IMPACT AND MANAGEMENT OF MARINE BIOFOULING

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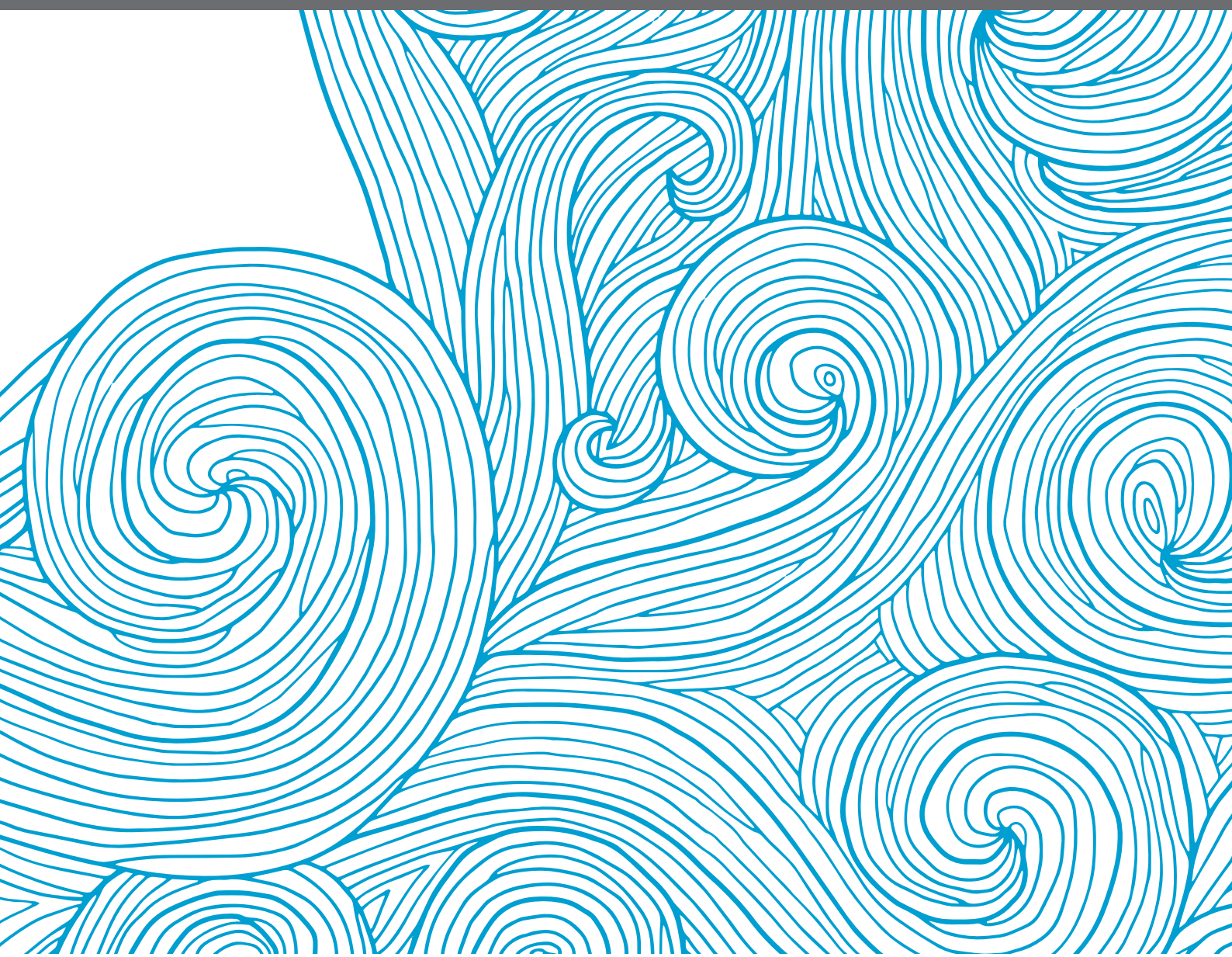

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IMPACT AND MANAGEMENT OF MARINE BIOFOULING

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EDITORIAL
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Editorial: Impact and Management of Marine Biofouling

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Editorial on the Research Topic

Impact and Management of Marine Biofouling

Biofouling, the accumulation of organisms on wetted surfaces, is a ubiquitous challenge for submerged anthropogenic marine structures that can result in reduced efficiency, increased fuel consumption, and greenhouse gas emissions from vessels. In addition, it causes the weakening and corrosion of maritime infrastructure, detrimental effects to aquaculture, and translocation of harmful non-indigenous species. The impacts of marine non-indigenous species (including macro and micro-organisms) on economic, environmental, and socio-cultural values have led to the formation of national and international regulations and guidelines to manage the risks associated with the vessel biofouling pathway.

The objective of this Research Topic was to address the impact and management of marine biofouling towards sustainable transportation and energy, to fill the gaps in the literature, and hence contribute to and update the evidence-base in this field (**Figure 1**).

Due to the aforementioned negative effects, it is desirable to minimize the accumulation of biofouling on marine vessels (Davidson et al., 2021; Riley et al., 2022; Chan et al., 2022) and structures (Hopkins et al., 2021; Briand et al., 2022). Fouling-control coatings are predominantly used to manage marine biofouling on vessels. An ideal coating should be effective against biofouling organisms over the duration of the in-service period and remain smooth enough to improve the surface properties of the vessels and structures to which they are applied. Biocidal active ingredients continue to receive considerable scrutiny at national and international regulatory levels, however, a balance must be achieved regarding the benefits of coating efficacy, which includes environmental benefits, and potential impacts to environmental values (Arabshahi et al., 2021; Gomez-Banderas et al., 2022). Other management strategies being considered across the variety of marine structures include, but are not limited to, marine growth prevention systems (Davidson et al., 2021), native species enhancement (Hopkins et al., 2021), bubble nets (Hopkins et al., 2021), acoustics (Hopkins et al., 2021), ultra-violet light (Whitworth et al., 2022), and proactive and reactive in-water cleaning technologies (Tamburri et al., 2021; Swain et al., 2022; Ralston et al., 2022).

The application of new biofouling management strategies for marine vessels, and mobile and immobile structures, require assessments of efficacy, practicality, feasibility, and environmental impacts (Arabshahi et al., 2021; Hopkins et al., 2021; Gomez-Banderas et al., 2022; Ralston et al., 2022)

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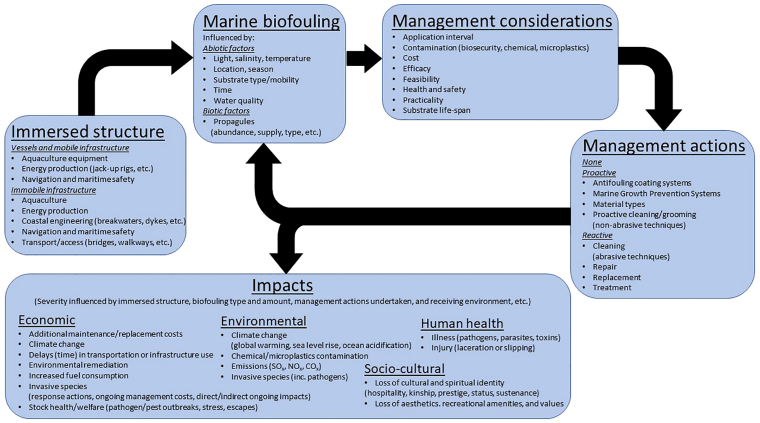

FIGURE 1 | Influences, management considerations, and impacts of marine biofouling on immersed structures.

to enable evidence-based decision making (Tamburri et al., 2021; Swain et al., 2022; Gomez-Banderas et al., 2022).

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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REVIEW
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Managing Biofouling on Submerged Static Artificial Structures in the Marine Environment – Assessment of Current and Emerging Approaches

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The number, extent, diversity, and global reach of submerged static artificial structures (SSAS) in the marine environment is increasing. These structures are prone to the accumulation of biofouling that can result in unwanted impacts, both immediate and long-term. Therefore, management of biofouling on SSAS has a range of potential benefits that can improve structure functions, cost-efficiency, sustainability, productivity, and biosecurity. This review and synthesis collates the range of methods and tools that exist or are emerging for managing SSAS biofouling for a variety of sectors, highlighting key criteria and knowledge gaps that affect development, and uptake to improve operational and environmental outcomes. The most common methods to manage biofouling on SSAS are mechanical and are applied reactively to manage biofouling assemblages after they have developed to substantial levels. Effective application of reactive methods is logistically challenging, occurs after impacts have accumulated, can pose health and safety risks, and is costly at large scales. Emerging technologies aim to shift this paradigm to a more proactive and preventive management approach, but uncertainty remains regarding their long-term efficacy, feasibility, and environmental effects at operational scales. Key priorities to promote more widespread biofouling management of SSAS include rigorous and transparent independent testing of emerging treatment systems, with more holistic cost-benefit analyses where efficacy is demonstrated.

Keywords: antifouling, aquaculture, biocorrosion, biosecurity, energy, marinas, ports, coastal infrastructure

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INTRODUCTION

A burgeoning human population is dramatically increasing the number, extent, diversity, and reach of submerged static artificial structures (SSAS) in the marine environment (Firth et al., 2016; Todd et al., 2019; Floerl et al., in press). Over the last century, floating pontoons, industrial water-use structures (e.g., power plants), oil and gas rigs, desalination plants, marine-farming installations, wave buoys, turbines, and other renewable-energy infrastructure have added to increasing amounts of traditional structures (Bugnot et al., 2020; Floerl et al., in press). All of these structures are subject to constant colonization pressure by microorganisms, macroalgae, and invertebrates – commonly

referred to as biofouling (Wahl, 1989) – with wide-ranging consequences for SSAS functions and the environment.

Although the adverse effects of biofouling on ships have been well known and subject to management activities since ancient times (e.g., Phoenicians 1300 BCE, Woods Hole Oceanographic Institute [WHOI], 1952), recognition of biofouling impacts on SSAS are relatively recent. For example, engineers in the Netherlands in the 1730s were caught unawares by disastrous effects of shipworm (*Teredo navalis*) on wooden revetments of the country's seawalls (Sundberg, 2015), even though ships had been actively managing against these effects for centuries (Woods Hole Oceanographic Institute [WHOI] 1952). Contemporary parallels are occurring in Venice as biofouling accumulations hamper the installation and functioning of its novel flood barrier system (MOSE; see Giovannini, 2017). Sea-water intakes and associated internal pipework of coastal factories, desalination plants, and power stations also have a well-documented history of problems from biofouling, particularly bivalves (Satpathy et al., 2010; Polman et al., 2013). In aquaculture operations, biofouling can result in biocorrosion (Li and Ning, 2019; Omran and Abdel-Salam, 2020), increased weight loading and hydrodynamic drag (Macleod et al., 2016; Vinagre et al., 2020), and production losses (e.g., occlusion, disease, competition, escape, or stock drop off) (Georgiades et al., 2016; Bannister et al., 2019). In nuclear power plants, biofouling has caused significant pressure drops in cooling water systems that impose serious production penalties and can instigate safety concerns (Neitzel et al., 1984, Satpathy, 1999). All of these direct outcomes necessitate heightened engineering considerations prior to installation to cope with loss of hydrodynamic performance, weight loading scenarios, and impaired functioning that requires increased initial investment, operating, and maintenance costs (Jenner et al., 1998; Polman et al., 2013).

The environmental effects of SSAS and ecological role of their biofouling communities are becoming increasingly important as hardened coastlines (e.g., seawalls and revetments) and other types of artificial structures proliferate worldwide. SSAS biofouling affects functioning of nearshore marine ecosystems including population and community distribution processes (Chapman, 2003; Bulleri and Chapman, 2010), and biogeochemical processing (Malerba et al., 2019). SSAS (particularly in ports and harbors) are often the first point of establishment for marine non-indigenous species (NIS) (Floerl et al., 2009; Bell et al., 2011; Woods et al., 2019) and associated pathogens (e.g., *Bonamia ostreae,* Howard, 1994; Ostreid herpesvirus, Whittington et al., 2018; Pagenkopp-Lohan et al., 2020), and subsequently act as 'reservoirs' that facilitate spread to the surrounding natural environment (Glasby et al., 2007). The down-stream implications of NIS and pathogens can be catastrophic and irreversible, impacting other industries, the environment, and cultural practices (International Maritime Organization [IMO], 2004, 2011; Molnar et al., 2008; Mineur et al., 2012; Georgiades et al., 2021). As a result, vector management requirements for ballast water have been established worldwide (International Maritime Organization [IMO], 2004), several jurisdictions have enacted stricter border standards regarding vessel biofouling (Ministry for Primary Industries, 2014; California Code of Regulations, 2017), and domestic vessel movements among regions are increasingly scrutinized and managed (Cunningham et al., 2019). Despite these enacted and emerging vector controls, biofouling of static infrastructure remains an important node of species populations that requires further research to understand and mitigate risk. For example, to support the early detection and response to NIS incursions, surveillance programs have been developed to determine the presence and distribution of NIS in urbanized environments, with emphasis on submerged coastal infrastructure (e.g., Seaward et al., 2015; Woods et al., 2019; McDonald et al., 2020).

Minimizing biofouling on maritime vessels remains a focus of significant global research and development – forming the basis of a multi-billion dollar "antifouling coating" industry – because of impacts on speed, manoeuvrability, operability, and durability (Woods Hole Oceanographic Institute [WHOI], 1952; Yebra et al., 2004; Callow and Callow, 2011). More recently, vessel biofouling management has been further incentivized by guidelines and regulations regarding NIS translocations (Davidson et al., 2016; Georgiades et al., 2020a). By comparison, managing biofouling on SSAS has received limited attention, with a *status quo* of resigned acceptance of impacts and consequences. This dichotomy reflects clear economic incentives, which are well-documented for maritime vessels where even 'light' biofouling causes hydrodynamic penalties that can dramatically increase power requirements, fuel usage, and associated emissions (Townsin, 2003, International Maritime Organization [IMO], 2011; Schultz et al., 2011). The benefits of preventing biofouling on SSAS are less apparent or longer term, but there is a growing awareness that unmanaged fouling on these structures can also threaten economic, environmental, and socio-cultural values (e.g., Polman et al., 2013; Atalah et al., 2020). Proactive biofouling management of these structures is often context specific or experimental, but there is an emerging array of approaches and tools that could offer viable solutions for broad applicability, scalability, and cost-effectiveness.

The objectives of this assessment are to highlight the current impacts of biofouling on SSAS and to review current and emerging approaches to biofouling management on these structures. In the context of this article, "management" is restricted to approaches for preventing, removing and/or rendering biofouling on SSAS non-viable. While ecological engineering approaches can also be used to limit the abundance of unwanted or harmful biofouling organisms (e.g., NIS) – by enhancing the development of desirable species on SSAS – this was not included in our review, whose emphasis is on prevention or minimization of all biofouling. Ecological engineering is a growing field in marine science and conservation and opportunities, frameworks, and methods have been reviewed and presented elsewhere (Dafforn et al., 2015; Airoldi et al., 2021; Floerl et al., in press).

Firstly, an overview of motivations for biofouling management is provided for various categories of SSAS, along with existing biofouling management approaches. Then, emerging approaches are identified, including some that are untested at operational scales or yet to be applied to SSAS (e.g.,

vessel biofouling management approaches that may solve SSAS issues). For each existing and emerging approach identified, prospective performance characteristics are qualitatively evaluated against criteria of feasibility, effectiveness, biosecurity, and collateral effects. These criteria can inform a set of research and development priorities to guide technological development and enable industries, governments, and other agencies to minimize the consequence of SSAS biofouling.

EXISTING APPROACHES TO BIOFOULING MANAGEMENT ON STATIC INFRASTRUCTURE

Three categories of SSAS were the primary focus of this review: aquaculture, energy production, and port and marina infrastructure (see **Figure 1** for example images). There are SSAS that fall outside of these categories (e.g., recreational jetties, bridges, outfalls, dykes, and some groins and breakwaters) and some of them have relevance with regard to biofouling and the spread of NIS (Airoldi et al., 2015). However, in most cases they are either treated using the same approaches identified for the three categories assessed or not presently managed for biofouling within their service life.

Two approaches were taken to build a reference library of accounts of SSAS biofouling and its management. Firstly, a standardized literature search was performed using Web of Science, Google Scholar, and Google Search. Keywords selected were "marine infrastructure," "pontoon," "wharf," "pile," "piling," "jetty," "sea wall," "seawall," "break wall," "breakwall," "rig," "installation," "marine farm," "aquaculture," and "turbine" associated with any of the following (with fouling or biofouling as a prefix): "management," "maintenance," "inspection," "cleaning," "defouling," "removal," and "control." Secondly, unpublished documents and information from websites were obtained from various companies (globally) who either manage or own infrastructure (e.g., marina companies), or undertake maintenance on coastal infrastructure (e.g., commercial divers).

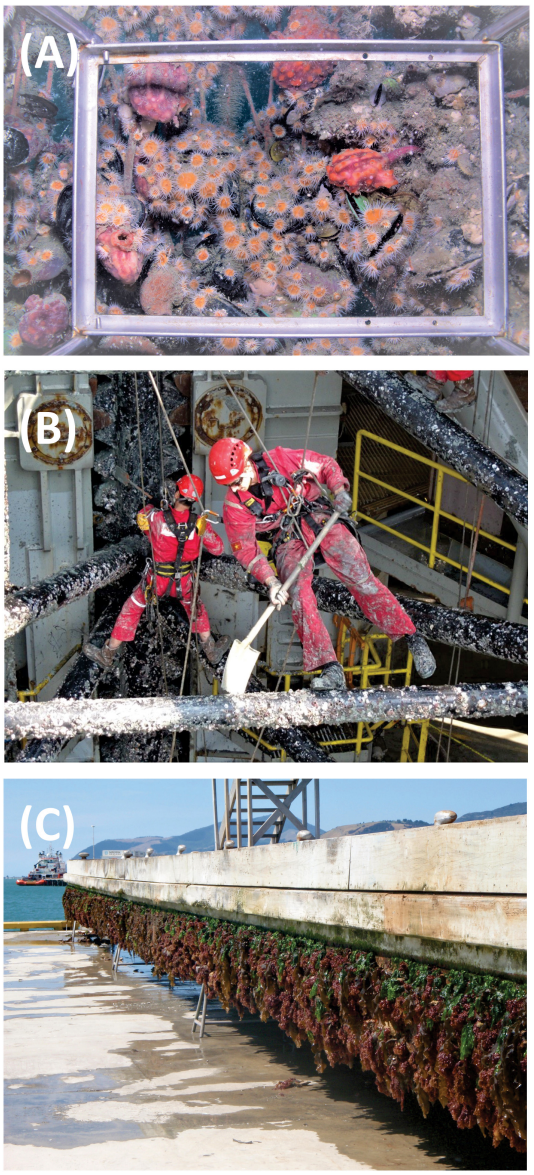

FIGURE 1 | Biofouling occlusion of a salmon farm predator net **(A)**, removal of biofouling from a jack-up drilling rig while it is being transported on-board a heavy-lift vessel **(B)**, and a superyacht marina berth removed from the water to undergo maintenance **(C)**. Image credits: Cawthron Institute.

Aquaculture

The intensity of biofouling management on marine farms varies by the species cultured and environmental conditions, and can include management of infrastructure (e.g., anchor warps, lines, nets, and cages) and the stock itself (e.g., bivalves). The key motivations for biofouling management on finfish farms include reduced water flows and dissolved oxygen levels via net pen occlusion, increased disease risk due to increased stress and direct interactions with biofouling communities, and impairment of infrastructure due to loading and impingement (Fitridge et al., 2012; Bannister et al., 2019). Direct operational impacts are the primary driver of management to reduce the likelihood of these types of impacts. Bloecher and Floerl (2020) calculated direct biofouling management costs for a typical Norwegian salmon farm (eight production pens) to be US$420,000 to $493,600 per production cycle (excluding farm personnel costs), equating to 2.2% of production costs for individual sites. Biofouling species common to aquaculture structures can act as reservoirs and amplifiers of pathogens (Costello et al., 2021), which can impact stock or broader disease dynamics within a seascape. There is growing recognition of the role of aquaculture in spreading marine pests and pathogens (Sim-Smith et al., 2016), and industry codes of practice and government guidance documents have been developed to improve outcomes in New Zealand and elsewhere (Georgiades et al., 2016, 2020a,b; Ministry for Primary Industries and Aquaculture New Zealand, 2016).

Biofouling on shellfish farms can result in physical damage to stock, mechanical interference with harvesting gear, competition for food and space, environmental modification, and

TABLE 1 | Existing approaches to managing biofouling on static submerged marine infrastructure.

Method	Category	Frequency	*In situ* or Removed	Examples/References
Mechanical brushes/scraping/abrasion	Physical	Periodic	Both	Do (1991); Hodson et al. (1997), Guenther et al. (2011), and Li et al. (2020)
High pressure jets and power washing	Physical	Periodic	Both	Forrest and Blakemore (2006) and Hopkins et al. (2010)
Air exposure/desiccation	Physical	Periodic	Both	Forrest and Blakemore (2006) and Hopkins et al. (2016)
Encapsulation (also with chemical additives)	Physical and chemical	Periodic	*In situ*	Inglis et al. (2012); Atalah et al. (2016), and Ammon et al. (2019)
Traditional biocidal paints	Chemical	Continuous	Removed (majority)	Braithwaite et al. (2007); de Nys and Guenther (2009), Guenther et al. (2011), and Bloecher et al. (2015)
Sprays and dips	Chemical	Periodic	Both	Denny (2008); Guenther et al. (2011), Fitridge et al. (2012), and Sievers et al. (2019)

In situ or removed' refers to whether the method can be applied to an existing structure as they are found (submerged), or only apply to new builds, or otherwise require removal from water.

hydrodynamic drag and weight on both stock and infrastructure (Dürr and Watson, 2010; Fitridge et al., 2012; Georgiades et al., 2016; Bannister et al., 2019). Adams et al. (2011) found that management costs for biofouling on US shellfish farms approximates 15% of total operating costs (excluding any loss in productivity). As with finfish farming, disease outbreaks are a major disruptor for shellfish farm operations and non-stock biofouling can exacerbate those risks while also occupying space that would otherwise be profitably taken by stock species.

A broad range of approaches has been used to manage biofouling growth on marine farm stock and infrastructure (**Table 1**), including the use of biocidal paints (de Nys and Guenther, 2009; Bannister et al., 2019), high pressure jets/power washing (Forrest and Blakemore, 2006), chemical sprays and dips (Denny, 2008; Guenther et al., 2011; Fitridge et al., 2012), encapsulation (Atalah et al., 2016), and removal for cleaning (e.g., cleaning of finfish farm predator nets). Physical and mechanical removal of biofouling are the main management approaches for shellfish aquaculture, while finfish operations tend to use antifouling coatings (e.g., copper-based paints), undertake regular net changes and/or cleaning and, in rarer cases, use biological control (Fitridge et al., 2012; Georgiades et al., 2016; Bloecher and Floerl, 2020).

Most measures employed are reactive (i.e., address biofouling after it has established), inefficient, can have undesirable consequences (e.g., chemical contamination and pathogen transfer, Sandberg and Olafsen, 2006; Erkinharju et al., 2020), and can be too costly to apply at an appropriate frequency (Cahill et al., 2021). There is a trend for aquaculture activities to move away from coastal zones into more open seas, partly to reduce adverse effects on the environment (Carballeira Braña et al., 2021). This is likely to make it more difficult to remove stock and infrastructure from the water for biofouling management and, consequently, encourages more proactive or continuous approaches. One such approach is the development of a system to "flip" oyster baskets (at the surface) periodically to desiccate biofouling[1] (accessed June 25, 2020).

[1]https://www.flipfarm.co.nz

Energy Production

Accumulation of biofouling on energy production infrastructure can increase drag (Macleod et al., 2016; Vinagre et al., 2020), affect cathodic protection (leading to corrosion), create micro-environments that encourage microbial corrosion (Blackwood et al., 2017; Li and Ning, 2019), reduce water flow in cooling systems (Venkatesan and Murthy, 2009), compromise health and safety of operators (e.g., sharp or slippery fouling on stairs and ladders), and endanger the entire plant (Satpathy and Rajmohan, 2001). Considerable costs are associated with over-engineering to combat hydrodynamic impacts and weight loadings during SSAS development as well as the requirements for extensive inspection and ongoing post-deployment maintenance (Klijnstra et al., 2017; Loxton et al., 2017).

Several approaches have been developed for preventive or reactive treatment of biofouling within land-based industrial water-cooling systems (e.g., power plants and water treatment plants) that use bulk seawater (Rajagopal and Van der Velde, 2012). Treatments based on chlorine and heat exposure are the most common due to their versatility and cost effectiveness (Jenner et al., 1998; Venkatesan and Murthy, 2009; Satpathy et al., 2010; Costa et al., 2012).

For the offshore energy sector, the impacts of biofouling on submerged infrastructure also include harboring and spreading NIS (Yeo et al., 2010; De Mesel et al., 2015; Capel et al., 2019; Iacarella et al., 2019; Coolen et al., 2020). Many offshore structures, such as drilling rigs and oil platforms, remain static for extended periods during operations and layups, typically leading to extensive biofouling growth (Hopkins and Forrest, 2010; Georgiades and Kluza, 2017; Gormley et al., 2018). This can represent a major invasion risk that can involve translocations of entire communities, including biofouling or reef-forming species and associated mobile fauna (Foster and Willan, 1979; Wanless et al., 2010; Yeo et al., 2010). When biofouling becomes extensive, or when structures require mitigation prior to deployment to meet biosecurity requirements (e.g., Ministry for Primary Industries 2014), there are limited or challenging options available for removal and/or treatment (Hopkins and Forrest, 2010; Iacarella et al., 2019). A key difference between drilling

rigs and other energy infrastructure, such as wind farms and wave turbines, is that some rigs can be relocated to maintenance facilities or to other sites of operation. Biofouling removal can occur while a rig remains in-water or while on-board a heavy-lift vessel (Hopkins and Forrest, 2010; Hopkins et al., 2011).

Removal of established biofouling assemblages often requires long-term planning due to the limited facilities that can accommodate energy production structures. This "reactive" practice is typically mechanical (e.g., hydro-blasting, cavitation, diver-, and remotely operated vehicle (ROV)-operated brushes) and must be re-applied frequently, often leading to high overall cost and feasibility issues. Because antifouling coatings are designed almost exclusively for use on ship hulls and require water flow to remain effective (Dafforn et al., 2011; Larsson et al., 2016; Xie et al., 2019), they are rarely applied in energy production. Instead, coatings seen on energy production structures are designed to limit or manage corrosion (e.g., epoxy-polyurethane duplex coating systems or metal sprayed coatings; Versowsky, 2005; Sørensen et al., 2009; Eom et al., 2020) as stipulated by ISO standards (International Organization for Standardization [ISO], 2016). Biofouling on niche areas of rigs, such as sea chests and internal pipework, is generally managed similarly to vessels, e.g., regular inspections, chemical dosing, cathodic protection, physical removal, and the application of antifouling paints for sea chests (Davidson et al., 2016; Growcott et al., 2017; Georgiades et al., 2018). Omran and Abdel-Salam (2020) proposed future avenues to combat biofouling in the oil and gas industry, including plant-based biocides (e.g., aqueous extracts of lupin seed and citrus peels) to treat micro- and macro-fouling, and polymer coatings to combat corrosion (e.g., polymers combined with biocides, antibacterial polymers containing quaternary ammonium compounds, and conductive polymers).

We are aware of one product, Marine Growth Protector Rings, that have been developed to continually clean surfaces on offshore installations (Do, 1991). These devices consist of buoyant rings, linked by vertical linkages, that are powered up and down column-shaped surfaces by waves and tides. While performance evaluations were unavailable, it is claimed that these devices can remove existing fouling (reactive) and prevent fouling from reoccurring (continuous).

The planned service life of an offshore wind structure typically exceeds 20 years; therefore, to avoid large-scale and costly repairs, they are designed to resist mechanical damage, physical and environmental loadings, and chemical deterioration (Price and Figueira, 2017). Intertidal and splash zones of an offshore wind structure are typically protected from corrosion by applying multi-layer epoxy-based coatings with a polyurethane topcoat prior to installation (Momber et al., 2015). Subtidal components typically use a combination of coatings and cathodic protection. Repairing coatings on offshore wind structures can be very difficult and expensive, costing up to 50 times more than the initial application (Price and Figueira, 2017). These issues could be overcome with more resilient or self-healing coatings (Cho et al., 2009; Stankiewicz et al., 2013; Zhang et al., 2018).

Wave-power generation (e.g., through wave energy convertors) is not a widely employed commercial technology compared to other established renewable energy sources, but there are now installations off the coasts of the United Kingdom, Portugal, Australia, Sweden, and the United States. Simulations by Yang et al. (2017) showed that biofouling on mooring lines and power cables associated with wave energy converters reduced energy performance (up to 17%) and caused significant reductions (up to 76%) in the fatigue life of moorings. Wave energy converters have been designed for 20–25 years of maintenance-free service, however, supporting infrastructure such as safety lines, underwater cables, and subtidal equipment may be prone to biofouling (Rémouit et al., 2018). As an emerging technology, there is little information on biofouling management approaches for wave energy converters, although manual or mechanical cleaning via diving and ROVs may be required (Rémouit et al., 2018) and should be considered in economic forecasting.

Port and Marina Infrastructure

Port and marina environments contain vast arrays of SSAS, including breakwalls, pontoons, wharves and jetties, seawalls, navigational markers, and launching ramps. These structures are typically made of concrete, steel, plastic and wood, and serve a variety of functions to harden shorelines, protect maritime access and infrastructure, or support on-water activities. A recent assessment found that shipping and boating related SSAS occupied on average 20% of coastlines associated with 30 coastal urban centers around the world, with marina pontoons making up the highest proportion, followed by commercial wharves and jetties (Floerl et al., in press).

The need for proactive biofouling management in port and marina environments varies substantially among the types of structures. For example, loose rock or concrete breakwalls that protect coastal transit routes are not managed for biofouling accumulation. Biofouling is rarely managed unless it affects the operation of floating jetties or pontoons. For most marina structures, biofouling growth is either not managed during the service life of submerged structures, periodically removed by scraping (by surface personnel), or they are opportunistically cleaned (often by water blasters) when removed to land for other maintenance needs. In some cases, coatings, preservative treatments (treated wood), or 'sleeving' is employed as a proactive treatment of materials to prevent or reduce deterioration (including deterioration caused by biofouling and bioeroders). Damage from ship worms (*T. navalis*) and wood boring isopods (e.g., *Sphaeroma* spp.) to timber structures (e.g., wharf piles) can lead to complete failure of structures (Tsinker, 2004), meaning chemical treatments (e.g., linseed oil, biocides, and creosote) or physical barriers (e.g., plastic or copper sheathing) are deployed. However, no treatment method has been found to be completely effective to date (Sundberg, 2015).

In commercial ports, concrete or wood pilings can number in the thousands and are continually monitored by port dive teams as part of annual inspection schedules, which often includes removal of biofouling to assess structural integrity of materials (Tsinker, 2004). In this case, biofouling is removed in patches as it hinders inspection, although the broader impacts of biofouling for increased weight, drag forces, and acceleration of

TABLE 2 | Emerging approaches to managing biofouling on submerged marine infrastructure.

Method	Category	Frequency	*In situ* or Removed	Examples/References
Autonomous and remotely operated cleaning systems	Physical	Both	*In situ*	https://www.jotun.com/us/en/b2b/news/hull-skating-solutions/; https://www.ecosubsea.com/(September 12, 2020)
Laser radiation	Physical	Periodic	*In situ*	Kostenko et al. (2019)
Bubble streams	Physical	Continuous	*In situ*	Scardino et al. (2009); Bullard et al. (2010), Lowen et al. (2016), and Hopkins et al. (2021)
Ultrasound	Physical	Periodic	*In situ*	Guo et al. (2013, 2011) and Legg et al. (2015)
Electrical fields	Physical	Continuous	*In situ*	www.electroclear.co.nz (accessed May 13, 2020)
Heat	Physical	Periodic	Both	Wotton et al. (2004); Piola and Hopkins (2012), Cahill et al. (2019b), and Sievers et al. (2019)
Novel coatings and surface materials	Physical and chemical	Continuous	Removed (majority)	Hodson et al. (2000; silicone), Scardino et al. (2003), de Nys and Ison (2004; wax), Carman et al. (2006); Schumacher et al. (2007), Bakker et al. (2011; food grade oils), Carteau et al. (2014); Azemar et al. (2015), Gibson and Arun (2016); Yang et al. (2018), and Ye et al. (2019)
Biological control	Biological	Continuous	*In situ*	Enright et al. (1984); Lodeiros and García (2004), Ross et al. (2004); Switzer et al. (2011), and Atalah et al. (2014)

corrosion are also acknowledged in port construction (Tsinker, 2004). Navigational buoys are periodically maintained (e.g., physical scraping and water blasters) to prevent sinking due to excess loading (Mitchem et al., 2007). Permanent systems to proactively manage biofouling are largely absent for the majority of infrastructure found in ports and marinas. A lack of biofouling removal or prevention can be compounded by the semi-enclosed nature of these environments leading to vastly increased rates of biofouling development in ports and marinas compared to adjacent areas (Floerl and Inglis, 2003).

Wrapping or encapsulation in plastic or fabric has been used to eliminate NIS on SSAS associated with ports and marinas, and on vessels (Anderson, 2005; Coutts and Forrest, 2007; Roche et al., 2015). To date, this approach has primarily been adopted to remove particular NIS as part of eradication or incursion response efforts rather than a biofouling removal technique, even though the effect is typically all encompassing (i.e., wrapping affects all target and non-target taxa). There can be questionable efficacy without the addition of chemical treatment within the wrap and some maintenance and monitoring of encapsulations (Inglis et al., 2012). The potential loss of plastic to the environment is also a drawback, as is the amount of waste generated over time (in most cases, wraps are not reusable). Encapsulation materials are likely to become fouled over time unless they have antifouling properties, and there is some time sensitivity to the approach to ensure they do not become heavily fouled themselves, potentially exacerbating the problem. The development of improved encapsulation fabrics to assist with ease and speed of deployment would be beneficial, especially if they can be reused and reapplied without significant cost or environmental risk (e.g., detachment of NIS, see Coutts et al., 2010).

EMERGING BIOFOULING MANAGEMENT APPROACHES

Our review of the literature identified a range of existing and emerging approaches to prevent and manage biofouling on static infrastructure, including approaches initially developed for vessels that could be refined for SSAS (**Table 2**).

Physical/Mechanical Methods

Remotely operated marine robotics have been developed to remove biofilms and macrofouling from vessel hulls[2,3] (accessed September 12, 2020). Similarly, Kostenko et al. (2019) reported on the use of ROVs to inspect and clean vessel submerged surfaces using laser radiation. Remotely operated brush systems are used to remove fouling from finfish farm production nets (e.g., Bloecher and Floerl, 2020). It is reasonable to expect that systems could be developed to continuously inspect and clean other categories of SSAS. However, there are challenges to overcome, such as heterogeneous surfaces, the sometimes disconnected nature of SSAS, and power supply for devices.

Continuous bubble streams, or micro-bubbles, have shown promise in preventing biofouling accumulation on vessels (Scardino et al., 2009) and submerged marina materials (Bullard et al., 2010). More recently, Hopkins et al. (2021) undertook laboratory and field trials to determine the efficacy of continuous bubble streams over surfaces, focusing on materials typically used to construct marina pontoons (concrete and polyethylene) and with and without foul- release coatings typically used on vessels. No macroscopic fouling developed on treated panels after exposure in the marine environment for 4 months. By contrast, untreated control panels were completely covered by mature fouling assemblages. While there are several barriers to upscaling this approach, including the initial cost of installation, power consumption, and ongoing maintenance (e.g., keeping bubble diffusers fouling-free), the approach is promising as a potential 'set-and-forget' proactive antifouling solution for static structures (Hopkins et al., 2021).

Legg et al. (2015) reviewed acoustic methods for biofouling management and concluded that ultrasonic (>20 kHz) frequencies were preferable to audio frequencies (20 Hz to

[2]https://www.jotun.com/us/en/b2b/news/hull-skating-solutions

[3]https://www.ecosubsea.com/

20 kHz) due to potential environmental effects associated with underwater noise and the potential to increase biofouling settlement at certain frequencies on sound generating surfaces (see Wilkens et al., 2012; McDonald et al., 2014). Equipment used to generate ultrasonic frequencies typically include a signal generator/power amplifier and transducers, with antifouling effectiveness decreasing with distance from the transducer (Legg et al., 2015). Results indicate that lower ultrasonic frequencies may be suitable for treating vessel hulls, but optimal operating parameters must be refined to cater for a broad range of fouling organisms (Legg et al., 2015). Legg et al. (2015) also highlighted the need for greater rigor when testing efficacy to ensure proper replication, controls, quantitative response variables, and photographic documentation of responses. These considerations apply broadly, including for some emerging possibilities using electric fields to prevent biofouling accumulation[4] (accessed April 12, 2021).

Heat has shown promise as an environmentally friendly tool to manage biofouling on exterior hulls (e.g., Hull Surface Treatment[5] accessed August 2, 2021), internal pipework (Cahill et al., 2019b), and sea chests (Piola and Hopkins, 2012) of vessels. Use on SSAS has been limited but has included an eradication attempt of the Asian kelp *Undaria pinnatifida* found on a shipwreck (Wotton et al., 2004), and on natural shoreline habitats (Hunt et al., 2009). Sievers et al. (2019) tested combinations of treatment (heat and two acids) on two aquaculture species (a mussels and oyster) as well as three common biofouling pests (a hydroid and two ascidians). They observed varying levels of success with some of the pest species targeted, and for all but one species (the ascidian *Styela clava*), were able to attain effective treatments without adversely affecting the culture species.

Biological Control

Biological control to manage biofouling on marina pontoons, wharf piles, and aquaculture structures has shown promise (Enright et al., 1984; Lodeiros and García, 2004; Ross et al., 2004; Switzer et al., 2011). Atalah et al. (2014) found that two species of gastropod (*Cookia sulcata* and *Haliotis iris*) could largely prevent biofouling development over 3 months on pre-cleaned marina pontoons, including inhibiting colonization of several high-profile NIS. *Cookia sulcata* was also effective in reducing established fouling cover and biomass on wharf piles by ≈70% over a 3-month period. However, retention rates for *C. sulcata,* the most promising candidate on pontoons, was relatively low (<50% after 20 days and <20% after 60 days) when not contained within a cage. Retention without additional infrastructure is likely needed for widespread adoption since fouling of the cage must also be considered.

Biological approaches to marine biofouling management have the potential to result in unanticipated impacts to the wider ecosystem (Atalah et al., 2013). The use of native natural enemies, termed augmentative biocontrol (Eilenberg et al., 2001), undoubtedly reduces this risk compared to classical biocontrol approaches (Atalah et al., 2014), but nonetheless the potential for non-target effects must be considered prior to implementation.

Novel Coatings and Surface Materials

Ideally, extensive global research to develop alternative coating technologies (Gu et al., 2020; Yan et al., 2020) will provide non-polluting alternatives that reduce biofouling build up on SSAS. There has been a wealth of research into environmentally benign alternative antifouling biocides based on bioactive natural products. A wide range of antifouling bioactives have been discovered from marine and terrestrial invertebrates, plants, and microbes (Qian et al., 2015; Liu et al., 2020), with varied modes of action (Qian et al., 2013). The potency of some natural products compares favorably to available biocides and booster biocides, and in some instances synthetic or semi-synthetic derivatization has further improved potency relative to the parent molecule (e.g., Moodie et al., 2017; Wei et al., 2017; Almeida et al., 2018). However, natural products have not yet translated into useable antifouling products.

Common barriers from research and development to a marketable product (Ravel, 2020) include limitation of supply (i.e., many natural products are present in small quantities in nature and are difficult to access synthetically); inappropriate physico-chemical characteristics (i.e., incompatible with coating matrix systems or suboptimal stability); and regulatory challenges (i.e., data requirements and approvals to bring a new biocide to market). A related approach that has seen some success has been development or repurposing of synthetic bioactives. For example, medetomidine is a synthetic drug first developed as a surgical anesthetic and analgesic and is now used as a deterrent of barnacle settlement (Loxton et al., 2017). This bioactive has been successfully registered and marketed as Selektope™ by ItechAB (Sweden) as the first novel antifouling biocide to enter the global market in recent times (Chaabane et al., 2019). Examples of other synthetic bioactives being pursued or developed include cationic short peptides that disrupt membrane integrity of biofoulers (Trepos et al., 2015) and anti-parasiticides (e.g., ivermectins) incorporated in soft coatings to 'contact kill' biofouling (Pinori, 2013).

Another rich area of research and development is novel non-biocidal antifouling coatings and materials. A diverse range of approaches have been trialed and progressed to varying degrees for three main classes of coating/material: foul-release, surface topography, and physico-chemical. Novel foul-release coatings are diverse, with approaches ranging from high-tech amphiphilic polymer composites and fluropolymers (Selim et al., 2017; Rahimi et al., 2021) to comparatively low-tech coatings impregnated with silicone (Hodson et al., 2000), wax (de Nys and Ison, 2004), or food-grade oils (Bakker et al., 2011). Antifouling surfaces with engineered surface topographies mimic natural fouling resistant surfaces such as mussel shells and shark skin (Myan et al., 2013; Sullivan and O'Callaghan, 2020). Surface topography effects work by producing slightly smaller surface configurations than target biofouling larvae to prevent easy contact with the surface and hierarchical topography theoretically provides protection against a range of biofouling organisms

[4]https://www.auckland.ac.nz/en/news/2018/11/14/bioengineers-tackle-underwater-fouling-in-new-way.html

[5]https://commercialdiving.com.au/wp-content/uploads/HST-Research.pdf

(multi-scale roughness spanning micrometers to nanometers; Schumacher et al., 2007). Antifouling activity is influenced by texture design and the ratio between the height, width, or pitch of the surface features and settling organism length. The approach is intuitively attractive but has often failed to translate from laboratory to real-world (Carve et al., 2019). A possible reason for this is the lack of taxonomic diversity of biofouling organisms explored in laboratory studies, compared with highly unpredictable recruitment patterns found in nature encompassing a diversity of taxa with settlement preferences that span multiple orders of magnitude of topographical length-scales.

Physico-chemical surface properties that deter or exclude fouling settlement are also widely studied. Examples include chemical modification of surface wettability (Li and Guo, 2019), photocatalytic surface chemistry (Zhang et al., 2019; Szeto et al., 2020), oil- or air-trapping polymers (Arnott et al., 2014; Ware et al., 2018), and sol-gels (Richards et al., 2019; Wanka et al., 2020). Many foul-release, surface topography, and physico-chemical approaches show promise, presenting potential avenues for truly environmentally benign and long-lasting antifouling protection but product commercialization is low compared to research outputs (Sullivan and O'Callaghan, 2020). Common hurdles to be overcome include cost, scalability, and the requirement of specific physical environments (e.g., foul-release typically requires strong water flow sufficient to dislodge fouling). Combining multiple approaches (e.g., surface topography plus photocatalysis; Vucko et al., 2013) could overcome challenges presented by the range of biofouling attachment mechanisms and dynamic environment in the sea (Yan et al., 2020).

It is important to highlight that biocidal and non-biocidal coatings would need to last the lifetime of the structure or be refreshed or reapplied at appropriate frequencies to remain effective. This may be feasible for certain infrastructure such as aquaculture installations with growing cycles in the order of months to 1 or 2 years, but represents a formidable challenge for structures like wharf piles, marina pontoons, and renewable energy infrastructure where reapplication is not possible nor cost-effective under most scenarios. Other major challenges for emerging biocidal coatings for SSAS include registration procedures and regulatory requirements for new compounds and, for aquaculture, meeting strict food-safety requirements, and satisfying consumer perceptions. Ensuring that novel coatings and materials are environmentally acceptable is also a key barrier to implementation. Novel biocides are, by definition, intended to kill or otherwise exert control over organisms, resulting in overall high likelihoods for environmental harm (de Campos et al., 2021). Moreover, antifouling formulations typically contain multiple bioactive constituents that can have additive environmental risks to significantly complicate risk assessments, and this also applies for non-biocidal coatings that can contain an array of ingredients with bioactive potential (e.g., preservatives, catalysts, and fluorinated compounds; Piazza et al., 2018). Accordingly, regulatory data package requirements to bring any new antifouling active or formulation to market are extensive.

FRAMEWORK FOR PERFORMANCE EVALUATION

Approach

For there to be high levels of uptake, biofouling management methods must be simultaneously cost-effective, environmentally benign, and pose a low risk to human health and safety. The performance of existing and emerging approaches to biofouling management on SSAS was qualitatively evaluated (**Table 3**) using modified criteria initially developed by Cahill et al. (2019a). Each management method was evaluated against the following performance criteria: Effectiveness (Effective against the broad range of biofouling taxa typically found on artificial surfaces), Biosecurity (The approach is unlikely to exacerbate risks posed by existing marine NIS), and Collateral Effects (The approach is unlikely to impact values such as esthetics, noise, environment, and ethics). For this assessment, three levels – yes, no, and questionable – were used to score whether a method met a criterion under normal operating conditions, which assumed operation by a suitably trained person, appropriate personal protective equipment, and acceptable levels of quality assurance. A fourth criterion, Feasibility (The resource intensiveness, expense, and infrastructure requirements of the approach over the intended lifetime of the SASS), was qualitatively evaluated for each existing and emerging category by industry/infrastructure type (**Table 4**).

Findings

Only a third of existing management methods met the Effectiveness, Biosecurity, and Collateral Effects criteria (**Table 3**). High-pressure washing and mechanical methods (e.g., brushes, discs, or scraping) failed the Biosecurity and Collateral Effects criteria due to risks associated with the potential release of viable propagules, organic material, or chemical contaminants during treatment. The biosecurity criterion could be fulfilled if biofouling species (whether native or non-native) can be released into the local environment without increasing population sizes. By contrast, only one emerging method (AUVs/ROVs) was considered suitable by all three criteria, primarily because of a lack of information to support the assessment of other methods. Although novel coatings and materials were considered effective, efficacy will be dependent on the surface it is applied to, and the environmental conditions experienced on surfaces. Not all surface types will be amenable to coatings, while fouling release coatings, for example, will only be effective if adequate water currents at the site operate in tandem with the coating (Hu et al., 2020).

Encapsulation approaches to biofouling management on SSAS failed two assessment criteria (**Table 3**). While proven highly effective in response scenarios (e.g., see Atalah et al., 2016), the use of large amounts of encapsulation material, usually plastics, poses environmental risks. Loss of wraps to the wider environment is possible (e.g., during storms), adding to broader plastic pollution and has long term consequences and potential to impact a broad range of organisms (Wayman and Niemann, 2021). The outer surface of encapsulation materials used to date is

TABLE 3 | Performance evaluation of existing and emerging management methods and approaches to manage static marine infrastructure.

		Mechanical brushes/ discs/ scraping/ abrasion	**High pressure washing**	**Air exposure**	**Encapsulation**	**Traditional biocidal coatings**	**Sprays & dips**	**Novel coatings and materials**	**AUVs/ ROVs**	**Laser radiation**	**Bubble streams**	**Ultrasound/ cavitation**	**Electric fields**	**Heat**	**Biological control**
Criteria	**Description**	**Existing**							**Emerging**						
Effectiveness	Effective against the broad range of biofouling taxa typically found on artificial surfaces	✓	✓	✓	✓	✓	✓	✓	✓	**?**	**?**	**?**	**?**	**?**	✓
Biosecurity	The approach is unlikely to exacerbate risks posed by existing marine non-indigenous species	**x**	**x**	✓	**x**	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Collateral effects	The approach is unlikely to impact values such as esthetics, noise, environment, and ethics	**x**	**x**	✓	**x**	**?**	✓	✓	✓	**?**	✓	**?**	**?**	✓	✓

✓, *yes, meets this criterion;* **x**, *no;* **?**, *not known/unclear.*

TABLE 4 | Evaluation of the feasibility of existing approaches in relation to resourcing, costs, and infrastructure requirements over their intended lifetime.

	Biocidal coatings	**High pressure washing**	**Mechanical brushes/ discs/scraping**	**Sprays and dips**	**Air exposure**	**Encapsulation**
	Continuous	**Reactive**	**Reactive**	**Reactive**	**Reactive/ Continuous**	**Reactive**
Aquaculture	Feasible for nets, but not effective for most other structure types and would be impractical or too costly to apply at a suitable frequency	Widely used reactively – sub-optimal, but industry perceives lack of alternatives. Potential to be used as a continual practice	Widely used reactively – sub-optimal, but industry perceives lack of alternatives. Potential to be used as a continual practice	Feasible for nets and shellfish, but not for structures	Feasible for nets and buoys, ropes, etc., but not for larger structures	Not feasible to be applied as a continuous approach in a cost-effective manner
Energy	Feasible – is used extensively in many sub-sectors, but for large structures that are difficult to remove it would be impractical or too costly to apply at a suitable frequency	Widely used – sub-optimal, but industry perceives lack of alternatives	Widely used – sub-optimal, but industry perceives lack of alternatives	Not feasible to apply regularly, if at all, due to the scale of structures	Not feasible for infrastructure where periodical removal from water is impractical. However, could be applied to oil rigs and other structures where periodic removal is possible. Practical for those structures in transit	Not feasible to be applied as a continuous approach in a cost-effective manner
Ports and marinas	Not feasible for most structures found in these environments	Not feasible to apply regularly due to the scale of structures	Not feasible to apply regularly due to the scale of structures	Not feasible to apply regularly, if at all, due to the scale of structures	Not feasible to regularly remove major infrastructure from the water for periodic exposure	Not feasible to be applied as a continuous approach in a cost-effective manner

also prone to biofouling, meaning inappropriate application can lead to biosecurity risk if biofouling cannot be retained during removal/replacement.

Among the four criteria assessed, Feasibility proved to be the most common hurdle for existing tools (**Table 4**). Encapsulation methods were considered unfeasible for all SSAS categories for general biofouling prevention or removal, reflecting the limited applicability of this approach to pest eradication or management activities over short timeframes (i.e., days, weeks to months, not years; see Atalah et al., 2016). Structures associated with aquaculture were most amenable to treatment, reflecting their size, the nature of farm activities that often allow structures to be treated when stock are temporarily absent from structures or sites, and possible removal to the water surface or land for treatment. It also reflects the significant amount of mitigation investment by this industry given the direct and quantifiable impacts of biofouling on their operations (Adams et al., 2011; Fitridge et al., 2012; Bloecher and Floerl, 2020). We note, however, that many permanent structures associated with aquaculture (such as anchors and pylons) face the same issues as other SSAS. Ports and marinas posed the greatest feasibility challenge, with none of the existing tools considered feasible to apply at a scale or frequency that would prevent biofouling accumulation. The complexity and large scale of these underwater built environments poses a high barrier to feasible biofouling treatment and the cost of biofouling to operations is not acute unless a particular pest causes damage (e.g., heightened bioerosion) or precipitates other risks (e.g., to amenity use or human health).

There was insufficient information to assess Feasibility of emerging approaches listed in **Table 3**. In fact, for most emerging approaches there is very limited evidence of testing under realistic conditions, and many unknowns remain regarding compatibility with surface types and environmental conditions, scalability, longevity, cost, and regulatory compliance. Biological control has been used in aquaculture operations for decades (e.g., cleaner wrasse in salmon aquaculture; Gonzalez and de Boer, 2017), but recent studies have shown that control of broad biofouling is challenging and retention of control agents on SSAS will certainly need to be addressed (Atalah et al., 2014). For uniform, flat areas (e.g., marina pontoons), engineering approaches to retain biocontrol agents could be simple, such as the attachment of shelves and barriers to prevent escapes. For larger or more complex structures (e.g., predator nets on finfish farms, wind turbines, oil rigs), biocontrol applications may be unfeasible. AUVs and ROVs have potential for use on flat, uniform areas of large structures (e.g., marina pontoons and the base of wind farms) and on ropes and nets associated with aquaculture (Ohrem et al., 2020).

