; Poirel et al., 2005; Potron et al., 2011). In addition, significant releases of human- and animal-derived antibiotic-resistant bacteria into marine environments have been observed worldwide (Al-Bahry et al., 2009; Fistarol et al., 2015; Sadowy and Luczkiewicz, 2014). However, it is not known how antifouling paint affects bacterial communities with regard to antibiotic resistance. If

antifouling paint would promote antibiotic resistance in the marine environment, painted hulls could not only serve as breeding grounds for antibiotic-resistant bacteria but also contribute to global dissemination of such bacteria like that seen for pathogens present in ballast water (Cohen et al., 2012; Ruiz et al., 2000).

In this study we hypothesized that antifouling paint can promote antibiotic resistance development by selecting for antibiotic-resistant bacteria in the marine environment and that ARGs would be enriched in such selected bacterial communities. To address this, we have collected biofilm communities established on surfaces painted with a copper and zinc-containing antifouling paint and unpainted control surfaces 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 älv 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°40'73.0"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 (Filtropur S, Sarstedt, Nümbrecht, 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

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