DISCUSSION

Our review and assessment highlighted that biofouling management of SSAS is only prioritized when impacts are high and directly affect structural performance, operations, or profitability. There is a relatively strong economic motivation for biofouling management in the aquaculture industry, but biofouling management is rarely, if ever, considered in the design of port and marina infrastructure. The accumulation of biofouling does not usually affect day-to-day operations of ports and marinas and biosecurity risks associated with these facilities

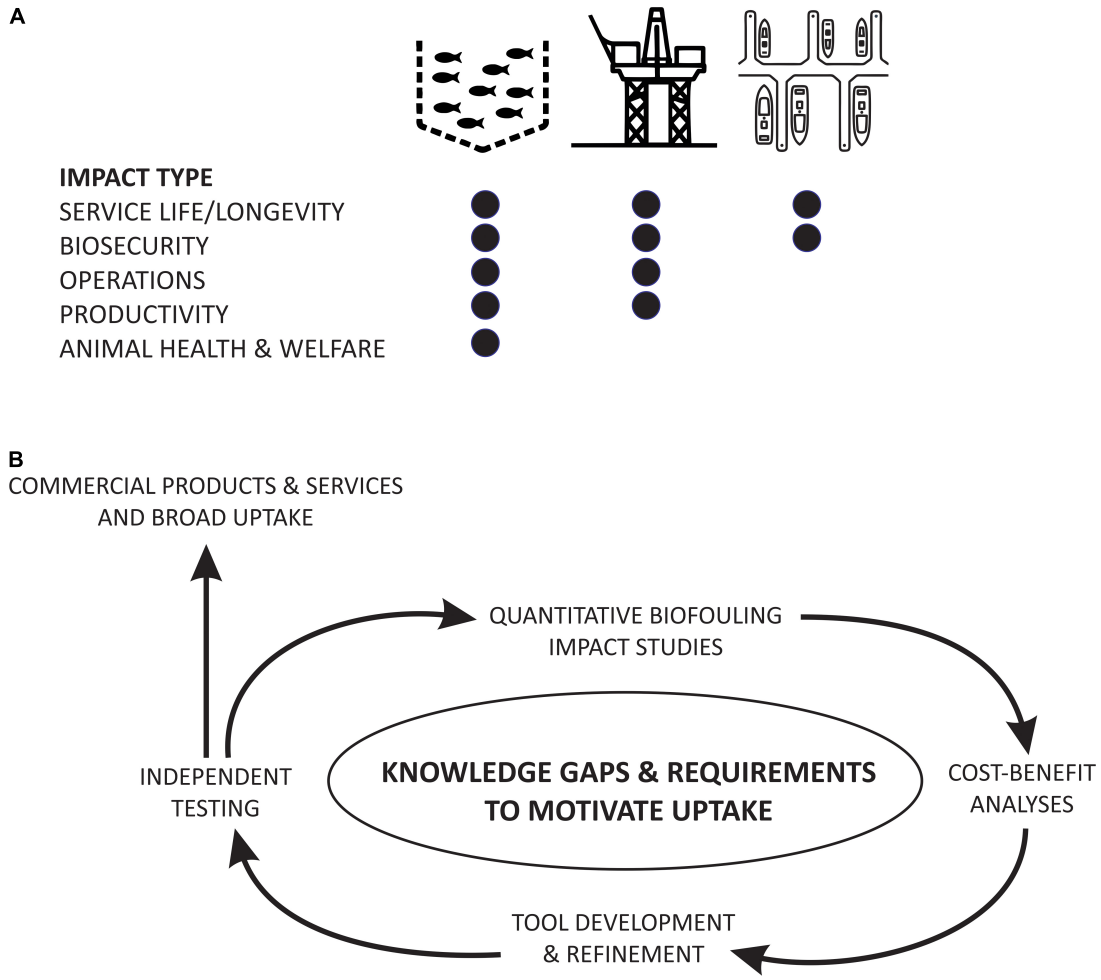

FIGURE 2 | (A) Impact types assigned to the categories of SSAS focused on in this review: aquaculture, energy production, and port and marina infrastructure, and **(B)** knowledge gaps and requirements to facilitate or motivate the uptake of existing and emerging biofouling management tools. Impact types assigned in panel **(A)** are based on normal operations and could change during a biosecurity response (e.g., closure of a marina for treatment).

(establishment and proliferation of NIS) are not typically managed by the facility operators. For the energy and ports and marina industries, the Feasibility criterion was the biggest implementation hurdle, as many of the present-day tools would be cost-prohibitive or impractical to apply at scale or to SSAS types associated with these industries. Nonetheless, there are motivations for proactive and sustained biofouling management for most maritime infrastructure that could reduce chronic impacts of biofouling to improve the longevity or function of structures and improve environmental and biosecurity outcomes associated with the built environment. It is noteworthy that 75% of existing management methods were categorized as "reactive," applied after biofouling has accumulated or has become problematic (**Table 4**). Only biocidal coatings, non-biocidal coatings, and desiccation (i.e., the FlipFarm approach used in oyster farming[6] accessed June 25, 2020) provide proactive and continuous protection from biofouling. By contrast, all emerging methods represented proactive and continuous approaches, highlighting that innovation in this field is trending toward preventing problems rather than responding to them.

Antifouling coatings in conjunction with marine growth protection systems represent the primary line of defense used by vessels to prevent colonization and growth of biofouling on submerged laminar surfaces and internal seawater systems, respectively (International Maritime Organization [IMO], 2011; Georgiades et al., 2018; Davidson et al., in press). These antifouling technologies have been optimized for operational vessels and as such are not directly transferable to static infrastructure. Foul-release coatings are only effective on vessel surfaces that provide shear stress above threshold values that prevents adherence of biofouling organisms (Larsson et al., 2016; Georgiades et al., 2018). Similarly, self-polishing and soluble-matrix biocidal coatings rely on water movement to continually erode thin layers of surface coatings to expose fresh biocide (Xie et al., 2019). The ecological impacts of biocidal

[6]https://www.flipfarm.co.nz

coatings include continual release of broad spectrum and persistent biocides (Amara et al., 2018; Richir et al., 2021) which are regulated in many jurisdictions (e.g., New Zealand Environmental Protection Authority, 2013). Given their functional limitations in static environments and role in chemical pollution, broad use of biocidal coatings on SASS is questionable.

Advances in biofouling management in the shipping industry are gradually moving toward less reliance on biocides and untreated discharges to marine systems with persistent environmental effects. For example, antifouling coatings used on commercial ships have shifted away from highly effective but highly toxic TBT-based products to less-toxic formulations, as well as phasing out several co-biocides (International Maritime Organization [IMO], 2001, New Zealand Environmental Protection Authority, 2013). Motivations for biofouling management for vessels began as operational (e.g., improved hydrodynamic performance, Schultz et al., 2011), but over time, legislation has added biosecurity considerations and responsibilities to bear on the industry (International Maritime Organization [IMO], 2011; Georgiades et al., 2020a). Regulatory requirements are driving attention and improvements in biofouling management on vessels for surfaces that do not incur significant hydrodynamic or operational penalties (Davidson et al., 2016). This is driving research and development toward biofouling management of heterogeneous surfaces and hydrodynamically complex vessel surfaces that may have carry-over benefit for SSAS management in future. A similar trend has improved ballast water management over time, including ballast exchange and the more recent installation of treatment systems, derived from a history of municipal or land-based water treatment, to manage biosecurity risks (Balaji et al., 2014; Davidson et al., 2017).

Unlike established operational and legislative biofouling standards for vessels, standards for SSAS are less developed and largely exist as consent or permitting conditions for an activity (e.g., a requirement to keep marine farming structures free of listed pest species), as a safety consideration (e.g., suction pressure for intakes) or, more broadly, as industry guidelines or best practice (e.g., Australia's Biofouling Management Guidelines for the Petroleum Production and Exploration Industry). Relatively last-minute responses to offshore platform movements, usually on a case-by-case basis, underscores how little forethought is applied to biofouling management for these structures (Wanless et al., 2010; Hopkins et al., 2011). In the absence of regulation, decisions to manage biofouling proactively or reactively on SSAS will ultimately focus on net benefits in terms of cost. Determining whether SSAS management measures are cost effective is straightforward where direct benefits of managing biofouling accumulation can be quantified. For example, reduced biofouling could lead to increased yield or a superior product from a marine farm (Bloecher and Floerl, 2020). For a drilling rig or power plant, a greater number of operational days per year could be realized if delays or temporary shutdowns associated with biofouling impacts are avoided. Drilling rigs could also benefit from freedom to operate in regions that have biosecurity measures associated with biofouling management (Scott et al., 2017). By contrast, for some categories of SSAS, such as marinas and port infrastructure (pontoons and pilings, etc.), the cost of biofouling impact is usually not considered, or measured in decades, or the impacts are indirect. This does not mean that costs or benefits cannot be quantified or modeled. For example, the multi-year or decadal cost of structure degradation under biofouling pressure can be combined with indirect costs associated with heightened fouling rates on vessels and the costs associated with NIS incursion and proliferation at these hubs of vessel activity.

Likewise, boaters have voiced concern and frustration that levels of biofouling in marina environments often vastly exceeds the levels desirable or required for vessels (Newton, 2019). There is a growing body of work that describes these broader impacts, their costs, and the management responses that are enacted to address them (Bax et al., 2002; Coutts and Forrest, 2007; Groeneveld et al., 2018). An additional advantage of cost-benefit analyses is that they can be used to determine the "break even" management cost ("external cost") per unit area of SSAS, below which a net positive benefit is achieved. This can be used to inform the design, material types, and technology needed for various structure types. For some structures, it will be a challenge to develop management approaches within cost-benefit parameters that meet the performance criteria considered in this review without factoring indirect benefits into decision making.

The performance evaluation framework applied in this study was used to identify capabilities and limitations of existing and emerging biofouling management methods. The range of data and information available to assess methods against criteria varies dramatically, sometimes including marketing material, personal anecdotes, and "educated guesses" because robust testing data or evidence of performance is lacking for many methods. This lack of evidence is arguably the largest hurdle for uptake by end users. To facilitate uptake, independent testing to verify effectiveness, safety, and feasibility – such as type approval processes that occur in many industries, including ballast water treatment – is paramount (**Figure 2**). Such system testing is widespread and for our Biosecurity criterion, technical advice to inform evaluation procedures for in-water cleaning systems to remove or treat vessel biofouling have been developed and demonstrated (Morrisey et al., 2015; Growcott et al., 2019; Tamburri et al., 2020) and could be adapted for SSAS applications where applicable.

Concluding Comments

This review highlights that the arsenal of tools currently available to manage marine biofouling on SSAS lacks proactive options to limit the associated consequences to infrastructure and the environment. For SSAS that can be removed from the water without too much difficulty, environmentally friendly antifouling coatings hold promise as a cost-effective approach. However, for SSAS that are difficult or impossible to remove for maintenance, novel approaches are needed that can be applied prior to structure deployment or constantly while the structure is in water. Environmental and operational benefits can be realized if biofouling management of static infrastructure can be applied in a cost-efficient manner, with innovation in emerging approaches

trending toward preventive or continuous management rather than reactive cleaning of surfaces. More holistic cost-benefit analyses that include indirect costs of inaction and benefits of biofouling management will provide a stronger framework for implementation using cost estimates that more accurately reflect the broad effects of biofouling on SSAS throughout the seascape.

AUTHOR CONTRIBUTIONS

GH developed the original concept with subsequent refinement by all other authors. GH and PC wrote the first draft of the manuscript. All authors contributed to manuscript's preparation, revision, and read and approved the submitted version.

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REVIEW
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A Review of Biofouling of Ships' Internal Seawater Systems

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Internal seawater systems (ISS) are critical to the proper functioning of maritime vessels. Sea water is pumped on board ships for a broad array of uses, primarily for temperature control (e.g., engine and electrical systems), cooling capacity (e.g., air conditioners and refrigeration), and water provision (e.g., drinking, firefighting, steam, and ballast). Although sea water may spend only a brief period within ISS of a vessel, it can carry microorganisms and larval stages of macroorganisms throughout the system leading to biofouling accumulation that can impair system function or integrity. ISS can also act as a sub-vector of species translocations, potentially facilitating biological invasions. This review describes ships' ISS with a focus on operational impacts of biofouling and current drivers and barriers associated with ISS biofouling management. As ISS internal components are difficult to access, reports and studies of ISS biofouling are uncommon and much of the dedicated literature is decades old. The impact of biofouling on ISS and vessel operations is based on increased surface roughness of pipework and equipment, restricted water flow, corrosion and subsequent component impingement, reduced surface functional efficiency, and potential contamination by pathogens that can affect human and aquatic animal health. Biofouling management is primarily achieved using antifouling coatings and marine growth prevention systems, but independent and accessible data on their efficacy in ISS remain limited. Further research is required to resolve the extent to which biofouling occurs in ISS of the modern commercial fleet and the efficacy of preventive systems. Such information can ultimately inform decisions to improve operational efficiency for vessel operators and ensure any biosecurity risks are appropriately managed.

Keywords: internal seawater systems, biofouling, ship pipework, engine cooling, operational impacts, biosecurity

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INTRODUCTION

Biofouling is a ubiquitous and enduring problem for the maritime shipping industry, requiring constant management to optimize operational performance (Townsin, 2003; Dafforn et al., 2011; Davidson et al., 2016). Coating roughness, biofilms, and algal and animal biofouling all contribute to a gradient of operational drag that can increase annual vessel running costs by several millions of dollars (Townsin, 2003; Schultz et al., 2011). The hydrodynamic deficiencies of increased surface roughness can drive up fuel costs to levels that greatly exceed biofouling management costs

(Schultz et al., 2011). As such, the incentive for proactive, or preventive, biofouling management of external submerged surfaces is obvious for vessel operators. An equivalent understanding of biofouling occurrence and impairment of ships' internal seawater systems (ISS)—drawing from various fields of engineering, ship operations, biology, and economics—has not been developed despite long-standing questions on the topic (Houghton and Gage, 1979; Carlton, 1985). The current lack of quantitative knowledge on the impacts of biofouling within ISS leads to an underappreciation of potential direct and indirect benefits of ISS biofouling management. Direct benefits are likely to include increased operational efficiency and reliability, while indirect benefits include reducing biosecurity risks associated with species translocations (Georgiades et al., 2020).

Ships' ISS deliver ambient sea water to a range of on-board locations via a network of pipes and pumps. This seawater delivery system is used for a variety of purposes critical to the proper functioning of ships, including engine cooling, ballasting, firefighting, freshwater production, air conditioning, and other specialty functions dependent on the ship type (Coutts et al., 2003; Gust et al., 2018b). In extreme situations, impairment of these systems can threaten the seaworthiness of vessels, endanger crew and passengers, and damage cargo. For example, damaged and blocked ISS have resulted in complete power loss (blackouts) and engine room flooding of ships with subsequent running aground or sinking (UK Marine Accident Investigation Branch, 1999, 2010, 2013; Transportation Safety Board of Canada, 2014; United States Coast Guard [USCG], 2018). As such, ISS and associated equipment are considered critical safety elements (International Maritime Organization [IMO], 1993) because their impairment or abrupt failure creates hazardous conditions or can prevent the operation of other critical systems.

Impairment of ISS occurs when the system or its components are compromised due to blockages, equipment failure, or operational failure. Blockages can occur in the form of abrupt occlusion due to ice, marine life (e.g., jellyfish and krill), marine debris (e.g., plastic bags) or from the gradual accumulation of sediments within ISS components. Equipment failure typically occurs because of electrical or mechanical impairment, corrosion, or a combination of these (Edyvean, 2010). Because raw sea water enters ISS, the internal surfaces of ISS are also subjected to constant biofouling pressure which can itself lead to ISS impairment or exacerbate other sources of impairment (e.g., blockages and corrosion) (Jones and Little, 1990a; Grandison et al., 2011).

Even though ISS perform a range of critical functions on board commercial ships, difficulties in accessing these systems is a widely acknowledged limitation that contributes to the lack of studies of biofouling accumulation and associated impacts, costs, and inefficiencies (Scianni and Georgiades, 2019). The purpose of this review was to:

- Briefly describe ISS of ships;
- Synthesize reports of biofouling accumulation within these systems;
- Assess the relationship between biofouling and ISS structural and operational performance;
- Summarize maintenance procedures to mitigate and manage biofouling impairment of these systems and;
- Examine information or data on the benefits and costs of ISS maintenance on commercial ships.

THE PURPOSE, CONFIGURATION, AND FUNCTION OF ISS

Sea water has been used in large quantities on board ships since the early 20th century when steel ships replaced wooden ones, combustion engines became the dominant propulsion for ocean-going vessels, and sea water became a dominant source of ballast (Carlton, 1985; Stopford, 2009). In addition to being plentiful, cheap, and easily accessible, sea water has a range of beneficial properties that can be exploited for shipping purposes, including thermal conductivity, density, fire-quenching, and a source for freshwater generation. A key ISS function requiring continual waterflow is the removal of heat from engine equipment or conversion of gases to liquids in condensers. Sea water absorbs heat from engine systems and its abundance means the heat can be diffused and discharged rather than recirculated or subjected to treatment. ISS also deliver sea water on board to provide ballast, in contrast to earlier times when the more laborious process of solid ballasting dominated (using sand, rocks, discarded port debris; Carlton, 1992). Sea water in ballast tanks provides trim and balance that ensures correct buoyancy, submerged running gears, and optimal ship maneuverability (David, 2015). The main firefighting capacity and sprinkler systems on board ships are also supplied with sea water, as are general service outlets that use sea water intermittently for cleaning and deck wash (Gust et al., 2018b). Ships extract their freshwater supplies using desalination plants, and air conditioning and refrigeration systems require sea water as a coolant in condensers. More recently, sea water is used as part of exhaust scrubber systems that remove greenhouse gases from ships' emissions (Andreasen and Mayer, 2007). There are many other uses that pertain to various vessel types, often involving cooling for mechanical equipment (e.g., auxiliary engines, thrusters, and emergency generators), or seawater supply for distillers, deluge pumps, cleaning equipment, and toilet systems (Gust et al., 2018b).

The configuration, construction, and scale of ships' ISS vary greatly among vessel types, with most ISS being tailored installations that broadly adhere to classification society requirements for material type, minimum critical dimensions, and labeling (e.g., DNV GL, 2018; Gust et al., 2018b; Cahill and Floerl, 2019). Accounting for the inherent diversity of ISS is essential to effectively understand and manage ISS biofouling, but common features of ISS can illustrate the nature and extent of systems and provide context for biofouling within them (**Figure 1**).

The Flow of Sea Water Through ISS

A sea chest is a recessed compartment ($\approx$ 1–3 m^3 on large commercial ships) within the hull through which sea water is drawn and delivered to ISS pipework and equipment by

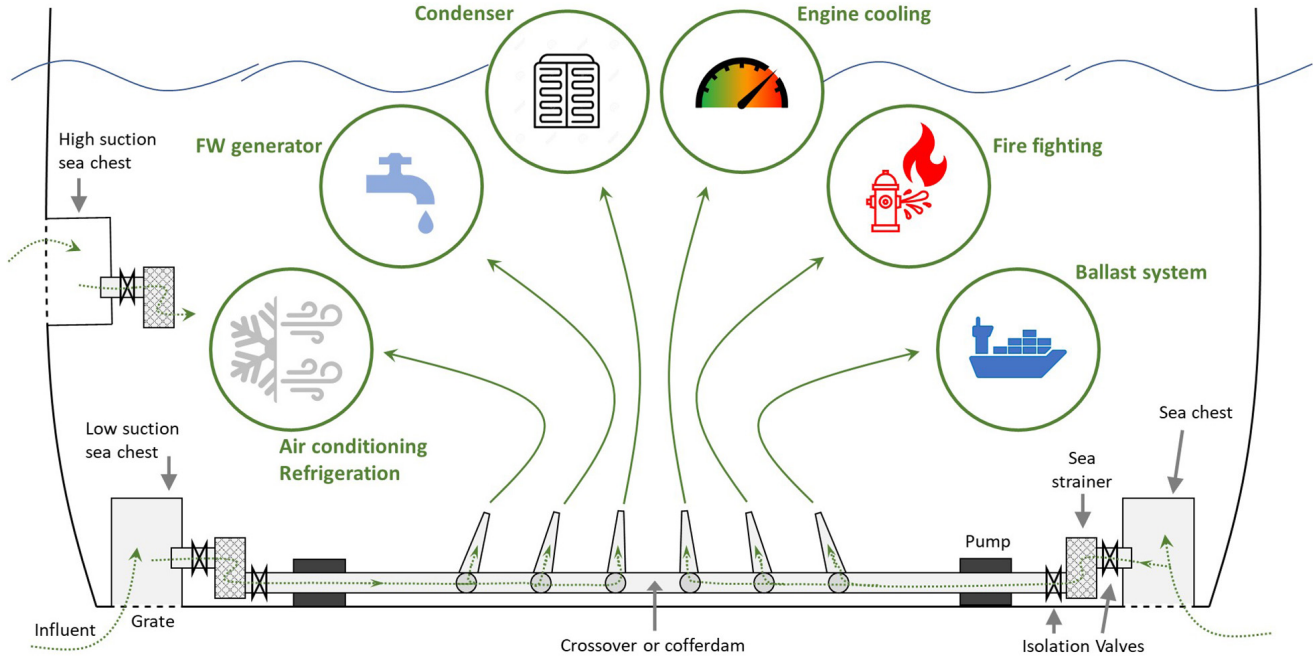

FIGURE 1 | Schematic of initial sea water passage through an integrated ISS, depicting the range of uses for sea water on board a ship. Diagram based on Coutts and Dodgshun (2007); Kongsberg (2014); and Gust et al. (2018b). FW refers to fresh water.

pumps. Grates at sea chest entrances prevent coarse material from entering ISS (Dodgshun and Coutts, 2002). The sea chest acts as a reservoir or "halfway house" for sea water, straddling the space between the 12 to 20-knot laminar flow adjacent to a ship's external hull and the suction side of intake pipes. The chest prevents countervailing forces that would cause water cavitation that reduces or disrupts pumping efficiency of incoming water while also increasing propulsion drag for the ship (Coutts et al., 2003). The size and number of sea chests varies among commercial ships, with larger ships typically having larger and more numerous sea chests (Frey et al., 2014; Davidson et al., 2018). Naval ships, cruise ships, and some other specialty vessels may not adhere to this generalization as they have configurations and operational tempos that differ substantially from cargo vessels (Moser et al., 2017; Polglaze, 2019). Sea chest redundancy is commonplace, with "high" and "low" sea chests referring to their position on the hull: low sea chests typically draw water at or near the flat bottom of the hull and high sea chests are usually located on the vertical side of a ship's hull. Ship operators switch intake suction between sea chests based on water depths and available clearance to the seafloor, meaning high sea chests are often used in ports to reduce the likelihood of sediment entrainment while low sea chests are used when the ship is underway or in deeper anchorage (Coutts and Dodgshun, 2007).

From the sea chest, water moves into an intake pipe, bypassing an open valve before reaching an internal sea strainer. This is the second filter through which incoming water passes and is often a removable basket- or bucket-shaped metal filter that sits in a housing. After the strainer, sea water passes another open valve and through the main pump. Sea chest grates (≈ 15–35 mm gaps) and sea strainers (≈ 5–20 mm holes) prevent larger unwanted material from reaching, damaging, or clogging this pump (Coutts et al., 2003; Coutts and Dodgshun, 2007). Pumps are often centrifugal or single-entry impeller pumps (Gust et al., 2018b) that accelerate water through a chamber or impeller casing and onward from the pumps' discharge piping. For ISS servicing multiple on-board functions (integrated ISS), sea water moves from the pump into a crossover pipe or cofferdam, both of which act as a common seawater reservoir for use in ISS throughout the ship. Crossover pipes or cofferdams often span the width of a ship, connecting the sea chests on both sides. For ISS servicing a single function (independent ISS), sea water travels directly from a sea chest to perform a function without a shared intake pipe or crossovers.

After passing through suction pumps, sea water is used to perform various functions, of which heat exchange for engine equipment is a primary use by overall volume. Engine cooling systems are found on almost all motorized vessels (the terms heat exchangers and coolers are used interchangeably in the maritime industry). In integrated ISS, sea water typically travels from the crossover pipes and often through secondary pumps and filters into an ever-decreasing range of pipe diameters. When it reaches plate or shell-and-tube heat exchangers, sea water is confined inside a chamber that is separate from the hot fresh water (or coolant) from which heat is being transferred. Sea water entering plate-type heat exchangers can occupy 1.5 mm-thin spaces within a chamber separated by titanium or stainless-steel plates through which heat is advected from fresh water and diffused by sea water departing the chamber (Gust et al., 2018b). Modern plate designs include varying degrees of herringbone patterns on plate

surfaces (theta chevron angles) which can increase water velocity and turbulence in the narrow gaps between plates to increase heat exchange efficiency while reducing fouling risk (Ahn et al., 2019). Likewise, shell-and-tube coolers can have pipe diameters of <1 cm with heat from fresh water in the chamber (shell) being transferred and removed by sea water flowing through a bundle of pipes (tubes) (Gust et al., 2018b). The sea water traversing this equipment can experience dramatic shifts in temperature, up to 15°C, in a short time span (US Environmental Protection Agency, 1999). After passing through heat exchangers, sea water can move directly through overboard pipes that typically take the shortest distance for discharge at the exterior hull.

Sea water cooling systems are designed to be as short and direct as possible due to acknowledged issues of ISS biofouling and the expense of corrosion-resistant materials. Sea water is pumped by the main sea water pumps through the main heat exchanger(s) where low temperature (LT) fresh water is kept at a constant temperature (typically 32–34°C). LT fresh water is then pumped by LT pumps to equipment that needs cooling. This decoupling from sea water makes the temperature control of secondary systems easier as it avoids or reduces problems caused by (1) biofouling of internal surfaces; and (2) the influence of seawater temperature fluctuations.

Similar heat transfer principles are employed for sea water that traverses through refrigeration and air-conditioning systems. In these systems, sea water is used within condensers, typically turning a chemical refrigerant from a gaseous to a liquid state (Eames et al., 1992). Sea water also flows from crossovers or independent ISS to seawater supply systems, including ballast tanks, freshwater makers, and firefighting systems (Gust et al., 2018a). Unlike sea water that travels through heat exchangers, these systems can be used intermittently and result in sea water that is stationary for varying periods. Ballast tanks represent one of the few situations where sea water travels from relatively constricted spaces into large tanks. Sea water traveling through freshwater makers is subjected to evaporators and condensers, or passes through membranes in reverse osmosis systems, to convert sea water into a ship's freshwater supply, with salty brine discharged overboard (Gust et al., 2018b). Ultimately, most sea water entrained in ISS travels through various routes and equipment until discharge to the sea from overboard pipes (US Environmental Protection Agency, 1999).

Overall, ISS are complex systems whereby sea water passes grates, chambers, pipes, fittings, valves, strainers, pumps, seals, gaskets, filters, plates, tubes, tanks, and/or membranes. These components can be made of (or contain) a huge diversity of material types, including, but not limited to carbon steel, stainless steel, brass, bronze, aluminum, copper-nickel, titanium, rubber, neoprene, epoxy, nylon, polypropylene, plastic, silicone, coatings, and anodes (Gust et al., 2018b; Cahill and Floerl, 2019). Sea water flowing through these systems can contain traces of copper, iron, aluminum, zinc, nickel, tin, titanium, arsenic, manganese, chromium, lead, oil, and grease (US Environmental Protection Agency, 2013). Sea water can undergo a range of temperature fluctuations, including discharges of 60°C (US Environmental Protection Agency, 1999) and conversion to steam. Up-taken sea water can traverse ISS spaces of varying sizes, ranging from millimeters wide (in heat exchangers) to large tanks several meters high, wide, or long (Krata et al., 2012; David, 2015). Water residence times vary from minutes for continuous flow-through cooling systems (US Environmental Protection Agency, 1999) to days or weeks for ballast systems (Verling et al., 2005).

Functional Components of ISS

Ship Engine Cooling

Internal combustion engines generate heat sufficient to melt engine materials or set fire to lubricants and equipment (Ezgi et al., 2014). Using sea water in engine cooling systems makes it possible to have practically sized heat transfer equipment on ships within a "single pass" open system that transfers the heat through seawater (typically via a heat exchanger rather than direct raw water cooling) that is released overboard or redirects this energy to other purposes (Balaji and Yaakob, 2012; Kongsberg, 2014; Garcia and Trueba, 2019). Efficient heat exchange prevents catastrophic engine failure and reduces the rate of fuel consumption that helps maintain power. This critical function explains the need for continuous seawater flow and the large volumes required to maintain these systems. For example, an aircraft carrier can process 170,000 gallons (644 m^3) of sea water per minute when underway (US Environmental Protection Agency, 2013).

There are two main types of heat exchangers to service engine cooling: plate coolers and shell-and-tube (or pipe) coolers. Both types work to transfer heat from hot fresh water within a closed-loop engine cooling system to the colder sea water traversing the cooling chamber within an open seawater system. In addition to onboard heat exchangers, ships can have box (or keel) coolers which are essentially recessed compartments (similar to sea chests) on the external hull. Closed-loop freshwater piping is housed in the "box" and is cooled by ambient sea water before following the pipelines back inside the hull. These compartments are not accessible from inside the ship and while they are typically coated with antifouling or foul-release coatings to reduce biofouling accumulation, the pipework for heat exchange often remain uncoated. Box coolers are rare in cargo ships but relatively common in certain types of tugs, fishing vessels, and other small vessel types (Gust et al., 2018a).

Ballast

Floodable cargo holds are used as ballast tanks on some commercial ships (e.g., bulk carriers) but dedicated ballast tanks between the hull and internal superstructure (i.e., double bottoms, wing- and deep tanks) on all ships can be numerous, widely distributed, and contain complex internal configurations (Hewitt et al., 2009; Krata et al., 2012). Longitudinal and transversal structures, baffles, ledges, stairs, struts, and platforms contribute to convoluted flow patterns during flooding and emptying, including localized low-flow or dead zones (Hewitt et al., 2009; Guney et al., 2020). Tank sizes and ballast operations vary among ship types (Verling et al., 2005), but cumulative surface areas of internal tank walls and interconnecting pipes are typically thousands of square meters per ship. Ballast pumps are often some of the largest within ship ISS and must cater for potentially vast amounts of water movement over

relatively short docking timeframes (David, 2015). For example, single deballasting events of 103,000 m^3 have been reported (Minton et al., 2005). Pumps are also activated during open-ocean exchanges when ballast water from ports is removed and oceanic water is entrained in its place for biosecurity purposes (International Maritime Organization [IMO], 2004; Hewitt et al., 2009). More recently, ballast treatment systems have been installed on ships which are intended to control the numbers of organisms in ballast tank water (Davidson et al., 2017). The waste heat from engines can be redirected via ISS to treat ballast water (e.g., Bawat A/S, 2021).

Firefighting

Firefighting systems on ships are connected to crossover pipes or independent ISS (usually both) to supply sea water to main firefighting installations (emergency mains with hose connections), sprinkler systems, and water spray systems (Gust et al., 2018b). The capacity and configuration of the firefighting system is highly regulated, including placement and numbers of pumps, hydrants, and hoses. Hoses and couplings are internationally compatible systems, but regulations vary by ship type and potential fire hazards (i.e., cargo and operational considerations). Valves of these systems can only be closed for maintenance or icing risk, and corrosion of steel piping is typically monitored. Because water for these systems is not in continuous demand, standing sea water can lead to unacceptable corrosion problems. For this reason, sprinkler systems that are supplied by ISS typically sit filled with fresh water via a header tank (Murdoch, 2012). When in use, sea water is used after the fresh water is discharged and the sub-system is backfilled with fresh water after use. Ultimately, the firefighting system is reliant on functioning ISS pumps and pipes.

Freshwater Makers

Desalination plants on board ships provide on-demand fresh water. There are two main types of desalination plant supplied with raw sea water from ISS: distillation generators and reverse osmosis systems. For distillation generators, sea water passes through an evaporator and the subsequent steam through a condenser to produce fresh water. Distillation water makers operate in a vacuum to enable evaporation at 40–45°C and use waste heat energy from the engine's cylinder cooling water as a heating source. Reverse osmosis systems apply pressure to the seawater side of a membrane-separated chamber to produce fresh water on the other side. The latter systems can have seawater heaters upstream of the unit to improve efficiency for ships operating in cold waters. Both systems can be affected by biofouling and entrained biota at filters just upstream of the systems.

Air Conditioning and Refrigeration

Air conditioning and refrigeration systems operate similarly to engine cooling systems. A chemical refrigerant (e.g., freon and ammonia) is converted to a gas in an evaporator and then compressed and returned to liquid form by a fresh water or an ISS-fed condenser (Eames et al., 1992; Gust et al., 2018b). The sea water running through the condenser cools the gas and is discharged overboard. These systems cater to crew needs on many ships (e.g., comfort and food storage) and are essential to maintain safe and comfortable working conditions in engine rooms (and living areas). Larger versions of air conditioning and refrigeration plants can be required on cruise ships and some fishing vessels to cater to larger numbers of people or maintain low temperature to prevent catch spoilage (Hafner et al., 2018).

Other Systems

Internal seawater systems are also used for steam condensers, general service (i.e., deck wash and toilets), lubrication oil and fuel oil cooling, cargo pump turbines, some specialty functions on certain ship types, and exhaust scrubber systems (Andreasen and Mayer, 2007; Kongsberg, 2014). Exhaust scrubber systems are a relatively recent innovation used to remove nitrogen oxides (NO_x), sulfur oxides (SO_x), and particulate matter from ships' exhaust emissions to adhere with recent regulations from the International Maritime Organization (Ji, 2020; Teuchies et al., 2020). In these systems, the natural alkalinity of sea water is used to remove SO_2, for example, from the exhaust plume, producing an increase in the amount of sulfate in discharged sea water (Andreasen and Mayer, 2007). Systems involving sea water generally push exhaust gas through a chamber in which sea water is sprayed to remove SO_2. Cleaner exhaust gas is emitted, while the scrubbing effluent (liquid) is further treated or discharged overboard (Issa et al., 2019). Open-loop scrubber systems are considered effective and dominate the market but require large pumping capacities to maintain high amounts of water passing through the system (Teuchies et al., 2020). However, the amount and possible impacts of contaminants in discharged effluent are being further scrutinized (Teuchies et al., 2020) and a growing number of jurisdictions have restricted or banned scrubber effluent discharge in their waters (Britannia P&I, 2020).

BIOFOULING IN ISS

Biofouling throughout ISS can impair the flow of water, the integrity of the system or its component equipment, and the function of those component systems (Houghton and Gage, 1979; Jones and Little, 1990a; Coutts and Dodgshun, 2007; Gust et al., 2018a).

Biofouling Accumulation in ISS

The internal surfaces of ISS are subjected to biofouling pressure because raw sea water, in particular sea water associated with coastal marine (and port) environments, carries microorganisms and larval stages of a broad range of marine macroorganisms from the surrounding environment (Carlton, 1985; Frey et al., 2014). While the range of conditions biofouling organisms could be exposed to within ISS is very broad, localized conditions at a particular point of settlement are likely to be comparatively stable. For example, a given settlement site in pipework upstream of an engine-cooling heat exchanger will be subject to a continuous flow of ambient sea water, even though seawater temperatures along the entire engine cooling ISS will range from ambient up

to 60°C (US Environmental Protection Agency, 1999). As such, biofouling organisms may experience quite stable conditions, and there may be scenarios where conditions are more favorable and stable within ISS than on external hull surfaces and niche areas (e.g., because it is a "protected" space with a constant source of sea water for food and respiration, or if heat exchangers yield more suitable temperatures for given biofouling organisms).

In general, there is likely an inverse relationship between biofouling accumulation within ISS components and distance from suction sea chests. That is, biofouling accumulation probably decreases through the system from sea chests to overboard pipes because of water filtration and processing through various equipment, but this has yet to be explicitly tested. After passing through the sea chest, settlement sites within ISS tend not to have surface coatings to prevent colonization (Cahill and Floerl, 2019) but some pipework and other ISS components (e.g., impeller housings and valve bodies) are made of copper-nickel (cupronickel) alloys. Cupronickel alloys are increasingly widespread in critical ISS components, primarily due to their excellent resistance to seawater corrosion (Powell and Michels, 2000). Most cupronickel grades also confer antifouling activity against both macrofouling and microfouling akin to copper-based antifouling coatings or elemental copper (Schutz and Scaturro, 1991). Wet and dry periods within certain components likely reduce macrofouling amount and viability (e.g., ballast tank walls), and the absence of light in ISS, which does not penetrate these systems far beyond a sea chest grate, prevents the occurrence of marine algae (i.e., seaweeds).

The occurrence of biofouling within ships' pipework was acknowledged some time ago (Newman, 1963; Carlton, 1985), however, it remains poorly understood relative to more easily accessible external biofouling (Visscher, 1928; Woods Hole Oceanographic Institution, 1952; Davidson et al., 2016). Despite this limitation, ISS biofouling and the ways in which microfouling and macrofouling can impinge on the functioning and integrity of ISS components has been a focus of some naval and applied research (Edyvean, 2010). Houghton and Gage (1979) characterized the role of biologists in applied shipping fleet research as resolving "problems which are peculiar to ships and their operation" which could be classified as "exterior to the vessel" and "internal to the structure." The impacts were understood to be both critical to ship structure and functioning but also more subtle, cryptic, or gradual impacts that reduce overall efficiency. Hull biofouling and the impact of microorganisms (including biofilms) on fuel and hydraulic fluids and their tanks were serious concerns, but biofouling in ISS was also considered important. Houghton and Gage (1979) reported macrofouling—primarily mussels, barnacles, tubeworms, hydroids, and ascidians—variously occurring extensively in end-boxes of heat exchange elements, reducing the bore of pipes (internal space) within and adjacent to these systems, inducing "impingement attack" on heat exchanger tubes with subsequent leakage, and filter blockage leading to greatly reduced flow rates.

Likewise, Alberte et al. (1992) described the overriding approach for decades of biofouling research at the United States Office of Naval Research (ONR) covering bioadhesion, biofilms, biofouling succession, and antifouling coatings. Most of the attention was directed to external hull surfaces based on fleet readiness to reach design speeds, increases in acoustic noise, and estimates of biofouling management costs that increased from $200 million USD to $360 million USD per annum between 1974 and 1981. Their estimated cost-benefit of 10–60% fuel savings exceeded the remediation costs but was only based on external hull fouling. ISS were mentioned in terms of needing research and fouling control strategies that catered for sea chests and cooling water systems, but similar scoping of biofouling in these systems or its impact was not reported.

A study by Gust et al. (2018a) collated observations from 170 inspections of 126 vessels predominately engaged in the maritime extraction industry in Australia. While these vessels may not reflect broader trends for in-service commercial (cargo) ships, the report described seawater systems and nodes susceptible to biofouling accumulation, with significant (>5%) levels of biofouling associated with 80% of ISS inspected. The literature also provides some information, which is largely qualitative, about the occurrence of biofouling in ISS for individual ships.

Sea Chests

Sea chests and their grates are a relatively frequently reported contributor to ship biofouling communities (e.g., Coutts and Dodgshun, 2007; Inglis et al., 2010; Frey et al., 2014). Sea chests have been described as ideal environments for biofouling, biofilms, and mobile marine species because they are protected spaces with supplies of nutrients and clean sea water, but without strong current flows that could cause dislodgement (Jones and Little, 1990a). Antifouling and foul-release coating efficacy is also challenged by these conditions, notably via baffles, curves, and corners that can create low water flow "dead zones" on sea chest walls (Leary et al., 2016; Georgiades et al., 2018). Several studies that evaluated biodiversity or abundance of marine organisms in sea chests have found a broad array of taxa that are not often found elsewhere in ship biofouling assemblages (Coutts et al., 2003; Coutts and Dodgshun, 2007; Frey et al., 2014; Lewis, 2016). Naval vessels with long port residence times accumulated significant and problematic levels of biofouling in sea chests, primarily mussels, hydroids, and serpulids (Jones and Little, 1990a). Several studies have provided inventories of species from sampled ships' sea chests, including 150 taxa from 53 sea chests examined in New Zealand, with a range of 1–33 species per sea chest (Coutts and Dodgshun, 2007). A single ship sampled in Sydney had 11 species with large clumps of biofouling at intake pipe entrances (Coutts et al., 2003). Frey et al. (2014) sampled 82 sea chests from 39 ships in Canada, finding 80% with biofouling encompassing 299 different species. Their analysis suggested in-service period since last cleaning and vessel voyage range (international versus domestic) influenced the abundance and richness of biofouling assemblages. More recently, Lewis (2016) reported images from dry dock surveys of sea chest grates that were entirely covered with hard-bodied macrofouling, indicating significant occlusion of the grate gaps. Gust et al. (2018a) recorded significant biofouling in over 60% of 738 sea chests surveyed in their collation of data from vessel inspections.

Sea Strainers

Sea strainers are one of the most accessible internal nodes of ISS and are situated within meters of the sea chest. Because they can be isolated, drained, and opened while the vessel is afloat, sea strainers can be routinely inspected and cleaned. This practice is not necessarily reflected in reports from the literature on biofouling occurrence in sea strainers and their housings. Anecdotal mentions of sea strainer biofouling occur in regulatory texts, but detailed records are few; for example, Jones and Little (1990a) reported heavy fouling of basket strainers of United States naval ships, with mussels a particular concern. One exception is the Gust et al. (2018a) study in which 34% of 773 sea strainer inspections had significant biofouling.

Pumps and Pipes

Crossovers and cofferdams are typically the largest pipes or seawater reservoirs (excepting ballast tanks) through which sea water continuously flows. Access to internal surfaces of pipes (for inspection or cleaning) is limited. Pipe sections adjacent to sea chests and sea strainers, but upstream of the primary suction pump, are slightly more accessible than pipework further downstream and may accumulate biofouling that reduces the bore of those pipes (Gust et al., 2018a). Jones and Little (1990a) reported biofouling near strainers at multiple nodes of an ISS of a single United States Navy ship. Gust et al. (2018a) ranked crossovers as the highest priority for treatment compared to box coolers, sea chests, downstream pipework, and sea strainers as they had 87% ($n = 116$) prevalence of significant biofouling. There were no reports of pump biofouling found in this review; copper-nickel impeller housings or pump casings may play a role in this.

Heat Exchangers and Cooling Systems

The impact of biofouling on cooling system efficiency drives cooling system monitoring, especially within heat exchanger components. As these systems are not easily accessible, there are relatively few studies relating to biofouling of ship cooling systems. Fouling from non-biological sources is also reported in these systems, including chemical reaction fouling, corrosion fouling, precipitate fouling, freezing fouling, and particulate fouling (Garcia and Trueba, 2019). While the source and mechanisms of fouling are important for diagnosing and resolving fouling problems, reports do not always distinguish among them.

Jones and Little (1990a) reported fouling and clogging of various oil coolers and condensate drain cooler tubes with mussels, barnacles, shells, and wood chips. These problems instigated their research on United States naval ships to better understand the locations and effects of biofouling and to determine best practices for resolving these problems fleet-wide. Likewise, biofouling by the serpulid polychaete *Hydroides elegans* and the hydroid *Tubularia ralphi* were responsible for problems in the seawater cooling systems of submarines in Sydney (Lewis and Smith, 1991). Gust et al. (2018a) recorded significant biofouling in 179 of 244 main engine cooling systems. Because this was a system-wide measurement, these results do not reflect biofouling in heat exchangers alone, but any point within a main-engine cooling system, including shared components. The broader literature provides generic reports on the issue of biofouling in cooling systems and the need to implement regular maintenance, but without reporting particular instances of biofouling accumulation. The US EPA regulations for incidental discharges from ships highlight the occurrence of biota (biofouling) and other debris that is discharged from a ship after "blowdown" (clearing) of cooling system strainer plates (US Environmental Protection Agency, 2013). This information implies that biofouling is commonplace in these systems, even though not reported in a systematic way.

Ballast Systems

Ballast tanks are large compartments in ships providing hundreds to thousands of square meters for potential biofouling colonization. Ballast tanks are typically associated with carrying organisms that live in the water column (i.e., plankton and larval stages of species) and sediments that can accumulate on ballast tank floors (Smith et al., 1999; Wonham et al., 2000). Ballast tank biota ranges in size from viruses to fish, including some specimens up to 25 cm long (Davidson pers. obs.) suggesting some uncertainty about longevity and growth for some species in ballast tanks and the size of organisms that can pass through ships' pumps (Gollasch et al., 2002). Benthic species on floating debris have also been sampled from ballast tanks (Carlton and Geller, 1993). Ballast sediments can be 30 cm deep and account for hundreds of tons per ship (Hamer, 2002) with a distinct soft-sediment community, including cysts and spores, occupying this discrete niche (Bailey et al., 2005).

Biofouling conditions within ballast tanks have been described as "dark intertidal zone" environments with variable hydrological conditions that nonetheless support various taxa (Drake et al., 2005). Studies suggest this environment rarely supports macrofouling and only about 10% of available surfaces are colonized by biofilms (Drake et al., 2007). Nonetheless, biofilms sampled from in-service ships consist of bacteria, virus-like particles, cysts of microalgae, and have included human pathogens (Drake et al., 2005, 2007). Ballast treatment of influent water is likely to further reduce the risk of biofouling in these systems.

Firefighting Systems

Firefighting capacity on ships is highly regulated, requiring specific numbers and capacities of pumps and outlet stations to ensure redundancy and ability to quickly control fire outbreaks wherever they occur on a vessel (International Maritime Organization [IMO], 2002; Gust et al., 2018b). There are usually pumps connected to main firefighting lines (pipe systems) that can be single main systems or horizontal loop systems (US Maritime Training Advisory Board, 1994). The feed lines upstream of freshwater-filled branch lines (e.g., sprinkler systems) are constantly filled with sea water unless draining is required to prevent freezing. Branch lines connected to these main lines may only service fire hydrants, other fire systems, and deck wash facilities. Biofouling could potentially reduce the flow of water in these systems, which are subject to volume (i.e., pipe diameter) and testing pressure requirements (US Maritime Training Advisory Board, 1994). Palermo (1992) reported

that firefighting systems of vessels operating in United States freshwater systems were susceptible to zebra mussel (*Dreissena polymorpha*) infestation, which affects compliance with safety regulations and can endanger life in emergencies. Significant biofouling was recorded in 64% of main firefighting systems and 47% of emergency firefighting systems (n = 98 and 122, respectively) inspected by Gust et al. (2018a).

Freshwater Makers

Freshwater makers that use reverse osmosis or distillation (evaporator/condenser) are unlikely to support biofouling organisms within the units themselves, but the piping and filters directly upstream of these units are likely to be sensitive to biofouling and biotic accumulation that restricts flow and affects water quality entering the units. In this review of literature, the only report of biofouling associated with freshwater makers was from Gust et al. (2018a), in which biofouling was recorded in 62% of 37 freshwater generating systems evaluated (systems included pipework and filters).

Overboard Piping

Biofouling in overboard pipes is largely unreported, except for points of discharge at the outer hull, which is a more heterogeneous surface than adjacent vertical hull surfaces. These point locations can be colonized by biofouling at the mouth of the pipe. This biofouling is part of the external biofouling community on a relatively minor external niche area rather than biofouling derived from water passing through ISS. Biofouling in this location can reduce the bore of these relatively small diameter pipes at their outlets (e.g., 5 cm), but ISS effluent blockage has not been reported in the literature.

IMPACT OF BIOFOULING ON ISS COMPONENTS AND FUNCTION

Biofouling can affect surfaces, disrupt water flow, and compromise the structural integrity of ships' ISS. There are several mechanisms by which these issues can occur (**Table 1**), and these can work in concert to exacerbate the scale or speed of impacts.

Surface Roughness

It is widely accepted that all seawater-immersed ship surfaces will be affected by biofouling (e.g., for outer hull surfaces, Schultz et al., 2011), with a continuous impact of performance deterioration below design specifications (i.e., idealized conditions). For ISS, the simple presence of biofouling changes how water flows over surfaces. Failures in pipework and components can occur because of seawater turbulence, flow impingement, and cavitation at elbows, joints, valves, pumps, and other orifices (Schutz and Scaturro, 1991). Schutz and Scaturro (1991) described such impacts on newly commissioned military vessels as "blatant examples" of accelerated failure of cupronickel pipework with less than one year of service (in this instance, the cause of turbulence was not explicitly tied to biofouling). The remedy—to increase the diameter size of pipework—had the knock-on effect of significantly increasing system cost and weight while not eliminating the flow-erosion-corrosion problem. An intended solution to replace the inner surfaces of pipes with titanium was thought to reduce pipe sizes, improve flow rates, reduce costs, and minimize surface biofilms, biofouling, and sedimentation.

Flow

A key impact of biofouling in ships' ISS is reduced water flow (**Table 1**). In general, smaller diameter pipework is more prone to clogging and will see proportionally greater restrictions in flow for a given thickness of biofouling compared to larger diameter pipework. In some cases, restricted flow resulting from biofouling is considered at the design stage of a seawater system component. For example, engineers of heat exchangers consider an allowable fouling resistance in their design calculations to ensure some leeway before maintenance is required (Ezgi and Ozbalta, 2012). Similarly, water flow within certain pipes should not exceed specified flow rates, based on pipe composition and diameter

TABLE 1 | Mechanisms of biofouling impact within ships' ISS.

Mechanism of biofouling impact	Context
Increased surface roughness	Biofouling alters the trajectory of water relative to clean conditions, resulting in changes to water behavior within pipework, grates, and pumps leading to turbulence, cavitation, and altered flow velocities that can cause system failure over time (Schutz and Scaturro, 1991; Polman et al., 2013).
Accumulation that restricts water flow	ISS components are often space-limited and designed to deliver pre-determined volumes and flow rates of water to suit ship operations. Biofouling on grates, grilles, strainers, and filters can reduce water flow and require more energy for pumping. A similar effect occurs in pipework with reduced bore of pipes and cooling systems whereby biofouling occupies narrow spaces or tube diameters (Polman et al., 2013).
Accumulation and subsequent mortality that restricts water flow	Biofouling is typically a chronic issue that develops over time. There are instances, however, when biofouling accumulation followed by subsequent synchronous mortality can block nodes of a system (Jones and Little, 1990a; Tamburri, pers. comm.).
Corrosion and subsequent component impingement, perforation, and leakage	Biofouling can cause or exacerbate "impingement" attack of metal surfaces. This problem occurs under biofouling when organism cement, byssuses, or structures cause very localized weakness in surfaces leading to rapid corrosion (Edyvean, 2010). When this occurs in association with biofilms it is termed "microbiologically influenced corrosion" (MIC) (Machuca, 2019).
Reduced surface functional efficiency	Surfaces within ISS are often key to functioning of components. Biofilm and biofouling coverage of heat transfer surfaces reduces the capacity of those surfaces to exchange heat, with knock-on effects to equipment or materials requiring temperature control (Faes et al., 2019).
Contamination	Pathogens have been recorded on the surfaces of pipework and within water samples from seawater systems (e.g., ballast tanks)—these can threaten human health (Drake et al., 2007) or aquatic health and contribute significantly to their long-distance spread (Pagenkopp Lohan et al., 2020; Georgiades et al., 2021).

(Croatian Register of Shipping, 2013). This can happen if the bore of pipes is reduced by biofouling. Nonetheless, it appears most pipework and components within ISS are designed and manufactured to deliver a specified capacity, largely discounting biofouling impairment, as is the case with hull design and static water-use industries (Schultz et al., 2011; Polman et al., 2013).

Sea chests and their grates are prone to dense aggregations of biofouling that cover entire grates and the entry ways to pipes, significantly impeding flow (Lewis, 2016). However, reports of biofouling acting as a complete barrier to water movement within these components were not found. Severe occlusions in these systems almost certainly require much higher work rates for pumps, increasing the overall workload of an affected ship's equipment and energy usage (Polman et al., 2013). Biofouling also increases the risk of full occlusion by other means (e.g., trash or marine life) because a partial blockage (by barnacles, mussels, hydroids, or other organism types) is already in place.

Restricted flow has been anecdotally reported by several authors (see Grandison et al., 2011). Houghton and Gage (1979) observed mussels, tubeworms, and barnacles fouling heat exchangers, reducing flow, and reducing the bore of pipes upstream from those units. They also reported ships' strainer and filter occlusion by ascidians. Heat exchangers are sensitive to restricted flow and can cause serious problems for engine output. Ezgi et al. (2014) examined the performance of a ship's shell-and-tube heat exchanger immediately after biofouling was cleaned from the unit and after 2500 subsequent working hours. They recorded significant negative effects on water pressure in the system, temperature of cooling fluids, and overall system effectiveness over time. These thermohydraulic effects can accumulate substantial costs over the service duration of a cooling system and should be considered when planning proactive and ongoing maintenance schedules.

While restricted flow is a key parameter that can affect seawater systems and components, biofouling is typically a chronic issue because accumulation and growth of larger organisms on surfaces occurs over time (i.e., days, weeks, and months; Greene and Schoener, 1982; Davidson et al., 2020). Acute occlusion of systems, including clogging of strainers, filters, and pumps occurs with sudden blockage. In the worst-case scenario, acute occlusion can result in total power loss and can have catastrophic impacts on ship and crew safety. Such scenarios are most often attributed to icy conditions, trash, and free-swimming marine life (e.g., fish, krill, and jellyfish; Transportation Safety Board of Canada, 2014; United States Coast Guard [USCG], 2018), but acute flow restriction can occur from biofouling when mass mortality causes the release of organisms from surfaces that subsequently clog and completely occlude strainers or filters. Such catastrophic blockages occur in other seawater-use industries (e.g., hydroids in Chesapeake Bay are known to clog power plant intakes; Tamburri, pers. comm.) and is possible on ships, particularly after reactive treatment of biofouling (Jones and Little, 1990a; Cahill et al., 2019). Transition from sea water to a freshwater area (e.g., Panama Canal or river passages) can kill marine life that then accumulates in strainers or can block narrow ISS passages (Hinz, pers. obs.).

Corrosion

Sea water is well known to accelerate corrosion, and biofouling (both micro- and macro- fouling) has been shown to initiate and enhance the aggressiveness of corrosive attack on pipework and within ISS components (Edyvean, 2010). The contribution of biofilms to this problem is well described in the literature (e.g., Jones and Little, 1990a,b; Machuca, 2019). Pipe erosion occurs when very localized changes in fluid velocity occur. This can be triggered by biofouling organisms when their mechanism of attachment or adhesion undermines the surface boundary layer (US Environmental Protection Agency, 1999). Sea strainers have been observed to be highly corroded under heavy macrofouling, requiring strainer replacement (Jones and Little, 1990a). Similarly, heat exchangers for refrigeration units, main engine cooling, and other system coolers and condensers have been undermined by combinations of macrofouling affecting flow distribution and aggressive microbiologically induced corrosion (Jones and Little, 1990b; Eames et al., 1992). These effects can be relatively rapid (within weeks) and serious corrosion problems require expensive unscheduled maintenance (Grandison et al., 2011) or cause flooding if left unattended (UK Marine Accident Investigation Branch, 2013).

Microbiologically influenced corrosion (MIC) is corrosion of metallic surfaces and structures that is initiated or enhanced by microorganisms (Machuca, 2019). MIC was responsible for a *trans*-Alaskan pipeline failure in 2006 that caused the leak of 200,000 gallons of crude oil, a major loss of production, and an impact on global oil prices (Machuca, 2019). MIC contributes to more than 20% of total corrosion loss worldwide, including 40% of internal corrosion of underground pipelines, and its impact is so great that it is measured at the scale of lost proportions of national and global Gross Domestic Product (Hashemi et al., 2018; Li and Ning, 2019).

It is long known that biofilms can accelerate corrosion by factors of 1,000 to 100,000 (Costello, 1969) and severely compromise pressurized water systems. Examples of heat exchanger and ISS failure due to MIC have been reported. Eames et al. (1992) described MIC-damaged seawater-cooled refrigerator condensers used for air conditioning, resulting in complete failure within months of installation. This case highlights the rapid effects of bacterial enhanced corrosion of tubes, resulting in end plates damaged by a mixture of refrigerant and leaked sea water. Considerable deposits of sediment and debris combined with biofilm activity to undermine the unit and cause it to fail. Jones and Little (1990a,b) reported multiple nodes of "under-deposit" corrosion and MIC. As such, the effects of MIC promote serious and sustained problems for ships, including damage to cargo, fuel systems, and ISS, including total failure of propulsion systems (Edyvean, 2010). Furthermore, inadequate maintenance or last-minute design alterations within ISS can exacerbate MIC attack, which can lead to stress corrosion cracking or contribute to corrosion fatigue. The outcome may be substantial repair work to a sub-section of a pipe or catastrophic failure along the entire pipe length (Edyvean, 2010).

Function

In addition to impaired water flow and structural integrity issues, biofouling can alter functional aspects of ISS surfaces (**Table 1**). Functional impacts mainly pertain to heat exchangers, where surface fouling or deposits on heat exchange plates or tubes can lower the thermal conductivity of surfaces and thus greatly reduce efficiency of heat passed from high to low temperature water (Faes et al., 2019; Kah Hou et al., 2019). Biofouling can provide substantial thermal resistance, with biofilms of 1-mm thickness reported to reduce heat transfer efficiency by 50%, which exceeds the impact of most types of mineral fouling (Muilenberg and Candir, 2013).

Contamination

Pathogens occurring in ISS can affect the health of vessel crews and passengers, and ISS can act as dispersal vectors for local and global spread of human and aquatic animal pathogens. While such concerns are usually associated with ballast water organisms (McCarthy and Khambaty, 1994; Pagenkopp Lohan et al., 2017) or external hull biofouling (Howard, 1994; Whittington et al., 2018; Pagenkopp Lohan et al., 2020), pathogens have been sampled from biofilms inside ships (Drake et al., 2007). Hosts of disease agents that are of concern for external biofouling could also occur in ISS biofouling (e.g., *Mytilus* spp., *Crassostrea gigas, Carcinus maenas, Styela clava*; Coutts et al., 2003; Coutts and Dodgshun, 2007; Georgiades et al., 2021). Outbreaks of human diseases linked to water systems on board cruise ships, such as *Vibrio parahaemolyticus* and *Legionella pneumophila*, have had serious implications for those affected (Lawrence et al., 1979; Pastoris et al., 1999). In one case, a direct link to seawater systems was made because of seafood preparation using sea water from ISS, a practice that was discontinued following this discovery (Lawrence et al., 1979).

Operations

The range of biofouling impact mechanisms and their outcomes for ISS are often overshadowed by concerns for external hull biofouling, but they are significant nonetheless (**Table 2**). Biofouling can exert a penalty on the efficient operation and structural integrity of ships' seawater systems. These penalties range from chronic underperformance of systems or ISS components to critical losses of integrity that can threaten the safety of the entire vessel and its crew (**Tables 1**, **2**). Considering the known impacts of biofouling on land-based industrial cooling systems (e.g., Rajagopal et al., 2012), it is likely that the relative lack of information for ISS reflects the difficulties accessing ISS to quantify biofouling and assign impacts, rather than a lack of impacts (Grandison et al., 2011; Growcott et al., 2017).

Biofouling incurs costs across the lifetime of equipment: capital expenditure, energy costs, maintenance costs, production loss, and environmental management costs (Steinhagen et al., 1993; Kah Hou et al., 2019; **Table 2**). Some direct costs are straightforward to quantify but indirect costs are difficult to estimate and there appears to be a lack of corresponding ship-scale models. For example, it is not clear how much initial capital outlays cater for expected biofouling pressure. For cooling systems at least, that cost is incurred in the form of larger heat-exchange units, increased space and weight in the engine room, and increased installation costs (Ezgi and Ozbalta, 2012). There are known costs to installation and upkeep of marine growth prevention systems (MGPS) (Grandison et al., 2011). Production losses may vary broadly across a range of impacts from reduced capacity or offline systems, including ship scheduling issues. Throughout the life cycle of a ship, ISS will incur energy losses, lost productivity, and maintenance and personnel expenses that each cause substantial cumulative costs (Stopford, 2009; Kah Hou et al., 2019). Losses occur as systems get older and no longer run as new, with biofouling and a range of other causes contributing to higher maintenance and running costs. Environmental management costs to operators are usually restricted to direct compliance costs, i.e., costs associated with meeting regulatory standards. Broader environmental costs are perhaps the most difficult to quantify as they are indirect and largely external to industry (vessel owners and operators). However, potential environmental costs could be large, possibly involving costly management interventions to respond to biosecurity incidents or environmental contamination breaches. There have been at least two biosecurity responses partially or fully funded by maritime liability insurance, albeit the circumstances involved lost or run-aground vessels (Wotton et al., 2004; Wanless et al., 2010).

TABLE 2 | Consequence of operational impacts of biofouling within ships' ISS.

Operational cost or consequence of biofouling impact*	Description
Capital outlays	Components and pipework that will be exposed to biofouling pressure may need to be designed and built to cover the likely effect of biofouling relative to ideal (non-fouled) conditions. The additional space, weight, surface, and operational issues incur higher costs. Outlays on surface coatings and MGPS can also be significant. Outlays on asset management and replacement of assets can be significant to extreme.
Energy costs	Surface and flow impacts change the work rate required to pump water through the system, increasing the energy use and emissions of the ship. Cooling systems directly affect engine efficiency and fuel costs.
Maintenance costs	Prevention (coatings, MGPS) or removal of biofouling (e.g., reactive treatments) or replacement of equipment or parts.
Production loss	Planned or unscheduled maintenance often require partial or complete shutdown, or slow down, reducing the performance and increasing the running costs of the system. Not meeting targets in the charter party (cargo delivery schedules) can incur loss of income.
Environmental and safety compliance costs	Biofouling may affect the biosecurity status of a vessel resulting in altered itineraries or refusal of vessel entry. Biofouling can have safety implications for the ship and the people on board, increasing costs to manage these issues.
Loss of ship – worst case	Cryptic or unattended biofouling problems can contribute to rapid deterioration of engine room or ship integrity.
Loss of life – worst case	Biofouling (or associated pathogen exposure) can be a contributing factor for failures leading to illness or loss of life.

**The top five categories listed here follow Steinhagen et al. (1993) and Kah Hou et al. (2019) referring to industrial heat exchangers.*

While chronic impacts often have underlying costs that go unmeasured, there are also acute negative consequences if biofouling affects critical safety elements or otherwise violates safety standards. Worst-case scenarios result in the loss of ships or loss of life at sea. Annual reviews of maritime casualties and incidents may prove useful for identifying human health implications of equipment failure, but thus far (in the incident reports and summaries examined for this review) there is insufficient detail about the causes or circumstances of equipment failure to make a strong link to a role of biofouling or maintenance issues. For example, governmental organizations provide evidence that equipment failure and engine rooms are an important driver of marine casualties and incidents at sea (European Maritime Safety Agency [EMSA], 2016). However, they do not always provide levels of detail that pinpoint those incidents occurred because of ships' ISS specifically, much less that biofouling may have been a contributing factor.

BIOFOULING MITIGATION AND MANAGEMENT WITHIN ISS

Several reviews have been conducted on proactive and reactive management approaches for ISS biofouling (Lewis and Dimas, 2007; Grandison et al., 2011; Bracken et al., 2016; Growcott et al., 2016, 2017; Cahill and Floerl, 2019). These studies highlight the range of tools that can be installed prior to the development of ISS biofouling or as a remediation measure if problems associated with biofouling have occurred. In general, ships' ISS are currently managed proactively using coatings (usually antifouling or foul-release paints) applied to the surfaces of sea chest grates and the sea chest itself, with questionable efficacy based on the range of hydrodynamic conditions at these locations (Lewis, 2016). Due to issues including access, compatibility, and utility, these coatings are not applied throughout the system and are usually limited to ISS components most exposed to ambient seawater conditions. Some critical ISS components are also constructed of (or lined with) fouling-resistant materials, mainly cupronickel. In addition, MGPS are installed within the sea chest or sea strainer as a means of chemically treating the influent of ISS to reduce biofouling accumulation throughout the system (Grandison et al., 2011; Lewis, 2016). The most common MGPS use sacrificial copper anodes or electro-chlorination dosing systems that release biocide into the influent stream to treat pipework and component systems (Grandison et al., 2011; Growcott et al., 2017). There are, however, very few independently verified, published, and accessible data on the efficacy of these systems (Lewis, 2016; Growcott et al., 2017). Reactive treatments involve a range of chemical additions (e.g., descalers, chlorine, bromine, acetic acid, and quaternary ammonium compounds) or physical treatments (such as manual cleaning, steam/hot water, fresh water, deoxygenation) to kill or remove biofouling in ISS (Growcott et al., 2017; Cahill and Floerl, 2019). While there are some promising approaches and existing commercially available reactive treatments, these can be prohibitively expensive, lack information on efficacy as well as safety or compatibility with ISS components, and/or may not be feasible at ship-scale (Bracken et al., 2016; Cahill and Floerl, 2019). Treatments may also inadvertently contribute to occlusion problems if reactive techniques remove biofouling from pipework surfaces and drive debris to block pumps, filters, or equipment (Grandison et al., 2011; Cahill et al., 2019).

There are few explicit accounts or data that show the cost-benefit of ISS biofouling management in the real world (Grandison et al., 2011). Ezgi et al. (2014) conducted a ship-scale measurement and modeling study and determined savings of USD $16,500 per 2,500 h of usage if their recommended cleaning schedule (every 600 h) was implemented. Similarly, Pamitran et al. (2016) modeled the effect of biofouling on a ships' cooling system and determined a monthly cost of USD $464,000 in excessive fuel use is incurred from biofouling-caused performance loss of the main-engine heat exchanger. In terms of proactive treatments, Coutts and Dodgshun (2007) recorded a significant difference in species richness of sea chest biofouling when comparing MGPS presence versus absence (7 ± 1.1 species versus 11 ± 1.1 species). This comparison was not controlled for other factors, such as duration since last cleaning, vessel history (such as voyage routes, stationary periods, etc.), or MGPS operational factors. Best practice recommendations for preventing biofouling accumulation on intake grates and within sea chests suggest that foul-release coatings be applied to intake grates and soft biocidal antifouling coatings be applied to internal sea chest surfaces (Lewis, 2016; Georgiades et al., 2018). Lewis (2016) recorded no effect of copper anodic, chemical dosing, or sonic MGPS on sea chest biofouling. This finding aligned with a study in Canada that observed significant biofouling in sea chests with MGPS installed (Frey et al., 2014). In earlier studies, questions were raised regarding the efficacy of MGPS copper dosing in submarines (Lewis et al., 1988) and an experimental system was fitted on a submarine in Sydney that kept an ISS cooling system free of fouling over two summers (Lewis and Smith, 1991).

Management of ships' ISS is governed directly and indirectly by various regulatory systems at international, national, and regional levels. Seawater systems fall under management requirements for operational safety reasons, for technical operation standards, or for environmental reasons related to discharges. The International Maritime Organization introduced the International Safety Management (ISM) code in 1993 to "provide an international standard for the safe management and operation of ships" (International Maritime Organization [IMO], 1993) as part of the Safety of Life at Sea (SOLAS) Convention. The code includes a requirement for companies to "*identify equipment and technical systems the sudden operational failure of which may result in hazardous situations. The SMS [safety management system] should provide for specific measures aimed at promoting the reliability of such equipment or systems.*" By any standard, ISS components would fit this description. Classification societies are a key oversight body within this system and provide very detailed specifications and auditing requirements on ships' ISS (Murdoch, 2012; DNV GL, 2018). Examination and recording of biofouling do not play an explicit role within classification society procedures, however, which aligns with hull requirements and specifications. Dry

docking schedules that occur based on classification society rules are an opportunity to manage ISS biofouling rather than a specific requirement, meaning that out-of-water biofouling maintenance is an added value of dry-docking schedules rather than an explicit reason for dry docking. International Standards Organization (ISO) standards exist to identify piping systems by their purpose and contents (International Standards Organisation [ISO], 2008), although these are not required across all jurisdictions and different systems can exist from place to place (Gust et al., 2018b). ISS are also regulated in national and international waters with respect to discharges from ships, which include permitting requirements to minimize incidental effluents or compliance with biosecurity regulations in various countries (International Maritime Organization [IMO], 2011; US Environmental Protection Agency, 2013; New Zealand Ministry for Primary Industries, 2014). The overarching effect of these regulations is that ISS biofouling may carry a compliance burden for ship operators whereby a benefit of ISS management is likely compliance with port state controllers and international standards.

Ship owners acknowledge the importance and potential value of maintaining low levels of biofouling on ships' ISS. In a survey of ship owners, Strietman and Leemans (2019) reported fuel costs, regulatory compliance, and protection of seawater cooling systems as the main (self-reported) benefits of biofouling control on ships. Auditors also recognize the importance of ships' ISS for safe and efficient vessel performance and the "hidden danger" of pipework management that is often neglected or weakly developed (Murdoch, 2012; International Association of Classification Societies [IACS], 2018). There is clearly a demand for products and techniques that reduce biofouling impairment of ISS and manage biosecurity risks associated with these systems.

DISCUSSION AND CONCLUSION

The occurrence and impact of biofouling in ships' ISS is a curious case whereby the significance of the issue is widely acknowledged but is not supported by broadly available literature or supporting data from appropriate sample sizes of ships. This contradiction may be because data on ISS operations and functioning are scattered among private industry and navy accounts, industry bulletins and paywalled sources, and standards and code texts (Kah Hou et al., 2019). Most of the examples in the literature describe a phenomenon of ISS problems linked to biofouling (without biofouling data) or individual case-study examples to highlight the issue. Some exceptions are driven by accessibility of ISS components and interests in biofouling that are more environmental than operational in nature (e.g., sea chest studies; Coutts et al., 2003; Coutts and Dodgshun, 2007; Frey et al., 2014; Lewis, 2016). Further insights are also driven by an understanding of strong impacts of fouling on heat-exchangers (Ezgi et al., 2014; Pamitran et al., 2016) and a renewed consideration of the importance of MIC as a source of corrosion problems more generally (Machuca, 2019).

Limited accessibility to the internal surfaces of most ISS while vessels are afloat is undoubtedly a factor in a lack of ISS biofouling sampling, which contributes to a poor understanding of biofouling impacts on these systems. This poor understanding was noted decades ago in relation to transport of non-indigenous species with a presumption that such instances were rare because ISS were not permitted to become heavily fouled (Newman, 1963; Carlton, 1985). Since then, although few studies have been conducted, the role of certain components of ISS has been highlighted as biofouling hotspots and thus sub-vectors for species translocations, notably sea chests and easily accessible pipework (Lewis and Smith, 1991; Coutts and Dodgshun, 2007; Frey et al., 2014). Hence niche area management, including ISS, is heavily emphasized in regulations and guidance materials (International Maritime Organization [IMO], 2011; California State Lands Commission, 2017; Georgiades et al., 2018; Georgiades and Kluza, 2020).

It is not clear how much of a role biofouling plays in the design stage of many ISS components, apart from heat exchangers. Notably, hull surfaces and propeller systems are also currently designed to consider fluid dynamics and power (Carlton, 2018), but not biofouling, and thus rely on post-construction remedies to manage biofouling which reduces efficiency to below design specifications (Owen et al., 2018). Unlike external biofouling, however, quantitative ship-scale models of the impacts of biofouling on ISS do not appear to exist. Some measurement and modeling have occurred for certain components (e.g., heat exchangers), providing a possible template for much broader evaluations across modern commercial ships under a variety of environmental and operational conditions. There are also anecdotal reports of individual issues or incidents, broader understanding of drivers of problems (e.g., MIC), and larger macro-scale estimates of impact of corrosion and biofouling on other water use industries (e.g., energy production industries).

Ship-scale assessments of biofouling impacts across ISS that include costs and benefits of management approaches at relevant maritime time scales (voyage itineraries, inter-dry-docking periods, or the lifetime of a ship) are absent from the accessible literature. Possibilities for this absence include:

- The complexity of the issue;
- The issue cannot be readily observed or measured and is therefore "out of sight, out of mind;"
- The issue is overshadowed by external hull biofouling and other engineering concerns for a ship's operations;
- A lack of effect or impact on modern ships (possibly linked to antifouling technology and a cost-benefit ratio that leans heavily toward continuing with operations until planned maintenance events);
- Third-party contractors that carry out maintenance tasks occasionally when vessels are in port and that may clean or otherwise treat ISS without leaving detailed data or records with the ship (i.e., the client understands an issue has been resolved but the information may only be retained by maritime service contractors and is not accessible);
- A shifting baseline acceptance of reduced efficiency because of inevitable biofouling effects (as occurs to varying degrees for external hull biofouling); and/or

- A lack of options for effective mitigation and management (Grandison et al., 2011; Growcott et al., 2016).

Developing knowledge at the ship scale is essential to promote understanding of biofouling within ISS, the operational impact of its occurrence, and the benefits of ISS biofouling management. Reductions in biofouling for operational purposes would contribute to reductions in international and domestic biofouling transfers with ships and associated biosecurity risks. Incorporating direct and indirect economic components to ship-scale ISS models is likely to provide compelling evidence to improve alignment between industry and environmental priorities.

AUTHOR CONTRIBUTIONS

ID, PC, and EG conceived the idea for the manuscript which was drafted by ID. PC, AH, DK, CS, and EG contributed ideas and edits to the draft based on their areas of expertise. All authors contributed to manuscript revisions and finalized and approved the submitted version.

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Using Virtual AChE Homology Screening to Identify Small Molecules With the Ability to Inhibit Marine Biofouling

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The search for effective yet environmentally friendly strategies to prevent marine biofouling is hampered by the large taxonomic diversity amongst fouling organisms and a lack of well-defined conserved molecular targets. The acetylcholinesterase enzyme catalyses the breakdown of the neurotransmitter acetylcholine, and several natural antifouling allelochemicals have been reported to display acetylcholinesterase inhibitory activity. Our study is focussed on establishing if acetylcholinesterase can be used as a well-defined molecular target to accelerate discovery and development of novel antifoulants via sequential high-throughput *in silico* screening, *in vitro* enzymatic studies of identified compound libraries, and *in vivo* assessment of the most promising lead compounds. Using this approach, we identified potent cholinesterase inhibitors with inhibitory concentrations down to 3 μM from a 10,000 compound library. The most potent inhibitors were screened against five microfouling marine bacteria and marine microalgae and the macrofouling tunicate *Ciona savignyi.* No activity was seen against the microfoulers but a potent novel inhibitor of tunicate settlement and metamorphosis was discovered. Although only one of the identified active cholinesterase inhibitors displayed antifouling activity suggesting the link between cholinesterase inhibition and antifouling is limited to certain compound classes, the study highlights how *in silico* screening employed regularly for drug discovery can also facilitate discovery of antifouling leads.

Keywords: homology screening, *in silico* screening, *in vitro* enzymatic studies, cholinesterase, AChE inhibitor, antifouling

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INTRODUCTION

Marine biofouling organisms rapidly settle and colonise any surface submerged in the sea to form complex mixed biofouling communities that can impair both intended mechanical function and material durability (Vinagre et al., 2020). Effective biofouling management is essential for maintaining function of vessels and fixed infrastructure, with poor antifouling countermeasures having major economic and ecological repercussions (Yebra et al., 2004; Schultz et al., 2011). Historically, surfaces have been protected using antifouling coatings that operate via broad-spectrum biocidal properties. While effective, collateral damage to non-target marine

organisms and extended environmental half-lives and sediment accumulation has led to historic and emerging bans on many broad-spectrum antifouling biocides (Yebra et al., 2004; Dafforn et al., 2011). While innovative counter measurements such as iodine bubbles (Dickenson et al., 2017), UV-light (Richard et al., 2021) grooming strategies (Hearin et al., 2015) and functional coatings (Tian et al., 2020; Jin et al., 2021) are being researched, it is clear that more targeted and less toxic alternate antifouling technologies are urgently needed (Vinagre et al., 2020).

A key challenge to developing new antifouling technologies is the large taxonomic diversity implicated in the problem, including both micro- (e.g., bacteria, diatoms) and macro-organisms (e.g., tunicates, barnacles, mussels, seaweeds) (Vinagre et al., 2020). The realisation that a combination of several mechanisms of action may be needed to target the diverse range of organisms involved in biofouling communities has spurred research into innovative coating technologies that exploit strategies evolved by marine and terrestrial organisms to prevent competition, overgrowth, and colonisation by others (Lejars et al., 2012; Flemming, 2020; Maan et al., 2020). Physicochemical and mechanical material properties such as surface energy, hardness, charge, and hydrophobicity can be straightforwardly designed and probed (Lejars et al., 2012; Selim et al., 2020). The development and design of novel environmentally friendly antifouling chemicals is, however, less straightforward to address (Flemming, 2020; Maan et al., 2020).

Settlement repelling compounds, in general, interfere with biochemical signal transduction pathways used by biofouling organisms to select a site to settle and initiate attachment and metamorphosis (Herzberg et al., 2021). The approach is intuitively attractive and there are many examples of natural allelochemicals active against one or a few biofouling taxa (Qian et al., 2009; Moodie et al., 2017a,b; Liu et al., 2020). However, broadly effective settlement repelling compounds have been elusive to date and only a few common structural features stand out as clearly associated with antifouling activity. Brominated modified dipeptic derivatives represent an exception with numerous highly active natural and synthetic antifouling compounds being reported (Sjögren et al., 2004; Hanssen et al., 2014; Trepos et al., 2014; Labriere et al., 2020). One of the underlying reasons to this generally poorly established structure activity relationship is the wide range of fouling organisms involved in biofouling and thus a high number of possible molecular and mechanistic targets which limits the possibility for rational design and screening of compound libraries (Qian et al., 2013; Vinagre et al., 2020). It further highlights how challenging it is to develop a single "silver-bullet" type repelling antifouling compound that is not also a general biocide (Flemming, 2020).

The ability to target well defined receptors and biosynthetic pathways relies on understanding the mode of action, and to rationalise this challenge, initial *in silico* screening and docking studies of plausible leads is an important part of modern drug discovery and development (Terstappen and Reggiani, 2001; de Souza Neto et al., 2020). While the *in silico* approach is rarely employed outside of medicinal chemistry, chemical library screening can facilitate effective design of new bioactive compounds in diverse range of end-use scenarios including antifouling. The potential of this strategy is exemplified by the targeted screening of octopamine receptor activators which ultimately led to the development of α2-adrenoceptor agonist medetomidine into the commercial antifouling product Selektope® (Lind et al., 2010). Selektope® prevents the settlement of barnacle larvae by stimulating active swimming away from surfaces, illustrating how a targeted screen is applicable for the development novel repelling antifouling technologies (Lind et al., 2010). One bottleneck to applying this approach is a lack of well-defined molecular targets to prevent organism settlement.

Of the known plausible molecular targets for marine antifouling (Qian et al., 2013), several natural antifouling allelochemicals inhibit acetylcholinesterase (AChE) (Moodie et al., 2019), for example polymeric alkylpyridinium salts from the sponge *Reniera sarai* (Faimali et al., 2003b), barrettin and analogs form the sponge *Geodia barrette* (Olsen et al., 2016) and secondary metabolites from the marine bacterium *Salinispora arenicola* (Melo, 2016). Acetlycholine has likewise been demonstrated to play a role in how macrofoulers choose surfaces to settle on and subsequently attach. Inhibiting AChE has been shown in laboratory bioassays to prevent settlement and attachment of the barnacle *Balanus Amphitrite* (Faimali et al., 2003a; Blihoghe et al., 2011), the blue mussel *Mytilus edulis* (Dobretsov and Qian, 2003), the bryozoan *Bugula neritin*a (Yu et al., 2007), the ascidian *Ciona intestinalis* (Mansueto et al., 2012), and the goose barnacle *Pollicepes pollicepes* (Almeida et al., 2015). These observations suggest that the cholinergic system may modulate certain settling mechanisms, or it could indicate that the cholinesterase enzyme ligands display overlapping structural features with other molecular targets involved in the settlement (Moodie et al., 2019).

To probe the potential link between AChE inhibition and antifouling activity we have undertaken a rational *in silico* design, screening, and assessment approach for AChE inhibition of marine larvae using 10,000 compounds from the Chembridge screening library. The compounds were virtually screened for binding in an AChE homology model and several selection criteria were employed to produce theoretical binders which were evaluated *in vitro* as AChE inhibitors. The lead compounds were used to identify structural analogs, and these were rescreened experimentally to determine the binding affinity to AChE. Selected compounds, both potent AChE inhibitors as well as inactive controls, were finally screened *in vivo* against a set of microfouling bacteria and microalgae as well as against macrofouling tunicate *Ciona savignyi.* Our study provides a model of how modern computational approaches can be combined with high-throughput screening approaches to accelerate the generation of antifouling leads and how a well-defined molecular target can optimise outcomes.

MATERIALS AND METHODS

Homology Model

The 3D structure of the target protein was developed using homology modelling. The query sequence was obtained from *Crassostrea gigas* AChE (XP_011454985.2). A template for this

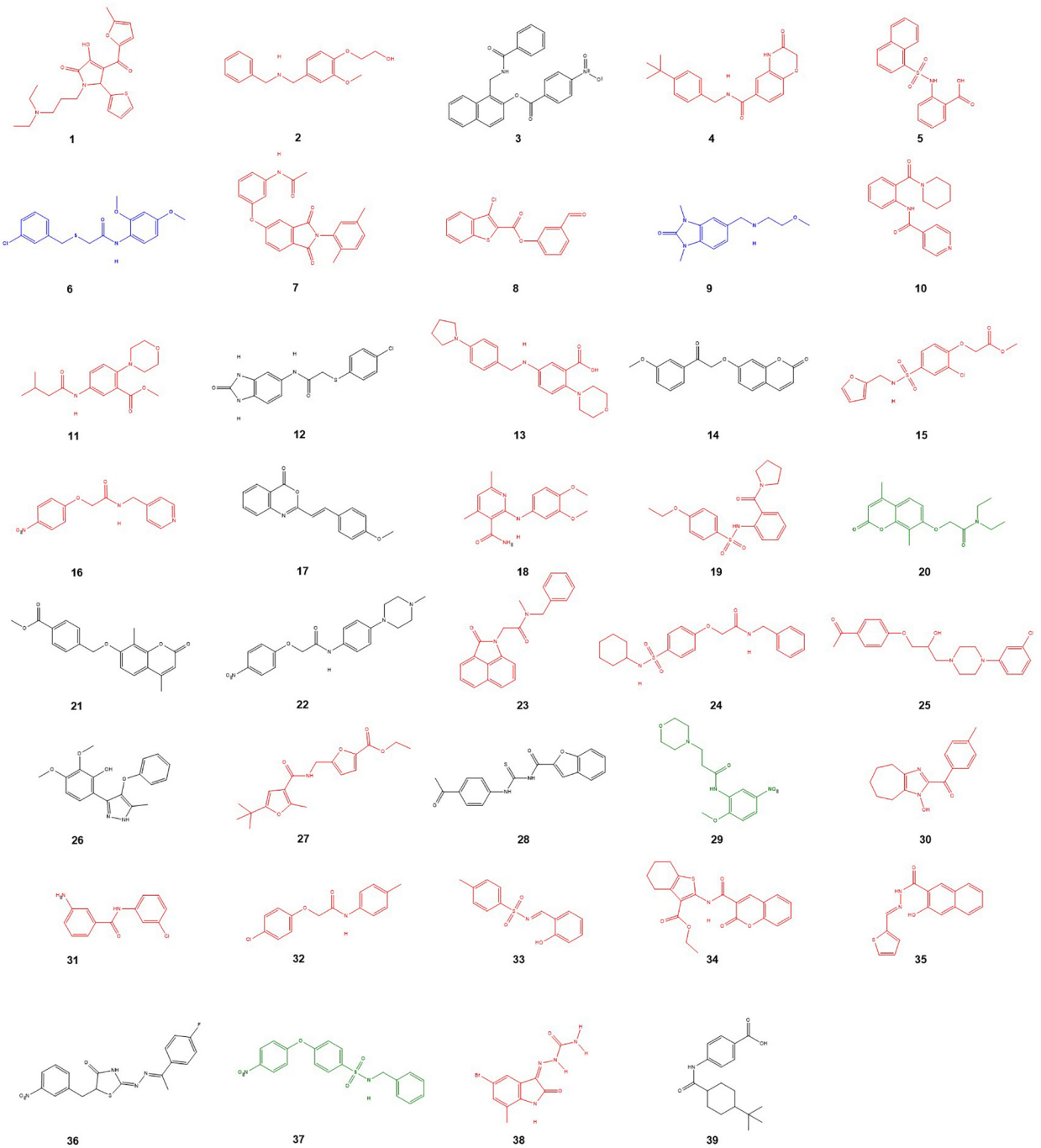

FIGURE 1 | Structures of the compounds selected for the first *in vitro* screen. Compounds in black were too insoluble for *in vitro* testing. Red compounds were inactive. Weakly active compounds **(6,9)** are blue and moderately active compounds **(20,29,37)** are displayed in green.

sequence was identified using Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990). The crystal structure for AChE (PDB ID: 6G1U, 1.79Å, *Tetronarce californica*) (Galdeano et al., 2018) was selected as the template and had an overall sequence similarity of 45% and an E-value of 7×10^{-166}. A sequence alignment was performed using the ClustalW server tool (Larkin et al., 2007). Homology models were generated using MODELLER 9.17 (Fiser and Šali, 2003), the model with the lowest MODELLER objective function was chosen for refinement and further validation. The SciGRESS FJ 2.9 program (Stewart, 2009) was used to prepare the homology model for docking by adding hydrogens, resolving any clashes, correcting missing

valences and hydrogen energy minimisation using the MM2 protocol (Allinger, 1977). Validation of the model was conducted using the Ramachandran plot and evaluation from the ProSA web server (Wiederstein and Sippl, 2007).

Virtual Screening

For the first screening run, a library of compounds was prepared using the SciGRESS FJ program using the MM2 force field (Allinger, 1977) to optimise the structures and remove any salts. The library selection was from the Chembridge EXPRESS screening library, which comprises small compounds with a mean molecular weight of 355 Da, mean cLogP of 1.6 and mean total polar surface area of 66.8 Å (Schultz et al., 2011). A majority of the compounds within this library are defined by drug-like and lead-like descriptors, making them suitable targets for further development. The centre of the modelled binding pocket was defined by the positioning of the catalytic triad of AChE, (x = –58.4790, y = 58.3950, z = 19.8190) with 10 Å radius. The compounds were initially screened at 50% search efficiency, and 10 runs per compound. The basic amino acids lysine and arginine were defined as protonated and aspartic and glutamic acids were assumed to be deprotonated. The GoldScore (GS) (Jones et al., 1997), ChemScore (CS) (Eldridge et al., 1997; Verdonk et al., 2003), ChemPLP (PLP) (Korb et al., 2009) and ASP (Mooij and Verdonk, 2005) scoring functions were implemented to validate the predicted binding modes and relative energies of the ligands using the GOLD v5.4 software suite. The output for the scoring functions is dimensionless, a greater score predicts better likelihood of binding success. A higher screening efficiency of 100% and 100 runs per compound were used for the second round of screening. A consensus model was used to evaluate the pose of each compound, compounds that had similar poses determined by at least three of the four scoring functions were considered as possible candidates (Charifson et al., 1999). The final step of the virtual screen was the visual inspection in which the position of the compound within the pocket, and all protein ligand interactions, were evaluated for binding potential. Compounds with unfavourable poses or interactions were deemed unsuitable for selection.

In vitro Cholinesterase Inhibition Assay

Cholinesterase activities were measured by the Ellman method (Ellman et al., 1961) adapted for microtiter plates, as described by Ristovski et al. (2018) using electric eel acetylcholinesterase (eeAChE), human recombinant AChE (hrAChE) or horse serum butyrylcholinesterase (BChE) (all Sigma-Aldrich, St. Louis, Missouri, United States), all dissolved in 100 mM potassium phosphate buffer (pH 7.4) to 0.0075 U/mL. Stock solutions of the tested compounds (1 mg/mL) were prepared in 100% ethanol and progressively diluted in 100 mM potassium phosphate buffer (pH 7.4) to a final volume of 50 μL. Acetylthiocholine chloride and 5,5′-dithiobis-2-nitrobenzoic acid were dissolved in the same buffer in the respective final concentrations of 1 mM and 0.5 mM, and added in 100 μL volumes to the wells of the microtiter plates. Aliquots of 50 μL of each of the cholinesterases were added to start the reactions, which were followed spectrophotometrically at 405 nm and 25°C over 5 min using a kinetic microplate reader (Dynex Technologies Inc, Chantilly, Virginia, United States). Blank reactions without the inhibitors were run in the presence of the appropriate dilution of ethanol in 100 mM potassium phosphate buffer (pH 7.4). The concentrations of the compounds inducing 50% inhibition of enzyme activity (IC_{50}) were determined as mean values ± SEM of three independent measurements. The data were analysed using the OriginPro software (OriginPro, 2020, OriginLab Corporation, Northampton, Massachusetts, United States). Active compounds were classified as either potent ($IC_{50} < 10$ μM), moderate (IC_{50} 10–100 μM) or weak inhibitors ($IC_{50} > 100$ μM) based on their IC_{50} values (Moodie et al., 2019).

In vitro Microfouling Screening

The active compounds from the first screen and their analogs were evaluated against five microfouling organisms, including marine bacteria, *Vibro harveyi* (DSM 19623, *Halomonas aquamarina* (DSM 30161) and *Pseudoalteromonas citrea* (DSM 8771), and the two microalgae *Porphyridium purpureum* (AC122) and *Exanthemachrysis gayraliae* (AC15). The adhesion and growth inhibiting potential against the microfoulers was investigated in a 96-well format at 10 and 100 μg/mL employing the methodology described by Hellio et al. (2015) Briefly, stock solution of the selected compounds (2 mg/mL) were prepared in 20% ethanol and diluted to 10 and 100 μg/mL. Aliquots (100 μL) of diluted compound were distributed in microplates and evaporated at 25°C under vacuum. For the marine bacteria, 100 μL of stock culture at 2×10^8 cells/mL in peptone marine broth were added to each well. Bacterial growth was monitored at 620 nm after incubation for 48 h at 25°C. The bacterial adhesion was quantified at 545 nm after crystal violet staining of residual biofilm after careful washing of the wells. For microalgae, 100 μL of algal culture (0.1 mg chlorophyll *a*/L) in F/2 medium were added to the wells as described for the bacterial growth experiments above. The culture was maintained for 5 days at 20°C and the microalgal growth was quantified via fluorescent analysis (excitation at 485 nm, emission at 645 nm) of chlorophyll *a* released upon addition of 100 μL of methanol to the wells. Chlorophyll extraction was also performed on the adhered cells, after using a multichannel pipette to eliminate all the non-attached cells, and quantified by fluorescence measurement to determine the algal adhesion.

In vitro Macrofouling Screening Against *Ciona savignyi*

The ability of the rescreened leads to inhibit the settlement and metamorphosis of the Pacific transparent sea squirt *Ciona savignyi* larvae was investigated. Compounds were screened for effects following methods described in Cahill et al. (2016). Adult brood stock were harvested from Nelson Marina, Nelson, New Zealand and held in a recirculating seawater system (18 ± 1°C, 33 ± 1 PSU) and fed bulk-cultured *Isochorysis glabana* until ready to spawn (as indicated by full egg and sperm ducts). Three individuals were surgically spawned, cross-fertilised in artificial seawater, and left for 24 h to hatch. Freshly hatched larvae were diluted in additional artificial seawater to

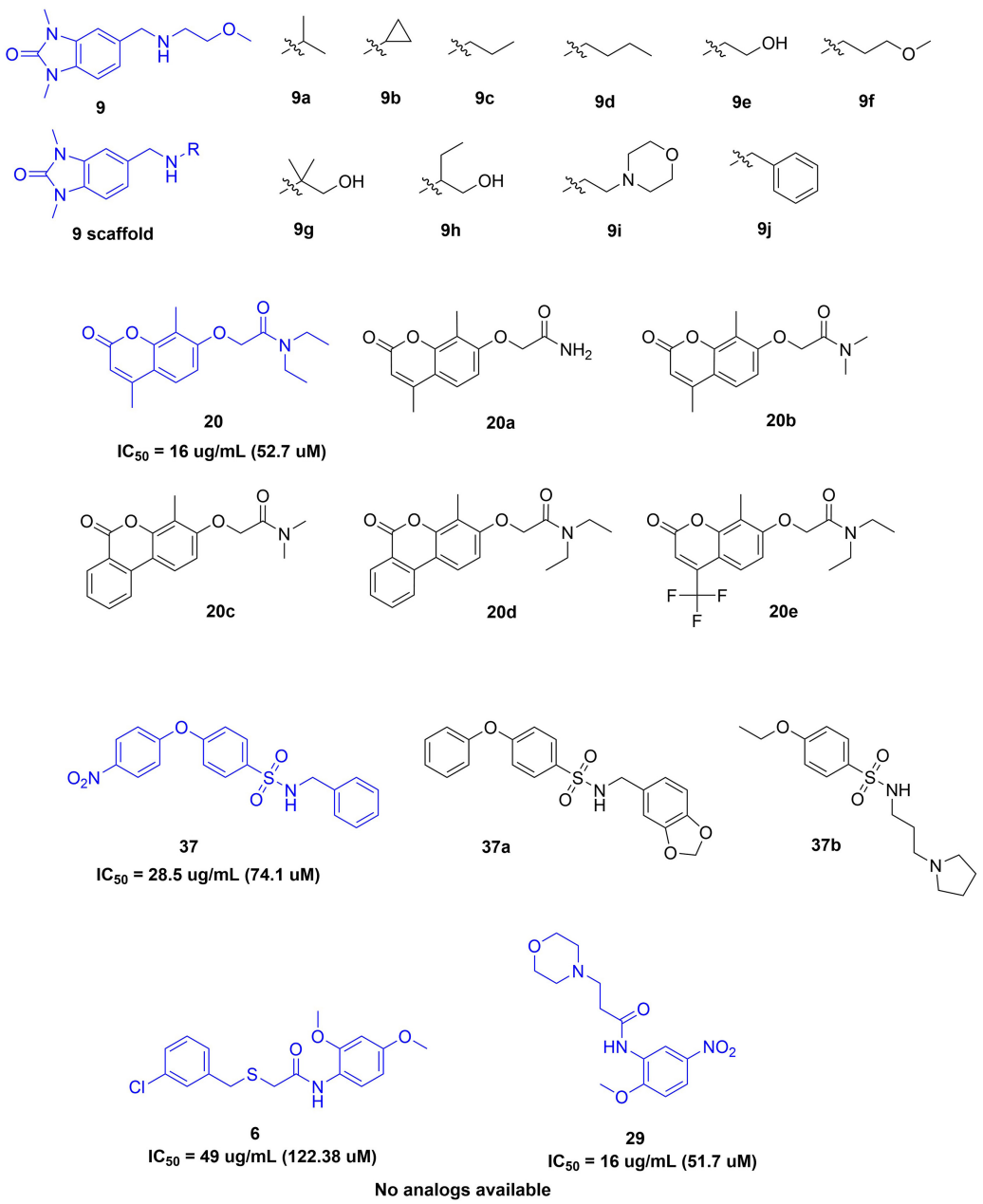

FIGURE 2 | Structures of analogs designed and selected for the second *in vitro* screen and for micro- and macrofouling evaluation.

yield final larval concentration of 3 ± 1 larvae/mL. Aliquots (7.1 mL) of this larval suspension were added to wells of 12-well tissue culture plates (Corning Costar) which contained serial dilutions of the test compound in small amounts (70 μL) of 20% ethanol. The range of concentrations of each compound assessed was 0.25, 0.5, 2.5, 5, 12.5, 25, 50, and 100 μg/mL. Blank and solvent controls were also included, and three replicates were performed in all cases (N = 3). Plates were held at 18 ± 1°C for 5 days, after which the number of successfully settled and metamorphosed individuals were counted. Dose-responses were modelled using Weibull curve fitting (as dictated by model fit) and nominal concentration estimates that reduced settlement and metamorphosis relative to the controls by 50% (IC_{50}) using R statistical software (R Core Team, 2020).

RESULTS AND DISCUSSION

Homology Modelling

Fifty models were built using the MODELLER software package (Fiser and Šali, 2003). The model with the lowest objective function was chosen for further refinement and

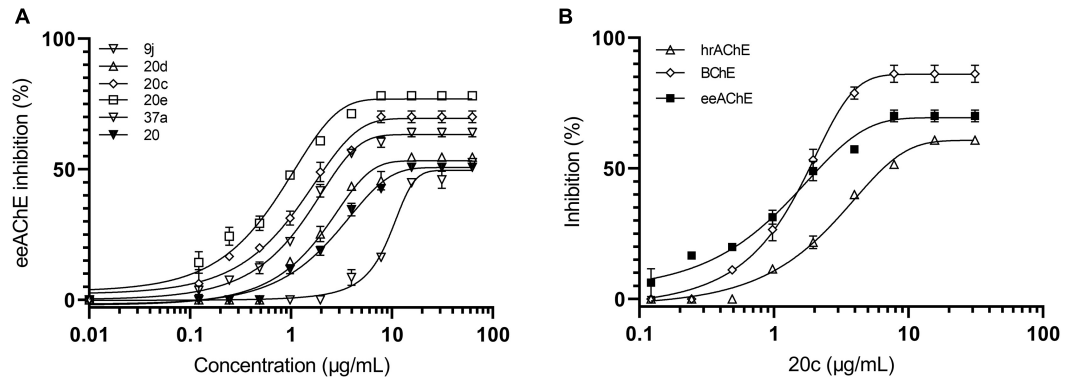

FIGURE 3 | Inhibition of cholinesterase enzymes. **(A)** Inhibition of eeAChE by compounds **9j**, **20**, **20c**, **20d**, **20e,** and **37a.** The IC_{50} values toward eeAChE were determined as 9.44 μM for **9j**, 52.7 μM for **20**, 7.23 μM for **20c**, 35.35 μM for **20d**, 3.36 μM for **20e** and 169.54 μM for **37a**. **(B)** Inhibition of eeAChE, hrAChE and BChE by compound **20c.** The IC_{50} values toward eeAChE, hrAChE and BChE were determined to be 7.23 μM, 22.81 μM and 5.94 μM, respectively.

validation. Stereochemistry of the model was evaluated using a Ramachandran plot. The plot identified that 97.9% of the amino acids were in their highly preferred regions while the remaining 2.1% were preferred observations. No amino acids were positioned in questionable locations. The ProSA server result generated a Z-score value of –9.49 indicating that the overall model quality is more comparable to an NMR structure rather than an X-ray structure. Given the low sequence similarity a lower Z-score was expected. However, given the limited options the homology model was used for the virtual screen.

In silico Screening – Cholinesterase Docking

Molecular docking has been employed in several studies to develop an understanding of novel cholinesterase inhibitors for neurological applications (Correa-Basurto et al., 2007; Farrokhnia and Nabipour, 2014; Moodie et al., 2019). To probe a virtual screening approach for generating antifouling leads, compounds with theoretical binding to the active site of an AChE homology model of a marine fouling organism (*C. gigas*) were searched for from 10,000-compound library. The compounds from the screening library were selected, prepared, and docked to the AChE homology model to identify potentially active compounds for further analysis.

Selection of compounds to proceed to the next round of screening was based on the scoring threshold generated for each scoring function. The first-round thresholds of 31 (GS), 41(CS), 43(PLP), and 21(ASP) resulted in a total of 1,058 compounds continuing for the second round of screening. The selected compounds were then redocked to the active site of AChE with more stringent scoring thresholds set at 51 (GS), 25(CS), 60(PLP), 35(ASP) resulting in 201 compounds with sufficient binding scores. These compounds were visually inspected and further evaluated based on their pose and bonding interactions within the binding pocket. Finally, 39 compounds displaying good theoretical fit were purchased from the commercial vendor for initial testing in the biological assays (**Figure 1**).

TABLE 1 | Cholinesterase inhibition of lead compounds generated from the virtual screening.

Compound	Cholinesterase inhibition (μM)		
	eeAChE	**hrAChE**	**BChE**
6	> 400	122.38 ± 3.19	na
9	> 400	na	na
9j	9.44 ± 0.85	na	na
20	52.7 ± 4.10	na	69.22 ± 1.05
20b	49.04 ± 1.88	na	na
20c	7.23 ± 2.14	22.81 ± 0.88	5.94 ± 2.36
20d	35.35 ± 0.90	na	5.6 ± 0.45
20e	3.36 ± 0.39	na	na
29	51.7 ± 2.80	na	na
37	74.1 ± 1.99	74.14 ± 5.87	91.05 ± 4.92
37a	169.54 ± 0.91	na	na

IC_{50}, concentration required to induce 50% inhibition of enzyme activity. Data are means ± SEM of three independent measurements. na = no activity.

In vitro Screening – Cholinesterase Enzyme Inhibition

The initial leads were tested for their ability to inhibit electric eel AChE. Compounds **3**, **12**, **14**, **17**, **21**, **22**, **26**, **28**, **35**, **36**, and **39** were shown experimentally to be too hydrophobic to allow dissolution in either water, ethanol or DMSO which excluded them from the *in vitro* evaluation. Of the remaining 28 compounds, five displayed either weak (IC_{50} > 400 uM, compounds **6** and **9**) or moderate inhibitory activity (IC_{50} 10–100 μM, compounds **20**, **29**, and **37**). The remainder of compounds were inactive. The five active compounds (**6**, **9**, **20**, **29**, and **37**) were used as the basis for further activity refinement via analog searches of the active scaffolds. A search for all commercially available analogs was conducted and 19 additional compounds were subsequently purchased for testing after taking compound hydrophobicity into account (ClogP < 4.0) as a key selection criterion (**Figure 2**).

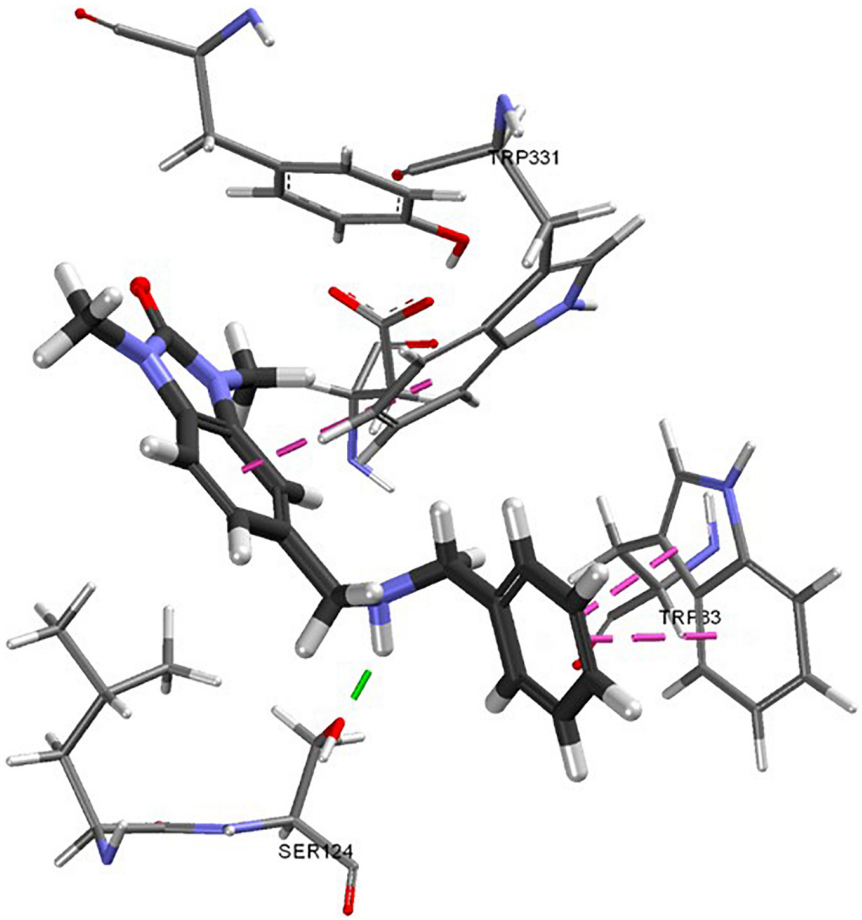

FIGURE 4 | Predicted binding of **9j** to the homology model. Green dashes indicate hydrogen bonding interactions, pink dashes indicate π-π interactions.

When the 19 compounds were rescreened for eeAChE, hrAChE, and BCh inhibitory activities, seven analogs displayed moderate or high inhibitory activity in a dose-response dependent manner (**Figure 3** and **Table 1**). IC_{50} values against eeAChE ranged from 3.36 ± 0.39 μM for **20e** to 169.54 ± 0.91 μM for **37a**. Most compounds were active against eeAChE only and only two compounds (**20c** and **37**) were active inhibitors of all three enzymes.

The activity seen for the most active compounds is high (Moodie et al., 2019) and shows how the virtual screening is able to guide the discovery of novel structures. For this study, the analog design/selection was based on commercially available compounds and no chemical modifications or synthetic procedures were employed to modify the leads. Given the inhibitory activities observed, these lead structures identified could form the foundation for future rational design and structure-activity relationship studies.

No suitable analogs of **6** and **29** were commercially available but several analogs of **9** with structural diversity in the N-alkyl chain were included in the second screen. Of these analogs (**9** – **9j**), only **9j** displayed pronounced cholinesterase inhibition with a potent inhibitory activity against eeAChE of 9.44 ± 0.85 μM. This experimental observation was supported by subsequent docking studies. Docking predictions indicated consistent poses for the non-active compounds based on the four scoring functions used to generate results. Compound **9j** had a different predicted pose (**Figure 4**), showing the opposite pattern, predicting a hydrogen bonding interaction with SER124 and Pi Stacking interactions with TRP83, and TRP 331.

Four of the five chromenone analogs of compound **20** displayed positive results in the cholinesterase bioassays with only terminal amide **20a** being inactive. Interestingly compound **20b** (49 μM) and **20e** (3 μM) appears to be selective for eeAChE while **20** and **20d** and showed activity against both eeAChE and hsBChE indicating they may be a less selective inhibitors. Finally, **20c** showed a high activity against all enzymes illustrating a high general cholinesterase inhibitory profile.

Selected analogs were redocked to investigate the difference in activity seen between evaluated enzymes. Compound **20b** showed a consistent predicted binding pose by all four scoring functions. The chromenone moiety was positioned deep in the pocket with the tail facing the surface (**Figure 5**). The chromenone carbonyl moiety has a predicted hydrogen bonding interaction with GLY119. The ester also has a predicted interaction with SER124

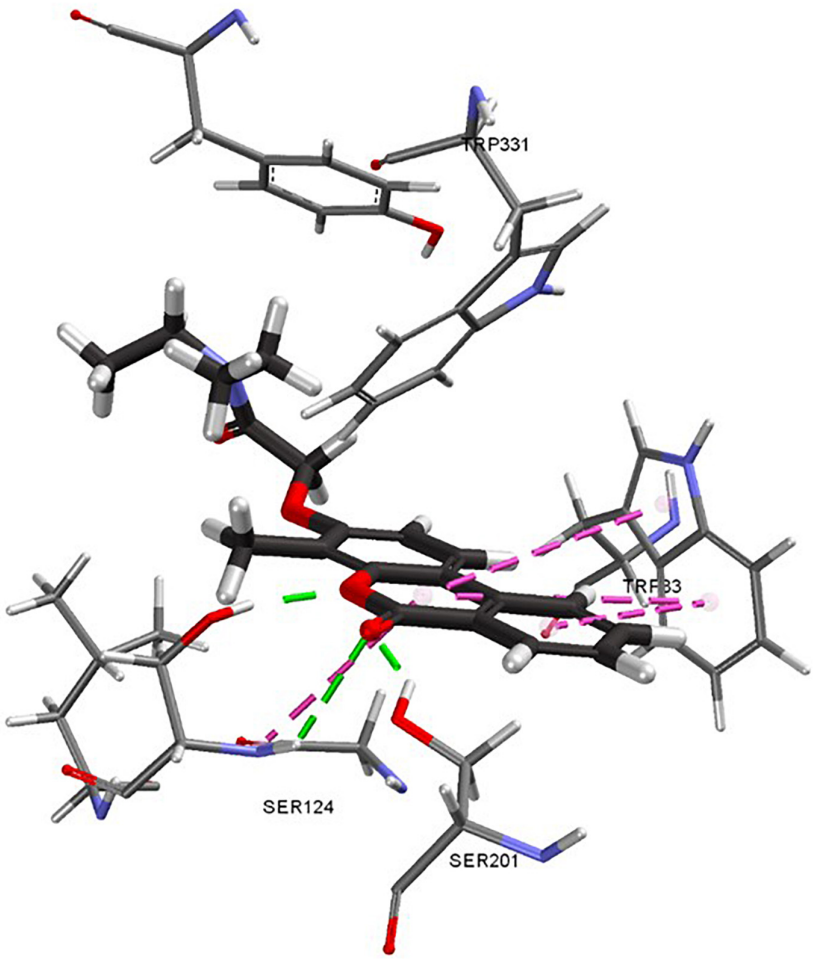

FIGURE 5 | Predicted docking pose of compound **20d**. Green dashes indicate hydrogen bonding interactions and pink dashes indicate π-π interactions.

positioned in the middle of the pocket. The chromenone moiety also features multiple hydrophobic interactions with TRP83.

Docking results of compound **20d** showed a predicted shift in position, aligning the chromenone moiety closer to allow for hydrogen bonding interactions with both SER198 and SER121 (**Figure 6**). The shift in positioning now indicates that the hydrophobic interactions of this moiety are taking place with TRP331. The interaction with SER198 may be the reason for increased activity in the eeAChE assay and new activity in the hsBChE assay, as the amino acid is known to play a part in catalytic activity.

Compound **20e** has structural similarities to **20d,** featuring a trifluromethyl group on the chromenone in place of the phenyl ring. This alternation reduced activity against hsBChE but increased activity for eeAChE. The pose prediction for **20e** compound varies from **20d** with the chromenone moiety rotated and predicted to have hydrogen bonding interactions with GLY119 and TYR132. The pi-pi interactions appear to stack more compared to **20d**. The impact of the trifluoromethyl can be observed when comparing **20** to **20e**, as the only difference is the substitution of the methyl with trifluoromethyl, resulting in a large increase of activity against eeAChE. This observation implies that the change has either altered the polarity of the compound, resulting in a less stable energy conformation within the pocket, or the halogen interactions and size had been more favourable. Chromenone class compounds are known for their activity in human neurotransmission (Piazzi et al., 2003; Najafi et al., 2016) so it is interesting that the virtual screen has generated compounds that are not active against the hrAChE which implies that these compounds may be worth further exploring.

Of the two analogs of compound **37** evaluated, only **37a** displayed any activity, representing a reduction in comparison to the generally active **37**. Both compounds share a very similar pose, indicating that the difference in activity could be due to the loss of the nitro group.

In vivo Screening – Biofouling Settlement and Metamorphosis

Having established a wide range of inhibitory activities against three cholinesterase enzymes, the compounds were subsequently experimentally assessed *in vivo* against five microfoulers

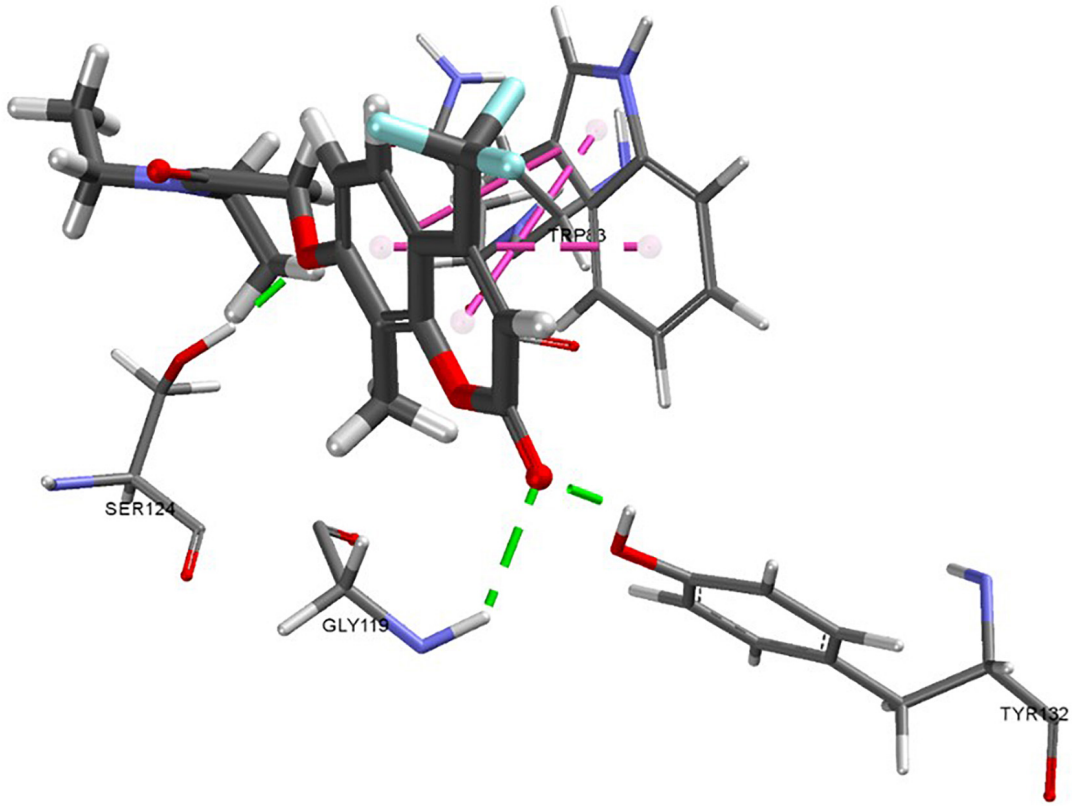

FIGURE 6 | Predicted docking pose of compound **20e**. Green dashes indicate hydrogen bonding interactions and pink dashes indicate π-π interactions.

and one macrofouler. Microfouling can cause microbially induced corrosion and it also leads to loss of hydrodynamic properties despite being caused by microscopic organisms. The microfouling screen assessed settlement/adhesion and growth of selected marine bacteria and microalgae. Both the active compounds from the second cholinesterase screen as well as the inactive ones were included. No reduction in either the settlement or growth of the five microfouling organisms was observed over the course of the experiments (data not shown) suggesting that the compounds are inactive at the two concentrations evaluated (10 and 100 μg/mL). While no specific antifouling activity was observed, the lack of effect on the growth of the microorganisms also indicate that these compounds are not generally toxic.

The 11 active compounds (**Table 1**) were also evaluated as inhibitors for the metamorphosis of larvae of the seasquirt *C. savignyi*, an invasive macrofouler that is problematic in scenarios including ship hull fouling and aquaculture. Out of the 11 compounds, **9j** was shown to be a potent inhibitor with an IC_{50} of 4.60 ± 0.39 μg/mL as determined from the dose-response curve (**Figure 7**). None of the other compounds displayed any activity against *C. savignyi* at the employed concentrations.

While **9j** is clearly a potent novel inhibitor of both *C. savignyi* larval metamorphosis and eeAChE, none of the other active cholinesterase inhibitor analogs were active in the antifouling screens. The initial lead structure **9** was only weakly active and it is likely that **9j** would not have been discovered without

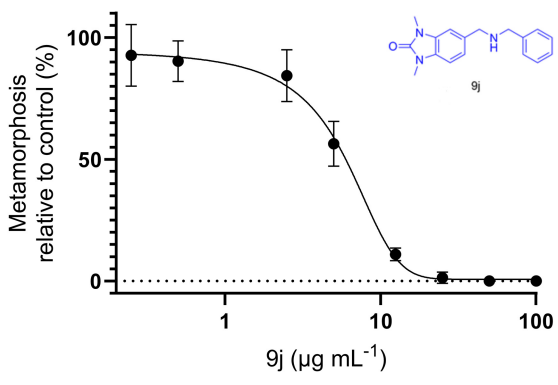

FIGURE 7 | Dose-response curve illustrating the effect of **9j** on the metamorphosis of *C. savignyi larvae*.

inclusion of analogs after the initial *in vitro* screen. Several of the other inactive antifouling compounds display similar or stronger cholinesterase inhibitory potential *in vitro* (**20c**, **20d**, and **20e**) and cholinesterase inhibition at low micromolar concentrations and our study shows that this does not automatically equal a strong antifouling effect even though several natural products have been reported with these dual bioactivities. Key structural requirements are at play and these warrants further research.

In addition, our study illustrates the importance of performing *in vivo* experiments to verify effects predicted from *in silico* and/or *in vitro* screening experiments.

The fact that none of the compounds were active against the microfoulers was more expected as these primitive microorganisms lack the nervous system of the eukaryotes. Prokaryotic cholinesterases exist, but despite catalysing the same chemical transformation, they share little structural homology with the eukaryotic counterparts and overlapping binding preferential of compounds from the virtual hrAChE screen is not expected (To et al., 2020). Nevertheless, this study highlights that the cholinesterase enzyme family represent a valid target for several types of antifouling organisms.

CONCLUSION

The current study probes the correlation between cholinesterase inhibition and antifouling and describes a computational approach to search for novel antifoulants *via* virtual homology screening. A selection of novel cholinesterase inhibitors was discovered through the *in silico* and *in vitro* screening process. From these leads a single novel antifouling compound was identified with strong inhibitory effects on *C. savignyi* larval metamorphosis. No compounds with activity against marine bacteria or microalgae were discovered. Our study illustrates a rational and rapid screening strategy to identify novel structural leads for antifouling lead development and it also highlights that a strong *in vitro* cholinesterase activity is not directly transferable to a high *in vivo* antifouling activity.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

HA, PC, and JS planned and conceived the study, interpreted the data, and selected compounds. HA performed the virtual screening and the computational experiments. TT, RF, and KS performed the cholinesterase studies and compiled the results. VF and CH performed the microalgal antifouling experiments. PC performed the macrofouling screen. JS compiled all data and drafted the initial manuscript. All authors contributed to generate the final manuscript.

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frontiers in Marine Science

POLICY BRIEF
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Technical Considerations for Development of Policy and Approvals for In-Water Cleaning of Ship Biofouling

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Submerged ship surfaces are often inhabited by diverse sessile and sedentary marine organisms, which can directly impact vessel operations and increase the likelihood of non-indigenous species (NIS) establishment and impacts. Ship in-water cleaning (IWC) systems are now being incorporated into biofouling policy, and rigorous, transparent, and predictive verification testing is vital to regulatory success. Performance criteria for IWC approval should focus on environmental protection goals by including: qualified and independent testing; quantitative, robust, and statistically sound data, rather than qualitative observations; water sampling at all critical control points to characterize the release of harmful materials, including dissolved and particulate biocides; measurable and protective endpoints, rather than percent reductions; determinations of presence or absence of macro-organisms, irrespective of species origins or physiological state; and appropriately trained IWC operators.

Keywords: ship biofouling, in-water cleaning, non-indigenous species, environmental regulations, verification testing

INTRODUCTION

The colonization of submerged surfaces by sessile and sedentary organisms, including microbes, invertebrates, and macroalgae, has long been a significant challenge for coastal and ocean-going ships (Woods Hole Oceanographic Institute, 1952). Biofouling of the global shipping fleet, which is responsible for transporting approximately 80% of the world's goods and materials

(UNCTAD, 2018), can result in increased vessel corrosion rates, hydrodynamic drag, fuel consumption, and exhaust emissions (Townsin et al., 1981; Schultz, 2007; Li and Ning, 2019). Beyond the direct impacts on vessel operations, maintenance, and associated legal/contractual requirements (e.g., BIMCO, 2013, 2019; Altarriba and Halonen, 2019), which drive advances in biofouling prevention/management systems (Scianni and Georgiades, 2019), biofouling management is the focus of current and evolving environmental protection regulations.

Greenhouse gas (GHG) emissions from ships have been regulated since 2005 under Annex VI (Regulations for the Prevention of Air Pollution from Ships) of the International Convention for the Prevention of Pollution from Ships (MARPOL 73/78, 1997). Therefore, biofouling, which even at the biofilm (i.e., slime layer) level requires ships to increase fuel use to maintain speed (e.g., Schultz et al., 2011), will result in amplified GHG emissions that may exceed required limits.

The role of ship biofouling in the introduction, establishment, and subsequent spread of non-indigenous species (NIS) is also a significant concern (Hewitt and Campbell, 2010; Ruiz et al., 2015; Davidson et al., 2018; Bailey et al., 2020; Georgiades et al., 2021). Marine NIS have caused a range of impacts to various economic, ecological, societal, and cultural resources (Ruiz et al., 1997; Grosholz, 2002; Hewitt et al., 2004; Bell et al., 2011; Georgiades et al., 2021). Thus, guidelines and regulations to prevent NIS impacts via ship biofouling are beginning to emerge.

In response to ship operational needs, and to a lesser extent emissions and biosecurity regulations, a sophisticated antifouling system (AFS) industry continues to evolve to minimize biofouling accumulation. The primary AFS employed by ships is surface coatings, which inhibit macrofouling attachment (using biocides) or reduce adhesion (foul-release) to wetted surfaces (Dafforn et al., 2011). Biocidal coatings typically require regulatory approval for use to prevent unintended environmental degradation (Dafforn et al., 2011). Although some biocides are now banned [e.g., tributyl tin (TBT)] due to non-target environmental effects (e.g., Sonak et al., 2009), copper- and zinc-based biocidal AFS remain the most commonly applied on commercial ships (Scianni et al., 2021).

Despite continuous improvements in AFS efficacy and safety, coatings have a limited service life (reapplied at 5–10 year dry-docking intervals) and do not prevent biofouling accumulation on all ship surfaces (Davidson et al., 2016; Georgiades and Kluza, 2017), especially if ships have extended stationary/immobile periods (BIMCO, 2013; Davidson et al., 2020). Biofouling "hotspots" also exist that include areas on ships which are difficult to paint (e.g., dry-dock support strips) or sub-optimal for antifouling coating performance (e.g., niche areas such as rudders and sea chests) (Coutts and Taylor, 2004; Davidson et al., 2009, 2016). In-water removal of established macrofouling (i.e., individual organisms or colonies visible to the eye) is a common practice for many ships, primarily to increase fuel efficiency (e.g., Schultz et al., 2011).

Traditionally, ship in-water cleaning (IWC) has involved divers or remotely operated vehicles (ROV), which use scraping tools or cleaning carts to remove macrofouling from hull and propeller surfaces without capture of released debris (i.e., fouling organisms and coating material) (Jones, 1999; McClay et al., 2015; Morrisey and Woods, 2015). However, without debris capture, IWC of macrofouling can directly lead to discharges of NIS and harmful AFS biocides (Scianni and Georgiades, 2019; Tamburri et al., 2020a). Thus, IWC technology is rapidly developing to either (a) capture and process debris removed from ships or (b) conduct periodic proactive IWC (i.e., reduction/removal of biofilms to prevent or inhibit/limit macrofouling growth) (Tribou and Swain, 2010; Scianni and Georgiades, 2019; Tamburri et al., 2020a). Although proactive IWC is typically less abrasive than macrofouling removal, substantial amounts of microscopic material (biological and chemical) can be released into the environment. However, proactive IWC is viewed as a relatively low biosecurity risk because it may ultimately minimize the translocation of macrofouling species, including associated pathogens (where present) (Department of Agriculture [DOA] et al., 2015; Georgiades et al., 2021).

The removal or prevention of macrofouling through reactive IWC with capture or proactive IWC, respectively, may represent rare win-win solutions for both the shipping industry and the environment. However, it is critical to ensure these practices do not result in unintended consequences, including: (a) increased biosecurity risk, (b) increased discharge of AFS biocides, and (c) diminished coating condition that reduces AFS performance or service life (Scianni and Georgiades, 2019; Tamburri et al., 2020a). Therefore, as various authorities develop new biofouling policies that include the use of approved ship IWC systems, comprehensive, evidence-based consideration of system efficacy and environmental safety is paramount.

This policy brief assesses the methods proposed for evaluating IWC systems, describes the challenges associated with quantifying IWC system performance and environmental safety, and proposes a series of practical and feasible recommendations for the verification testing and approval of ship IWC systems.

POLICY OPTIONS AND IMPLICATIONS

Ship biofouling management guidelines (IMO, 2011) and regulations (California Code of Regulations, 2017; MPI, 2018) have been developed at international, national, and regional levels (Georgiades et al., 2020; Scianni et al., 2021) to minimize biosecurity risks. Importantly, these guidelines and regulations identify vessel IWC or treatment as important tools for ship maintenance (IMO, 2011; Department of Agriculture [DOA] et al., 2015; Scianni et al., 2017; Georgiades et al., 2018), renewing the interest in appropriate and proven technologies.

The Australian and New Zealand antifouling and IWC guidelines were drafted in 2011 (Ministry of Agriculture and Forestry, 2011). This action was coincident with the development of IMO biofouling guidelines and movement toward biofouling management regulations in Australia, New Zealand, and California, and followed the 2001 International Convention on the Control of Harmful Anti-fouling Systems on Ships that banned TBT internationally (IMO, 2001). The intent of these guidelines was to provide a risk-based framework for approving biofouling management practices by considering both

chemical contamination and biosecurity risks. These guidelines were published in 2013 and were updated by Australia in 2015 (Department of Agriculture [DOA] et al., 2015).

New Zealand's Craft Risk Management Standard for Biofouling on Vessels Arriving to New Zealand (CRMS-BIOFOUL) requires ships to take preventive measures to maintain a "clean hull" prior to arrival (MPI, 2018). Compliance is through the presentation of documentary evidence showing that one of the following measures has been undertaken: (a) continual maintenance following best practice, or (b) cleaned within 30 days prior to arrival in New Zealand, or (c) scheduled arrangement with an MPI-approved provider for cleaning or treatment within 24 h of arrival. While there are no MPI-approved providers for cleaning or treatment of international ships within New Zealand territorial waters, technical advice for evaluation of IWC systems has been produced for external hulls and niche areas (Morrisey et al., 2015) and internal niche areas (Growcott et al., 2019), with the former being recently tested (Jones and McClary, 2021).

In the United States, IWC is regulated under the National Pollutant Discharge Elimination System (NPDES) section of the Clean Water Act (33 U.S.C. 1342). IWC without capture is regulated as a discharge incidental to the normal operation of a vessel under the 2013 Vessel General Permit (USEPA, 2013), whereas IWC with capture requires a separate NPDES permit typically issued by a state or regional water quality regulatory entity. This arrangement will change with the adoption of regulations developed by the USEPA and U.S. Coast Guard under the authority of the 2018 Vessel Incidental Discharge Act (VIDA, 33 U.S.C. 1322). Final rules have not been published, and there remains uncertainty as to how these activities will be regulated once they are in place.

In 2021, the Baltic and International Maritime Council (BIMCO) and International Chamber of Shipping (ICS) proposed an industry standard for IWC with capture (BIMCO/ICS, 2021a,b). The intent of this standard is to help "ensure that the in-water cleaning of a ship's hull, and niche areas, including the propeller, can be carried out safely, efficiently and in an environmentally sustainable way." However, several components of the proposed standard and approval regime may limit the latter goal. These include, among others: (a) allowance of 10% of macrofouling to remain on the hull following cleaning, (b) reliance on percent reduction for removal of material from the effluent that allows for proportional increases of material release with increasing vessel size and fouling extent, (c) use of untested systems on substantial portions of vessels (up to 5%), and (d) option to not include measurements for AFS biocide release in evaluations/approvals.

The Australian Government consulted on their draft IWC standard in 2021 (DAWE, 2021). The draft standard includes water quality (biocide) testing, captured debris effluent filtration to 10 μm (or treatment to render organisms non-viable), and relies on a percentage cut-off for capture akin to (but a higher level than) BIMCO/ICS. Similarly, Transport Canada released draft voluntary guidance for relevant authorities on ship IWC for consultation (Transport Canada, 2021). Their draft guidelines introduce secondary treatment of captured debris, in addition to particle separation (10 μm post-2023), and also require the discharge to meet all legal requirements within the jurisdiction of the activity. Importantly, while independent testing is fundamental to both drafts, detailed guidance regarding quantitative IWC system evaluation is not provided.

ACTIONABLE RECOMMENDATIONS

Comprehensive, consistent, and rigorous performance verification of any technology, and its subsequent approval for use, is often a complicated process that requires: (a) careful consideration of policy goals, (b) clear communication of technology performance requirements, (c) availability of accepted/validated verification test methods and approaches, (d) defensible, independent test results, and (e) post-approval compliance and enforcement processes (e.g., Bedson and Sargent, 1996; USEPA ETV, 2010; Tamburri et al., 2020b). This complexity is particularly true for the diverse suite of novel IWC systems designed for use on various types of ships (e.g., numerous designs, coatings, ages, and operational profiles) and biofouling (e.g., assorted stages, types, coverages, and locations), and under diverse environmental conditions (e.g., variable visibilities, swells, currents, and ambient water qualities) (Scianni and Georgiades, 2019; Tamburri et al., 2020a). This section provides advice to help avoid pitfalls in verification testing and approval of IWC systems, which can undermine the success of emerging environmental regulations.

The likelihood of NIS introduction and establishment from ship biofouling is driven by a variety of factors (e.g., volume of traffic; vessel operational profiles; antifouling measures employed; time since dry-docking; fouling type, extent, and maturity; and environmental conditions of fouling origin and recipient locations) (Coutts et al., 2010; Inglis et al., 2010; Davidson et al., 2020). While it is unrealistic to expect ships to remain completely free of all biofouling at all times, minimizing the species richness, abundance, and maturity of macrofouling lowers the biosecurity risks (Georgiades and Kluza, 2017; Georgiades et al., 2021). As different authorities consider acceptable measures for minimizing ship biofouling and acceptable biofouling levels, statistical comparisons of biofouling on various areas of ships (i.e., hull and niche areas) before and after IWC, including treated (cleaned) and control (not cleaned) areas, remains key to protective and robust IWC system approval.

Detailed methods for quantitative assessments of ship biofouling for NIS inspections, and in association with both reactive and proactive IWC performance testing, are available (e.g., Morrisey et al., 2015; ACT/MERC, 2019, ACT/MERC, 2020; Growcott et al., 2019; Georgiades and Kluza, 2020; Tamburri et al., 2020a). While these approaches vary to some extent, they all require a sufficient level of biofouling sampling and analysis that will produce the statistically sound and predictive data needed for meaningful IWC system assessment. While a balance between testing practicability (in particular, time and cost) and comprehensive data for IWC approval is needed,

the use of qualitative estimates of biofouling (e.g., inconsistent, non-numerical video assessments) as part of IWC system approval is ineffective and discouraged.

Ship biofouling is an open system, with live organisms and coating biocides directly exposed to local waters. Nevertheless, IWC activities can exacerbate the release of harmful material to the environment through: (a) the lack of, or ineffective, capture of debris at the cleaning unit/vessel interface, (b) cleaning operations related disturbances (e.g., support boat movement, diver or ROV movement, umbilical or hose management), and (c) incomplete/ineffective processing and disposal of captured debris (if attempted) (**Figure 1**). Therefore, to identify measurable environmental impacts, if any, verification testing of IWC systems should include appropriate water sampling and analysis at all points critical for potential harmful material release, and for statistical comparisons to be made against ambient water not influenced by IWC (e.g., ACT/MERC, 2019,ACT/MERC, 2020; Tamburri et al., 2020a; Jones and McClary, 2021).

It has been demonstrated that water quality sampling and analyses targeting both chemical contaminants of concern (e.g., copper and/or zinc when cleaning a biocidal coating) and total suspended solids (TSS, as a proxy for particulate debris, including macrofouling organisms) is a feasible and effective approach (e.g., Tamburri et al., 2020a). Importantly, both the particulate and dissolved forms of coating biocides should be assessed to enable a complete understanding of environmental impacts (e.g., Tamburri et al., 2020a; Jones and McClary, 2021). Examination of possible chemical contamination during IWC should only be an optional consideration for non-biocidal coatings.

Visual approaches to detect the release of harmful material (e.g., visible plumes) during IWC are also under consideration. While such observations can help to identify areas of concern, they are subjective, qualitative estimates that are highly influenced by environmental conditions (e.g., sea state, water clarity/visibility, amount and angle of light, distance of observations). Visual observations of debris capture or release should, therefore, only be considered supplementary information, and not as approval criteria.

Determination of measurable environmental impacts as a result of IWC system use avoids the drawbacks associated with requiring a percent reduction in levels of potentially harmful materials. IWC performance standards based on percent reductions (whether in biofouling removal or in debris capture and disposal) are inappropriate because quantitative, mass balance measures of: (a) what is removed from a ship's submerged surface, (b) what is captured, and (c) what is released over the entire cleaning process (e.g., from cleaning unit to shore processing waste disposal and discharge) would be required but not currently (nor ever likely to be) practical or feasible. Furthermore, while arbitrary criteria of 90, 95, or 99% reductions would clearly decrease risks, if initial biofouling levels or debris loads are high, particularly in the case of larger vessels, even > 99% capture and removal can lead to considerable discharges of harmful material, which may significantly impact local waters (**Figure 2**).

The physiological state of dislodged organisms, and their origins, are also being considered as part of biofouling policy and IWC approval. Ideally, effective reactive IWC would release very few, if any, live macrofouling organisms (and their propagules or associated pathogens) into the environment. However, because no technology (especially new and complex technologies) is perfect, interest in requiring the release of only dead, non-viable or local/native organisms would appear reasonable. The problem remains that methods for appropriate levels of species identification, and for definitive determination of live vs. dead and/or viable vs. non-viable (including propagules and tissue fragments but excluding residual baseplates or basal shell material remaining after reactive IWC) are in dispute and often challenging and error-prone (e.g., Zaiko et al., 2016; Blatchley et al., 2018). However, where IWC systems are required to minimize discharges of the dissolved and particulate components of biocidal coatings

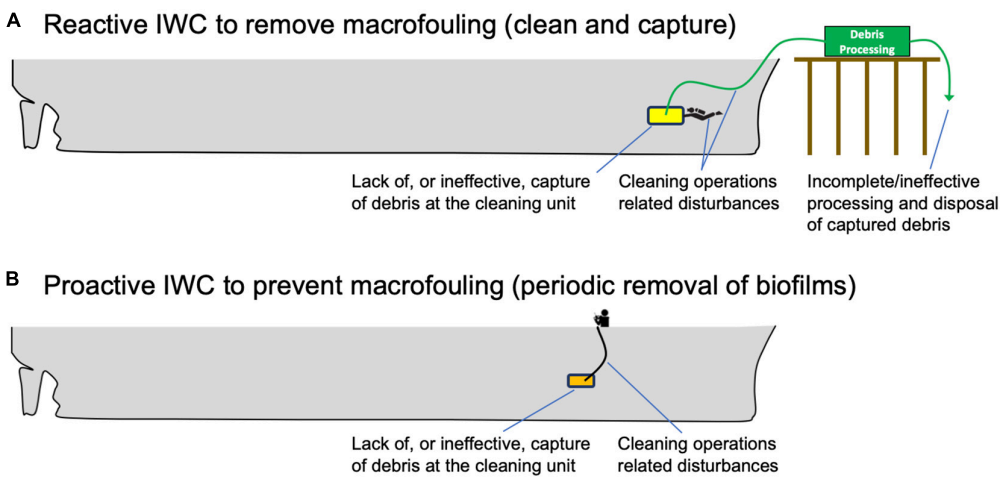

FIGURE 1 | Possible sources for the release of harmful material during ship **(A)** reactive and **(B)** proactive in-water cleaning (IWC).

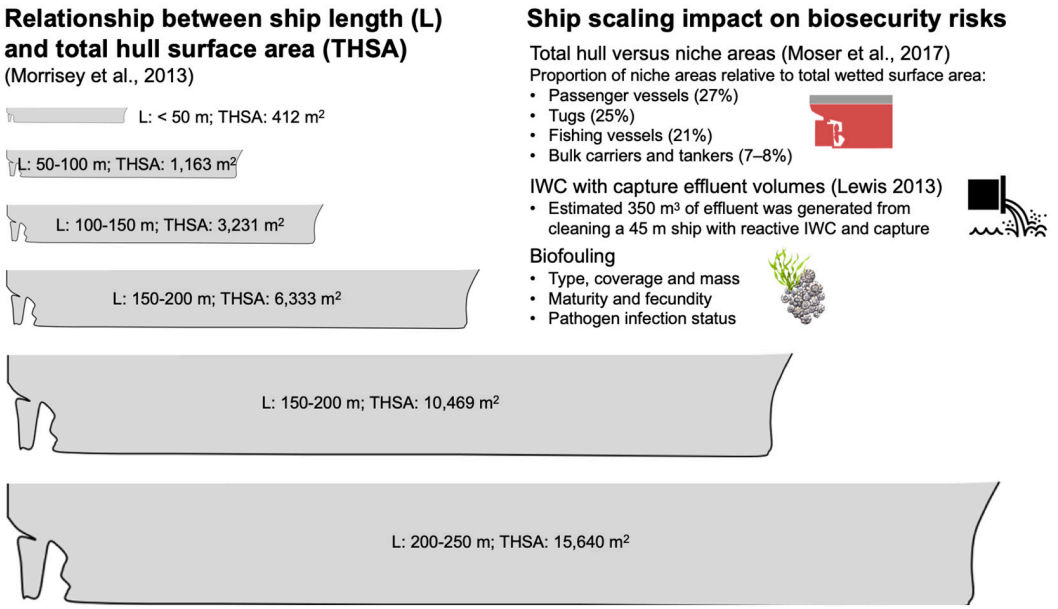

FIGURE 2 | Considerations of ship size in the development of in-water cleaning standards.

to appropriate levels, or to treat/disinfect debris to prevent the release of possible pathogens, this secondary processing of debris will likely have a similar effect on macrofouling organisms and their propagules (Scianni and Georgiades, 2019; Georgiades et al., 2021).

Emphasis on proactive IWC and "clean-before-you-leave" approaches may eliminate many of the organism-based concerns, compared to reactive IWC (Department of Agriculture [DOA] et al., 2015; Scianni and Georgiades, 2019; Georgiades et al., 2021). However, to date, no proactive IWC system has been independently assessed for efficacy and environmental safety. The applicability of clean-before-you-leave is also dependent on: (a) the vessel's prior itinerary, (b) NIS and pathogen status of areas previously visited since last dry-docking or cleaning event and the recipient area, and (c) proximity to high-value areas (Department of Agriculture [DOA] et al., 2015; Georgiades et al., 2021). While not insurmountable, it requires authorities to determine the boundaries for what would be considered "local/regional" biofouling (Department of Agriculture [DOA] et al., 2015; DAWE, 2021) noting that the larger the area, the less protective it will be in terms of NIS spread (Outinen et al., 2021). This determination should be underpinned by suitable knowledge of the presence/absence of macro-organisms and pathogens (e.g., surveillance programs; Sim-Smith and Diggles, 2019; Woods et al., 2019), while also recognizing that NIS continue to occur and their distributions constantly shift (Bailey et al., 2020). Further, authorities should continue to be mindful of the potential for chemical release associated with all IWC activities and potential for AFS damage (Scianni and Georgiades, 2019; Tamburri et al., 2020a).

Finally, all current IWC operations are conducted by divers or ROV pilots (with support staff). Therefore, any IWC approval should also explicitly consider both operator safety and proficiency. It is recommended that, at minimum, standards be developed for required levels of diver and ROV pilot training, expertise, and experience to ensure consistent and effective IWC.

CONCLUSION

Independent, transparent, and predictive verification testing of IWC systems is fundamental to regulatory success of emerging biofouling policies. Performance criteria required for IWC approval should focus on the most environmentally protective variables, including presence or absence of macro-organisms (irrespective of species origins or physiological state), and measurable impacts to local water quality (as opposed to percent reductions in the release of debris). Equally important is the need to measure the selected performance criteria with scientifically and statistically sound, quantitative methods that provide regulatory agencies, approval bodies, ship owners/operators, and the public with the confidence needed that IWC of ship biofouling is safe and effective.

AUTHOR CONTRIBUTIONS

MT led the overall project design, execution, and drafting of this manuscript. EG, CS, MF, GR, and CJ contributed to information, expertise and insight, and assisted in drafting various sections of the publication. All authors contributed to the article and approved the submitted version.

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Experimental Analysis of Survival and Recovery of Ship Fouling Mussels During Transit Between Marine and Freshwaters

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Ships and boats may transport whole communities of non-indigenous species (NIS) through hull biofouling, some members of which may become invasive. Several studies have evaluated the diversity of these communities, but very few have analyzed the survival of organisms after their voyages into different and potentially inhospitable conditions. This factor is important to consider because the last port of call approach for risk assessments assumes that if the conditions observed in the last port of call are different from those observed in a receiving port, risks are diminished or null. Using an innovative experimental system, we tested the survival and recovery of the marine blue mussel (*Mytilus edulis*) and the freshwater zebra mussel (*Dreissena polymorpha*) by exposing them to adverse salinity conditions at varying temperatures to simulate ships and boats transiting to ports or marinas with contrasting environmental conditions. Both mussel species, which are well-known for their adaptability to new environments as aquatic NIS, survived better at colder temperatures, with blue mussels surviving up to 14 days in freshwater, and zebra mussels up to 8 days in marine water. This highlights the importance of considering the resistance of fouling organisms to adverse conditions in vector and species risk assessments.

Keywords: biofouling, environmental tolerance, non-indigenous bivalves, risk assessment, shipping, boating, survival

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INTRODUCTION

Aquatic invasive species are non-indigenous species (NIS) that have considerable impacts on ecosystem function, biological community composition, and global economies in marine coastal and freshwater environments (Bax et al., 2003; Molnar et al., 2008; Havel et al., 2015; Gallardo et al., 2016). Important introduction vectors include vessels (e.g., commercial ships and recreational boats) that may transport organisms into novel environments where they can become invasive (Molnar et al., 2008; Seebens et al., 2013). Biofouling and ballast water are the two most important means by which NIS may be transported to different geographic locations by ships and boats (Molnar et al., 2008; Sylvester et al., 2011; Williams et al., 2013; Chan et al., 2016). Regulations have

been adopted to prevent the discharge of ballast water (used to maintain ship trim) near coastal habitats since the realization that it can disperse organisms of various life-stages that may establish (IMO [International Maritime Organization], 2004, 2021; Simard and Hardy, 2004; Firestone and Corbett, 2005; Bailey, 2015; Scriven et al., 2015). Although most work on shipping-related introductions of NIS has focused on ballast water (Bailey, 2015), biofouling by a diverse assemblage of fouling organisms (Bailey et al., 2012; Chan et al., 2012; Adams et al., 2014; Linley et al., 2014) on submerged ship surfaces, including hulls, sea chests, etc., may be an equally important vector (Coutts et al., 2010a,b; Sylvester et al., 2011; Chan et al., 2016, 2019). Likewise, biofouling of smaller boats (recreational, fishing, etc.) may be an important source of NIS introductions in many areas (e.g., Davidson et al., 2010; Ashton et al., 2014; Zabin et al., 2014; Pelletier-Rousseau et al., 2019).

Although there are no federal regulations concerning the control of biofouling in Canadian waters, Canada has adopted the voluntary International Guidelines for the Control and Management of Ships' Biofouling to Minimize the Transfer of Invasive Species proposed by the International Maritime Organization (IMO [International Maritime Organization], 2011) and is a strategic partner to the 2017 GloFouling partnership (Chan et al., 2015; IMO [International Maritime Organization], 2017, 2021). These guidelines focus mostly on the prevention of attachment and removal of organisms from submerged ship surfaces *via* anti-fouling systems and cleaning programs, respectively. The efficiency of anti-fouling systems varies and cleaning is done on a voluntary basis and thus does not entirely prevent the risk of transferring organisms that accumulate over time and locations (Drake and Lodge, 2007; Sylvester et al., 2011; Chan et al., 2016; Tamburri et al., 2020).

Risk assessments (RAs) are an effective way to identify, evaluate, and estimate the level of threat of a potential NIS or pathway (Bell et al., 2011; Lodge et al., 2016). Ideally, RAs for biofouling include detailed information on all ports of call visited by a vessel along with its travel history to best understand the potential fouling communities associated with a given ship or boat. Normally, information on last port-of-call (LPoC) is used to assess the relative risk of introduction of NIS (Floerl et al., 2005; Bailey et al., 2012; Chan et al., 2012; Adams et al., 2014; Linley et al., 2014; McDonald et al., 2015; Shucksmith and Shelmerdine, 2015), under the assumption that vessels arriving from areas with differing environmental conditions (e.g., from marine to freshwater or temperate to polar waters) will be of low risk as associated organisms are assumed to be killed by inhospitable conditions. However, species within hull fouling communities may be very different from those in the LPoC, given that organisms may have accumulated over time, over voyages to multiple destinations, and subjected to a variety of environmental conditions (Drake and Lodge, 2007; Sylvester et al., 2011). In addition, vessels may only briefly transit to LPoC in zones with contrasting conditions and then quickly return to similar initial conditions (e.g., marine A to freshwater to marine B; risk to marine C would be evaluated as very low since the LPoC was freshwater; **Figure 1**), although the effect of such brief incursions to inhospitable conditions remains poorly understood (Miller et al., 2018). Taxa such as mollusks (bivalves and gastropods) and barnacles can resist salinity changes by closing their shells, which not only interrupts feeding and ventilation (Foster, 1970; Schoffeniels and Gilles, 1972; Hoyaux et al., 1976; van der Gaag et al., 2016) but also protects them from desiccation and osmotic and temperature stressors (e.g., Borthagaray and Carranza, 2007; Nicastro et al., 2010; McFarland et al., 2015). Many marine invertebrates can also adapt physiologically to a wide range of water temperatures (Harley et al., 2006; Sanford and Kelly, 2011; Sunday et al., 2012). If organisms can endure brief transits into inhospitable conditions, then the risk for certain classes of voyages may be greatly underestimated. Furthermore, there is evidence that certain biofouling species, including bivalves, resist exposure to high temperatures (Rajagopal et al., 2005b; Piola and Hopkins, 2012; Lenz et al., 2018). The extent to which fouling organisms may survive incursions into different inhospitable conditions, after challenges such as a combination of adverse salinity and temperature, remains unknown. This information is crucial as only organisms able to withstand these variations have the potential to invade a new location (Bailey et al., 2012; Bailey, 2015; Schimanski et al., 2016).

Bivalves are aquatic organisms that are likely to be transported *via* biofouling and can have great impacts on introduced areas (Strayer et al., 1998; Lee and Chown, 2007; Herbert et al., 2016). These bivalves include freshwater mussels, such as the zebra mussel *Dreissena polymorpha* and other members of the family Dreissenidae, and marine mussels of the family Mytilidae, including *Mytilus edulis* (e.g., Fofonoff et al., 2021). The objective of this study is thus to investigate the survival of biofouling organisms in environmental conditions simulating those encountered by ships during transits and upon arrival, using these two well-known biofouling bivalves as model species. We used a laboratory system to recreate conditions experienced by hull fouling organisms during transit and measured survival of both mussel species. Blue mussels were (1) acclimatized in saltwater at different temperatures, (2) exposed to freshwater at different temperatures, and (3) returned to initial conditions for recovery; activity and survival were monitored during the different phases. Zebra mussels were exposed to the reverse conditions, i.e., freshwater to marine water and back to freshwater.

MATERIALS AND METHODS

Study Organisms

Two species of mussels were used for this study, the marine blue mussel (*M. edulis*) and the freshwater zebra mussel (*D. polymorpha*), which are well-known for their invasiveness and biofouling capabilities (Berntsson and Jonsson, 2003; Drake and Lodge, 2007; De Ventura et al., 2016). Blue mussels are native to the intertidal shores of the northern Atlantic (Gosling, 2003; Moreau et al., 2005; Gaitán-Espitia et al., 2016) and part of a complex of hybridizing *Mytilus* species that also includes *M. trossulus, M. californicus*, and *M. galloprovincialis* (Koehn, 1991). *Mytilus* species are known for their plasticity which allows

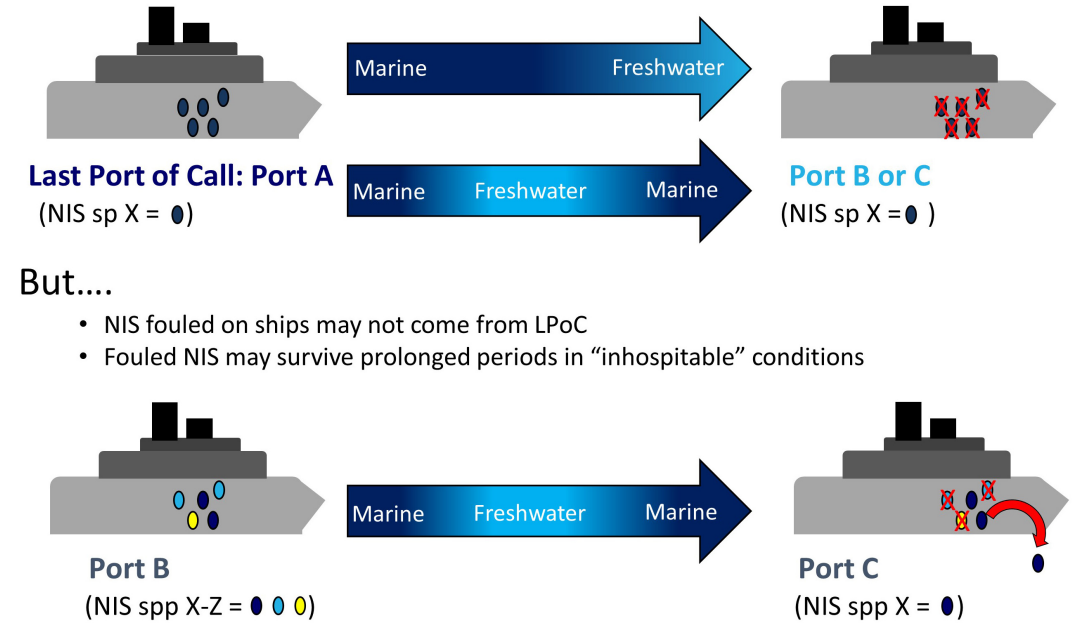

FIGURE 1 | Conceptual diagram of transit between ports. Biofouling risk assessments only consider Last Port of Call (LPoC), but biofouling organisms from other ports with similar conditions can also be found. NIS, non-indigenous species.

them to adapt to new environments and *M. galloprovincialis* and *M. edulis* have been introduced by aquaculture operations on the Pacific coast of North America and many other areas worldwide (Moreau et al., 2005; Gaitán-Espitia et al., 2016; Mathiesen et al., 2017). *M. edulis* is now established in South America (Hickman, 1992; but see Gaitán-Espitia et al., 2016), Asia (Tang et al., 2002), Africa (Ajani and Oyebola, 2010), and Oceania (Westfall and Gardner, 2010; Colgan and Middelfart, 2011). Blue mussel species are believed to withstand salinities ranging from 4 to 36 PSU (Gosling, 2003; van der Gaag et al., 2016).

Zebra mussels are extremely successful invaders that have spread, in only a few decades, over large regions through ballast water and hull biofouling in fresh- (Karatayev et al., 2015a,b) and brackish water (Mackie and Schloesser, 1996; Strayer et al., 1996; Minchin et al., 2002; Carlton, 2008). Zebra mussels originate from Eurasia and have invaded freshwater bodies in most European countries and in eastern North America, including the freshwater section of the St. Lawrence estuary and the Great Lakes (Johnson and Carlton, 1996; Kwan et al., 2003; Casper et al., 2014). Adult zebra mussels can withstand higher salinities than those usually found in freshwater environments at colder temperatures—up to 16 PSU in natural settings (Mackie and Schloesser, 1996; Hayward and Estevez, 1997).

Sampling Locations

Over 4,000 blue mussels (commercial size, 5–6 cm in length) were purchased from two different mussel farms from the Gaspésie (Quebec, Canada) region: Carleton (June 2018) and Gaspé (October 2018). Both mussel farms are located offshore of estuaries and exposed to seawater conditions [(Carleton (June 2018): 11.5°C, 27.1 PSU and Gaspé (October historical data): 8.0°C, 25.8 PSU)].

Zebra mussels were collected from the Parc Nautique de Lévis (Lévis, QC, Canada) marina on two occasions in June 2018 (20.8°C, 0.1 PSU) and October 2018 (10.9°C; 0.1 PSU). Over 4,000 individuals (1.9–3.0 cm in length) were obtained by SCUBA divers who scraped mussels from the underside of floating wharves.

Both blue and zebra mussels were transported by road (maximum of 4 h) to the laboratory at Maurice-Lamontagne Institute (MLI, Mont-Joli, QC, Canada). They were placed in coolers without water but kept moist with wet towels and cool with ice packs during transport. All organisms were collected and transported with the required permissions and permits. Upon reaching MLI, animals were removed from the coolers, and dead or damaged individuals were discarded. Mussels were considered dead when the shell gape was wide, there was no reaction to direct probing, and body tissues showed signs of decomposition along with a putrid odor (Nichols, 1992). The remaining zebra mussels were placed directly in the freshwater husbandry tanks of the experimental set-up, which was equipped with a high-performance filtration system. Blue mussels were placed in a 1,000 L quarantine tank filled with continuously running freshwater (~8°C, 0.4 PSU) for 24 h to remove potential

associated marine invasive species (Carman et al., 2016). All mussels were subsequently removed from the quarantine tank and any dead individuals were discarded. We recognize that this may constitute a bias (i.e., artificial selection for individuals better adapted to freshwater exposure), however, biosecurity protocols were required by MLI and very little mortality (less than 5%) was observed in blue mussels.

Coolers and all the materials used for both species were soaked for 72 h in a 0.5% chlorine solution for disinfection (Invasive Mussel Collaborative, 2017).

Mussel Husbandry

Mussels were held in meshed rectangular plastic baskets placed in large flow-through tanks (3 × 250 L of freshwater for zebra mussels and 1 × 500 L of filtered saltwater for blue mussels) with discharged water passing through the high-performance filter system, where used water was first filtered by a series of cartridges (100, 50, 5, and 1 micron) to remove particles and gametes, and then circulated through UV-C neon lights (254 nm; 30,000 $\mu Ws/cm^2$). The light regime consisted of 12 h light and 12 h dark (Walz, 1978; Nichols, 1992). Tanks were cleaned twice weekly to remove feces and dead individuals, and mussels were fed [40 mL Reed's shellfish 1,800 and 40 mL Nanno 3,600 (*Nannochloropsis* sp.)] three times daily through slow drip-systems which kept the algae in suspension in the water column. Blue mussels were kept at ∼ 4°C (pH 8 and ≥ 27 PSU) in marine water pumped from the St. Lawrence estuary at a depth of ∼15 m, 2 km from shore. After a few weeks, post-catch mortality decreased and stabilized to less than 10 individuals per week. Zebra mussels were kept at 8°C (pH 7, 0.4 PSU), a temperature at which mussels are prevented from releasing gametes, as 12°C is the minimum temperature for spawning (Borcherding, 1991). Temperatures in husbandry tanks were the same as that of the water sources. Calcium levels and pH remained in the range of values that are adequate for survival (calcium levels ≥ 12 mg of Ca^{2+}/L and pH values of 7.4–9.4; McMahon, 1996). Freshwater was supplied from a nearby municipality (Price, Quebec, Canada) and kept 48 h in large reservoirs to allow chlorine to evaporate before being used in the experimental system. All mussels were kept a minimum of 4 weeks in the laboratory prior to their use in trials, as post-catch stress may affect individual mortality up to that period (Kilgour and Baker, 1994). Post-catch mortality of zebra mussels decreased and stabilized to less than 50 individuals per week.

Experimental Set-Up

To recreate conditions experienced during ship transits, we manipulated temperature and salinity using a system of four water circuits: cold freshwater, warm freshwater, cold seawater, and warm seawater (**Figure 2**). Temperature was manipulated at the inflow of each replicate tank by mixing water from the cold and warm circuits, and salinity was manipulated by a complete change from the freshwater to the marine circuits or vice-versa. Each circuit was composed of a 250 L header tank and a 1.5 horsepower (HP) pump that continuously circulated water through conditioning and distribution loops (**Figure 2**). In the condition loop, water was first circulated through a sand biofilter and then either chilled with two heat pumps (0.75 and 1.5 HP) mounted in series or warmed using a 24 KW inline heater before returning to the header tank through a degassing column. Another heat pump (5 HP) was occasionally used to supplement system heating or chilling capacity. Distribution loops fed the 40 replicate tanks (15 L plastic buckets) equipped with standpipes; the loop returned excess water to header tanks through the degassing columns. The water used in the experimental tanks was either returned to a header tank through a drain or discarded. Water was renewed in each header tank using float valves to ensure that water was replaced at the same rate as it was disposed of.

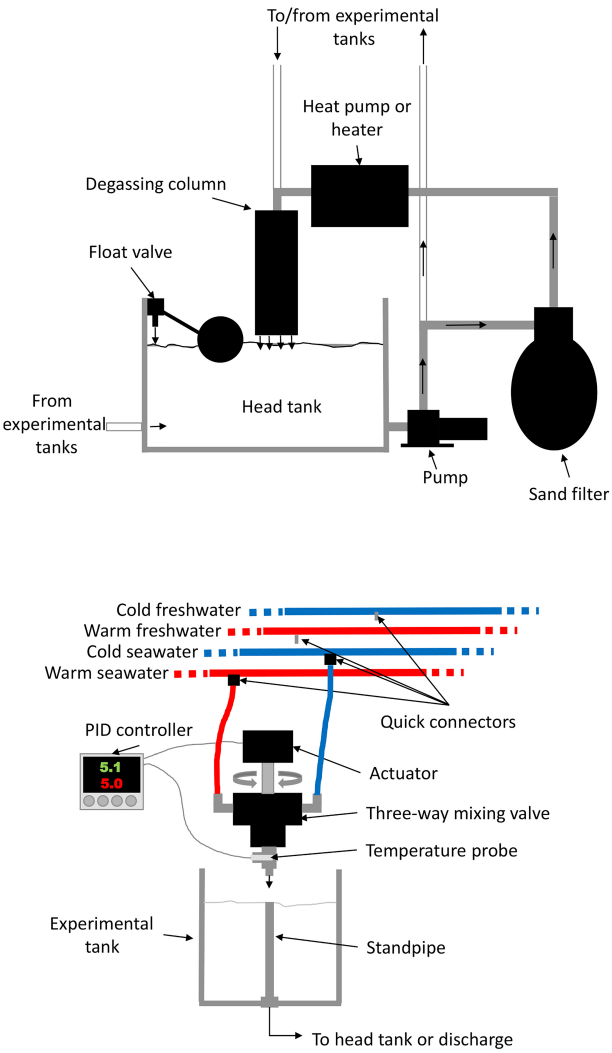

FIGURE 2 | Diagram showing one of the 40 systems used to manipulate temperatures and salinity in the experimental tanks and one of the four systems used to condition the water.

Distribution loops brought water to each experimental tank using an individual automated system (**Figure 2**). Each system consisted of a three-way ball valve, with one side connected to a cold circuit and the other to a warm circuit, using flexible tubing and quick connectors. The proportional opening of each

valve (thus, changing temperature over a continuum of possible temperatures within the range of the difference between the cold and warm circuits) was controlled by an actuator activated by a proportional-integral-derivative temperature controller. Desired temperatures were programmed into the controllers and the system continuously adjusted the valve opening to maintain the target temperature. Water sources were easily changed from saltwater to freshwater (or vice-versa) using quick connectors, allowing mimicking transitions between marine and freshwater environments. Independent data collectors (Onset HOBO, model UA-001-08) were placed in each aquaria and header tanks at the beginning of each experimental run to gather temperature measurements at 15 min intervals.

Treatments and Experimental Runs

We used the experimental laboratory system to mimic various conditions endured by hull fouling organisms during transits between marine and freshwater systems. Mussels were first transferred from the husbandry tank to experimental tanks (20 mussels per tank) filled with water at conditions identical to those in husbandry tanks. Water temperature was then gradually increased or decreased by maximum daily increments of 2°C (Kilgour et al., 1994; Clements et al., 2018), until the desired treatment temperature was reached ("Acclimatization A" in **Figure 3**). Once reached, it was held constant until all replicates reached their own target ("Acclimatization B" in **Figure 3**). Twenty-four hours later, water sources were switched and temperature settings adjusted; this resulted in a ∼20-min transition period to the exposure conditions ("Exposure" in **Figure 3**). These conditions were maintained until > 50% of the mussels (of those alive at the end of the acclimatization period) died for a particular replicate (LC_{50}; Waller et al., 1993). Once this was reached, original conditions were restored for 5 days ("Recovery" in **Figure 3**) or until all individuals were dead.

We used a total of 13 transit scenarios with temperatures ranging from 5 to 25°C. For blue mussels, treatments included acclimatization in marine water at 5°C, exposure to freshwater at 5°C, and recovery in marine water at 5 °C; this treatment was termed 5–5 (i.e., Acclimatization-Exposure); using the same terminology, the other treatments were 5–10, 10–5, 10–10, 10–15, 15–10, 15–15, 15–20, 20–15, 20–20, 20–25, 25–20, and 25–25. The treatments were the same for zebra mussels, but the order of exposure to freshwater and marine water was reversed in these scenarios. Recovery conditions were the same as those during acclimatization. In addition, control treatments, where the acclimatization conditions were maintained for the duration of the experiment, were conducted at 5, 10, 15, 20, and 25°C. Because the range of water temperatures (5–25°C) exceeded the capacities of our heating and cooling devices, the treatments were divided into two experimental blocks: the cold blocks with temperatures of 5–10–15°C (conducted in winter 2019) and the warm blocks with temperatures of 15–20–25°C (conducted in fall 2018). Within each temperature block, all treatments and controls were replicated in two separate tanks for both species, and the procedure was repeated over two separate runs, resulting in four replicates of each treatment. Note that the 15–15 treatments and controls at 15 °C were completed for each block.

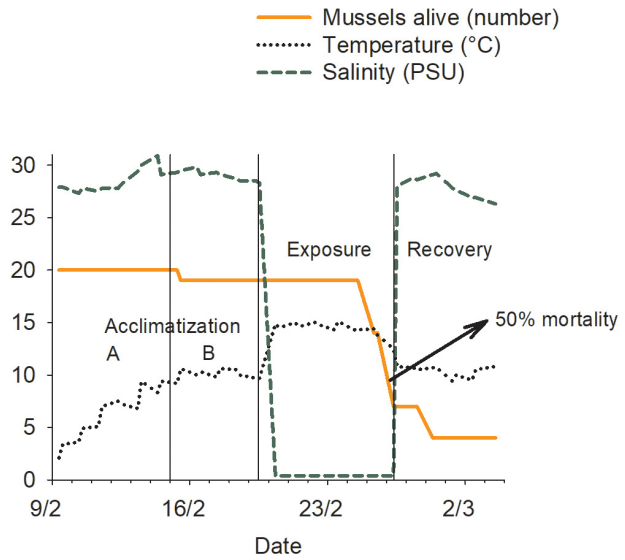

FIGURE 3 | Representation of an experimental trial involving blue mussels (*Mytilus edulis*) in a 10–15 treatment replicate, showing the variation in water temperature and salinity and the timing of the experimental phases. Units for the different variables are presented in parentheses in the figure legend.

Over the course of the experiment, the salinity of marine water varied between 25 and 30 PSU whereas the salinity of freshwater remained constant at 0.4 PSU. From the transfer of mussels from the husbandry tanks to reaching the initial treatment temperature in experimental tanks, mussel activity was observed to verify that they were able to adapt and function (filter, feed, defecate) for all treatments. The presence of feces or pseudofeces was taken as a sign of normal mussel functioning.

Measurements

Shell lengths of all mussels were measured with digital calipers before being placed into experimental aquaria (mean ± SD = 54.83 ± 2.89 and 24.56 ± 2.23 mm for blue and zebra mussels, respectively, n = 1,600). Once all mussels were in their respective aquaria, temperature and pH were taken twice daily. Ammonia and calcium levels were checked regularly and randomly in aquaria using commercial water testing kits. Mussels were fed once per day with the same mixture of liquid algae as in the husbandry tanks during all phases of the experiment, and feces were removed daily. Observations of mussel filtering activity were recorded following a chart based on stress evaluation (Nichols, 1992), and dead individuals were counted and removed from the experimental tanks. This was performed twice daily over the entire course of the experiment.

Statistical Analyses

Non-negligible mortality was observed in the control treatments for zebra mussels; we therefore used survival analysis to evaluate the effect of temperature on survival, with a separate analysis for each temperature block. We used the proportion of individuals still alive over time (pooled over all replicates from both runs)

and ran Kaplan-Meier log-rank tests followed by Holm-Sidak multiple comparisons.

The proportion of individuals filtering (taken as a proxy for mussel activity) for control treatments was analyzed using two-way factorial ANOVAs with the fixed factor *Temperature* and the random factor *Run*. Similarly, the proportion of individuals filtering during the acclimatization, exposure, and recovery phases were analyzed separately with two-way factorial ANOVAs with the fixed factor *Treatment* and the random factor *Run*. The dependent variable for the analyses of filtering activity was the average number of individuals observed filtering during the twice-daily observations for each replicate. Time to > 50% mortality and proportion of individuals still alive after the recovery period were also analyzed with two-way factorial ANOVAs with the fixed factor *Treatment* and the random factor *Run*. All ANOVAs were conducted for each species and block combination separately. Assumptions of homoscedasticity and normality of residuals were evaluated using Bartlett and Kolmogorov-Smirnov tests, respectively, and appropriate transformations were applied when assumptions were not met. Non-significant ($p > 0.25$) interactions between *Treatment* and *Run* were pooled with the error term and calculations of the F-ratio for *Treatment* were done using this pooled error term. Following detection of a significant *Treatment* effect, multiple comparisons were done using Tukey *post hoc* tests.

RESULTS

The experimental system successfully recreated the desired temperatures for the colder treatments (cold block); although some outliers were detected, means and ranges were close to the targets with no overlap among treatments (**Figure 4**). More deviations from the target temperatures were detected in the warmer treatments (warm block). In particular, variability was high for the 20°C target temperature for both marine and freshwater and the 25°C target could not be reached (mean ± SD; marine 23.58°C ± 1.49; $n = 28$, and freshwater 23.77°C ± 1.47; $n = 28$). Still, means among target conditions were largely different from one another and only a few replicates overlapped with other treatments (**Figure 4**).

In general, blue mussel survival was higher than that for zebra mussels in the control and experimental tanks for all temperatures. Blue mussels in the control treatments showed high survival with ∼ 2% mortality (10 out of 480 individuals) during the experimental periods (hence data not analyzed nor presented). In contrast, higher mortality was observed in zebra mussels control tanks with up to 55% dying in the warmer conditions (**Figure 5**). Survival of zebra mussels differed among temperature treatments for both the cold and warm blocks (Cold: Log-Rank Test = 7.62, df = 2, $p = 0.02$; Warm: Log-Rank Test = 16.20, df = 2, $p < 0.001$). In the cold block, multiple comparisons revealed that survival was lower at 5°C compared to 10°C; all other pairs of treatments did not differ significantly. In the warm block, all temperature treatments were significantly

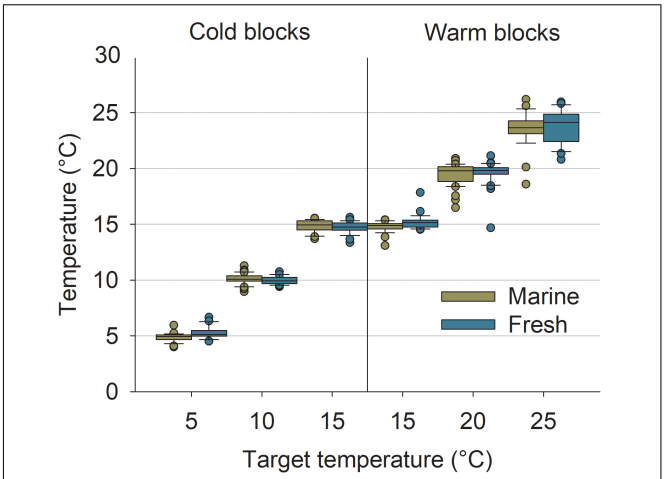

FIGURE 4 | Measured temperatures in the experimental tanks in relation to the different target combinations of temperature and salinity. Replicates represent the mean temperature of a phase (i.e., Acclimatization B, Exposure, or Recovery). Boxes represent 25th and 75th percentiles, lines show median, error bars show 5th and 95th percentile, and dots show outliers.

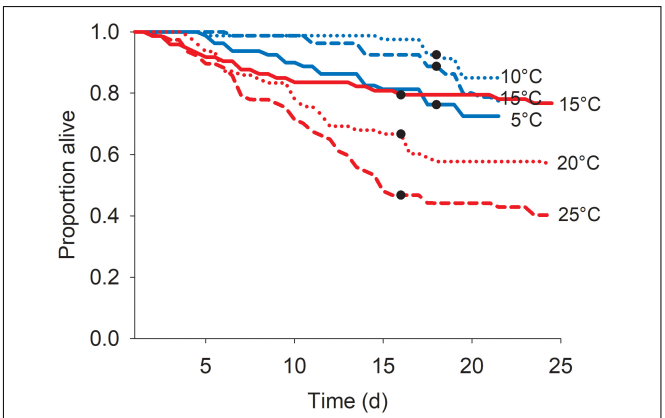

FIGURE 5 | Survival of zebra mussels, *Dreissena polymorpha*, in control treatments at the various acclimatization temperatures for the cold (blue lines) and warm (red lines) blocks. Values represent the proportion of the total individuals alive over time out of 80 individuals (2 runs × 2 replicates × 20 individuals). Dots show the moment when the shortest of the two runs ended.

different from each other with decreasing survival with increasing temperature (**Figure 5**).

Blue mussels showed high filtering activity, regardless of temperature. Blue mussel activity in the control treatments was unrelated to temperature (**Table 1**) with ∼50 and 40% of individuals filtering in the cold and warm blocks, respectively (**Figure 6A**). For control zebra mussels in the cold block, between 20 and 30% of individuals were observed filtering with a significant increase in activity with increasing temperatures (**Table 1** and **Figure 6A**). In the warm block, control zebra mussels spent most of their time with their valves closed, with ∼15% of individuals observed filtering, irrespective of temperature (**Figure 6A**).

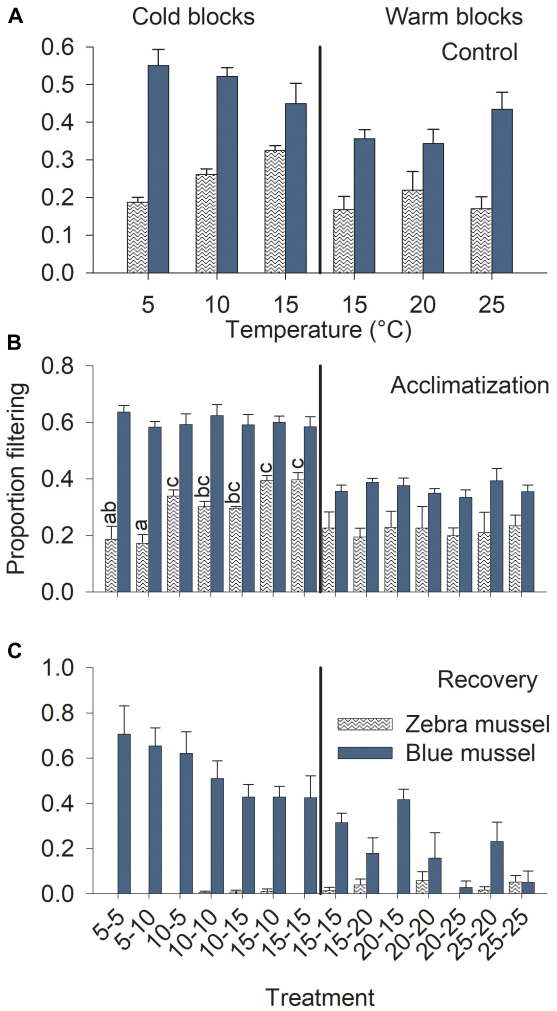

FIGURE 6 | Filtration activity of marine blue mussels (*Mytilus edulis*) and freshwater zebra mussels (*Dreissena polymorpha*) in the control treatments at different temperatures **(A)** and during the acclimatization **(B)** and recovery **(C)** phases of the experiment. Bars show mean (+SE, $n = 4$) proportion of individuals observed filtering and columns not sharing a common letter (within a Block and Species combination) are significantly different (Tukey *post hoc* tests). Vertical lines show the separation between the cold and warm blocks.

TABLE 1 | Results of two-way factorial ANOVAs evaluating the effect of temperature on the filtration activity of blue mussels (*Mytilus edulis*) and zebra mussels (*Dreissena polymorpha*) in the control treatments.

Source of variation	df	MS	F	*p*
Blue mussels				
Cold blocks				
Run	1	8.76 E-3	2.06	0.20
Temperature	2	1.10 E-2	0.75	0.57
Run × Temperature	2	1.46 E-2	3.43	0.10
Residuals	6	4.26 E-3		
Warm blocks				
Run	1	3.87 E-4	0.06	0.81
Temperature	2	9.67 E-3	1.60	0.26
Run × Temperature	Pooled with error term			
Residuals	8	6.04 E-3		
Zebra mussels				
Cold blocks				
Run	1	2.58 E-4	0.43	0.54
Temperature	2	1.88 E-2	12.72	0.07
Run × Temperature	2	1.48 E-3	2.41	0.17
Residuals	6	6.13 E-4		
Warm blocks				
Run	1	4.80 E-2	46.81	**<0.001**
Temperature	2	3.31 E-3	3.22	0.09
Run × Temperature	Pooled with error term			
Residuals	8	1.03 E-3		

Significant effects ($p < 0.05$) are indicated in bold.

During the acclimatization phase, treatment had no influence on filtering activity of blue mussels in either block (**Table 2**). Irrespective of temperature, mussels spent about 60 and 40% of the time filtering in the cold and warm blocks, respectively (**Figure 6B**). Filtering of zebra mussels during the acclimatization phase was significantly influenced by temperature in the cold, but not the warm block (**Table 2**). In the cold block, there was a gradual increase in filtering activity from ∼20 to 40% with increasing temperature whereas ∼20% of individuals were filtering in all treatments during the warm block (**Figure 6B**). With the exception of one specimen, all mussels of both species had their valves closed during the exposure phase, thus the data was not analyzed nor presented. Finally, during the recovery phase, treatment had no significant effect on blue mussel activity in either block (**Table 2**) although there were trends of decreasing activity with increasing temperature in both blocks (**Figure 6C**). Very few zebra mussels were filtering during the recovery phase and the data was thus not analyzed (**Figure 6C**).

Time to reach 50% mortality following exposure to adverse conditions varied significantly among treatments for both species and block combinations (**Table 3**). Blue mussel LC_{50} was up to 2 weeks in the coldest treatments, whereas the maximum LC_{50} observed for zebra mussels was 1 week. The general trend for both species and blocks was a gradual decrease in survival time with increasing temperatures (**Figure 7**). In particular, exposure temperature, rather than acclimatization temperature, was the main determinant of survival time. Generally, survival times of mussels were more similar among treatments with a common exposure temperature (e.g., 5–5 and 10–5) than among treatments with similar acclimatization temperatures (e.g., 10–10 and 10–15; **Figure 7**).

Of those individuals that survived the exposure phase, the proportion that survived through the recovery phase was generally higher for blue mussels in colder conditions than those in warmer ones and it tended to decrease with increasing temperatures (**Figure 8**); the trend was significant in the cold (data arcsin-square-root transformed), but not the warm block (**Table 3**). Very few zebra mussels survived to the end of the recovery phase in the cold block and none in the warm block (**Figure 8**); Treatment had no significant effects for the cold block (**Table 3**) and data was not analyzed for the warm block.

TABLE 2 | Results of two-way factorial ANOVAs evaluating the effect of treatments on the filtration activity of blue mussels (*Mytilus edulis*) and zebra mussels (*Dreissena polymorpha*).

Source of variation	df	MS	F	*p*	MS	F	*p*
		Cold blocks			Warm blocks		
Acclimatization (blue mussels)							
Run	1	9.27E-3	2.44	0.13	1.00E-6	3.9E-4	0.98
Treatment	6	1.70E-3	0.45	0.84	1.88E-3	0.47	0.81
Run × Treatment	Pooled with error term				4.04E-3	1.67	0.20
Residuals	20	3.81E-3			2.42E-3		
Acclimatization (zebra mussels)							
Run	1	3.51E-3	1.27	0.27	0.18	95.32	**<0.001**
Treatment	6	3.27E-2	11.82	**<0.001**	9.53E-4	0.15	0.98
Run × Treatment	Pooled with error term				6.30E-3	3.29	0.03
Residuals	20	2.77E-3			1.91E-3		
Recovery (blue mussels)							
Run	1	9.09E-3	0.29	0.59	3.09E-2	2.11	0.17
Treatment	6	5.78E-2	1.88	0.13	7.67E-2	3.07	0.10
Run × Treatment	Pooled with error term				2.49E-2	1.71	0.19
Residuals	20	3.08E-2			1.46E-2		

Significant effects ($p < 0.05$) are indicated in bold.

TABLE 3 | Results of two-way factorial ANOVAs evaluating the effect of treatments on the survival of blue mussels (*Mytilus edulis*) and zebra mussels (*Dreissena polymorpha*).

Source of variation	df	MS	F	*p*	df	MS	F	*p*
		Cold blocks				Warm blocks		
Time to 50% mortality (blue mussels)								
Run	1	78.89	66.14	**<0.001**	1	1.51	2.56	0.13
Treatment	6	41.43	34.74	**<0.001**	6	12.06	20.43	**<0.001**
Run × Treatment	Pooled with error term				Pooled with error term			
Residuals	20	1.19			20	0.59		
Time to 50% mortality (zebra mussels)								
Run	1	7.51	6.44	**0.02**	1	1.08	3.85	0.06
Treatment	6	14.73	12.64	**<0.001**	6	1.41	5.04	**0.002**
Run × Treatment	Pooled with error term				Pooled with error term			
Residuals	20	1.17			20	0.28		
Recovery (blue mussels)								
Run	1	1.53E3	4.97	**0.04**	1	8.28E2	0.26	0.62
Treatment	6	1.07E3	3.48	**0.02**	6	2.02E3	2.31	0.17
Run × Treatment	Pooled with error term				6	8.75E2	2.71	0.06
Residuals	20	3.07E2			14	3.22E2		
Recovery (zebra mussels)								
Run	1	1.59E2	2.78	0.12				
Treatment	6	2.20E2	1.79	0.25				
Run × Treatment	6	1.23E2	2.15	0.11				
Residuals	14	5.71E1						

Significant effects ($p < 0.05$) are indicated in bold.

DISCUSSION

Using an innovative experimental system, we demonstrated that well-known biofouling organisms may survive for days to weeks through a combination of inhospitable conditions, particularly at cooler temperatures. This is, to our knowledge, the first time that survival and recovery of biofouling organisms have been tested experimentally in scenarios recreating transits between freshwater and marine environments using two variables (temperature and salinity). These results are important for RA exercises, which have been used as tools to target interventions where the potential to prevent invasions is the greatest

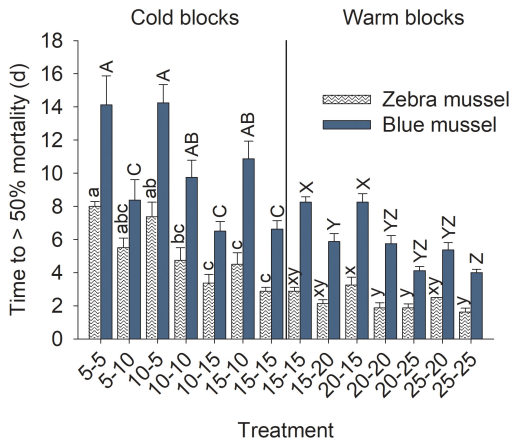

FIGURE 7 | Time to > 50% mortality of marine blue mussels (*Mytilus edulis*) and freshwater zebra mussels (*Dreissena polymorpha*) during the exposure phase of the experiment. Bars show mean (+SE, $n = 4$) and columns not sharing a common letter (within a Block and Species combination) are significantly different (Tukey *post hoc* tests). Vertical lines show the separation between the cold and warm blocks; capital letters are for blue mussels and lowercase for zebra mussels.

(Lodge et al., 2016; Roy et al., 2018). RAs typically base the risk of a pathway on the level of similarity in environmental conditions between the origin and recipient systems (Hayes and Barry, 2008; Floerl et al., 2009; Chan et al., 2012). Emphasis has been put on temperature and salinity as those variables are usually considered key in delimiting geographic ranges of aquatic organisms (Keller et al., 2011; Chan et al., 2013; Havel et al., 2015). Our results suggest that RAs tend to underestimate the risk associated with a transit when it involves a transition between freshwater and marine environments. For example, in previous assessments, a ship with a LPoC in freshwater would be assigned as "low risk" when entering a marine port (Bailey et al., 2012; Chan et al., 2012; Adams et al., 2014; Linley et al., 2014). Survival was affected by temperature during the exposure phase, with both species showing greater survival in colder conditions. This may be due to the bivalves' defense mechanism, by which they can shut their valves tight under stress and isolate themselves from adverse conditions for an extended time period (Kramer et al., 1989). In such situations, bivalves would be dependent on the oxygen reserves they were able to capture in their valves; given that metabolic activity decreases with decreasing temperatures (Seed and Suchanek, 1992; Braby and Somero, 2006; Anestis et al., 2010), temperature during the exposure phase would determine how long individuals can last on such reserves. While substantial mortality occurred during simulated transits, a significant proportion of individuals survived and recovered in most scenarios tested. These results highlight that exposure to marine or freshwater (for freshwater and marine species, respectively) is no guarantee of a risk-free transit, in particular during short trips in cold waters. We therefore recommend that future RAs take into consideration the travel history of a ship (voyage length and general environmental characteristics of transit route) and the identity of the species being assessed rather than simply the LPoC. Furthermore, RAs are tools used to reduce the risks of NIS being transported to, and established in, new environments. However, in cases where high numbers of NIS are already present (likely established through biofouling), such as in California and New Zealand (Cranfield et al., 1998; Kospartov et al., 2008; Ruiz et al., 2011), RAs have supported the implementation of biofouling regulations (Georgiades et al., 2020; Scianni et al., 2021). However, reactive measures to remove or treat biofouling on ships in an environmentally responsible manner, and testing methods for jurisdictional approvals, are still under development (Scianni and Georgiades, 2019; Tamburri et al., 2020, 2021).

The effects of salinity and temperature variation have been evaluated for many types of organisms (Hoyaux et al., 1976; Berezina, 2003; Braby and Somero, 2006; Calliari et al., 2008; van der Gaag et al., 2016; Bertrand et al., 2017). Such experiments typically evaluate how organisms are affected when one or the other of these variables is increased or decreased, how they react to long exposures to various environmental conditions or a combination of the two. The innovative approach we used allowed changes in salinity and temperature to be performed rapidly to recreate transitions between different environments as experienced by fouling organisms during transits along vessel pathways. That is, to evaluate the capacity of fouling aquatic NIS to survive and acclimate to realistic scenarios of increasing/decreasing salinities and temperature, followed by a return to the initial conditions (e.g., marine A to freshwater to marine B).

This study focused on bivalves, which are known for their tolerance to adverse conditions, including rapid changes in salinity and temperature (Schoffeniels and Gilles, 1972; Hoyaux et al., 1976). Moreover, for mussels (including *M. edulis*), sequential heat stress events can even enhance their survival and make them more resistant to subsequent exposures (Lenz et al., 2018). Even when heat treatments are used and extreme temperatures are applied as a biofouling treatment technique, mussels have shown to be extremely resistant, with larger mussels generally being more resistant than smaller ones (Rajagopal et al., 2005b; Piola and Hopkins, 2012). Zebra mussels can survive extended periods of high temperatures (around 30°C) but only if their acclimatization temperatures are also elevated (between 15 and 20°C; Spidle et al., 1995). Our results agree with this observed tolerance to higher temperatures in blue mussels, however, zebra mussels did not perform well in the warmer treatments.

Additionally, zebra mussels can adapt to increasing salinities, especially at cooler temperatures (3–12°C) and can gradually acclimate to a combined 20°C/8 PSU, but only after a year of incremental changes in the environment (Mackie and Kilgour, 1992; Kilgour et al., 1994). They have also been shown to live at 8–12 PSU in the Caspian region (Strayer and Smith, 1992), and survived 6 days at salinities of 7.5 PSU and higher (van der Gaag et al., 2016). Our treatments were performed to mimic vessel transit and the acute changes in environmental conditions that a hull fouling community may experience, with changes in salinity occurring at a much faster rate. Any potential tolerance shown by zebra mussels in the Kilgour et al. (1994) study would likely be diminished by the speed of change at which

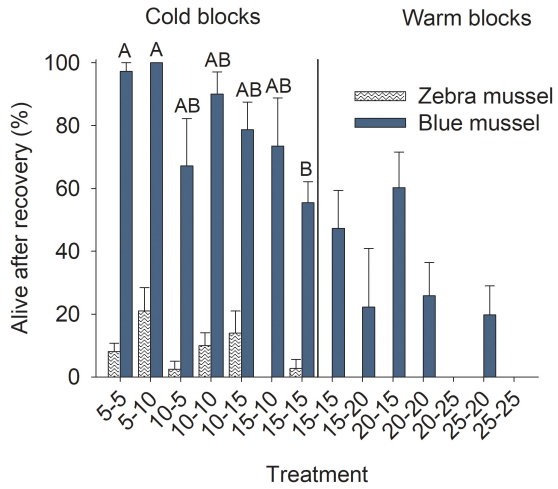

FIGURE 8 | Proportion of marine blue mussels (*Mytilus edulis*) and freshwater zebra mussels (*Dreissena polymorpha*) still alive after 5 days of the recovery phase. Bars show mean (+SE, $n = 4$) and columns not sharing a common letter (within a Block and Species combination) are significantly different (Tukey *post hoc* tests). Vertical lines show the separation between the cold and warm blocks.

they are exposed while in-transit. Nevertheless, for short transits between colder regions, we show that this species can still tolerate rapid salinity changes. Therefore, zebra mussels accumulated on transiting vessels may be subjected to selection pressure where individuals that have a higher resistance to temperature (Spidle et al., 1995) may actually increase this tolerance through acclimation, similar to what was observed in blue mussels by Lenz et al. (2018); similarly, an increase in tolerance to salinity was observed in the freshwater golden mussel *Limnoperna fortunei* (Sylvester et al., 2013).

Biofouling communities are diverse and their resistance to inhospitable conditions likely varies widely among species. Organisms that possess ways to physically isolate themselves from harsh environmental conditions, such as bivalves, gastropods, barnacles, and some bryozoans, may weather inhospitable conditions for several days (e.g., up to 18 days for *M. galloprovincialis*; Atalah et al., 2016 and 34 days for the oyster *Crassostrea gigas*; Hopkins et al., 2016). By contrast, mortality may occur very rapidly in soft-bodied taxa such as tunicates, sponges, or cnidarians and in taxa that lack a capacity to osmoregulate, such as echinoderms (Coutts et al., 2010a,b; Chan et al., 2016). Survival time under different environmental conditions for most taxa is still unknown and this information would be valuable to include in RAs for biofouling species (Keller et al., 2011; Ibáñez et al., 2014). Once the tolerance of a wide array of species is known, future RAs could include a weighting resistance factor in their calculation according to species type. For instance, a higher risk score to shelled organisms (e.g., bivalves and barnacles), and a lower score to non-shelled/soft tissue organisms (e.g., tunicates and cnidarians). Such adjustments could address assumptions that are commonly used in RA exercises (Bailey et al., 2012; Chan et al., 2012; Adams et al., 2014; Linley et al., 2014) where it is assumed that survival of fouling communities is low when the environmental conditions of the last port of call differ from recipient ports.

The higher survival of mussels under colder conditions may have important consequences from a NIS management point of view. With Arctic ice cover decreasing, the Northwest Passage is expected to see an increase in international shipping traffic (Dawson et al., 2020; Copland et al., 2021). While the Arctic was historically deemed a region with comparatively lower risk to invasion, it may play an increasing role in the spread of NIS. Since water remains cold, even during the summer months when most shipping activity occurs, biofouling organisms may be able to survive for much longer periods of time (when compared to more temperate locations), due to decreased metabolic activity. Many organisms may spawn upon arrival in coastal areas where conditions are somewhat warmer or offer more nutrition than more offshore locations (Minchin and Gollasch, 2003). Combining this with good survival due to cold temperatures in transit could lead to significant spawning events within recipient ports or harbors. Yet, factors other than temperature and salinity may influence the survival of fouling organisms and, as a result, their risk profiles. Factors such as vessel speed, proximity to major vectors or bioinvasion hotspots, or antifouling systems (Coutts et al., 2010a; Sylvester et al., 2011; Ulman et al., 2019) were not considered in this study. Furthermore, it is unknown how changes in water temperature and salinity could affect the assemblage of biofouling organisms, and how this community may in turn affect fouling mussels, considering their tertiary level in the temporal succession of biofouling phases (preceded by biofilm as primary and hard encrusting organisms and some soft algae as secondary fouling phases; Arndt et al., 2021, and the references therein).

Zebra mussel survival was likely underestimated in our study. We encountered difficulties at maintaining captive zebra mussels in good condition, as illustrated by the steady mortality rate in control treatments and husbandry tanks, as well as limited filtering activity. It has been previously shown that tolerance of zebra mussels maintained in experimental setups can be affected by collection season, water quality conditions of locations where mussels are collected, type of food, and length of time in captivity (Kilgour and Baker, 1994, and the references therein). Kilgour and Baker (1994) also observed sub-optimal health in mussels kept under laboratory conditions, possibly due to inadequate temperature and/or feeding. Although temperatures between 14 and 26°C are believed optimal for zebra mussel feeding (Lei et al., 1996), zebra mussels have been maintained under laboratory conditions at temperatures between 4 and 24°C (Nichols, 1992). In our study, we verified, through filtering and production of feces, that the food given was adequate. It was, however, observed that zebra mussels in all experimental treatment and control tanks produced very little byssus, compared to blue mussels which produced byssus in both; thus, zebra mussels may have exposed themselves more often to inhospitable conditions when trying to produce byssus, leading to greater sensitivity (Rajagopal et al., 2002, 2005a). Thus, we hypothesize we may have used individuals that could have been affected by any of

these stress sources, making it possible that mussels in better condition would have yielded even longer survival times.

Results of this study clearly show that LPoC protocols for RAs of hull fouling organisms may underestimate the resistance of certain biofouling organisms which transit between freshwater and marine environments. Overly simplistic assumptions may affect overall assessment reliability, possibly resulting in misleading conclusions, depending on the organism being assessed. Hull fouling risk assessment accuracy could be improved by the inclusion of ship travel history (in addition to LPoC) and a resistance factor depending on species type and life history. This study also offers new insight on the role of low temperature during transit, highlighting the future potential role of the Arctic as a route for the spread of NIS. These factors may play an important role in increasing the probability of introduction, and thus calculated risk, since organisms may successfully transition from ports that would previously have been considered to have a low probability for successful transfer due to poor environmental match.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical review and approval were not required for the animal study because there are no animal ethics requirements for animal studies involving mussels.

AUTHOR CONTRIBUTIONS

CR, DD, JG, KH, CHM, NS, and CWM contributed to the design and concept of the study. CR was in charge of the experimental setup, with active contributions from DD and M-FL. JMH, CWM, and NS provided scientific advice. DD performed the statistical analyses. CWM and KLH obtained funding. DD, CWM, and KLH provided resources. CR was in charge of writing the original draft of the manuscript, together with DD, JG, and CWM. All authors reviewed and edited the manuscript contributing to the final version.

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frontiers in Marine Science

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Surface Characteristics Together With Environmental Conditions Shape Marine Biofilm Dynamics in Coastal NW Mediterranean Locations

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Microbial colonization of artificial substrates in coastal areas, which concerns hull ships, sensors as well as plastic debris, is of huge significance to attain a rational environmental management. Some surface and environmental drivers of biofilm development have previously been described but their relative impact on the formation of biofilms remains unknown while crucial. Especially, there is no evidence of the relative importance of physical surface properties (wettability, roughness, smoothness) compared to seawater characteristics in driving biofilm abundance and diversity. In addition, few studies have considered the temporal evolution of this complex form of colonization, which often prevent to globally understand the process. Using experimental facilities in two Mediterrranean locations, a multidisciplinary approach including surface characterizations as well as seawaterquality analyses, flow cytometry and 16S rDNA metabarcoding, allowed for the identification of the main drivers of colonization for two antifouling (AF) coatings. One AF coating released copper (SPC1) while the other limit colonization thanks to physical properties, namely a low surface energy, roughness and smoothness (FRC1). Results were obtained over 75 days and compared to a control surface (PVC). Biofilm development was observed on all surfaces, with increasing density from AF coatings to PVC. Pionneer bacteria were dissimilar within all three surface types, however, communities observed on FRC1 converged toward PVC ones overtime, whereas SPC1 communities remained highly specific. A remarkably low and unique diversity was found on SPC1 during the experiment as *Alteromonas* accounted for more than 90% of the community colonizing this substrate until 12 days, and remained one of the co-dominant taxa of mature biofilms. Moreover, clear differences were found between geographical locations. Low nutrients and higher hydrodymanics in Banyuls bay resulted in less dense biofilms overall compared to Toulon, but also in a the slower dynamic of biofilm formation. This is illustrated by the

persistence of pioneer *Alteromonas* but also Hyphomonadacae after 75 days on SPC1. We concluded that, even if local environmental conditions influenced the composition of biofilm communities, particular physical features may control the biofilm density but not the diversity, while copper releasing coating controlled both. In addition, it is evident from these results that sequential biofilm dynamics should carefully be considered as initial processes of formation differed from the long-term ones.

Keywords: biofilm, polymer surface, antifouling coatings, microbial ecotoxicology, molecular ecology

INTRODUCTION

Microbial colonization of immersed surfaces, i.e., biofilm growth, is a crucial process to control for the management of coastal areas. Antifouling (AF) coatings are designed to control the biofouling of ship hulls in order to limit the increase in hull roughness and vessel weight associated with hull colonization, which leads to an increase in fuel consumption and associated greenhouse gas emission, but also to reduce dry-docking and associated costs (Salta et al., 2013; de Carvalho, 2018). The colonization of surface by multicellular eukaryotes has been the focus of many studies but surfaces immersed in seawater always exhibited biofilms well before propagules reach them, due to high abundance of microorganisms compared to invertebrate larvae or algae spores. Then, interactions between biofilms and propagules influence macrofouling development (Hadfield, 2011; Salta et al., 2013; Lema et al., 2019), which strengthen the relevance to better decipher biofilm community drivers. Besides, biofilms are also involved in the transport of invasive species by ship hulls (Piola et al., 2009; Sardain et al., 2019), as well as in the degradation of polymers with the recent massive interest in marine plastic pollution (Jacquin et al., 2019; Oberbeckmann and Labrenz, 2020) or marine sensors maintenance (Delgado et al., 2021).

Some studies have reported a selective effect of substrate type on biofilm communities (Webster and Negri, 2006; Lee et al., 2008; Frere et al., 2018; Oberbeckmann et al., 2018; Ogonowski et al., 2018; Ding et al., 2019; Kesy et al., 2019; Muthukrishnan et al., 2019). Differences among conventional polymers are often limited, especially when immersion time exceeds weeks (Kirstein et al., 2018; Pinto et al., 2019). However, physico-chemical substrate characteristics are scarcely measured in field studies and, consequently, conclusions on their role in shaping biofilm community composition remained putative as they are only based on laboratory studies using bacterial strains (Genzer and Efimenko, 2006). Studies on biofilm communities colonizing AF coatings have essentially targeted biocidal paints (Self Polishing Coatings or SPC), generally showing specific effects after end-point studies with different immersion time, from weeks (Briand et al., 2012; Camps et al., 2014; Sathe et al., 2016; Yang et al., 2016a,b; Briand et al., 2017; Sathe et al., 2017; Dobretsov et al., 2018) to months (Muthukrishnan et al., 2014; von Ammon et al., 2018; Catão et al., 2019). Fouling Release Coatings (FRC) are AF coatings with specific physical surface properties such as a low roughness and elastic modulus. They were developed to decrease the impact on marine ecosystems associated to the release of biocides by conventional AF coatings (Lejars et al., 2012). FRC remained poorly studied in terms of colonizing community, even if they are known to limit their colonization to microorganisms. Their study represents a great opportunity to specifically decipher the relative physical and chemical (biocide) effects that surface characteristics have on biofilm communities.

Highly abundant and diversified communities have been described on artificial surfaces, exhibiting dissimilar profiles depending on immersion locations (Briand et al., 2012; Oberbeckmann et al., 2016; Curren and Leong, 2018; Lema et al., 2019; Muthukrishnan et al., 2019; Zhang et al., 2019). However, (i) only a few studies included several locations with relevant measures of seawater parameters; (ii) early stages of bacterial colonization were mostly sampled; (iii) the relative influence of the substrate properties, never measured, and (iv) the environmental conditions remained often difficult to interpret as immersion time and location differed from one study to another.

In this study, we intend to decipher the relative contribution of substrates and environmental parameters in prokaryotic biofilm community dynamics. Polyvinyl chloride (PVC) was chosen as a reference, and immersed together with a FRC and a SPC at two North-western French Mediterranean sites (Toulon and Banyuls bays). FRCs are surfaces with a low surface energy, roughness and elastic modulus which limit the adhesion of organisms and enhance the fouling release properties. FRC1 is an ambiguous smooth surface composed of a polydimethylsiloxane (PDMS)-based elastomer and an amphiphilic additive, which is able to diffuse at the surface to provide both hydrophilic and hydrophobic properties (Duong et al., 2015) and disturbs the settlement of marine organisms. SPC1 contains biocides, mainly copper derivatives (dicopper oxide, copper pyrithione and zinc oxide), which are released into the seawater at a constant rate (Marceaux et al., 2018). Immersion during 75 days was monitored by eight sampling times at Toulon while only toward the end of the experimental Banyuls. Wettability and surface roughness of the substrates, as well as seawater parameters including trace metal elements, were measured. Biofilm dynamics was studied both in terms of abundance (Flow cytometry) and diversity (16S rDNA metabarcoding).

MATERIALS AND METHODS

Experimental Design, Immersion Sites, and Environmental Variables

Forty two panels (5 × 5 cm) for each of the sandblasted PVC, the commercial biocidal coating called SPC1 and the commercial

fouling release coating called FRC1 were immersed for 1–75 days in a semi-enclosed basin in the military harbor in the Bay of Toulon (43°06′25″N; 5°55′41″E) (18th June–1st September 2015). A static permanent raft enabled the immersion of panels at 1-m depth and seven sampling times were chosen: 1, 4, 8, 12, 20, 28, and 75 days. The immersion of 6 panels of the three substrates was also carried out within the bay of Banyuls-sur-Mer with only one sampling time after 75 days. A metallic frame was hung under the Oceanographic buoy SOLA, allowing the immersion of panels at 2 m' depth. This site is located 1 km off the coast with a depth of 27 m (47°27′13″N; 03°32′360″ E). Due to the very low turbidity of the sites allowing similar light availability, and the lack of water column stratification, immersion at 1 or 2 m' depth should be considered as similar. In addition, at each site, all the coupons were directed in the same way without any possible rotation. FRCs are surfaces with a low surface energy, roughness and elastic modulus which limit the adhesion of organisms and enhance the fouling release properties. FRC1 is an ambiguous smooth surface composed of a polydimethylsiloxane (PDMS)-based elastomer and an amphiphilic additive, which is able to diffuse at the surface to provide both hydrophilic and hydrophobic properties (Duong et al., 2015) and disturbs the settlement of marine organisms. SPC1 contains biocides, mainly copper derivatives (dicopper oxide, copper pyrithione and zinc oxide), which are released into the seawater at a constant rate (Marceaux et al., 2018). For all the substrates and conditions, half of the panels were dedicated to flow cytometry analyses and the other to metabarcoding analyses, all in triplicates.

For each sampling time point at Toulon, water temperature, pH and salinity were measured using a Hydrolab® DS5X probe (Hatch Hydromet, United States). Dissolved organic carbon (DOC) and total nitrogen (TN) were analyzed on a TOC-VCSH analyzer (Shimadzu) (Oursel et al., 2013). Nutrients (NO_3^-, PO_4^{3-}, $Si(OH)_4$) were analyzed using standard colorimetric methods for seawater (Coclet et al., 2018). Data from the same environmental variables were obtained at Banyuls-sur Mer from the SOMLIT website.[1]

Finally, trace metal (Cd, Cu, Pb, Zn) concentrations were determined at the two sampling sites by voltammetry on fully automated Metrohm/Ecochemie system (Cindric et al., 2015).

Initial Substrates Characterization

Wettability was evaluated by measuring static water contact angles θstat, using a DIGIDROP contact angle meter from GBX Scientific Ltd. (United Kingdom) equipped with a syringe and a flat-tipped needle. The substrates were previously cleaned with deionized water and dried under ambient conditions for 48 h before measurements. The reported θstat values were an average of five individual measurements, made on 1 μL-droplets of deionized water, deposited on different regions of the same substrate. Dynamic contact angle measurements experiments were also carried out by the advancing-receding drop method. A 1 μL-droplet is first deposited at the surface, keeping the needle inside the droplet. Then, the liquid is slowly dispensed until a final drop volume of 5–10 μL, followed by a withdrawal at a rate of 0.75 μL.s-1. For each coating, the reported advancing (θa) and receding (θr) water contact angles were the average values obtained from 1 cycle of advancing-receding of 5 deionized water drops. The hysteresis H (H = θa-θr) was also calculated.

Surface roughness profiles were measured by a contact type stylus surface profiler Formtalysurf 50 from Taylor Hobson Ltd. (United Kingdom) using a 2 μm-radius tip and a 0.1 μm-radius diamond tip with a minimum applicable 1 mN-stylus load. The stylus moved across 15 mm-length of the substrate at a constant velocity of 0.50 mm.s-1 to obtain surface height variations. The arithmetic mean deviation values of roughness (Ra) and waviness (Wa) were assessed from the average of three measurements. A microroughness filter λs is currently used to eliminate from the primary profile, the roughness which is not specific to the sample, but which is generated by the external environment, in particular by the background noise of the device. In other words, this filter makes it possible to compare the results provided by measuring devices of different manufacturers. A λs value of 0.0025 mm was taken for all samples. λc is an essential parameter since it allows to discriminate the roughness from the waviness of the primary profile. The choice of the cut-off theoretically depends on the nature of the profile (periodic or aperiodic) and its structure. In the ISO 4288-1996 standard, there are tables that allow you to know what value to give to the cut-off depending on the type of profile. Once the analysis is finished, the choice of the cut-off is checked again by comparing the Ra values obtained. In our case, the selected cut-off was λc = 0.8 mm because the Ra values of coatings were comprised between 0.1 and 2 μm.

Heterotrophic Prokaryotes Abundance

For each sampling points, three panels were used to estimate abundances of heterotrophic prokaryotes by flow cytometry (Camps et al., 2014; Briand et al., 2017; Pollet et al., 2018; Catão et al., 2019). Panels were totally scraped using sterile scalpels and the collected biofilms were fixed with 4 ml of a 2% formaldehyde–solution prepared in sterile artificial seawater (ASW). Samples were quickly frozen in liquid nitrogen and maintained at –80°C until analysis. Prior to the analysis, an experimental procedure was applied to release and separate the microbial cells from the EPS matrix, including sodium pyrophosphate and Tween® 80 addition and centrifugation step (Camps et al., 2014). Results were then gated by cell size using fluorescent beads and only cells under 2 μm were considered. Abundances of hetrotrophic prokaryotes were estimated using a BD Accuri™ C6 flow cytometer (BD Biosciences) using Sybr green. Data were acquired using BD Accuri CFlow Plus software and abundances were expressed as number of cells per cm^{-2}. Data on PVC were published alone before (Pollet et al., 2018).

DNA Extraction, Amplification, and Sequencing

For each sampling points, three panels were used for DNA extraction. Panels were totally scraped and biofilms were immediately dropped into liquid nitrogen and maintained at –80°C. DNA was extracted using PowerBiofilm DNA isolation Kits (Qiagen) following the supplier's instructions.

[1]https://www.somlit.fr/

The V4 region of the bacterial and archaeal 16S rRNA genes was targeted. The 16S forward primer 515Y (GTGYCAGCMGCCGCGGTAA) and the 16S reverse primer 926R (CCGYCAATTYMTTTRAGTTT) (Parada et al., 2016) were chosen due to the high coverage of the prokaryotic diversity, including Archaea (Pollet et al., 2018). The PCR reaction (50 μL) contained 10 μL of 5 × HotStar HiFidelity PCR buffer, 1μM of each primer, 2.5U of HotStar High Fidelity DNA polymerase and approximately 2ng of DNA. The following thermal cycling scheme was used: initial denaturation at 95°C for 5min, 25 cycles of denaturation at 95°C for 45s, annealing at 50°C for 1 min, followed by extension at 72°C for 1 min. The final extension was carried out at 72°C for 10min. Negative controls were performed by using the reaction mixture without template. Following the PCR step including controls, amplicons were cleaned and concentrated using 1X magnetic Agencourt AMPure XP beads (Beckman Coulter, Brea, CA). Concentrated DNA was quantified by PicoGreen fluorescence assay (Quant-iT^{TM} PicoGreen® dsDNA Assay Kit, Thermo Fischer Scientific) and pooled at equimolar concentrations. The equimolar mix was sequenced by GENOSCREEN (Lille, France) using Miseq Illumina 2 × 250 bp chemistry.

Sequencing Data Processing

Sequence processing and quality control were used using DADA2 software on R (Callahan et al., 2016). DADA2 was performed on primer-free reads to eliminate poor quality reads. After dereplication and learn error rates determination using default settings to remove sequences containing potential errors, chimeras were also removed. Paired end reads were merged to construct amplicon sequence variants (ASVs) for prokaryotic communities. ASVs were taxonomically assigned using SILVA 16S rDNA gene reference database (release 138). Chloroplast, mitochondrial, unclassified and singletons sequences were excluded from final dataset. Data on PVC were published alone before but using MOTHUR (Pollet et al., 2018).

Statistical Analyses

The ASV table from DADA2 was converted to biom format and imported in phyloseq (v.1.36.0) in R for data visualization and statistical analysis. Samples were rarefied with relative normalization method and ASV read counts were aggregated on the genus level. Using the rarefied dataset, alpha and beta diversity analyses were conducted with Vegan package (v.2.5-7). We examined the diversity models by exploring three different metrics which are the chao1, Shannon and Pielou indices. Differences in the ASV diversity, as well as prokaryotic densities determined by flow cytometry, were analyzed using one-way ANOVA followed by Tukey Honest Significant Difference (HSD) test ($p < 0.05$). Bray Curtis dissimilarity matrices were computed and used to generate non-multidimensional scaling (NMDS) to visualize the distribution of samples. Permutational analysis of variance (PERMANOVA) tests were carried out following Site, Material and Time as factors. Regression of supplementary environmental variables to ordination axes in unconstrained ordination (NMDS) was calculated with the envfit function (Vegan). Biomarkers, corresponding to differentially abundant taxa between coatings and sites, were identified with linear discriminant analysis (LDA) with effect size (LEfSe) (Segata et al., 2011) in the Galaxy environment (GENOTOUL Platform). LDA > 3.5 was applied for selection.

Network co-occurrence analysis was performed with CONET plugin (Faust and Raes, 2016) within Cytoscape version 3.8.2 (Shannon et al., 2003). Significant correlations between ASVs (positive and negative) were obtained by a combination of Spearman and Pearson correlations and Bray-Curtis or Kullback-Leibler dissimilarities, selecting the 200 top and bottom edges in the automatic threshold. CoNet was used for rows shuffling and detection of only correlations with p-value < 0.05 after Bonferroni test correction and bootstrap filtering of unstable edges (Brown method for p-value merge).

RESULTS

Seawater Parameters in the Two Mediterranean Areas

Temperature, salinity, pH, DOC and TN were similar at the two sites showing typical North-Western Mediterranean values (**Supplementary Table 1**). Conversely, both nutrients and dissolved metals exhibited higher concentrations at Toulon. While nitrates and phosphates were near the detection limits (0.01μM) at Banyuls, they reached values as high as 1.98 and 0.14μM at Toulon, respectively. The enrichment factor in Toulon with reference to Banyuls was higher for metals, reaching 10–20 for Cd, Pb and Cu and as high as 30–60 for Zn.

Initial Surfaces Roughness and Wettability

PVC and SPC1 were rougher (highest Ra values) than the FRC1 (**Supplementary Table 2**). In addition, the waviness (Wa) showed that the rough PVC surface had more peaks (lower Wa) than the SPC1 surface and the FRC1. FRC1 presented a hydrophobic surface (θstat and θa > 100°), and a high hysteresis (H) indicated an heterogeneous surface. Due to the presence of amphiphilic additives, the FRC1 surface is expected to have a chemical surface reorganization upon contact with water, with different degrees of hydrophilicity depending on the amount of hydrophobic and hydrophilic phases (Duong et al., 2015). Despite its inert surface, the PVC presented similar behavior than FRC1 with high hydrophobicity and hysteresis. This behavior could be explained by its higher roughness which limits the spreading of the water drop and retains water in asperities during its removal. SPC1 exhibited a hydrophilic surface with a lower hysteresis.

Contact angle measurements could not be carried out after the colonization occurred.

Density Dynamics of Biofilm Hetrotrophic Prokaryotic Communities for 75 Days

Densities as high as 3.5 × 10^4 cells/cm^2 could be observed after a day for both the reference PVC surface and FRC1 whereas SPC1 value remained significantly lower (6.7 × 10^3 cells/cm^2,

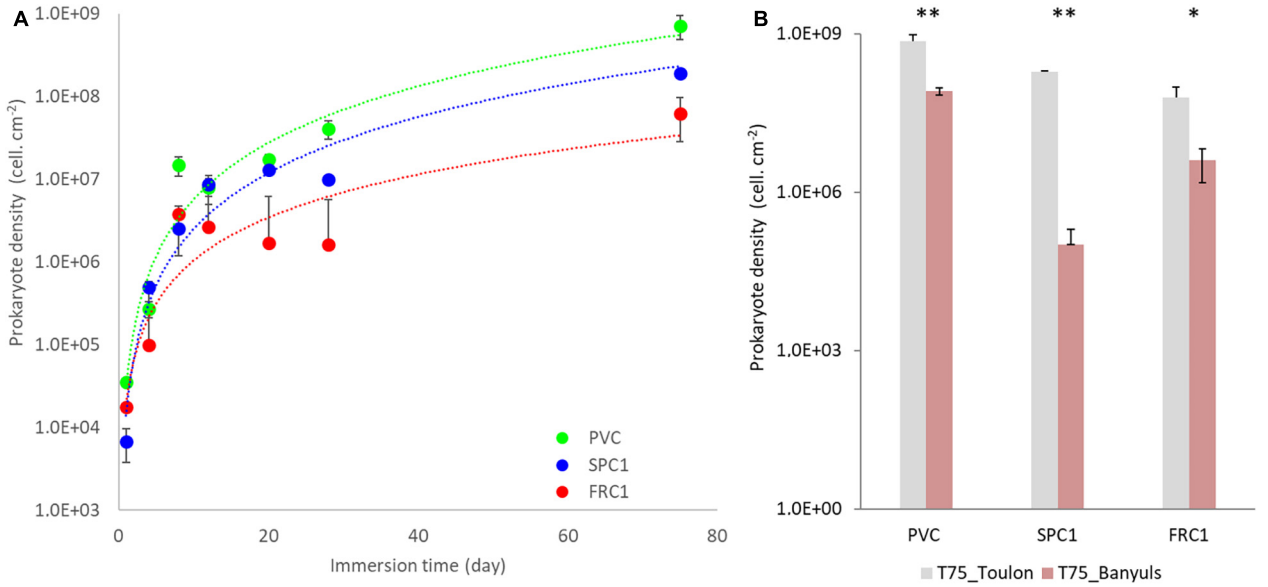

FIGURE 1 | Prokaryotic density colonizing PVC as well as two AF coatings (SPC1 and FRC1) from 1 to 75 days at Toulon bay **(A)** and after 75 days only at Banyuls bay **(B)** using Flow cytometry analyses. Statistical differences between sites were shown as follows: ** ($p < 0.01$), *($p < 0.05$).

$p < 0.05$) at Toulon (**Figure 1** and **Supplementary Figure 1**). The progressive increase of densities for all the surfaces led to significantly lower values for FRC1 (6.3×10^7 cells/cm^2) and SPC1 (1.9×10^8 cells/cm^2) compared to PVC (7.2×10^8 cells/cm^2) after 75 days ($p < 0.05$). At Banyuls, FRC1 showed higher densities compared to SPC1, both of them significantly lower than PVC at T75 too ($p < 0.05$). Whatever the substrate, densities at Banyuls were overall lower than their counterparts at Toulon ($p < 0.05$).

α-Diversity for 75 Days at Toulon and Banyuls

Numerous AF coatings samples exhibited low biomass, leading to amplification issues (SPC1 and FRC1 at T1, one PVC replicate at both T1 and T75 at Toulon, one FRC1 replicate at Banyuls) The Illumina Miseq run produced 829,149 paired-end reads. 2324 ASVs could be generated after clustering. Archaea were excluded (< 0.5%).

Both richness (Chao1) and diversity indices (Shannon and Pielou) showed the same temporal and surface relative trends at Toulon, with less significant differences for richness (**Figure 2**, ANOVA and *Post hoc* Tukey test, **Supplementary Table 3**). Alpha-diversity for the reference surface (PVC) rapidly increased and remained high and constant. Exhibiting very low values at T4 and T8 for SPC1, all the SPC1 and FRC1 indices showed a significant increase ($p < 0.0001$) with a lag of several days depending on the indices for the latter. Only after 75 days of immersion, induce showed no significant difference at both sites.

Dynamics of the β-Diversity for 75 Days at Toulon

Dissimilar temporal dynamics from T1 to T75 could be observed (**Figure 3**). Although communities on FRC1 and PVC tended to converge with time, SPC1 remained specific until T75, showing more similarity with early PVC ones.

At T1, the co-dominance of three genera was reported on PVC: *Oleibacter* sp., a potential hydrocarbonoclaste taxa among the Saccharospirillaceae (Gammaproteobacteria), *Alteromonas* spp. (Alteromonadaceae, Gammaproteobacteria) and *Tenacibaculum* spp. (Flavobacteriaceae, Bacteroidia) (37, 18, and 7% of the sequences, respectively) (**Figure 4**). Other Flavobacteriaceae represented also a significant part of the early community on PVC, with *Polaribacter* and *Dokdonia* or Rhodobacteraceae including *Tateyamaria*. Then *Oleibacter* and *Alteromonas* decreased rapidly with the late emergence of Candidatus *Endoecteinascidia* (Piscirickettsiaceae). The increase of Alphaproteobacteria was noticed in parallel, mainly Rhodobacteraceae (18%, especially unclassified, *Ruegeria* or *Tatayemaria*) and Cyanobacteria (17%, mainly *Xenococcus* and an unknown Phormidesmiaceae) together with the keeping of Flavobacteriaceae (13%, mainly *Tenacibaculum,* unclassified and *Gilvibacter*).

SPC1 exhibited a remarkable profile with *Alteromonas* (Gammaproteobacteria) accounting for more than 80% of the sequences until T8 (**Figure 4**). Among them, *A. genovensis*, *A. gracilis*, *A. macleodii*, *A. mediterranea* and unidentified species could be noticed. *A. gracilis* represented around 30% of *Alteromonas* reads at T4 and *A. genovensis* represented around 10% of *Alteromonas* reads at T4 and T8, before *Alteromonas* spp. was only detected (**Supplementary Figure 2**). *Aestuariibacter*, (Alteromonadaceae) and *Marinobacter* (Marinobacteraceae) remained between 4 and 23% of the reads all along the sampling time (highest% were reached at T12). A third Gammaproteobacteria, displayed similar percents. Together, the three Gammaproteobacteria represented 70% of the reads until

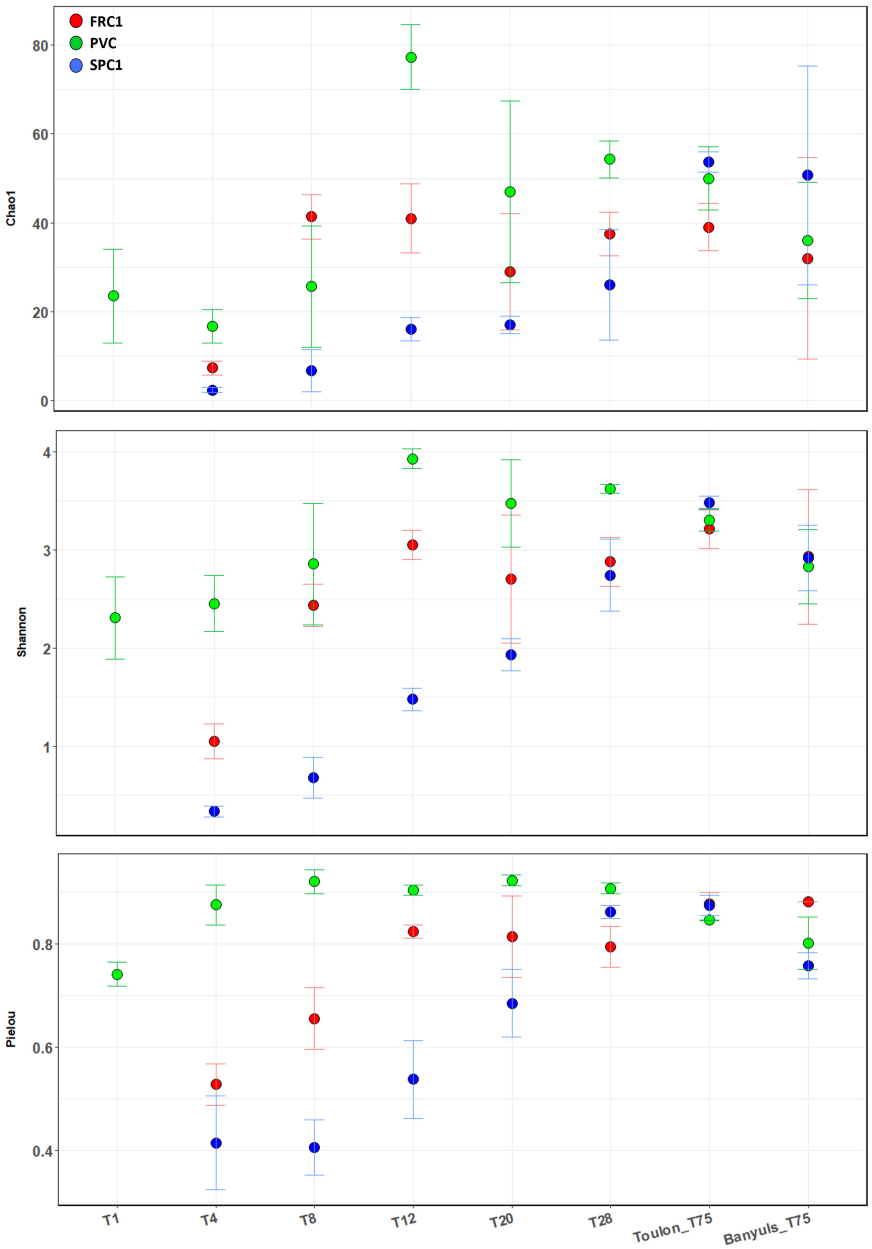

FIGURE 2 | Alpha-diversity indexes (Chao1, Shannon, and Pielou) corresponding to the prokaryotic communities on the different substrates (PVC and two AF coatings, SPC1 and FRC1), time and site (T1-T75 at Toulon bay and T75 only at Banyuls bay). Error bars stand for standard deviations. ANOVA and *post hoc* Tuckey tests were shown in **Supplementary Table 3**.

T20. The progressive diversification of the community observed from T20 lead to the dominance of the Flavobacteriaceae, reaching 23–32% of the reads at T75 (mainly *Aurantivirga, but also Aureisphaera and Croceitalea*). Together with *Alteromonas* (7%) and *Aestuariibacter* (7%), *Erythrobacter* (Sphingomonadaceae), SM1A02 (Phycisphaeraceae), Rhodobacteraceae, especially unclassified and *Loktanella,* were the most represented taxa.

Communities on FRC1 were dominated by *Alteromonas* ASVs as for SPC1 (73–26%) and also *Oleibacter* as for PVC (17–2%) until T8 (**Figure 4**). 15% of *Alteromonas* reads were identified as *A. gracilis* at T4 before *Alteromonas* spp. was only detected (**Supplementary Figure 2**). Similarly to SPC1, *Aestuariibacter* completed the early community (6–26%). Communities on FRC1 underwent a quicker and higher diversification compared to SPC1, and *Alteromonas* became nearly undetectable at T75. In addition, Methyloligellaceae (Alphaproteobacteria) reached 18% at T275. *Litorimonas* (Hyphomonadaceae) and *Cycloclasticus* (Cycloclasticaceae) reached 10 and 6% of the reads, respectively.

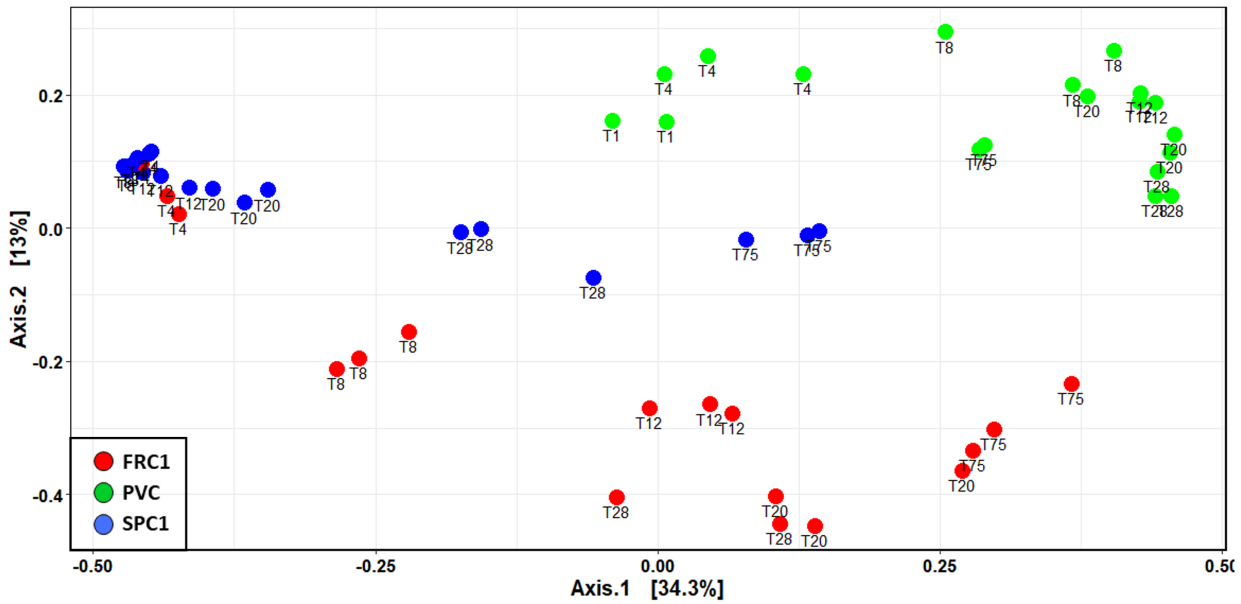

FIGURE 3 | NMDS showing dynamics of prokaryotic communities at Toulon from 1 to 75 days on the three substrates in triplicates (PVC and the two AF coatings, SPC and FRC) based on Bray and Curtis dissimilarity (Kruskal stress = 0.184).

Spatial Variations of Biofilm Communities

NMDS based on Bray-Curtis dissimilarities at T75 at the two sites (**Figure 5**) showed that the main discriminant factor was the presence of copper in coatings, while a significant site effect could be observed only secondarily (PERMANOVA, $p < 0.0001$).

The dissimilarity between sites on PVC could be assigned mainly to nutrients, metals and salinity, all higher at Toulon (**Supplementary Figure 3**). Difference in composition on PVC at the two sites was mainly associated with variations in the relative abundance of Cyanobacteria with a highest abundance at Banyuls (47% of reads compared to 16% at Toulon). In addition filamentous *Acrophormium* (Phormidesmiaceae) and Oscillatoriaceae dominated at Banyuls while *Xenococcus* and another Phormidesmiaceae represented the dominant Cyanobacteria at Toulon. *Marinicella* (Gammaproteobacteria), *Blastopirellula* (Planctomycetes), several genera among Flavobacteriaceae, *Ruegeria* and an unknown Rhodobacteriaceae and Saprospiraceae also distinguished biofilms at Banyuls compared to Toulon (**Figure 6**). SPC1 community at Banyuls differed from PVC at the same site or from the SPC1 at Toulon especially due to a significantly higher proportion of *Alteromonas, Aestuariibacter* and an unknown *Hyphomonadaceae*.

Linear discriminant analysis with effect size (LEfSe) was performed at T75 (**Figure 7**) showing that PVC biomarker at Toulon couldn't be identified. Cyanobacteria (especially Phormidesmiaceae) and *Rugiera* (Rhodobacteraceae) were specific to PVC at Banyuls, although detected on PVC and FRC1 at both sites. Similarly, Saprospiraceae and unknown Rhobacteraceae appeared as FRC1 biomarkers at Banyuls but were also observed at lower% on PVC. Caulobacterales (including *Litorimonas*) as well as Arenicellaceae displayed differential relative abundances on FRC1 at Toulon. Enteromonadales, especially *Alteromonas* and *Aestuariibacter*, together with Pseudomonadales, showed differential relative abundances on SPC1 at Banyuls whereas Cytophagales (mainly *Fabibacter*), *Erythrobacter* and *Marinicella* were more specific to SPC1 at Toulon

A co-occurrence network analysis was achieved to identify central ASVs among the mature biofilm communities (T75). 34 nodes (ASVs) constituted the major cluster of the network associated to 159 degrees or edges, corresponding to significant relationships between them (**Figure 8** and **Supplementary Table 4**). Ten genera were represented with specific characteristics. *Alteromonas* (6 ASVs) and *Aestuariibacter* (8 ASVs), which were both abundant on SPC1 at both sites, displayed positive edges among themselves while negative ones with *Ruegeria* and Cyanobacteria (*Leptolyngbya* and *Xenococcus*), conversely abundant on PVC and FRC1 at both sites. *Gilvibacter* (4 ASVs) displayed positive edges among themselves and with *Aestuariibacter* (1 ASV), while negative edges were observed with one Methyloligellaceae and *Leptolyngbia*, mostly abundant on FRC1. Hyphomonodaceae mostly exhibited positive edges with *Aestuariibacter* and negative edges with *Ruegeria* and *Xenococcus*. Finally, two *Cycloclasticus* ASVs, mostly abundant on FRC1 at Toulon, were separated from the main cluster.

DISCUSSION

Alteromonas, a Pioneer and Copper Resistant Prokaryote Genus

Alteromonas represented, after 4 days, the early dominant genus with 13, 70, and 89% of the reads on PVC, FRC1 and SPC1, respectively. However, PVC plates exhibited much more

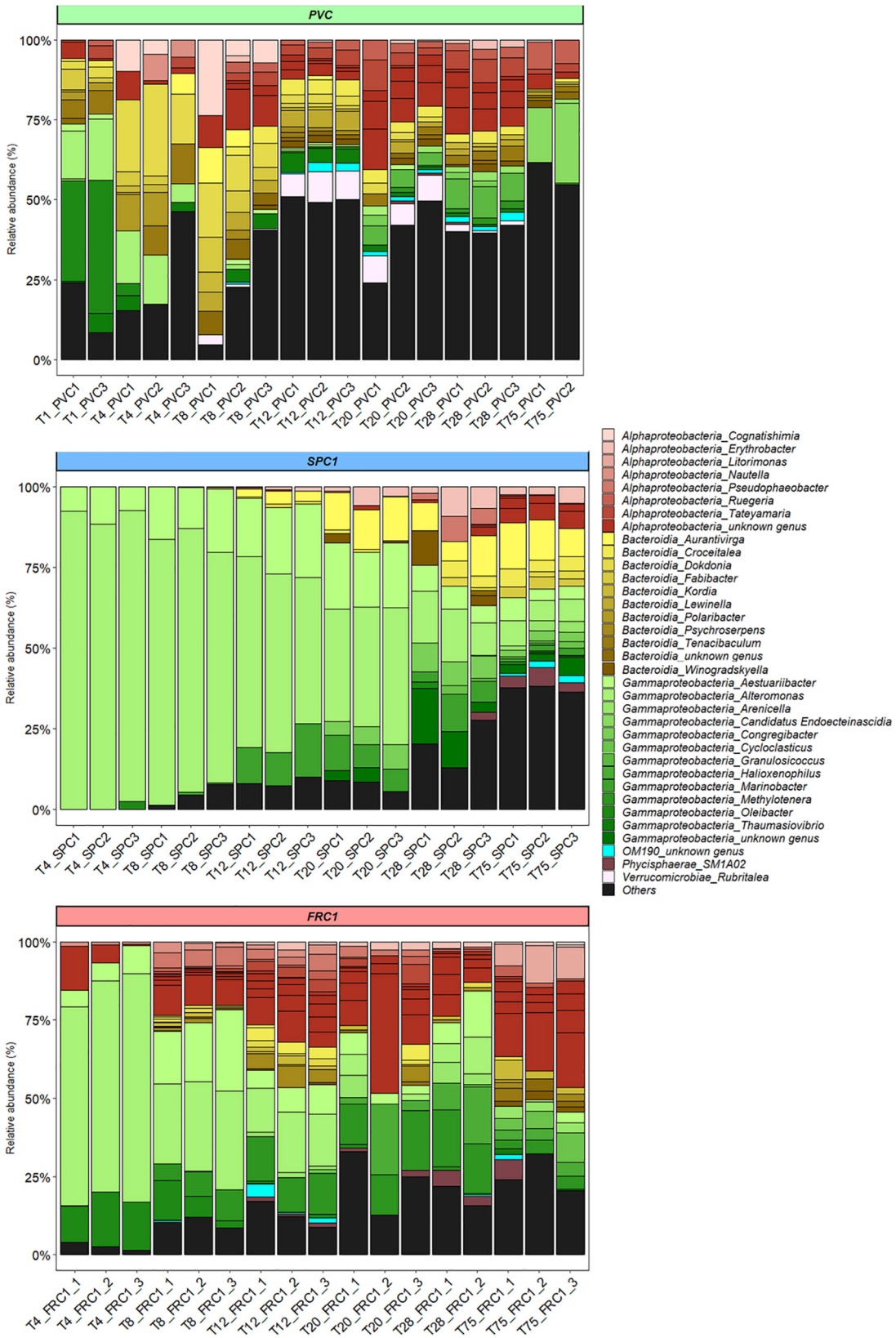

FIGURE 4 | Dynamics of the relative abundance of the bacterial genera from T1 to T75 for PVC, SPC1, and FRC1 (including replicates) at Toulon. Others corresponded to genera with a relative abundance lower than 0.5%.

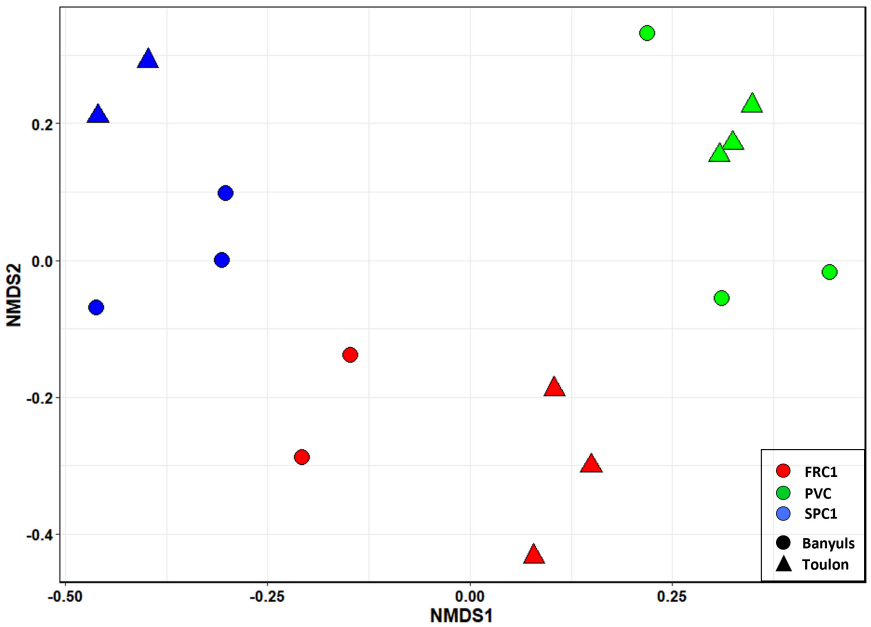

FIGURE 5 | NMDS of the prokaryotic communities on the three substrates in triplicates (PVC, FRC1, and SPC) after 75 days of immersion at Toulon and Banyuls.

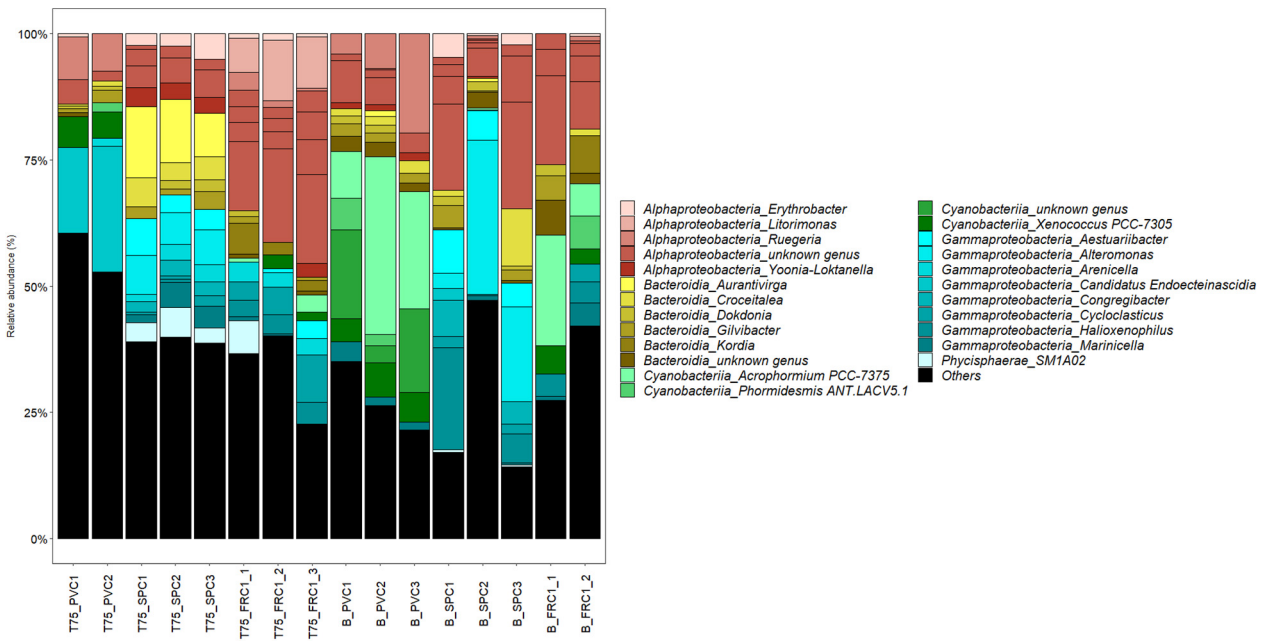

FIGURE 6 | Relative abundance at the genus level after 75 days of immersion at Toulon and Banyuls on the different surfaces (triplicates except PVC at Toulon and FRC1 at Banyuls that displayed only duplicates). Others were genera representing less than 0.5% of the reads.

diversified communities than SPC ones immersed 10 cm over. As there is no reason that all early colonizers were resistant to copper, *Alteromonas* were probably the only pioneers to be resistant to copper. This genus was already mentioned as a pioneer taxa in both temperate and tropical coastal seawaters on plastics on the South Carolina coast (Dang and Lovell, 2000) and the Northern Adriatic (Pinto et al., 2019), and other substrates (acryl, glass, steel, fiberglass) on the Korean coast (Lee et al., 2008) and Indian ocean (Rampadarath et al., 2017). *Alteromonas* spp. are mainly known as marine generalist copiotrophs, with the ability to degrade a broad range of organic substrates (Dang and Lovell, 2016), especially artificial surface conditioning films. Common adhesion properties like active swimming or surface adhesion mechanisms, involving outer-cell

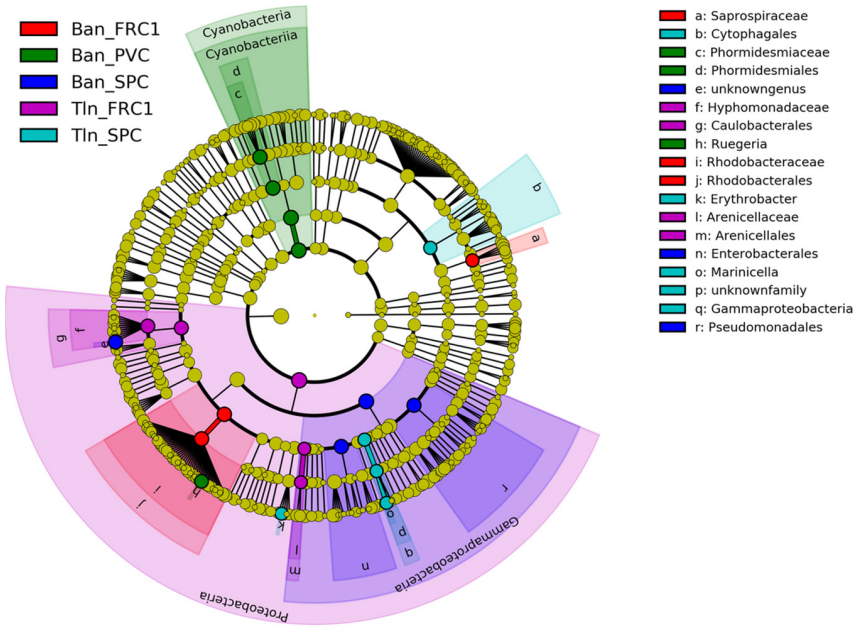

FIGURE 7 | LefSe analysis after 75 days of immersion at Toulon and Banyuls for the three surfaces (PVC, SPC1, and FRC1).

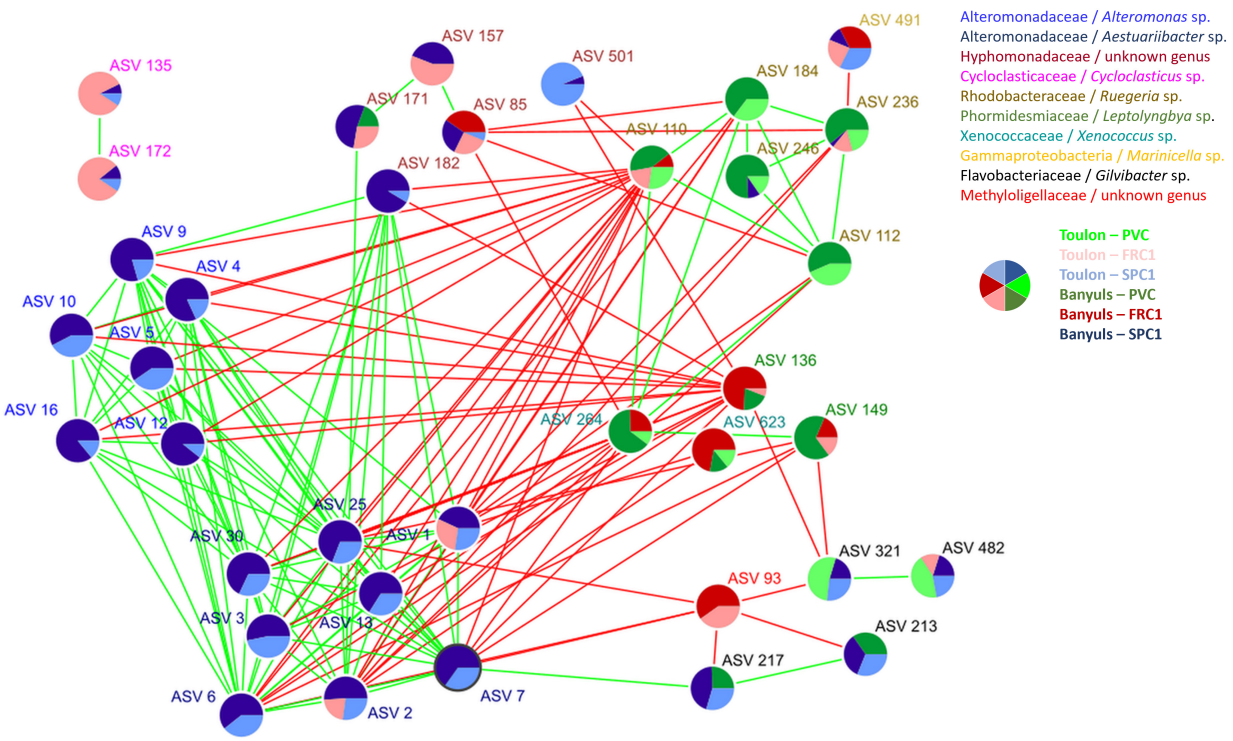

FIGURE 8 | Network co-occurrence analysis performed with CONET for samples after 75 days of immersion at both sites. Colors of ASVs corresponded to genera while pie charts indicated their relative abundance on the three coatings (PVC, SPC1, and FRC1) at the two sites (Toulon and Banyuls).

structures (as flagella, fimbriae, pili or curli) or adhesins are not particularly described for *Alteromonas*. However, the number of genes encoding diguanylate cyclases, which synthetize the c-di-GMP, an intracellular signaling compound that, among other functions, coordinates the transition from a motile to a biofilm lifestyle (Römling et al., 2013), was found higher in *A. macleodii* than *Pseudomonas aeruginosa* or *Escherichia coli* genomes, two biofilm forming model strains (Cusick et al., 2017).

A gene involved in the lipopolysaccharide synthesis, one of the main components of the outer membrane used to attach to substrates, was also reported by same authors to be highly induced. Moreover, additional cell characteristics may favor early colonization processes too. Its ability to be highly resistant to solar radiation, while non-pigmented (Agogué et al., 2005; Bacosa et al., 2015), may be an additional selective factor, not needed for later colonizers which take advantage of the EPS matrix produced and diatoms presence, in the biofilm community. As a matter of fact, the diversification of the biofilm community on SPC1 involved pigmented diatoms from T4 (data not shown). Moreover, *Alteromonas* was reported to be a dominant colonizer of copper-based SPCs after 1–4 weeks in marinas from two dissimilar environments, i.e., Singapore (Chen et al., 2013) and the Swedish West coast (Flach et al., 2017). Two hypotheses are generally proposed to explain such a metal resistance/tolerance mechanism: the occurrence of metal resistance genes (MRG) (Flach et al., 2017; Roberto et al., 2019; Catao et al., 2021) and the exopolymeric substances production (EPS) (Flemming et al., 2016). The former was recently demonstrated for several MRG, including copper specific (cusA, copA, and CueO) or not (czcA and pbrT), on substrates including copper-released coatings in the Bay of Toulon but also in a port of the French Atlantic coast in Brittany (Lorient) and in the tropical Indian ocean (Reunion island). *Alteromonas* showed the strongest correlation at the genus level with cusA whatever the site and coatings (Catao et al., 2021). However, the bay of Toulon appeared unique with high MRG abundance in all biofilm including on PVC after 30 days, together with particular communities on copper based coatings as also shown here. This could indicate that the selection on SPC coatings during the diversification that occurred with time will be based on alternative properties to manage copper like EPS production. Whole-genome sequencing of *A. macleodii* strains, isolated from copper alloy coupons but barely identified in our study, also indicated the presence of Cue, Cus, or Cop systems. They were identified in multiple megaplasmids which are known to be key vectors in horizontal gene transfer (Cusick et al., 2020).

Alteromonas was also identified at Banyuls on SPC1, but with higher relative abundance than at Toulon after 75 days. We may suppose that lower available inorganic nutrients at Banyuls could have slowed down the dynamics compared to Toulon and/or the higher hydrodynamic, experienced at Banyuls due to the location one km far from the coast with a 27 m depth, could have maintained unfavorable conditions for biofilm growth as also reflected by lower densities.

Copper-Releasing Coatings Strongly Control Biofilm Density and Diversity Dynamics

Previous studies have already reported that biocidal coatings exhibited dissimilar communities compared to non-biocidal ones using end-point studies (from a month to a year) in the Oman sea (Muthukrishnan et al., 2014; Dobretsov et al., 2018), the North Western Mediterranean sea (Camps et al., 2014; Briand et al., 2017; Briand et al., 2018; Catão et al., 2019), the Florida coast (Zargiel et al., 2011), the French Atlantic and Indian oceans (Catao et al., 2021) and the Auckland Marina (New Zealand) (von Ammon et al., 2018). However, sequential dynamics have never been studied. Early communities suffered a strong selective pressure due to copper as described above, which explained a lower density and diversity compared to the reference (PVC) for the 75 days of immersion. SPC 1 is a seawater-hydrolyzable coatings with self-polishing behavior (Marceaux et al., 2018). The release of copper ions from this coating relies on both the dissolution of Cu_2O and the erosion of the polymer matrix. Higher release rate of copper is commonly observed in the early stages of immersion (personal data) before equilibrium is reached between the dissolution kinetics of the biocides and the erosion kinetics of the hydrolyzable polymer matrix. This could result in a potential impact on early bacterial selection. Nevertheless, the biofilm diversity increased with time and *Alteromonas* spp. became only one of the co-dominant taxa at T75, together with a close phylogenetic-related genus *Aestuariibacter*, *Aurantivirga, Aureisphaera* and *Croceitalea* (Flavobacteriaceae), or *Loktanella* (Rhodobacteraceae). Some of them even became surface biomarkers like *Erythrobacter* (Sphingomonadaceae), Most of them were already reported in previous studies including in the Toulon bay like *Erythrobacter* (Dobretsov et al., 2018; Catão et al., 2019). As physico-chemical parameters didn't vary significantly during the time scale of the experiment (Redundancy analysis not significant, data not shown), additional factor would be involved. Then, as metal resistance genes were not exclusively described for these selected taxa, two hypotheses remained: (i) taxa without adhesion capacities but with copper resistant genes will progressively embed the biofilm community, taking advantages of the matrix generated by early taxa. As copper resistant genes could be shared by horizontal gene transfer among the prokaryotic community, taxa from diversified taxonomic origin were involved, (ii) taxa able to produce EPS, but without particular gene resistance occurrence, were progressively involved in the building of the biofilm structure, as EPS trapped copper released by the coating. The latter hypothesis could join the former as copper resistance genes from early taxa could be spread to the entire biofilm community by horizontal gene transfer which is known to occurred intensively in marine biofilms (Dang and Lovell, 2016).

Similar communities were observed on SPC1 at T75 and after a year of immersion in the same site the same year (Catão et al., 2019). This allowed to conclude that mature communities were reached between 28 and 75 days of immersion in the Bay of Toulon.

Physical Features May Control the Biofilm Density but Not Diversity

Biofilm dynamics on PVC have been already described in Pollet et al. (2018). Briefly, a rapid and intense colonization of the PVC occurred during the first days following immersion, including a rapid diversification of the biofilm community reaching a highly diversified and stable community after 4–8 days. Gammaproteobacteria from the genus *Oleibacter* strongly dominated microbial communities during the first hours of biofilm formation. These pioneer communities were quickly replaced by Alphaproteobacteria and Flavobacteria. We showed that Flavobacteria might be key actors in the functioning of

marine biofilms. Bacterial communities exhibited fast temporal dynamics with taxa displaying rapid increases and declines. With 2/3 of positive connections between bacterial OTUs, a network analysis showed that biofilm communities were more inclined to develop inter-specific cooperation than competition. The network analysis performed here, including metal-releasing coatings, was conversely dominated by negative connections as discussed below. Cyanobacteria mainly filamentous at Banyuls, but also unicellular (Xenococaceae) at Toulon, were reported as biomarkers of PVC. This implies that no heterotrophic abundant taxa was specific of this surface, which is supported by a high diversity and evenness characteristic of a stabilized (or mature) community, including typical biofilm taxa such as Deltaproteobacteria, Planctomycetes or Verrucomicrobiales. The former belongs to the sulfate-reducing bacteria which found anoxygenic conditions inside the biofilm. Planctomycetes are poorly studied "maverick" bacteria (Wiegand et al., 2018), highly represented in activated sludge and among algal surface associated community. Functions like metal binding extrapolymeric proteins or stress response could explain the success of this taxa among the biofilm community (Bondoso et al., 2017; Chen et al., 2019).

FRCs have been developed to be alternatives to biocidal coatings and more environmentally friendly AF strategies. FRCs aimed to minimize the adhesion of fouling organisms on ship hulls, so that they can be removed by hydrodynamic stress during navigation or by a simple mechanical cleaning. In addition, the smoothness of FRCs enables them to reduce the drag of the vessel and therefore reduce fuel consumption and greenhouse gas emissions (Lejars et al., 2012). FRC1 properties actually limited the prokaryotic colonization, exhibiting the lowest density throughout the study at Toulon, ten times less than PVC after 75 days of immersion. Community dynamics appeared also definitely slowed compared to the PVC, with early stages showing a lower alpha diversity with the dominance of *Alteromonas*, as for SPC1. This genus was already reported to be an early colonizer on plastics (Pinto et al., 2019). Its ability to attach to such smooth, soft and amphiphilic FRC1 surface confirmed a great potential of adhesion which would be interesting to better characterize, as several mechanisms including flagella, pili, curli, holdfast or EPS production could be involved (Flemming and Wingender, 2010; Cusick et al., 2017). Cusick et al. (2020) mentioned that metal resistance genes were not detected in all *Alteromonas* strains, but only in those isolated from copper alloy coupons. However, Catao et al. (2021) recently reported that MRG showed similar occurrence in biofilm from both AF and PVC surfaces especially in the Toulon bay, with probable link to a long history of metal contamination. This is in accordance with the fact that identical early colonizers could be identified on SPC1 and FRC1 here. *Oleibacter*, as for PVC and an additional Alteromonadaceae genus (*Aestuariibacter*), completed the early communities on FRC1, which allow to hypothesize that early colonizers on PVC were potentially not fully described with a sampling after 24 h, all the more that the alpha diversity was already high. Both local seawater (see below) and physical surface characteristics control the temporal dynamics, which could explain why early colonizers described in the literature were diverse. Only the study of the dynamics may allow to definitely identified early colonizers at one site. However, the diversification and the concurrent increase of the diversity of the biofilm community on FRC1 occurred during the 12–20 days of immersion, with a convergence of the assemblage on the PVC as clearly showed by the NMDS (**Figure 3**). Similar clustering was kept at very long immersion time (1 year), including in dynamic conditions (Catão et al., 2019). The similarity of mature communities on PVC and FRC1 appeared consistent with the already reported low influence of the plastic type on mature biofilm community structures when immersed at the same location (Lee et al., 2014; Kirstein et al., 2018; Pinto et al., 2019). Physical properties of substrates like wettability, roughness or softness have been described to limit the microbial density and select only early communities as also observed in South Carolina (Dang and Lovell, 2000), South Korea (Lee et al., 2008) or Croatia (Pinto et al., 2019). We clearly conclude that physical factors of substrate lose their influence on bacterial community structure with time.

Spatial Biofilm Community Dissimilarities Between the Two Mediterranean Sites Could Be Associated to Variations in Hydrodynamics and Seawater Metals Concentrations

Previous comparisons with sites in the Atlantic or Indian oceans already showed us that temperature, salinity, nutrients and metals could shape dissimilar biofilm communities on both plastic and AF surfaces (Briand et al., 2017; Catao et al., 2021). Salinity was also proposed to be a key biofilm community driver on plastics in the Baltic sea (Oberbeckmann et al., 2018). However, these sites exhibited several different environmental characteristics which implies a complex multifactorial control and we intended to test closer environmental conditions that could be found in the same Mediterranean region, where temperature and salinity are similar and mostly the anthropic impact changes with lower nutrients and metal contaminants availability in the Bay of Banyuls.

If SPC1 and FRC1 at both locations exhibited lower densities compared to PVC, they both seemed more efficient antifoulings at Banyuls, probably taking advantage of a higher hydrodynamics. Indeed, the location of the SOLA buoy, 1 km far from the coast with a 27 m depth, implies stronger currents that could limit biofilm densities, probably together with the lower nutrients availability, as also already reported on 1 year old biofilms (Catão et al., 2019). Moreover, it may have also accelerated the coating erosion and release of copper, increasing the SPCs efficacy (Bressy et al., 2009). As observed at Toulon, biofilms at Banyuls on SPC1 were clearly dissimilar from the ones on PVC and FRC1, the latter in addition showing a higher similarity. α diversity was similar on both locations for the different coatings but, as mentioned above, hydrodynamics and perhaps low nutrient availability, may have slowed biofilm dynamics on SPC1, explaining a higher *Alteromonas* relative abundance at Banyuls. In addition, the significant discrepancy between sites on SPC1 was also associated to the occurrence of an unknown genus of Hyphomonadaceae, which are known to produce a polar holdfast enabling the attachment on surfaces.

Such functional feature provides a selective advantage as pioneer taxa (Langille and Weiner, 1998; Dang and Lovell, 2016), which reinforces the hypothesis of a less mature biofilm on SPC1 at Banyuls. Considering FRC1, higher currents at Banyuls surely favorized the detachment of biofilm as such coatings are designed to minimize the attachment forces through low surface energy, roughness and elastic modulus. These high fouling release properties could explain its higher efficiency at Banyuls compared to Toulon.

Moreover, Desulfovibrionaceae (*Halodesulfovibrio*) and a tunicate endosymbiont (*Candidatus endocteinascidia*) (Pérez-Matos et al., 2007) were two of the five taxa explaining most of the dissimilarity between highly diversified biofilms on PVC at Toulon and Banyuls (SIMPER analysis), and representing higher relative percentages of reads at Toulon. Desulfovibrionaceae belongs to Deltaproteobacteria and especially anaerobic sulfate-reducing bacteria. They are well known to develop in the central part of mature biofilms which is characterized by low oxygen amounts (McDougald et al., 2011). Tunicates are invertebrate macrofoulers often observed at the bay of Toulon after several weeks of immersion on PVC (Briand et al., 2018) and consistent with visual observations (**Supplementary Figure 3**).

ASVs highlighted with the co-occurrence network analysis and that belong to PVC communities, were *Rugiera* (Rhodobacteraceae), *Gilvibacter* (Flavobacteriaceae) but also Cyanobacteria. Especially, Banyuls biofilms on PVC exhibited a large community of Cyanobacteria. If the coccoid *Xenococcus* occurred at both sites, filamentous Nostocales and Phormidesmiaceae displayed high relative percentages (around 47%) on PVC at Banyuls. Such specificity, compared to other sites in harbors or marinas studied elsewhere, could be linked to the low metal seawater concentrations, especially copper derivatives which are widely used as algicides. Using a 16S rRNA sequencing approach, only one other study reported Cyanobacteria as main contributors of biofilm community on artificial substrates in a marina in the Oman sea, but including AF coatings (Muthukrishnan et al., 2014). These may indicate that these diversified Cyanobacteria (coccoids and filamentous taxa including *Acaryochloris*, *Phormidium*, *Leptolyngbya,* or *Oscillatoria*) were probably adapted to high metal concentrations, conversely to our study.

Apart from density, nutrients availability, in the range we experienced in the mesotrophic Bay of Toulon and oligotrophic Bay of Banyuls, did not appear to be a strong driver of community structure as copiotrophs, especially among Gammaproteobacteria, reputed to be encouraged to grow in nutrient enriched systems (Lawes et al., 2016), which were not particularly more abundant at Toulon. When reported as biofilm community drivers in the Baltic Sea, inorganic nutrients displayed a much more higher range, reaching hyper eutrophic levels (Oberbeckmann et al., 2018; Kesy et al., 2019).

CONCLUSION

This study shows that both surface characteristics and environmental parameters control colonization. However, even if local environmental seawater characteristics influence biofilms, copper-releasing coatings apply a huge quantitative and qualitative selective pressure on microbial colonizers while particular surface physical characteristics control only density. Hydrodynamics could limit densities but also slow down biofilm dynamics. Finally, sequential biofilm dynamics should be carefully considered as initial processes differed from long-term ones.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA648348.

AUTHOR CONTRIBUTIONS

J-FB performed the immersion experiments, performed the sampling at Banyuls, formal analysis, and interpretation of the Illumina Miseq data with AP, wrote the original draft, supervised the project. TP executed the immersion experiments and sampling at Toulon ad Banyuls, the PCR for Illumina Miseq, formal analysis of the Illumina Miseq, review the draft. BM performed flow cytometry analyses and interpretation and reviewed the draft. CG analyzed and interpreted seawater chemical analyses. ML and MM painted the panels and executed the immersion experiments at Toulon. RB-M executed the immersion experiments and sampling at Toulon. J-FG reviewed and edited the draft. CB performed the immersion experiments, especially the choice of the antifouling coatings, supervised the global project, wrote, and edited the draft. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2021.746383/full#supplementary-material

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Importance of Duration, Duty-Cycling and Thresholds for the Implementation of Ultraviolet C in Marine Biofouling Control

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The introduction of a surface into the marine environment begins a process known as biofouling, which increases the weight and hydrodynamic drag of the fouled structure. This process is detrimental to maritime vessels and costs the industry ~$150B in fuel and maintenance spending annually. Preventing the settlement of fouling organisms mitigates these issues and limits the spread of non-indigenous species (NIS). This is primarily achieved *via* antifouling paints. Ultraviolet light is a sterilization method used in water purification, food storage packaging, and within medical fields. Ultraviolet C (UV-C) radiation interacts with DNA to prevent growth, proliferation, and survival of bacteria, and biofilm formation. Recent progress in microelectronics technology has advanced the range of commercially available light-emitting diodes (LEDs) to include the UV wavelengths, and the reduced size and cost has allowed their integration into previously inaccessible locales. This study builds on recent progress in integrating UV-C LEDs into UV-lucent silicone tiles for fouling control. The operational cycle needed to prevent growth of *Navicula incerta* cells was determined. Constant irradiance at a peak of 5.77 $\mu W/cm^2$ resulted in a significant reduction in diatoms within 2 h, and a 2 log and 3 log reduction after 48 h and 5 days, respectively. Duty cycling (pulsing) in all variations from 50 to 2.5%, indicated significant reductions in cell densities, and the lowest cycle could effectively reduce biofouling growth and increase the longevity of the LEDs for up to 45.6 years. Irradiance and exposure were altered over a set duration and indicated a restriction in growth between 0.01–0.82 J/cm^2 and an increased mortality at irradiances $>$ 2.65 J/cm^2, suggesting an effective antifouling threshold between these dosages. The effective dosage for 1 log reduction in fouling was estimated to be 25 J/cm^2 but varied according to irradiance delivery method. Effective dosage for a 1 log reduction between experimental methods was variable indicating that UV treatment of *N. incerta* departed from the Bunsen-Roscoe reciprocity law expectancy. The variation in densities at similar dosages could be explained with further investigation of DNA repair mechanisms. In conclusion, UV-B/C use was effective at all irradiances, including as low as 0.01 J/cm^2, and holds considerable promise for marine biofouling control.

Keywords: ultraviolet B/C, non-toxic antifoulant, marine biofouling, irradiance, fouling management, diatom

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INTRODUCTION

The accumulation of marine organisms on submerged surfaces, known as biofouling, is an ongoing challenge for the maritime industry. In aquaculture, biofouling increases the degradation of cages, can inhibit water exchange, and increases the potential of disease (Bannister et al., 2019). Powerplant cooling pipes that receive and deposit into the ocean can become clogged, reducing flow, and impacting internal temperature control (Flemming et al., 2009). Biofouling can occur on filtration membranes that are used for desalination of saltwater and reclamation of wastewater (Matin et al., 2021). The fouling of ships' hulls increases hydrodynamic drag, increasing fuel consumption and pollutant emissions (Munk et al., 2009). Fouling of hulls and internal ballast tanks also transports non-indigenous species (NIS), which can be ecologically detrimental to native species, as well as impacting upon farmed aquaculture species, submerged static artificial structures, and presenting risks to marine values (Sylvester et al., 2011; Piola and Hopkins, 2012; Georgiades et al., 2020, 2021; Hopkins et al., 2021).

Marine biofouling was estimated to cost the US Navy $56 million p.a. for the DDG-51 class alone, with estimates as high as ∼$150 billion for annual fuel cost savings to the global shipping fleet derived from effective antifouling coatings (Pritchard, 1988; Maréchal and Hellio, 2009; Schultz et al., 2011; Selim et al., 2017). Estimates can vary markedly based on the prevailing oil price. Since 2017 prices have ranged from $11.26 to $75.23 per barrel (WTI crude oil) and are currently ∼$25 (60%) per barrel more than when estimates were last calculated (Selim et al., 2017; MacroTrends, 2021), which could adjust current estimates to as high as ∼$240 billion.

Marine biofouling is an expansive industrial problem that is well-studied, with multiple reviews focusing on or around specific niches (Pritchard, 1988; Dobretsov et al., 2013). Methods to limit fouling on ships include biocidal treatment, interference with adhesion, settlement deterrence, and physical removal of foulers (Han et al., 2021; Liu et al., 2021; Pistone et al., 2021). Between these groups many strategies have been developed, ranging from electrochlorination to biocidal products (Growcott et al., 2017), self-polishing and controlled depletion paints (Almeida et al., 2007), fouling-release coatings (Lejars et al., 2012), and the development of new experimental approaches (Li et al., 2020). Currently, the most prevalent method for ships is the use of biocidal paints, which can be used on the hull and in niche areas, such as sea chests (Kiil et al., 2002). These paints are optimal for the hull but are often applied to sea chests, even though they are not designed for these areas, and perform poorly due to the different flow regimes (Coutts et al., 2003; De Castro et al., 2018). The hull experiences smooth flow whilst in motion, but niche areas (propellers, sea-chests, bilge intakes, etc.) are subjected to regimes that can remove or rapidly deplete paints, or restrict paint leaching (Coutts and Taylor, 2004; Coutts and Dodgshun, 2007; Sylvester and MacIsaac, 2010). The inhibition of early forming biofilms has been proposed as a means to counteract macrofouling, by interfering with the proposed successional development of a fouling community (Hadfield and Paul, 2001; Hadfield, 2011; Salta et al., 2013). Under this scenario, the biofouling process begins with the formation of a conditioning layer providing nutrients for subsequent adhering organisms (Wahl and Lafargue, 1990; Railkin, 2003; Grzegorczyk et al., 2018). Next, bacteria and diatoms use the nutrient-rich conditioning layer to produce an exopolymeric material resulting in a biofilm (Dang and Lovell, 2016). Diatoms are associated with early colonization of surfaces and are ubiquitous in their distribution (Hunsucker et al., 2014; Gómez-Ramírez et al., 2019). Diatomaceous biofilms can increase vessel hull friction by up to 70% based on the biofilm's thickness and percentage coverage; this can require increases in vessel power between 1.5 and 10.1% (Schultz et al., 2015). Biofilm formation is dependent on the physical and chemical conditions of the water, such as temperature and chemical input, and is vulnerable to unfavorable conditions (Cacabelos et al., 2020). Biofilms may then induce larval settlement and allow the formation of a macrofouling layer of juvenile barnacles, mussels, ascidians, polychaetes, and algal spores. While this process is generally considered to be successional (Scheer, 1945; Wahl, 1989; Bloecher et al., 2013; Chen et al., 2013), this is not always the case and many fouling organisms, which are opportunistic by nature, exhibit settlement inhibition or ambivalence in the presence of a biofilm (Roberts et al., 1991; Clare et al., 1992; Hadfield and Paul, 2001; Callow and Callow, 2011; Salta et al., 2013; Piola et al., 2016). As bacteria and single-celled organisms, such as diatoms, are particularly susceptible to ultraviolet (UV) light, its use as an antifouling strategy has generated interest both for the prevention of biofilm formation and possible knock-on effects to macroscopic biofouling prevention.

UV irradiation is used for sterilization in many industries including water purification, sewage treatment, medical surface cleansing, food preservation, and in scientific laboratories for routine sterilization of equipment. The UV spectrum is divided into ranges of wavelengths; 340–400 nm (UV-A), 280–340 nm (UV-B), and 100–280 nm (UV-C), and inactivates organisms most effectively at 254 nm (UV-C; Zelle, 1960). By isomerizing hydrogen bonds between purine and pyrimidine base pairs, when there are two of the same adjacent pyrimidines, the neighboring base pairs bond together producing a photolesion in the form of a cyclobutane pyrimidine dimer (CPD) or 6-4 photoproduct (6-4 PP; Schreier et al., 2009). In algae, these lesions cause a distortion in the structure of DNA, which fail a DNA assessment during photosystem II. This can lead to a failure in cell division and reproduction, and can cause cell apoptosis (Szabó and Ohshima, 1997). The impediment to proliferation has the potential to halt biofilm formation and inhibit fouling by organisms that require a biofilm for settlement. Additionally, evidence suggests that macrofouling larvae are more susceptible to UV light than adult phases, indicating that settlement could be prevented and macrofouling inhibited with continuous exposure (Kuffner, 2001; Hunsucker et al., 2019).

The use of UV sterilization has become more prevalent as technology has progressed and the costs and scale of the light sources have reduced. Mono- and polychromatic halogen lamps provide a high irradiance capacity but, due to their size, remain unfeasible for many applications. UV light-emitting diodes (LEDs) have improved since the early 2000s and are now

commercially available at a reasonable scale and cost (Bugbee, 2017). This has allowed for technological integration where UV use was not previously considered.

The use of UV-C LEDs for biofouling control was investigated by Piola et al. (2016), using a tile design that incorporated UV-C LEDs in a UV transparent silicone, allowing continual surface irradiance, and investigating settlement diversity over time. Unfortunately, some LEDs failed during operation. The LEDs that remained operational over the duration of the project maintained a fouling free surface within a radius of the light source. An estimated threshold irradiance of 1 $\mu W/cm^2$ was identified. The low power requirement and high efficacy of the UV sterilization technique confirmed the high potential of UV LEDs as a potential new avenue for the antifouling industry.

Using the initial findings of Piola et al. (2016), this study aimed to determine the efficacy of UV-B/C LED-embedded tiles in inhibiting biofouling over varying exposure durations, duty cycle periods, and fluence rates to establish a range of thresholds for these factors. For our experiments, we used a laboratory model biofilm-forming diatom, *Navicula incerta*. To determine thresholds, the minimum dosages required to attain a significant reduction in growth and removal followed by a 1 log reduction in the density of exposed organisms was investigated. By comparing settlement and survival, a novel UV-C based antifouling approach that is effective may be achievable.

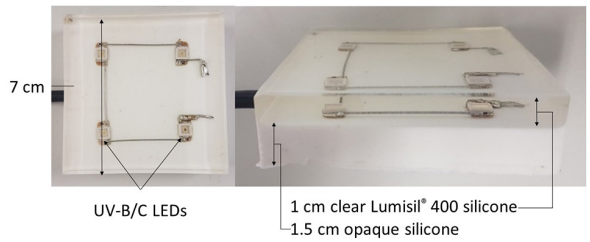

FIGURE 1 | Four low powered (24-volt, 0.17 amp) UV-B/C LEDs arranged in a 2 × 2 array on a 1.5 cm thick opaque silicone polymer and embedded in a 1 cm thick UV transparent Lumisil® 400 silicone.

MATERIALS AND METHODS

Units and Nomenclature

Throughout this manuscript, irradiances (a radiant flux which a surface receives per unit area—$\mu W/cm^2$) were measured using an ILT 950 spectroradiometer by taking a reading at the experimental surface. These measures were treated as the surface irradiance, which gave individual nm irradiances and total UV-B/C spectral range irradiances. The term fluence (units J/cm^2) is used interchangeably with UV dose. Fluence/dose is the amount of irradiance the organisms were exposed to over a given time (in seconds). The quantum yield, and how much of the dose was absorbed by individual organisms, was not measured. The UV dose was determined by taking an irradiance reading at the experimental surface and multiplying it by the duration of exposure in seconds. All rates and dose measurements were compiled and are supplied in **Supplementary Table 1**.

Methods and Materials

A total of four tiles (two irradiated and two control) were produced with a 2 × 2 array of UV (265–300 nm) LEDs embedded 1 cm beneath the surface of a 70 × 70 × 25 mm Lumisil® 400 (Wacker) silicone polymer (**Figure 1**). To degas the Lumisil® 400, it was cured at -0.1 MPa within a Medline Scientific™ Jeio Tech 665 L vacuum oven (OV-12) over 48 h at 70°C. This prevented bubble formation on the surface and allowed optimal internal cross-linking.

Supplier recommendations stipulated that LED power requirement was 24 V and 0.17 amps per LED to achieve an expected 10,000 h of dependable performance. This was used as the standard operating power supply and only altered in specific experiments, as described below.

Diatoms, *Navicula incerta* were chosen as a representative slime-forming species; a major challenge for contemporary non-biocidal fouling-release coatings (Schultz et al., 2015). *N. incerta* strain UTEX B 2046 from the culture collection at the University of Texas in Austin, United States was cultured following Kardela et al. (2019), and were used for experiments on day 5 of culture, at the peak of their exponential growth phase. Cells were agitated and suspended before experimentation, allowing the cell density to be calculated. The culture was then diluted with F/2 medium to give a 150,000 cells/mL culture. To the surface of the tiles, 20 mL of the 150,000 cells/mL culture was added and allowed 2 min to acclimate before irradiance was initiated. Tiles were split into irradiated and control, and operated in replicates of five for durations of 2 h, 1, 2, 3, 4, and 5 days. After the allotted exposure duration, tiles were transferred to fresh artificial seawater (ASW) made to 33 ppt using Tropic Marin® salts and placed on an orbital shaker for 5 min as a wash step. The wash step allowed any deceased and non-adhered *N. incerta* to be removed from the surface so that differences between viable cell densities could be determined.

Cells remaining adhered were photographed using an epifluorescence microscope (Leica DMi8 with Texas Red filter set, 570–600 EX and 604–644 EM). To control for possible uneven coverage, 30 fields of view were taken at randomly chosen locations (Callow and Callow, 2002), three of which were counted manually for comparison to subsequent automated counts. These were made using Image J software by converting images to 16-bit format, altering the threshold to isolate cells from the surface, and counting with a minimum pixel count of 35 and curvature of 0.2. All cell count survival and settlement data were then subjected to generalized linear modeling (GLM) and statistical analysis using R 4.0.0 (R Core Team, 2021).

The method described above was used for the pulsing study, which was conducted after the completion of the initial study but varied with irradiance supplied in periods of 20 min according to the following duty cycles: 10:10, 5:15, 2.50:17.50, 1:19, and 0.50:19.50 (irradiated: no irradiance). Experiments were run for 2 days, based on findings from the diatom settlement study.

To determine thresholds for survival of diatoms, the irradiance was altered by controlling the voltage to the tiles.

Diatom cultures and methodology otherwise followed the same protocol as above. Irradiance was measured using an ILT 950 spectroradiometer (**Supplementary Table 1**). The irradiance of the LEDs at the tile surface was 108 $\mu W/cm^2$ at full power over the UV-B/C range and peaked at 5.77 $\mu W/cm^2$ (**Figure 2**). Based on these findings, voltage experiments were conducted with increments from 18 to 24 V. Settlement and survival of *N. incerta* were measured and statistically compared for all voltage increments. Smooth line plots were generated using a t-based approximation, and GLM, where the normal confidence interval is constructed on the link scale and then back-transformed to the response scale within the ggplot2 package using the geom_smooth function (Wickham, 2016).

RESULTS

Tile Specifications

Tile irradiance was measured, using an ILT 950 spectroradiometer, at the water-tile interface directly over the LEDs and at the center of each tile. The mean irradiance from all tiles was 108.23 $\mu W/cm^2$ with an average peak irradiance of 5.77 $\mu W/cm^2$ at 281 nm, which did not vary significantly between the LEDs ($P > 0.05$), and had a wavelength range from 265 to 300 nm (**Figure 2**). There was some substantial drop off in fluence based on the tile surface location (**Figure 2**). These variations were taken into consideration in the design of experiments.

The irradiances for LEDs used in this study were determined at 18 V (0.07 $\mu W/cm^2$), 19 V (0.88 $\mu W/cm^2$), 20 V (4.76 $\mu W/cm^2$), 21 V (15.32 $\mu W/cm^2$), 22 V (36.04 $\mu W/cm^2$), 23 V (64.34 $\mu W/cm^2$), and 24 V (108.23 $\mu W/cm^2$). These gave fluence values over 48 h of 0.01, 0.15, 0.82, 2.65, 6.23, 11.12, and 18.70 J/cm^2, respectively (**Figure 3**).

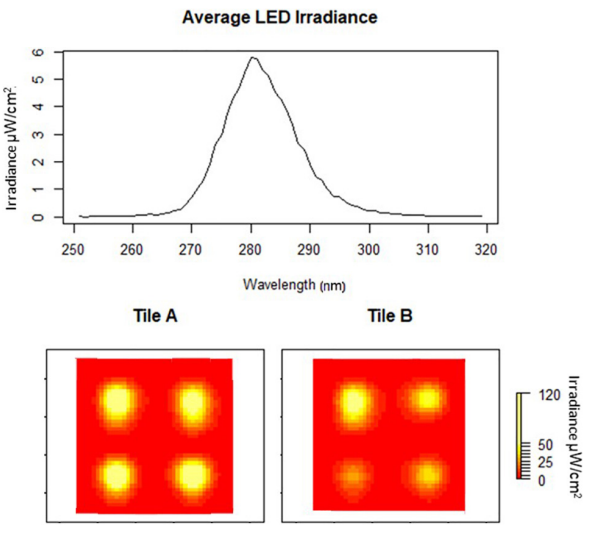

FIGURE 2 | Average peak wavelength of eight LEDs embedded in two tiles at 24 volts **(A,B)**. Heat maps of individual tile irradiances (yellow high and red low).

Duration of Exposure to Irradiance

Exposure durations of 2 h, 1, 2, 3, 4, and 5 days produced fluences of 0.78, 9.35, 18.70, 28.05, 37.40, and 46.75 J/cm^2, respectively (**Supplementary Table 1**). For the controls, of the total 150,000 cells/mL added, ∼1 million (∼209 ± 100 cells/mm^2) were able to settle and adhere within the first 2 h (**Figure 4**). Not all diatoms land on the raphe (which functions in adhesion and motility), and those that do not are able to reposition within 30–90 s for firm initial adhesion (Wetherbee et al., 1998). This indicates that the biofilm would sufficiently adhere within minutes, and that 2 h allowed before counting was adequate as a measure of initial settlement. Any irradiated treatment densities that were below this initial input level would be counted as a removal of cells, while higher levels would be counted as growth. The treatment controls represented the ability of the diatoms to proliferate with no UV treatment and indicated the population growth from the initial cell input. Comparing irradiated to both control and initial numbers enabled inhibition of reproduction and survival to be determined.

Control densities indicated population growth over time with increases of 257, 277, 551, 500, and 425% over the following 1, 2, 3, 4, and 5 days, respectively. Irradiated tiles showed a reduction of 35, 57, 93, 83, 99, and 98%, respectively, compared to initial settlement. Compared to the growth that accrued on the controls, there were, respectively, 35, 87, 98, 98, 99, and 99% fewer cells on the irradiated tiles (**Figure 5**).

Kruskal-Wallis and Wilcoxon pairwise tests were used for statistical analysis as data did not conform to assumptions of normality and equal variance after transformation. All doses resulted in a significant difference between the initial settlement, controls, and irradiated tiles (**Figure 4**).

Effect of Duty Cycling

Interval experiments were run for 48 h in periods of 20 min, allowing 144 duty cycles. Fluences were determined as 9.35, 4.68, 2.34, 0.94, and 0.47 J/cm^2 for each respective duty cycle of 10:10, 5:15, 2.50:17.50, 1:19, and 0.50:19.50 (irradiated: no irradiance).

The control treatments indicated a mean (± SD) diatom settlement of 1,352 ± 450 cells/mm^2, which was 989% higher than the initial cell input. Settlement differed significantly from the respective controls in all irradiance duty cycles $P < 0.05$ (**Figure 6**). Cell densities on irradiated tiles were reduced by 94, 90, 95, 85, and 78% of control tiles for the 10:10, 05:15, 02.50:17.50, 01:19, and 0.50:19.50 (irradiated: no irradiance) duty cycles, respectively. Duty cycles of 10:10, 05:15, and 02.50:17.50 had reductions of 67, 49, and 58%, respectively when irradiated tiles were compared to the initial settlement, whereas 01:19 and 0.50:19.50 had 19 and 24% growth, respectively. The point at which growth was inhibited, and reduction below initial input density observed, was between 0.94 and 2.34 J/cm^2 (**Figure 7**).

Effect of Voltage Changes

Voltage experiments were run to allow varying irradiance (**Figure 7** and **Supplementary Table 1**). All voltages indicated significant differences between control, initial, and irradiated samples (**Figure 8**). Low voltage irradiances resulted in diatom growth of 8, 74, and 285%, respectively, compared to the initial

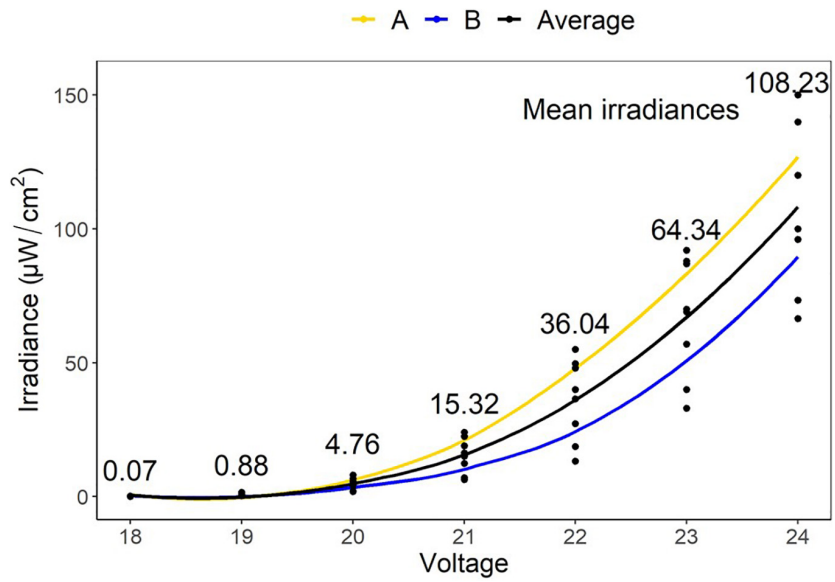

FIGURE 3 | Mean irradiances of eight LEDs embedded in two tiles **(A,B)** at varying voltages (18–24 V). These gave fluence values over 48 h of 0.01, 0.15, 0.82, 2.65, 6.23, 11.12, and 18.70 J/cm^2, respectively.

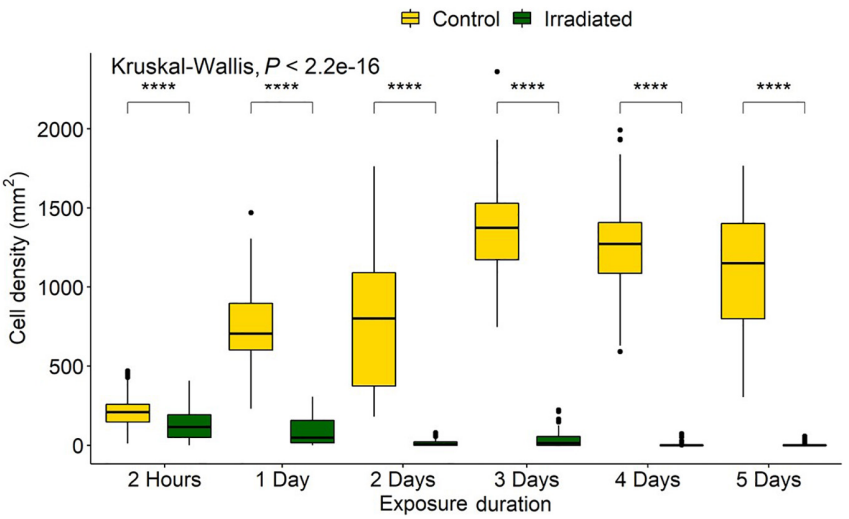

FIGURE 4 | *Navicula incerta* densities after UV irradiance over varying periods. Durations of 2 h, 1, 2, 3, 4, and 5 days produced fluences of 0.78, 9.35, 18.70, 28.05, 37.40, and 46.75 J/cm^2, respectively. Kruskal-Wallis value displayed with Wilcoxon pairwise comparison arranged above groupings (****$P < 0.0001$).

density for 18, 19, and 20 V. Significant reductions of 52, 70, 89, and 92% were also seen in the 21-, 22-, 23-, and 24-V treatments compared to the initial input. All voltages resulted in significantly lower densities (cell counts) than the growth controls, with one-volt increases from 18 to 24 V having 20, 31, 69, 88, 92, 97.5, and 98% lower densities, respectively. The intersect where the irradiated cell densities overlapped with the initial cell densities was between 20 and 21 V. This was between 0.82 and 2.65 J/cm^2 and represents a threshold of fluence to halt growth and, above this level, to initiate removal (**Figure 9**).

Comparisons at High and Low Dosages

Fluence is reported to have the same biological effect regardless of the method of delivery (Bunsen and Roscoe, 1863). Therefore, the three methods discussed previously should have resulted in reproducible reductions in diatom cell counts, if doses were the same (Sommer et al., 1996, 1998). High and low fluence groups were identified based on doses across the three experimental procedures. A low fluence of 0.78–0.94 J/cm^2 included the 2 h, 20 V, and 1:19 duty cycle samples, and a high fluence of 9.35–11.12 J/cm^2 included the 1 day, 23 V, and 10:10 duty cycle samples.

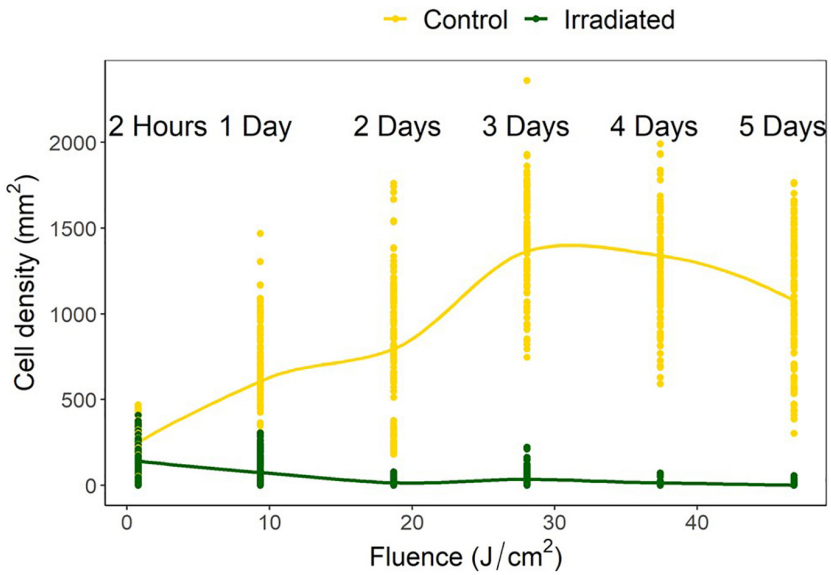

FIGURE 5 | *Navicula incerta* abundance after UV irradiance over varying periods with respective fluences. Durations of 2 h, 1, 2, 3, 4, and 5 days produced fluences of 0.78, 9.35, 18.70, 28.05, 37.40, and 46.75 J/cm^2, respectively.

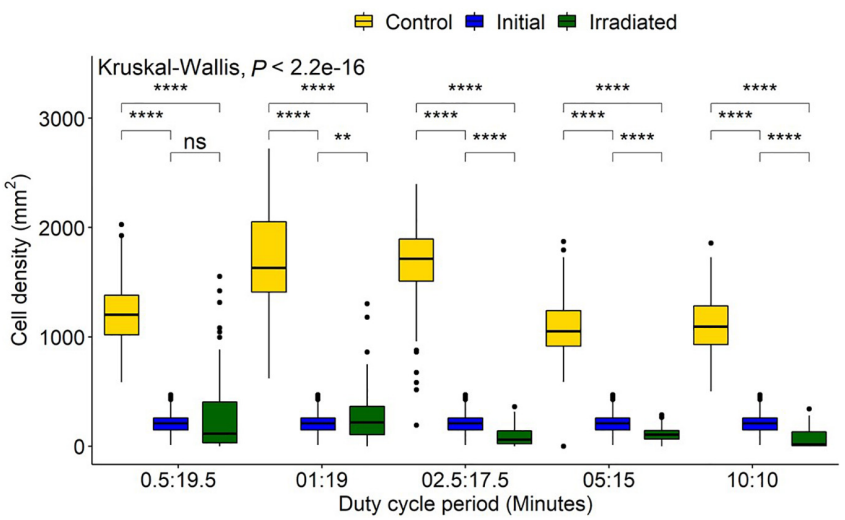

FIGURE 6 | *Navicula incerta* abundance after UV irradiance over varying duty cycles. Duty cycling periods display minutes in which the UV light was turned on to minutes it was turned off (On:Off). Fluences were determined as 0.47, 0.94, 2.34, 4.68, and 9.35 J/cm^2 for each respective duty cycle 0.50:19.50, 1:19, 2.50:17.50, 5:15, and 10:10 (irradiated: no irradiance). Significant differences indicated *via* Wilcoxon pairwise comparison above groupings (ns, no significance, $^{**}P < 0.01$, $^{****}P < 0.0001$).

There were no significant differences in cell densities between the 20 V and the 1:19 duty cycle method, however, the 2-h exposure was significantly more effective ($P < 0.05$). High fluence samples indicated that all treatment methods were significantly different from each other ($P < 0.05$), with 23 V having the highest removal and 1 day having the lowest removal (**Figure 10**).

Determination of Thresholds

The dose required to produce a 1 log reduction (lethal dose to achieve 90% cell reduction—LD_{90}) below initial densities was determined *via* GLM using predictive analysis based on the count data. Doses were compared separately by experiment and comprehensively by removing all control data, other than the initial settlement, and comparing irradiated data against the initial data. Only data that were below the initial density level were included as densities above this indicated growth and would produce predictions based on an inflated density. The duration experiment indicated that a fluence of 42 J/cm^2, with a range from 35 to 52 J/cm^2, would be required for a 90% reduction in diatom cell density (**Figure 11**). The pulsing data revealed

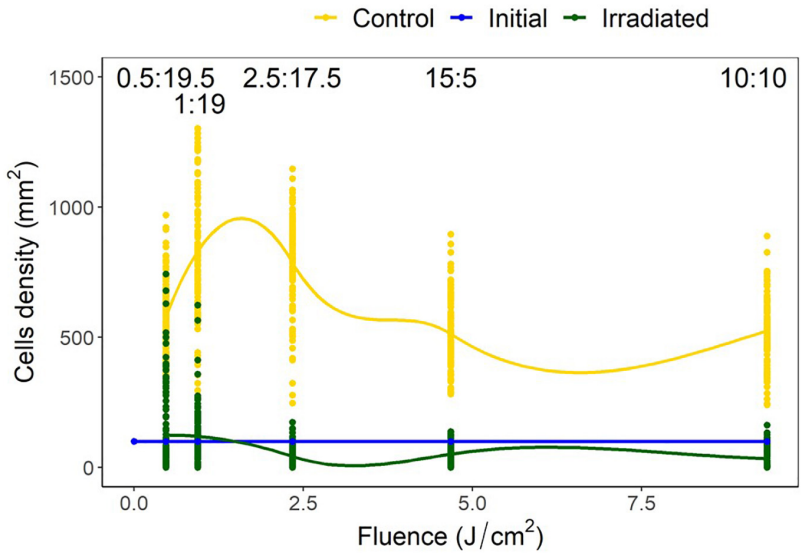

FIGURE 7 | *Navicula incerta* density and range after UV irradiance over varying duty cycles with respective irradiance dosages. Duty cycling periods display minutes in which the UV light was turned on to minutes it was turned off (On:Off). Fluence dosages were determined as 0.47, 0.94, 2.34, 4.68, and 9.35 J/cm^2 for each respective duty cycle 0.50:19.50, 1:19, 2.50:17.50, 5:15, and 10:10 (irradiated: no irradiance). Irradiated and initial intercept point (between 0.94 and 2.34 J/cm^2) indicates the fluence required to reduce density below initial levels.

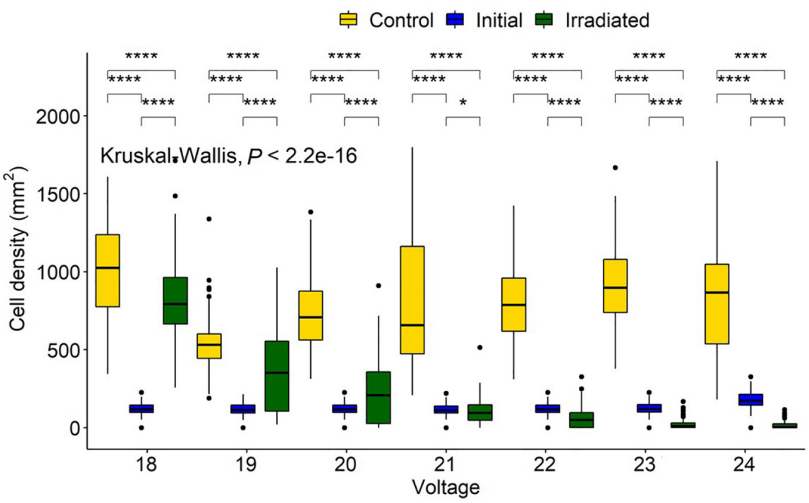

FIGURE 8 | *Navicula incerta* abundance and range after UV irradiance over varying voltages. Kruskal-Wallis value displayed with Wilcoxon pairwise comparison ranged above groupings (*$P < 0.05$, ****$P < 0.0001$).

increased cell densities in 0.5:19.5 and 01:19 duty cycles and these were thus removed from GLM. Modeling indicated that a fluence of 20 J/cm^2, with a range from 12 to 65 J/cm^2, would be required for 90% diatom removal. The voltage experiment indicated that cell numbers increased in the 18–20 V treatments, which were, therefore, not included in the predictive analysis. The LD_{90} of the voltage experiments was a fluence of 21 J/cm^2 with a range of 17–28 J/cm^2. The comprehensive LD_{90} was predicted to be 25 J/cm^2 with a range from 19 to 36 J/cm^2. All four data sets produced different LD_{90}s and were indicative of variation between irradiance delivery method and effect. The LD_{90} was predicted to be between 20 and 42 J/cm^2, with an overall range of 12–65 J/cm^2.

DISCUSSION

UV treatment is an effective form of sterilization used across diverse industries (Shaban et al., 1997; Tree et al., 1997; Bintsis et al., 2000; Shaw et al., 2008; Winward et al., 2008). The present results indicate the potential of UV treatment for control of biofouling of surfaces within the marine environment. In

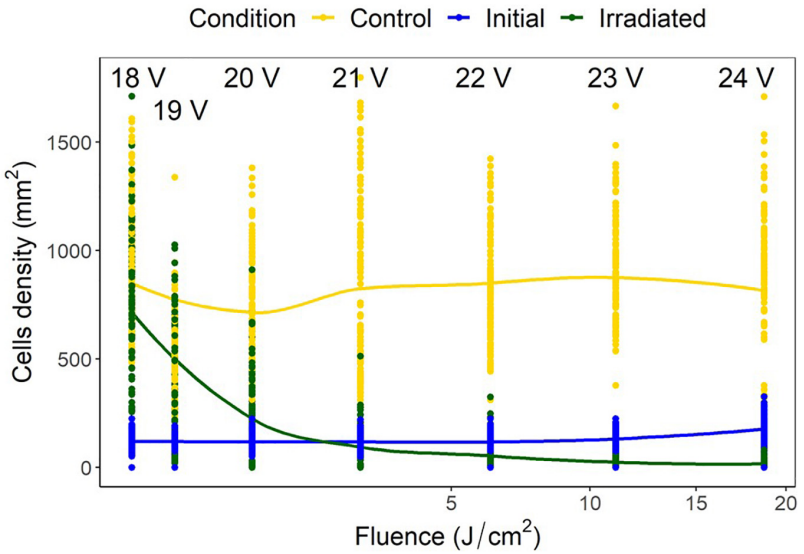

FIGURE 9 | *Navicula incerta* cell densities and ranges at different voltages, with the corresponding fluence on the *x* axis. Predictive generalized linear modeling indicated the intercept between irradiated density and initial density to be between 20 and 21 volts (0.82–2.65 J/cm^2).

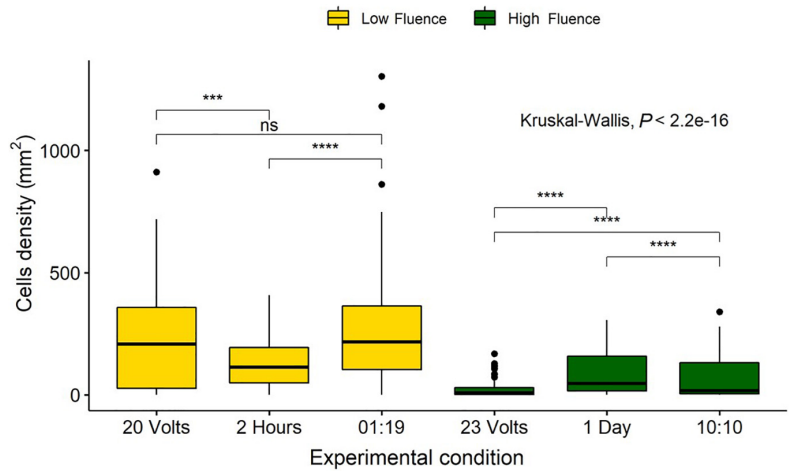

FIGURE 10 | Comparison of *Navicula incerta* cell densities remaining on the surface after low and high irradiance exposures. Low fluence grouped based on fluence of 0.82, 0.78, and 0.94 J/cm^2 for 20 volts, 2 h, and a 01:19 duty cycle, respectively. High fluence grouped based on fluence of 11.12, 9.35, and 9.35 J/cm^2 for 23 volts, 1 day, and a 10:10 duty cycle, respectively. Kruskal-Wallis value displayed with Wilcoxon pairwise comparison arranged above groupings (ns, not significant, ***$P < 0.001$, ****$P < 0.0001$).

the experiments conducted in our study, all diatom (*Navicula incerta*) samples, even those exposed to low fluences (0.01, 0.47, and 0.78 J/cm^2), demonstrated a significant reduction in densities when compared to control counts. The suppression of growth at the lowest fluence (<0.78 J/cm^2), combined with reduced cell densities above this level, highlights the efficacy of UV sterilization for biofouling control.

Effect of Duration of Irradiance

This study determined that diatom densities, compared to initial settlement, were impacted in all UV treatment durations. Cell densities in the controls (non-irradiated) increased above the initial settlement (after 2 h) establishing the ability of *N. incerta* to grow within the experimental conditions. Cell densities above the initial input, but below the experimental control cell densities, suggested a negative impact of UV treatment on cell proliferation, but biofilm growth was not entirely prevented. Cell densities, which were similar to initial densities after irradiance, implied a stop to cell division and an equilibrium between the repair mechanisms and the exposure dosage. Finally, when cell counts were lower than the initial cell count, following irradiance at fluences from 0.78 to 46.75 J/cm^2, the implication is that cell division stopped, and repair mechanisms could not keep pace with the damage caused by UV exposure. By

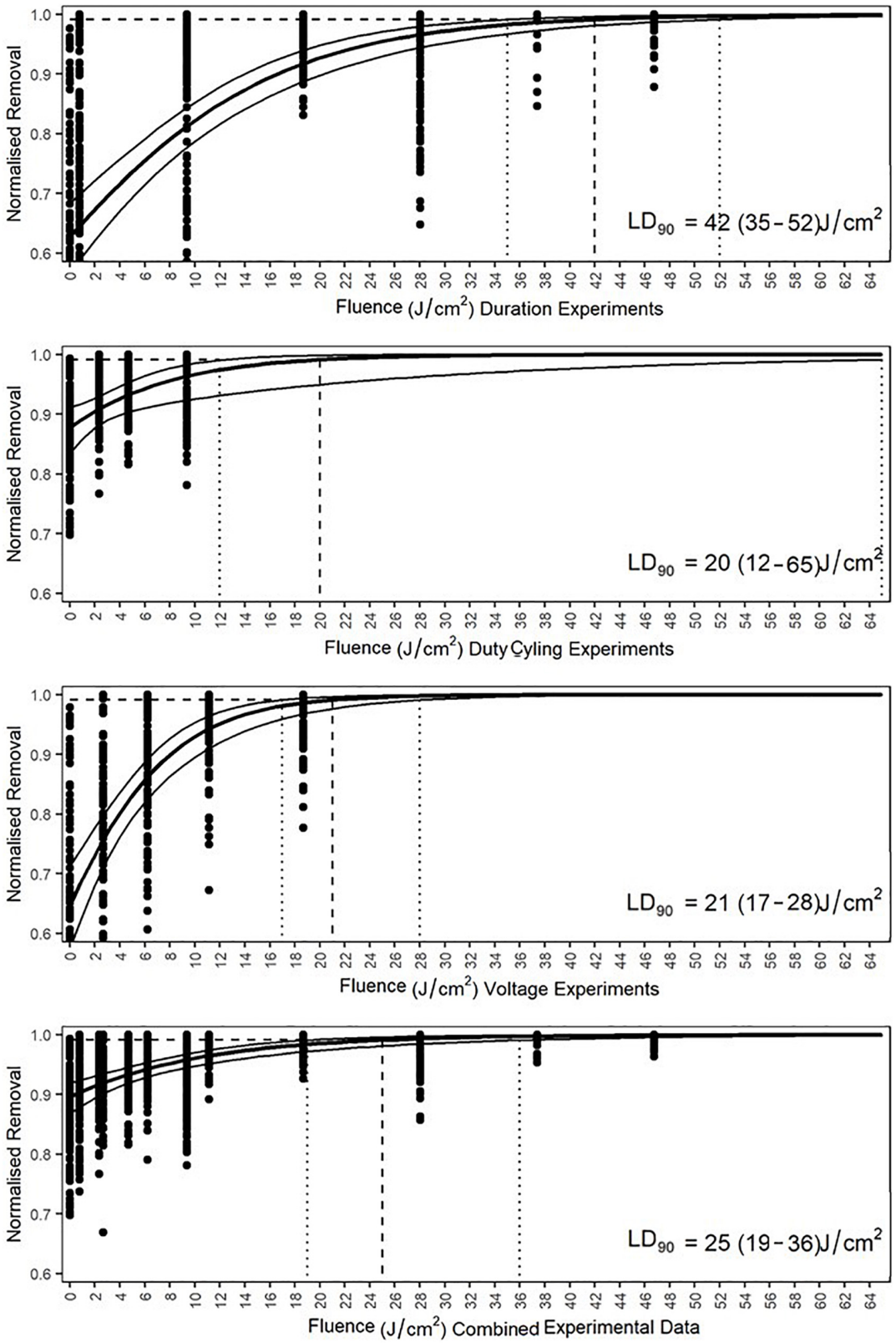

FIGURE 11 | Generalized linear model predicted lethal fluences, expressed as LD_{90}, of each experiment and the combined data to obtain a 90% reduction in cell density of *Navicula incerta* below the initial cell density. Dashed lines intercept at the LD_{90}. The 95% confidence interval ranges are indicated by the vertical dotted lines with the values in parentheses.

whatever mechanism, death and/or detachment, cells were lost from the surface.

The lowest fluences produced a reduction in cell densities, although a 1 log reduction compared to the initial settlement was only observed after 2 days of irradiance, equivalent to 18.70 J/cm^2 (**Figure 5**). After 4 days of irradiance, cell density was nearly 3 log lower than the control, suggesting that continuous irradiance was extremely effective, even at low fluence. This implied that fluence sufficient to negate growth and induce removal (i.e., above 18.7 J/cm^2), could be achieved to meet performance specifications required for antifouling approval by MIL-PRF-24647C and the European Chemical Agency (European Chemicals Agency, 2014; ECHA/EFSA, 2017). However, the methods required by the European Chemical Agency and MIL-PRF-24647C were mainly developed with chemical biocides in mind. There is currently no EU regulation under which an UV-based antifouling method could be appropriately certified (ECHA/EFSA, 2017).

Effect of Voltage

Voltage supply directly affected the irradiance of the LEDs with changes of a single voltage unit causing a reduction in irradiance (**Figure 3**). This had a substantial effect on the diatoms (**Figure 9**). Growth at the lowest voltages (18–20 V) was influenced by only small changes in voltage. Higher voltage supply (21–24 V) reduced cell density below the initial count, indicating a potential effective level for prevention of biofilm growth. This threshold is important for the optimized design of UV-B/C LED systems for biofouling control, though additional target fouling species need to be tested. An effective antifouling level (the required irradiance for preventing the growth of organisms on a surface) has been predicted to be 1 μW/cm^2 for UV-based antifouling systems (Piola et al., 2016). The fluence supplied to the tile surface in the current study had an average peak value which was 5.77 times higher than the predicted value at the recommended operating voltage (24 V), and a total UV-B/C fluence that was 109 times greater. Based on spectroradiometer readings, 19 V (**Figure 3**) supplied the closest irradiance for comparison to the reported level. Piola et al. (2016) stated that the level could be even lower than 1 μW/cm^2 and, for the current study, the irradiance threshold for *N. incerta* was validated as below 0.7 μW/cm^2. Although, as indicated by the intercept point in **Figure 9**, to achieve a reduction below initial cell density, a fluence between 4.76–15.32 J/cm^2 (between 20 and 21 V) would be required. Determining whether this can translate into the natural environment is imperative, as cultured organisms are known to be less resilient than their wild-type counterparts (Falciatore et al., 2020). Dissolved organic matter within the water can also impede UV treatment efficacy (Georgiades et al., 2021). Moreover, diatoms are just one component of a complex biofouling community. The extracellular polymeric substances secreted by diatoms, and the silicate structures left behind by dead cells, could attract and protect further colonizers. Wild-type resilience and population interactions may cause the effective level to differ from that currently indicated.

Effect of Duty Cycling

Developing UV as an antifouling strategy will require the method to meet some design criteria, one of which is prolonged efficacy with minimal maintenance. The manufacturers' specifications for the half-life (irradiance output) of the LEDs used in this study was 10,000 h (1.14 years), at constant irradiance. Visible light LEDs, on the other hand, can exceed 100,000 h of use and UV LED life expectancy will likely improve as technology develops (Muramoto et al., 2014). Several years of fouling protection under various conditions will be required to make the integration of UV LEDs appealing to the shipping industry.

Duty cycles were compared to explore lengthening the longevity of the LEDs. Outcomes demonstrated that even the shortest duty cycle of 2.5% could inhibit biofilm growth. Control tiles of the 02.50:17.50 and 01:19 duty cycles had higher diatom densities than the other duty cycle controls ($P < 0.05$). This apparent anomaly may be explained by the use of a new stock of F/2 growth medium, or small variations in cell density at the start of the experiment. This higher density may have allowed some variation within the irradiated samples. Since the same stock was used for both control and irradiated exposures, however, the differences may be compared.

A 12.5% duty cycle would be required to prevent growth and effectively reduce fouling. At the lower 2.5% cycle, longevity of the LEDs could be increased to 45.6 years, whilst at 12.5% cycling, 9.2 years could be achieved. Duty cycling of 0.01% (1 min/week) has been reported by Richard et al. (2021) to have a significant impact, however, for full macrofouling prevention, a duty cycle of 0.69% (10 min per day) was required. Rates as low as these could see effective antifouling *via* UV lasting the entirety of a vessel's lifespan. Differences in the effectiveness of the duty cycling between this study and the study by Richard et al. (2021) could be due to the different light sources used. Richard et al. (2021) used a 25 W Aqua UVC (254 nm) lamp rather than LEDs. This provided different irradiances than the current study and recorded an intensity of 1.31 μW/cm^2 at a peak wavelength of 254 nm rather than the 281 nm used herein. As biological impact is dose-dependent, a threshold would be advantageous in isolating specific duty cycles from individual light supplies.

Comparisons at High and Low Doses

Variation in dose and biological impact were comparable between the three methods of irradiance delivery used in the current study and provided effects of low and high fluence on cell density. Low fluence experiments included the 2-h experiment, which resulted in lower cell densities than the other methods. The lower cell density in the 2-h treatment, as opposed to 20 V and the 01:19 duty cycle, could be due to the fluence being below an effective inactivation level. This would enable the diatoms to proliferate during the 48-h experiment. According to the Bunsen-Roscoe reciprocity law (Bunsen and Roscoe, 1863), the total photochemical dose absorbed by an organism will have the same effect regardless of varying irradiance and/or exposure durations. Supporting evidence was discussed by Sommer et al. (1998) who found that a microbiocidal dose of 400 J/m^2 was required for a 4-log bacterial reduction regardless of delivery

intensity. Sommer et al. (1996) indicated that a low intensity and longer exposure duration was more effective at inactivation than short high irradiance on eukaryotic yeast cells. *Escherichia coli* and bacteriophages have displayed the opposite, reducing more over shorter durations at higher intensities (Sommer et al., 1998; Werschkun et al., 2012). UV damage is not described as being time dependent and is associated with the overall dose. However, diatoms require time to complete biological processes such as division and growth. Therefore, the biological processes may have counteracted some of the damage and be accountable for the differences. The densities may have been relatable if the 2-h experiment had the UV turned off and the diatoms left to grow for a further 46 h.

The longer experimental duration could explain why the high exposure 23 V experiment, in high fluence samples, had a lower cell density than the 1 day and duty cycle doses. If the irradiance is above an effective threshold for a longer time, then there would be more time for the population to reduce in number. This could explain why the 23 V method was significantly more effective than its similar dosage counterparts (**Figure 10**). The variance between sample counts in the duty cycle and the 1-day method was large, whereas the 23 V experiment had minor variance. This may indicate greater resilience during the pulsed method and could also result from repair and recovery during LED downtime. With regard to the 1-day experiment, the shorter duration may have different effects on diatoms of different sizes, as described by van de Waal and Litchman (2020). This could explain the variability within the counts and the deviation from the full exposure. The current results (**Figure 10**) contradict the Bunsen-Roscoe reciprocity law as similar irradiances produced significantly different effects. For *N. incerta* short, high irradiance treatments were more effective at inactivation than longer, lower intensity treatments which may indicate that biological factors can alter the overall outcome from a prolonged exposure.

It is important to consider that both high and low fluence had some variability and that the exact dose was not the same in all cases. The high doses, from the comprehensive analysis, had identical duty cycle and 1-day doses (9.35 J/cm^2), however, the 23 V dose was 1.77 J/cm^2 higher than these and could explain the lower cell density present within that treatment. Additionally, the low fluences were conducted with no two identical measures delivered, although they only varied by 0.16 J/cm^2 from highest to lowest. To clarify a fluence effect without variation, a repeat of the experiment with exact dose comparisons of the three different treatment methods would be necessary to determine if the small irradiance discrepancy was important.

The data in the current study suggest that the Bunsen-Roscoe law does not allow for interacting biological factors. Diatoms can self-repair photoactive lesions *via* excision repair mechanisms using photosynthetic light (De Tommasi et al., 2018). This enables them to excise the lesions from the DNA and combat the overall damage. A high intensity could overwhelm an organisms' repair mechanisms and a heavily outweighed system would be expected to inactivate quicker than a neutrally balanced or minimally overcome system. Additionally, if there was no interacting repair mechanism, the organisms would still be limited due to their quantum yield (Braslavsky, 2007). Organisms can only absorb a certain number of photons in a set period before reaching their maximum quantum yield, after which photons that the organism is exposed to become surplus (Li et al., 2006, 2010). Like enzyme reactions, there would be a limit to the reactions that could take place at any given time.

Threshold Analysis

The interaction between growth and density provides insight into a potential threshold of effect. To prevent growth, but not reduce below initial density, the threshold was determined to be between 0.01 and 0.78 J/cm^2. For densities to be reduced below the initial input levels, a threshold point between 0.78 and 2.65 J/cm^2 would be required. This is similar to the level reported by Piola et al. (2016). Using the densities and fluences from all three experiments and a combination of all the data, LD_{90}s were calculated, which suggest a predicted dose to reduce the initial input densities by 90% (Raikow et al., 2007). According to the Bunsen-Roscoe reciprocity law LD_{90} fluences should be the same regardless of methodology, however these were observed to vary in the current study. Cell density counts from pulsing (**Supplementary Table 1**) indicated that supplied irradiance doses did not reduce initial levels below 90%, but duration and voltage methodologies both indicated that 18.7 J/cm^2 produced over 90% removal. The predicted LD_{90} for guaranteed effect ranged from 12 to 65 J/cm^2 depending on delivery method, and 25 J/cm^2 when averaged across all of the data. Experiments with diatoms were conducted using a high nutrient growth medium (F/2), these nutrient concentrations are not expected to be encountered in the natural environment. Biofilm growth would therefore be slower than observed in laboratory conditions that are optimized for growth. Accordingly, a lower dose may be required for wild type removal. However, as both controls and irradiated experiments used the same medium, the results within the study are comparable. An adaption for more accurate thresholds would require a comparison between this study and field experiments. The predicted threshold for 90% removal, based on all data, was 25 J/cm^2. However, this is likely to be a conservative estimate when the fluences and their respective cell densities are compared. Duration and voltage treatments had $>$ 88% removal at 18.7 and 11.12 J/cm^2, respectively, which is considerably lower than the predicted value. The latter could have been impacted by the variability in densities and the pulsing not achieving 90% removal, which could explain why the threshold value was elevated. Adhering to an elevated fluence, similar to the conservative prediction, should result in effective antifouling prevention and removal but other factors that affect UV efficacy, not currently investigated, would need to be considered.

CONCLUSION

The effectiveness of UV-B/C for antifouling has been confirmed using a technology (LEDs) that could plausibly be incorporated using a cladding system. The effective levels determined by duty cycling could allow longer lifespans of LED equipment, and longer intervals between repair or replacement. Lower voltages of delivery would lower LED strain and could also

prolong longevity. By determining effective thresholds and doses, equipment can be developed to efficiently meet standards and protocols for the approval of UV B/C integration as an antifouling system. Exploration of ecosystem thresholds, under a range of field conditions (including non-target effects), is required to fine-tune this threshold level.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: Newcastle University Data Repository; doi: 10.25405/data.ncl.16811044.

AUTHOR CONTRIBUTIONS

PW was the primary researcher undertaking method development, experimentation, statistical analysis, figure production, and document scripting. KR was the technical lead aiding in method development and equipment production. AC and NA were in supervisory roles aiding in scientific theory and manuscript development. PD and JP aided in manuscript editing and technical partnering which drove the projects progress. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2021.809011/full#supplementary-material

Supplementary Table 1 | Experimental exposures, cell densities, and percentage differences.

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Proactive In-Water Ship Hull Grooming as a Method to Reduce the Environmental Footprint of Ships

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The application of a proactive grooming program to manage the fouling control coatings applied to ship hulls provides an opportunity to address the climate crisis, invasive species and the discharge of biocides into the marine environment. A large percentage of the total power required to propel a ship is to overcome the viscous drag created between the hull and the water. The powering penalty due to increases in coating roughness and the development of biofouling are well documented. In addition, poorly maintained fouling control coatings may lead to the transportation of invasive species. In-water hull cleaning is therefore an important part of ship operations; however, this is typically implemented as a reactive measure when fouling reaches a critical level and requires powerful machinery which damages the coatings, creates unwanted discharge and in many locations the discharge will require capture and disposal. Ship hull grooming is being developed as a proactive method to manage fouling control coatings that will ensure that they are maintained in a smooth and fouling free condition, there is no transport of invasive species or excessive discharge of material that occurs during cleaning. This manuscript will summarize the findings of many years of research and development.

Keywords: biofouling, fouling control coatings, grooming, ships, green house gas emissions, biocides, invasive species

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INTRODUCTION

The shipping industry is vital to trade, defense, and the world economy; however, it is under increasing pressure to reduce its environmental footprint in terms of CO_2 emissions, as a point source for biocides used for the control of biofouling, and the transportation of invasive species (International Maritime Organization, IMO). Our findings from several years of research investigating the proactive underwater maintenance of fouling control coatings (grooming), has demonstrated that such an approach offers the potential to ensure that the major areas of a ship hull can be kept in a smooth and fouling free condition for the lifetime of the coating. This in turn will reduce the environmental footprint of a ship and costs in terms of fuel and wear on machinery. There are many factors that determine how and when a ship hull is cleaned (**Figure 1**). These include:

- Vessel Specifics: the type of vessel, its schedule, utilization and speed
- Fouling Control Coatings: the type, condition and age of fouling control coating
- Environmental Conditions: physical and chemical properties of seawater and ecology.

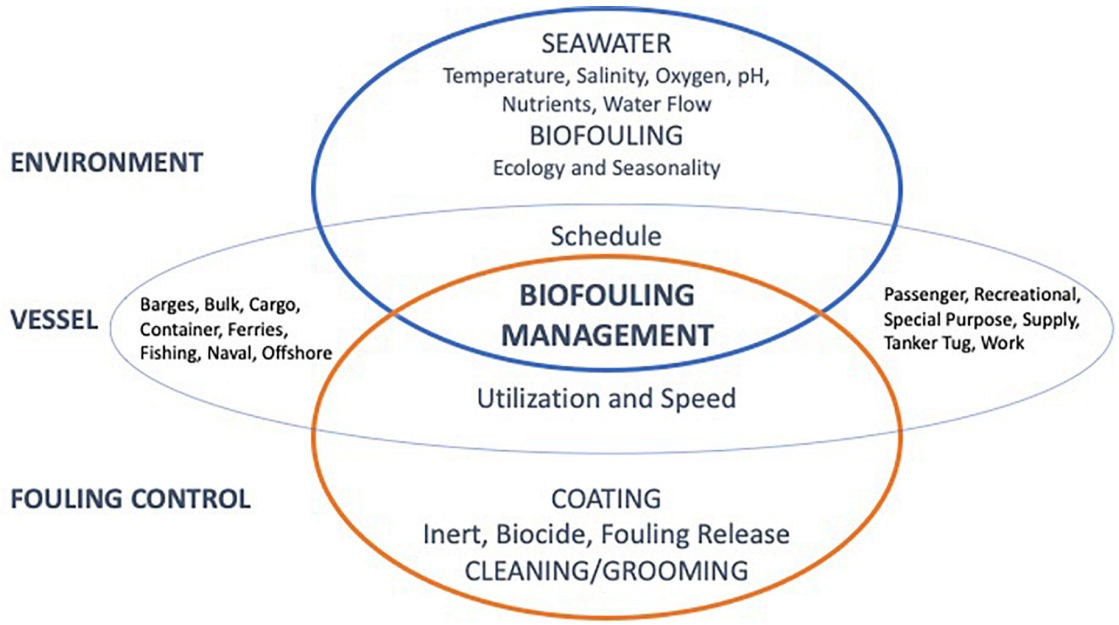

FIGURE 1 | Factors influencing the management of biofouling on ships.

In-water hull cleaning is routinely performed on ships in ports and harbors around the world, however, up until recently this has only been done using powerful devices as a reactive measure once fouling has reached significant levels (US Navy, 2006; United States Environmental Protection Agency [US EPA], 2011; McClay et al., 2015; Morrisey and Woods, 2015; Zabin et al., 2016; Song and Cui, 2020). This results in excessive discharges of paint and biofouling to the environment which may then require capture, treatment and disposal. It also damages the coating (Bohlander, 2009; Earley et al., 2014; Scianni and Georgiages, 2019; Oliveira and Granhag, 2020; Tamburri et al., 2020; BIMCO/ICS, 2021; Jones, 2021; Scianni et al., 2021). Ship hull grooming has been defined as "the gentle, habitual and frequent mechanical maintenance of submerged ships' hulls in order that they remain free from extraneous matter such as fouling organisms and particulate debris, with minimal impact to the coating" (Tribou and Swain, 2010). The purpose of this paper is to review our findings and to place them in context with the requirements to reduce environmental impacts and improve the operational efficiency of ships.

Fouling control coatings are known to foul when a ship is subjected to prolonged periods of inactivity. In 2003 SeaRobotics submitted a proposal to the Office of Naval Research "The HullBUG, A miniature Underwater Vehicle for Cleaning Ship Hulls" (Holappa et al., 2013). In 2005, the Office of Naval Research funded a ship hull grooming program. The concept was to develop fully autonomous vehicles that would proactively maintain fouling control coatings free of fouling (**Figure 2**).

Early research on small sized test panels demonstrated that proactive in water grooming may provide an effective method to prevent fouling (Tribou and Swain, 2010). This led to the construction of a large-scale test facility at Port Canaveral, Florida to develop and demonstrate the technology. It also found that the requirements for the grooming method were different for the biocide and biocide free systems (**Figure 3**). The performance of biocide-based systems is enhanced by a grooming method that maintains the active ingredient at a level that prevents fouling without causing excessive discharge into the environment (Swain and Tribou, 2014; Tribou and Swain, 2017). The performance of the fouling release coatings is enhanced by a grooming method

- Proactive method to maintain coatings as smooth and fouling free over the open expanses of the hull surface – combat ready.
- Applied by small inexpensive fully autonomous vehicles.
- Acts synergistically with hull coatings:
  - removes silt, organics and incipient fouling
  - maintains coating function
  - does not degrade the coating
  - develop coatings that are designed to be groomed.
- Does not require capture and disposal:
  - No risk of invasive species
  - No risk from biocide free coatings
  - No increase in output of active ingredients.
- Incorporated as a part of ship operations
- Frequency to match biofouling pressure and ship's operational schedule
- Removes divers from the water
- Extended time between dry docking (8-12 years)

FIGURE 2 | Concepts for the development of a fully autonomous ship hull grooming vehicle (Swain et al., 2020).

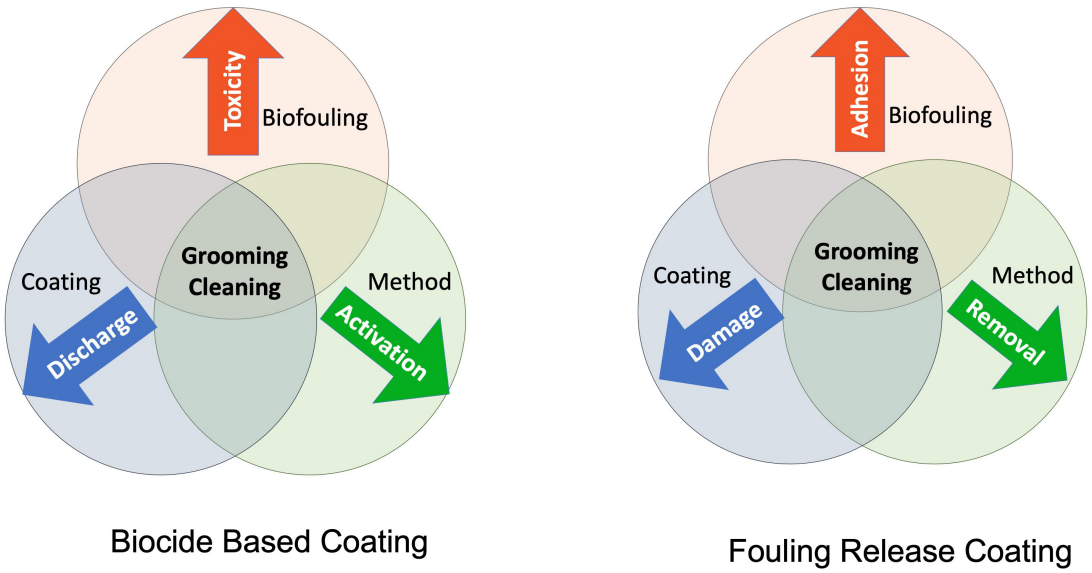

FIGURE 3 | Grooming method requirements for biocide based and fouling release coatings.

that provides sufficient force to remove the fouling without damaging the surface.

BACKGROUND

This research focused on the coating types and operational schedule of US Navy ships. About 96% of the US Navy ships are coated with copper ablative antifouling (AF) paint and the rest with copper free coatings including some fouling release systems. The majority of ships spend 40–60% of their time pier-side and this makes them vulnerable to fouling (Martin and Ingle, 2012). According to Chapter 081 of the Naval Ships' Technical Manual (**Supplementary Table 1**) a full hull clean for ablative and self-polishing paints will be required when a fouling rating of FR-40 or greater exists over 20 percent of the hull, exclusive of docking block areas and appendages. The fouling release coatings are treated differently and when a fouling rating of FR-50 or greater is observed over 10 percent of a hull NAVSEA Code 00C are contacted for cleaning advice. The challenges of waiting until the ship becomes covered by calcareous fouling before cleaning are that the ship is already operating with a drag penalty, that cleaning will require fairly high forces that may damage the coating and that the calcareous shells may become entrapped by the cleaning device causing further damage. Schultz et al. (2011) concluded that savings as high as $12 million/ship over a 15-year period could be achieved for the US Navy fleet of DDG-51 destroyers if the hull condition was maintained at a fouling rating of 10. Our research has demonstrated that regular grooming of BRA640 and IS1100 is able to maintain these coatings at a Navy Ship Technical Manual fouling rating of 0.

The findings from this study may also be used to help better manage the fouling control coatings of commercial shipping. Seaborne trade and the number of ships that are operating in the marine environment has increased dramatically in the last 50 years. According to the United Nations Conference on Trade and Development (2020) e-Handbook of Statistics the international seaborne trade grew from 2,605 million tons loaded in 1970 to 11,083 million tons in 2019. In 2019 there were 52,961 commercial ships with flags of registration of 1,000 GT and above. The environmental impacts from this number of vessels and the associated ports and harbors are enormous and the industry is now facing increased regulations to reduce harm to the environment. One option to lessen these impacts is by the improved selection and management of fouling control coatings (Swain, 2017). The condition and treatment of these surfaces have a significant impact on the power required to move a vessel (CO_2 emissions), the release of active ingredients to control fouling (biocides) and the transfer of marine organisms to new locations (invasive species).

LARGE SCALE TEST FACILITY

A large-scale seawater test facility was constructed in 2012 at Port Canaveral to evaluate the technology, provide a scientific understanding of the grooming process and to enable the development of grooming tools (**Figure 4**). The Port is subtropical and has year-round biofouling (**Figure 5**). The test surfaces were constructed from three 2.4 m × 4.57 m × 6.35 mm thick steel plates that were welded to 0.76 m diameter steel pipe for floatation. These were bolted together to form a continuous length of 13.7 m. The structures were coated with Intergard 264 epoxy anticorrosive paint and a topcoat of either Interspeed BRA 640 (BRA640) copper polishing or Intersleek 1100SR (IS1100) fouling release coating from Akzo Nobel. The steel pipes provided floatation and the panels were suspended vertically representing the vertical sides of a ship. A 10.4 m Mainship trawler acted

FIGURE 4 | Large scale test facility, Port Canaveral, Florida.

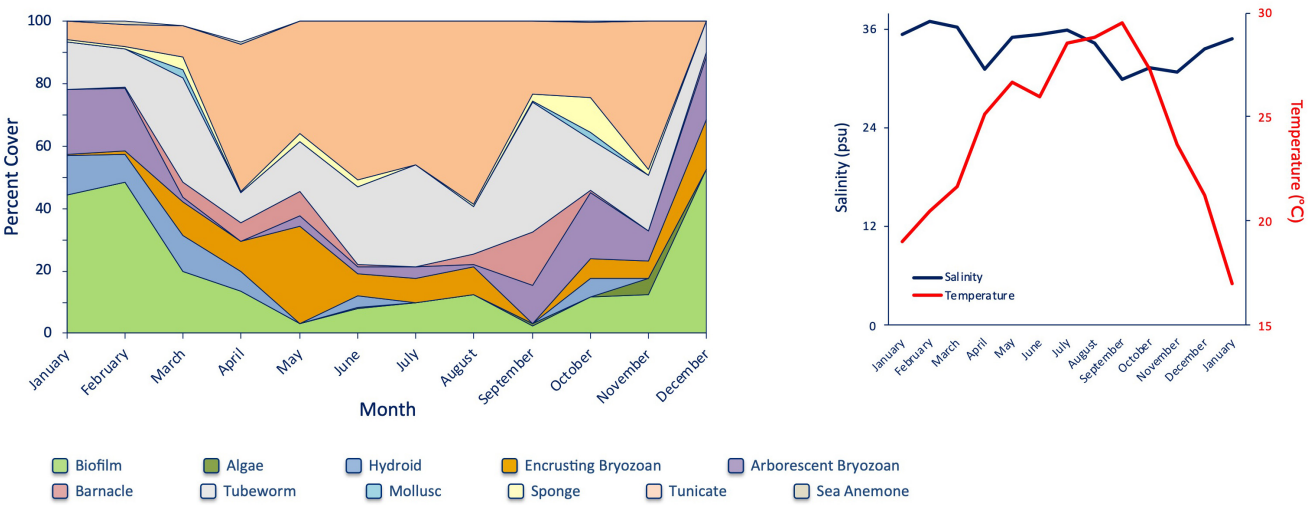

FIGURE 5 | Biofouling, temperature and salinity conditions at the large scale test site.

as a control center for the grooming vehicle and was moored adjacent to the pipe. The fouling control surfaces were groomed on a weekly basis and ungroomed areas acted as controls. The panels were inspected by divers and periodically, the panels were rotated to the horizontal position to enable visual inspection, dry film thickness and roughness measurements.

GROOMING METHOD

There are several different categories of cleaning devices available to remove biofouling from underwater surfaces (Akinfiev et al., 2007; Curran et al., 2016; Song and Cui, 2020). The concept for grooming required the design and fabrication of specialized

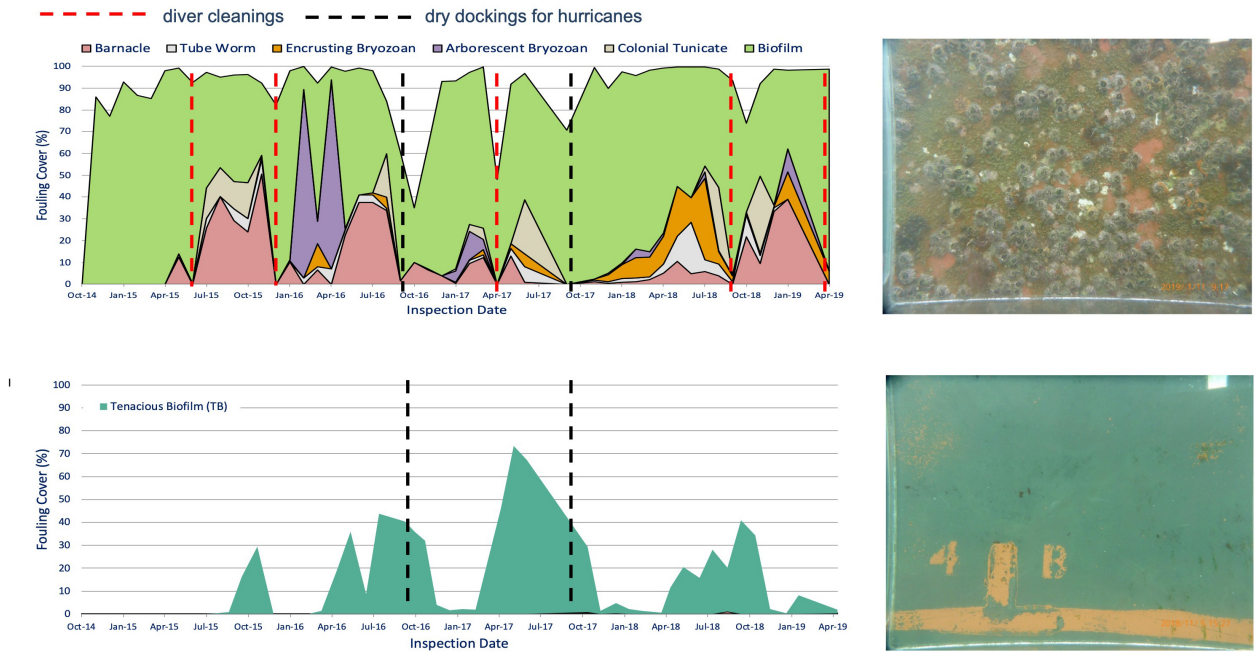

FIGURE 6 | Biofouling progression on ungroomed and groomed BRA 640 during a 54 months deployment.

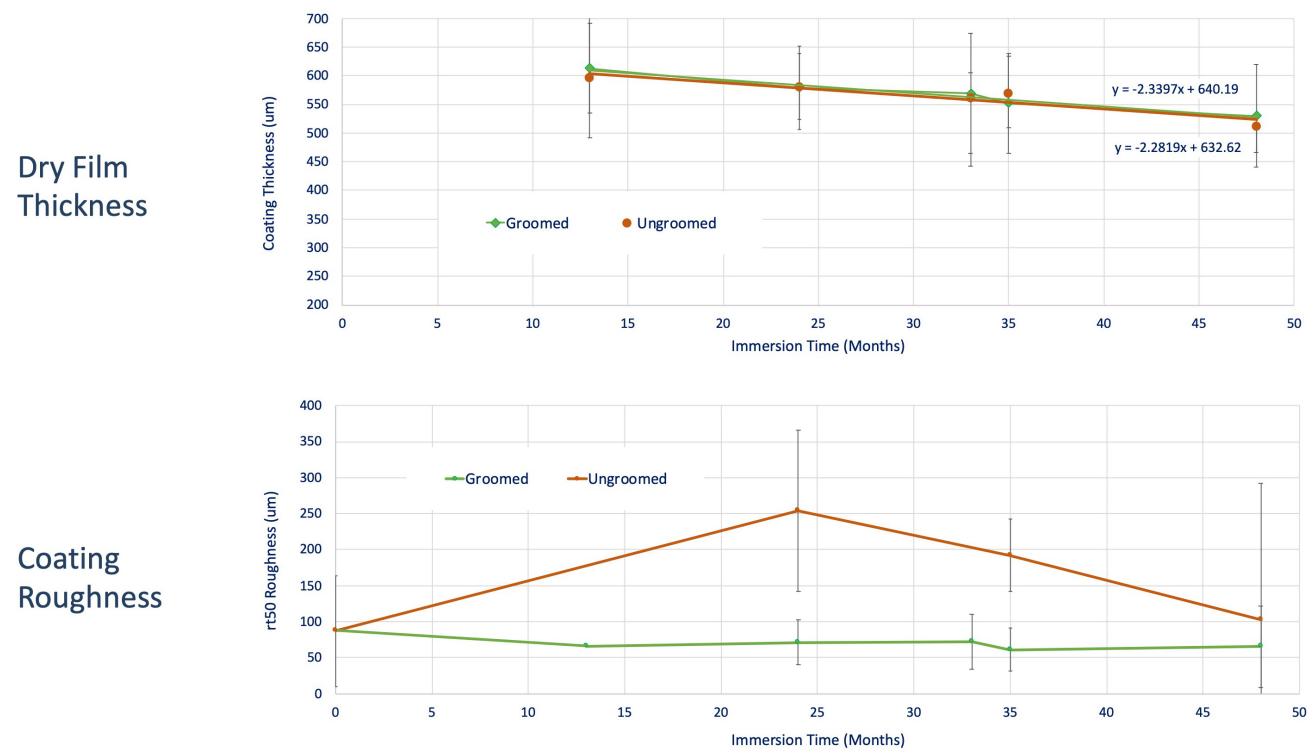

FIGURE 7 | Change in dry film thickness and coating roughness for BRA640 during a 54 months deployment.

tools that were engineered to apply the minimum force required to remove incipient fouling and biofilms without damaging the surface. Because the concept is to eventually develop fully autonomous vehicles the grooming tool was also designed to minimize power demands. Prior research guided our choice to favor small diameter (about 102 mm) vertically rotating

brushes rotating at between 300 to 600 rpm (Wathen, 1994; Schumacher, 1996; Harper, 2014). The brush rotation creates a low pressure in the entrapped water which causes suction forces and holds the brush to the surface. The vertical forces applied to the coating are controlled by the arrangement of the brush elements and the speed of rotation (Harper, 2014; Tribou, 2015; Erdogan, 2016). The variables included in grooming tool design are many and include: brush element material and size, element arrangement and mode of operation. For the grooming trials presented in this paper we used a 102 mm diameter hub populated with an outer and inner row of 24 tufts of nylon bristles. The grooming tool consisted of five brushes arranged with a slight overlap to give a swath of 560 mm and propelled by a remotely operated vehicle (SeaBotix vLBV). The vehicle was driven at about 0.25 m/s in a lawn mower pattern and a 50% overlap was visually maintained on reciprocal runs. Under these conditions an area of about 250 m^2 can be groomed each hour and it would therefor take about 12 h to groom an Arleigh Burke class destroyer (DDG-51) with a wetted surface area of $\sim$3000 m^2.

LONG-TERM GROOMING PERFORMANCE

To demonstrate the effectiveness of a grooming program the results from two long-term grooming studies and one cleaning study applied to Interspeed BRA640 and Intersleek 1100SR are presented (Swain et al., 2020). They follow the changes in biofouling, coating condition, dry film thickness (Elcometer digital coating thickness gauge) and roughness (TQC Hull Roughness Analyzer) for the duration of the deployment. Other results from grooming research have been published by Tribou and Swain (2010, 2015, 2017) and Hearin et al. (2015, 2016).

Interspeed BRA640

The BRA640 coating was subjected to weekly grooming for a period of 54 months (**Figure 6**). The groomed surfaces were maintained free of fouling, however, the ungroomed surfaces became fouled. The fouling included: biofilms, encrusting bryozoans, arborescent bryozoans, barnacles, tubeworms and colonial tunicates. The ungroomed surfaces were cleaned by divers using scrapers and brushes when the fouling rating reached FR-40 or greater over 20 percent of the surface (at 8, 14, 23, 30, 36, 47, and 54 months) and three times during dry dockings due to hurricane evacuations from the port.

The panels were inspected in the horizontal position at 12, 24, 33, 35, and 48 months immersion. Average dry film thickness measurements (DFT) showed a steady reduction which was similar for both the groomed and ungroomed surfaces (**Figure 7**). The change in DFT was used to calculate average copper output using the mass balance method presented in ISO 10890 (2010): Paints and varnishes – Modeling of biocide release rate from antifouling paints by mass-balance calculation (**Table 1**). The technical data for the BRA640 applied to the panel was as follows: % mass content of cuprous oxide 41.79; mass fraction of biocide in biocidal ingredient 0.86; density of paint 2.26 g/cm^3; volume solids content of paint 58.03. The average copper release rate was calculated to be 11μg/cm^2/day.

TABLE 1 | Formula to calculate mass biocide release rate from paint (ISO 10890, 2010).

M = L*a*w*p*DFT/NV. Where: M = Mass Biocide Released over lifetime of paint (micrograms/cm2).
L = 100 (Percent Biocide Released During Lifetime of Paint).
a = 0.86 (mass fraction of biocide in biocidal ingredient).
w = 41.79 (% by mass content of biocide in paint).
p = 2.26 (density of paint g/cm3).
DFT = ?? (dry film thickness μm).
NV = 58.03 (volume solids content of paint).

Coating roughness measurements using the hull roughness analyzer demonstrated that the groomed surfaces became smoother (average roughness decreased from about 90 to 70 microns). The ungroomed coatings increased in roughness due damage to the coating caused by cleaning and remains of fouling that was not totally removed.

Intersleek 1100SR

The IS1100 was groomed weekly for a period of 33 months (**Figure 8**). The groomed panel remained free of fouling except for occasional patches of tenacious biofilm and encrusting bryozoans that were removed during subsequent grooming sessions. The prevention of these types of fouling has been solved by modifying the brush design to better interact with the fouling release coatings. The ungroomed panel became fouled and required diver cleaning after 18 and 30 months. Another cleaning occurred during dry docking in October 2016 due to a hurricane. The fouling included: biofilms, encrusting bryozoans and tubeworms.

The panels were inspected in the horizontal position after 12, 24, 33, and 35 months immersion (**Figure 9**). Average dry film thickness values showed no significant difference during the immersion period. There was no significant change in coating roughness, however, the presence of small nicks in the coating caused by fish feeding on the fouling caused an increase in the standard deviation after 24 months. This was greater on the ungroomed surface and was attributed to fish feeding on the more abundant fouling.

CLEANING

BRA640 and IS1100 coated steel panels were left to foul over a one-year period and then subjected to diver cleaning. The BRA640 was heavily encrusted with barnacles, tubeworms and encrusting bryozoans and had a fouling rating of 90% FR100 and 10% FR30 (**Figure 10**). The IS1100 was not as heavily fouled including mainly biofilms, encrusting bryozoans and a few tubeworms with a fouling rating of 40% FR100 and 60% FR30.

The BRA640 was initially cleaned with a rotating polypropylene brush, but this was unable to remove the barnacle base plates and so a wire brush was applied. This removed most of the antifouling coating which had a DFT of

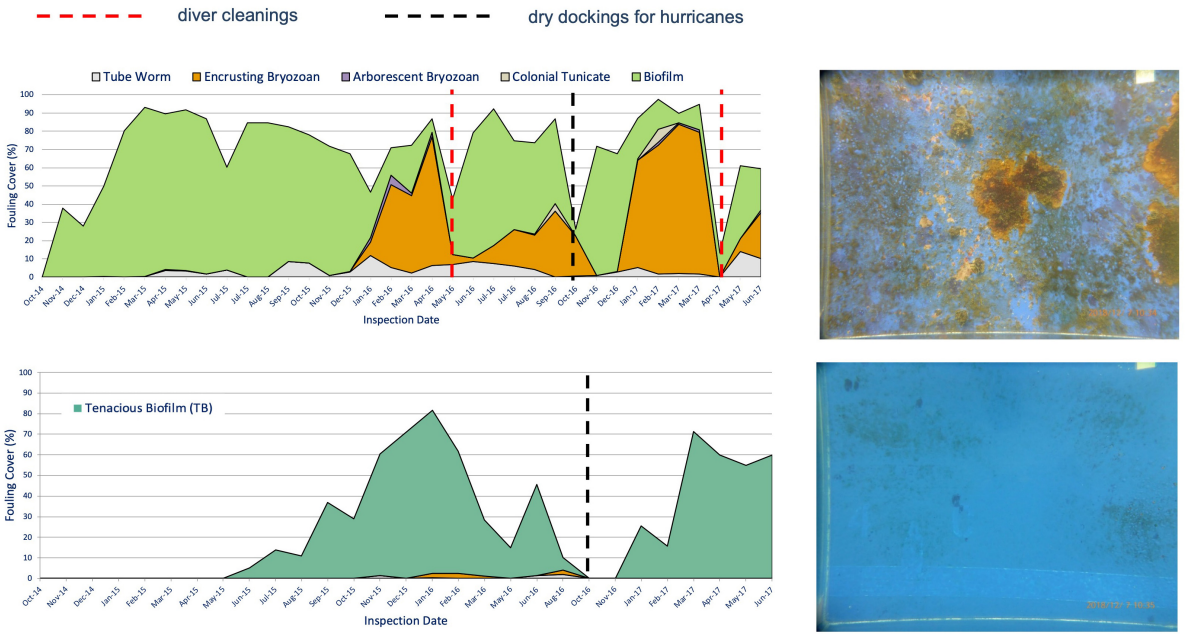

FIGURE 8 | The fouling progression on an ungroomed and groomed IS 1100 coating.

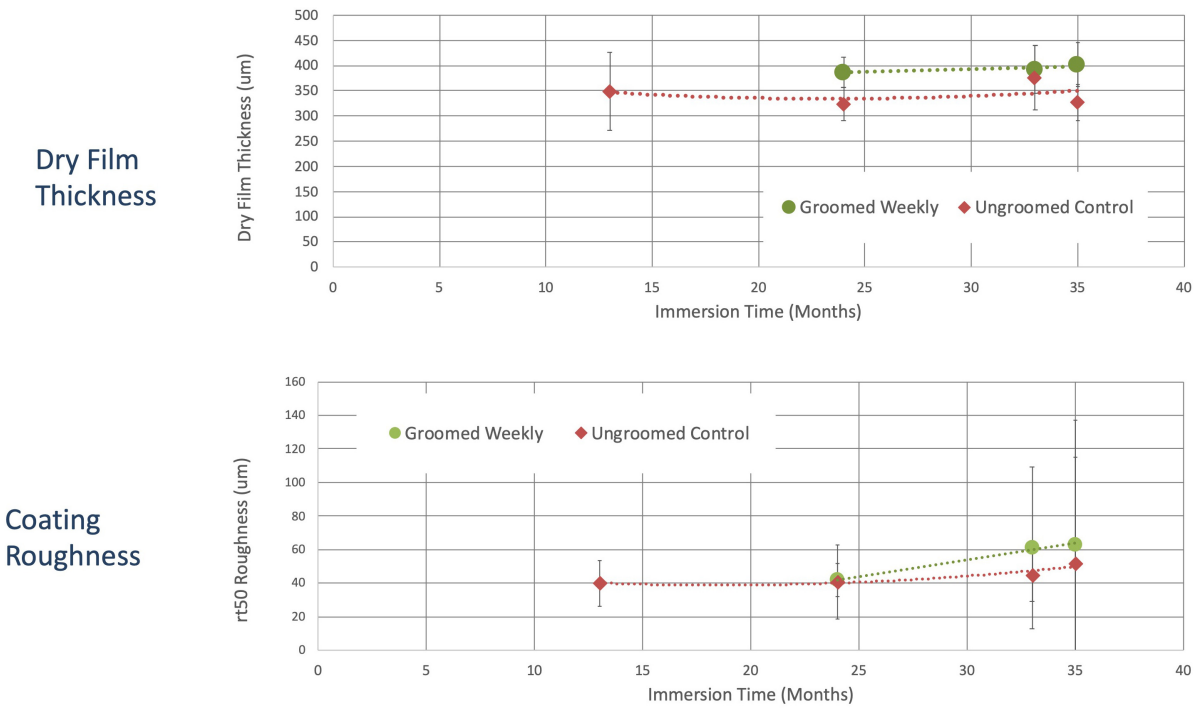

FIGURE 9 | Dry film thickness and roughness measurements on groomed and ungroomed IS1100 coating.

about 150 microns. The removal of 150 microns DFT BRA640 would release 0.2 kg copper/m^2 into the water.

The IS1100 was cleaned using the polypropylene brush. This removed most of the fouling, however, a small amount of biofilm remained, and some damage occurred to the coating where the brush filaments were allowed to dig into the coating and where calcareous fouling became entrapped in the brush causing damage to the coating before being ejected.

Whilst both these coatings were fouled at a much greater level than would normally be allowed, the damage to both the

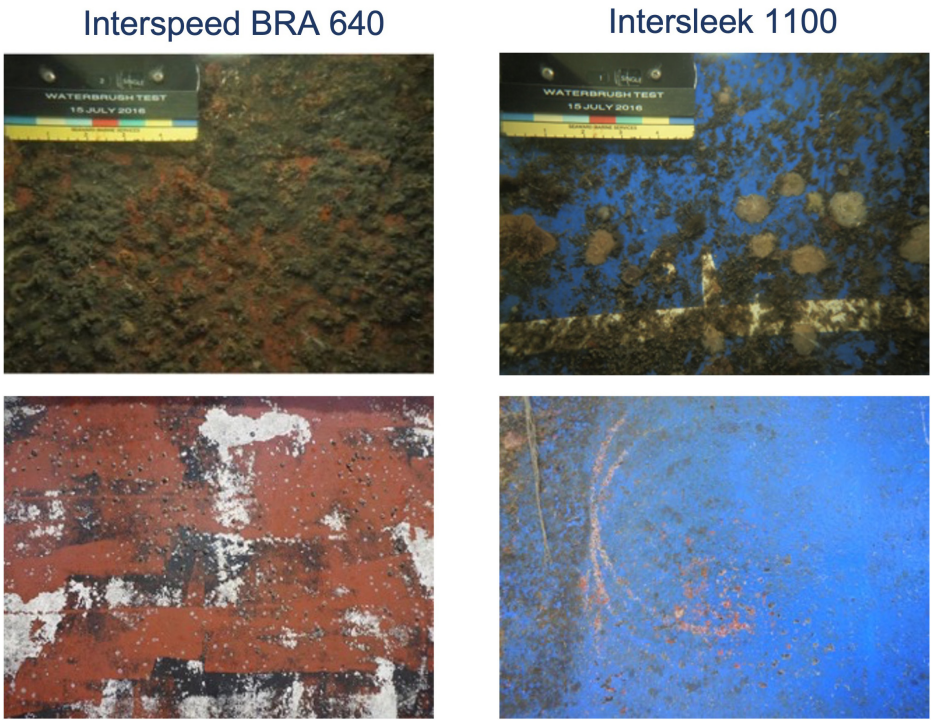

FIGURE 10 | The impact of underwater cleaning using a handheld polypropylene or wire brush.

BRA640 and the IS1100 coatings caused by the brush forces required to remove established biofouling demonstrated the negative impacts of a reactive ship hull cleaning program. The force required to remove a fouling organism is a function of the adhesion strength and the base area (Swain et al., 1994, 2007; Swain, 1996; Zargiel et al., 2011; Zargiel and Swain, 2014). A comparison of the typical force required to remove barnacles of increasing base diameter from a silicone fouling release surface, copper base antifouling, epoxy and cathodically protected bronze are presented in **Figure 11**. It not only demonstrates how different surfaces require different cleaning forces but also the exponential relationship between increasing barnacle diameter and the force required for removal. This emphasizes the importance of removing barnacles at an early stage which requires less force and prevents damage to the coating.

GROOMING AND COMMERCIAL FOULING CONTROL COATINGS

Whilst most of our research has focused on two US Navy qualified fouling control coatings (BRA640 and IS1100) we ran a one-year deployment of several commercial fouling control coatings (**Figure 12**). These were 150 × 300 mm panels of which one set were groomed once a week and the other set left to foul. All the groomed panels were kept free of fouling and only the copper free coating, which was very soft, showed signs of accelerated depletion due to grooming.

DISCUSSION

Long-term deployment of both biocide and fouling release coatings under static immersion in a location with high fouling pressure has demonstrated that a well-managed grooming program will maintain the coatings clear of fouling without damage or roughening of the surface. The adoption of a proactive inwater cleaning program has the ability to reduce greenhouse

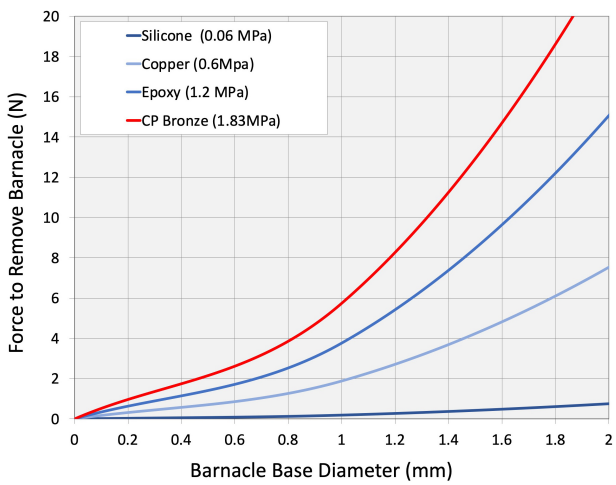

FIGURE 11 | The force required to remove a barnacle of increasing diameter from different surfaces.

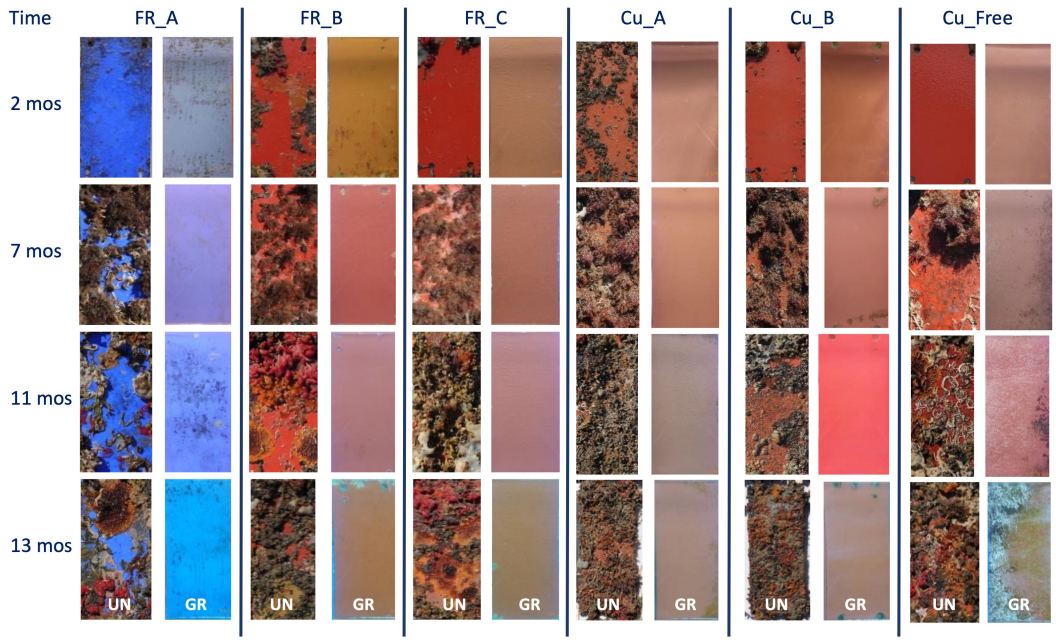

FIGURE 12 | The progression of fouling on three fouling release coatings and three biocide-based coatings subjected to static immersion: ungroomed vs. groomed once a week.

gas emissions, prolong the service life of fouling control coatings, reduce the point source discharge and the need for capture created by costly reactive cleaning programs and prevent the transport of invasive species.

Greenhouse Gas Emissions

The International Maritime Organization (2020a) Fourth Greenhouse Gas Study estimates that international shipping contributed about 2.89% of global greenhouse gas emissions or 1,076 million tons in 2018. The power to move a ship must overcome residuary resistance (which includes wave making resistance, form resistance, eddy resistance, and frictional form resistance) and frictional resistance. The frictional resistance may contribute between 40–90% of the power to move a ship. The contribution of frictional resistance for high-speed ships (cruise liners, passenger ships and navy vessels) will be less than for low-speed vessels (bulk carriers and tankers) (MAN B&W, 2004). The friction drag is a function of the seawater viscosity, the velocity gradients that develop in the boundary layer and the surface roughness. Surface imperfections in the form of coating roughness, weld beads, hull plate corrosion and biofouling all increase turbulence and mixing in the boundary layer which increases drag (Redfield and Hutchins, 1952; Townsin et al., 1981; Schultz, 2004, 2007; Swain, 2010). The absolute penalties incurred by hull roughness and biofouling are difficult to predict due to differences in hull form, hull speed and the heterogeneous nature of the hull condition and biofouling. However, assuming uniform roughness or biofouling Schultz (2007) developed a table that relates the hull condition to equivalent sand roughness height and maximum peak to trough height over a 50 mm sample length and applied them to the powering penalties for a 136 m long Oliver Hazard Perry class frigate (FFG-7) (**Supplementary Table 1**). These may be used to estimate the percent increase in viscous drag due to the hull condition. The data clearly demonstrates the importance of paying attention to everything from coating roughness (as applied 2% increase in resistance) the development of a biofilm or slime layer (11–21% resistance) and heavy calcareous fouling (86% resistance). According to the US Navy Technical Manual for Waterborne Underwater Cleaning of Navy Ships (2006) the decision to initiate a hull cleaning operation is based on the results of precleaning hull inspections. If a fouling rating of FR-50 or higher (over 10 percent of the hull) is observed for non-ablative paints or FR-40 (over 20 percent of the hull) higher for ablative and self-polishing paints (exclusive of docking block areas and appendages) is observed then a full hull cleaning is required. According to Schultz et al. (2011) this hull condition would increase the resistance of an Arleigh Burke-class destroyer (DDG-51) by 29 and 19% at speeds of 7.7 and 15.4 m s^{-1}, respectively, compared to the hydraulically smooth condition.

There are very few publications that provide information on the outer hull condition of the worlds fleet as most data are privy to the ship owners and paint industry. Munk et al., 2009 estimated that 1/3rd all vessels were in good condition, <20% added resistance; 1/2 all vessels in reasonable condition, 20–40% added resistance and the remainder in poor condition, >50% added resistance. More recently the Safinah Group published their findings for drydock inspections of nearly 270 ships where they found that 40% of ships had more than 20% hard fouling on the flats and that 10% of ships had more than 40% of their underwater area covered by hard fouling (Mihaylova, 2020). Clearly a significant reduction

in fuel consumption and CO_2 emissions can be gained by improving the maintenance of fouling control coatings.

From a global perspective, if the underwater portion of all the worlds shipping could be maintained in a smooth and fouling free condition, then the reduction in CO_2 and other exhaust gasses would be significant. Taking the estimates for CO_2 emissions from ships as 1,056 million tons/year (International Maritime Organization, 2020a) and assuming the average contribution of power from frictional resistance to move a ship is 70%, then using the estimated hull condition from Munk et al., 2009, we can estimate the reduction in CO_2 emissions if all vessels were maintained in a smooth and fouling free condition: 1,056 million tons /year × 0.7 friction resistance × [(33% ships with a 10% penalty) + (50% ships with 30% penalty) + (17% ships with 50% penalty)] = 198 million tons of CO_2 or 19% reduction of ship emissions.

Such calculations cannot be viewed as absolute but are presented as a demonstration of the importance of proactively managing the ship hull condition.

Point Source Discharge

Most fouling control surfaces use active ingredients to prevent biofouling. For most coatings this is the form of biocides which are incorporated into the paint and designed to be continually released at the minimum rate to prevent marine growth (Swain, 1999; Martins et al., 2020). These may reach concentrations in the water column or accumulate in sediments at levels that have a negative impact on marine life. This was a major setback for the biocide tributyltin which in the 1970s was being used as a very successful ingredient in antifouling paints (Champ, 2003; Dafforn et al., 2011). However, by the 1980s it was found to be negatively impacting non-target species at levels of less than 0.05 $\mu g\ L^{-1}$ (Laughlin and Linden, 1987) and ultimately the IMO introduced international regulations that prohibited its use (International Maritime Organization, 2008).

Copper has been successfully used in antifouling paints since the middle 1800s (Laidlaw, 1952) and today about 96% of the US Navy's fleet and 90% of the worlds ships use copper-based systems (Blossom, 2018). Copper is a naturally occurring element and required in trace amounts as a micronutrient. However, copper input from antifouling paints in areas of high boating activity may cause copper concentrations to reach undesirable levels (Srinivasan and Swain, 2007) and the National Recommended Water Criteria (USA) lists copper as a priority pollutant with recommended dissolved copper concentrations in the marine environment not to exceed 4.8 μg/l or an instantaneous concentration of 30 μg/l (Valkirs et al., 1994; United States Environmental Protection Agency [US EPA], 2016). The challenge to the chemist is to formulate the paint so that it releases the active ingredients at the minimum rate to prevent fouling under all service conditions of the ship. The minimum release rates for copper to prevent fouling has been quoted as between 10 to 20 $\mu g/cm^2$/day (Barnes, 1948) and 16 to 22 $\mu g/cm^2$/day (de la Court, 1988, 1989) to prevent barnacle and algal fouling, respectively (Swain, 2010). Actual release rates are highly variable (Seligman and Zirino, 1998; Haslbeck and Ellor, 2005; Haslbeck and Holm, 2005; Finnie, 2006) due to factors such as the type and age of paint, the ship activity and environmental conditions. Blossom (2002) estimated the annual copper input from all antifouling paints to be about $15x10^6$ kg/yr. Our estimate for 120,000 active commercial ships (including those < 100 gross tons) in the world fleet with an approximate wetted surface area of 325×10^6 m^2 (Moser et al., 2016) and assuming an average copper leaching rate of 10 $\mu g\ Cu\ cm^{-2}\ day^{-1}$ is:

$$10\ \mu g\ Cu/cm^2/day \times 325 \times 10^6\ m^2 \times 0.9 \times 365\ days/year \times 10{,}000\ cm^2/m^2 \times 10^{-9}\ kg/\mu g = 10.7 \times 10^6\ kg\ copper/year.$$

One of the challenges to managing a biocide-based coating is if the release rates drop below the threshold to prevent fouling, and the coatings become fouled. According to the US Navy Ship Technical Manual, the decision to clean ablative and self-polishing coatings is made when a fouling rating of 40 (**Supplementary Table 1**) or greater, is observed over 20 percent of the hull, exclusive of docking block areas and appendages. This will be done with the least aggressive method, however, experience has shown that the forces required to remove fouling, especially barnacle base plates, from a biocide based coating require vigorous cleaning that leads to coating loss and damage (United States Environmental Protection Agency [US EPA], 2011; Morrisey et al., 2013; Earley et al., 2014; Scianni and Georgiages, 2019; Tamburri et al., 2020). According to Morrisey et al. (2013) light cleaning may remove up to 650 $\mu g\ Cu\ cm^{-2}$ and aggressive cleaning up to 3,290 $\mu g\ Cu\ cm^{-2}$. This is a lot less than our observations for cleaning the BRA640 using a wire brush where up to 150 microns DFT coating were removed. This would be equivalent to 21,000 $\mu g\ Cu\ cm^{-2}$. Using these numbers then the aggressive cleaning of a very large ship with an underwater surface area of 10,000 m^2 (a 300+m cruise or container ship) may theoretically release between 329 to 2,100 kg of copper.

Whilst the environmental effects of copper are well understood, most copper-based paints also contain co-biocides to improve their performance. These may include: copper pyrithione, copper thiocyanate, cybutryne, dichlorooctylisothiazolinone, dichlorofluanid, medetomidine, tolylfluanid, tralopyril, zinc pyrithione, and zineb (Martins et al., 2020). The long-term environmental effects of these additives are less well understood. For example the co-biocide cybutryne (Irgarol-1051) may be added to paint at a weight percent of 2.3%. This may give a release rate of 2 μg cybutryne/cm^2/day (Netherlands, 2014) and environmental monitoring has shown it to be persistent in the environment and reach levels that are harmful to corals and other organisms (Owen et al., 2002; Sheikh et al., 2016). This has caused the IMO to a draft amendment to prohibit anti-fouling systems containing cybutryne (also known under its industry name Irgarol-1051) to apply to ships from January 1, 2023 (International Maritime Organization, 2020b).

Invasive Species

The translocation of species to new areas by biofouling on ships has long been recognized as a problem (Lewis, 2020) and this is recognized as one of the primary vectors of non-indigenous species (Hewitt and Campbell, 2010). It has been estimated

that 60% California's non-indigenous species and 80% of those present in New Zealand were transported by ship hull fouling (Kospartov et al., 2008; Ruiz et al., 2011; Miller et al., 2018; Scianni and Georgiages, 2019). One of the benefits of grooming is that a clean hull will not transport invasive species (Hunsucker et al., 2018a,b,c). They used the BRA640 and IS1100 associated with the long-term grooming study to record the presence and abundance of the non-indigenous organisms on the groomed versus ungroomed coatings. They found that non-indigenous species such as the Asian green mussel (*Perna viridis*), the striped acorn barnacle *(Balanus amphitrite),* arborescent bryozoan *(Bugula neritina),* calcareous tubeworm *(Hydroides elegans),* encrusting bryozoan (*Watersipora subtorquata complex),* and a filamentous bryozoan *(Zoo-botryon verticillatum*) recruited to the ungroomed coatings. None were present on the groomed surfaces. This demonstrates the benefits of a biofouling management strategy that incudes grooming.

The benefits of grooming or proactive cleaning to prevent the spread of invasive species is obvious, however, it must be remembered that robotic underwater cleaning of a ship hull is at present only applicable to the large open areas of a hulls' surface. This leaves small portions of the hull and niche areas that will still need intervention by divers to remove fouling.

SUMMARY

Large scale testing of an ROV equipped with a grooming tool has demonstrated that grooming (proactive, frequent light cleaning) can maintain fouling control coatings in a smooth and fouling free condition for extended periods without causing increases in the discharge of active ingredient into the environment. The practical and economic application of a successful grooming program for ships will require investment in new technology, hardware and a better understanding of the biofouling sequence in terms of ship operational schedules and fouling control coatings. It will require the development of inexpensive and reliable remotely operated or autonomous vehicles to move the grooming tool over the ship hull. Grooming tools will need to be designed to match the forces required to remove the fouling without causing excessive wear or damage to the coating. The hardness and durability of the major types of coatings must be matched to the grooming method. A guide to geographical and seasonal biofouling pressures and composition needs to be developed so that a digital twin may be linked to a ships' schedule and coating system to predict how and when grooming should occur. The development of such systems is now being considerd by several research teams and commercial companies. These include: Greensea (Kinnaman, 2019, 2020; Kyritsis and Arapkoules, 2021); Jotun SeaSkater (Oftedahl and Skarbø, 2021); International Paint Intertrac®HullCare and SeaRobotics. As these systems mature, so the costs and availability to the shipping community should provide commercially viable methods to apply proactive in water hull maintenance. This will reduce the environmental footprint and financial costs of shipping.

AUTHOR CONTRIBUTIONS

GS: principal investigator. HG and JH: research engineer. KH, ER, AS, and MT: research scientist. CE, LF, MH, JTH, KL, MN, BW, and AW: grad student.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2021.808549/full#supplementary-material

Supplementary Table 1 | Combing the fouling rating (RT) as given in the US Naval Ships' Technical Manual with the equivalent sand grain roughness height (k_s) and average coating roughness RT_{50} from Schultz (2007).

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Marine Natural Products: A Promising Source of Environmentally Friendly Antifouling Agents for the Maritime Industries

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Biofouling in the marine environment refers to an unwanted build-up of marine organisms on subsea surfaces including harbor docks, hulls of ships and offshore installations. The first stage of marine fouling occurs as a microbial biofilm which forms via the aggregation of bacterial, algal, and fungal cells. This biofilm provides a favorable substrate for the larval settlement of larger organisms such as mussels, barnacles and hard corals which accumulate to uncontrollable extents, causing issues for the maritime industries. Since the ban of tributyltin (TBT) in 2008 by the International Maritime Organisation, alternative antifouling agents have been used such as algaecides and copper-based coatings. Recent studies are showing that these can accumulate in the marine environment and have toxic effects against non-target species. Marine microbes and invertebrates are known to be prolific producers of bioactive molecules, including antifouling active compounds. These compounds are often produced by marine organisms as a means of chemical defense to deter predators and prevent fouling of their own surfaces, making them a promising source of new antifouling agents. This article discusses the effects of biofouling on the maritime industries, the environmental dangers of currently used antifouling compounds and why natural products from marine organisms could be a source of environmentally friendly antifouling agents.

Keywords: biofouling, antifouling, decommissioning, shipping, renewable energy, oil and gas, marine natural products

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INTRODUCTION

Biofouling in the marine environment can be described as an accumulation of marine organisms on subsea surfaces to undesirable extents. Marine bacteria quickly colonize submerged surfaces and aggregate to form microbial biofilms. These biofilms then attract the settlement of algal spores and the larvae of marine invertebrates such as barnacles, mussels, and hard corals (**Figure 1**). Microbial biofilms consist predominantly of bacteria, diatoms and an extracellular polymeric substance (EPS) matrix which is made up of polysaccharides, proteins, glycolipids, and extracellular DNA (e-DNA)

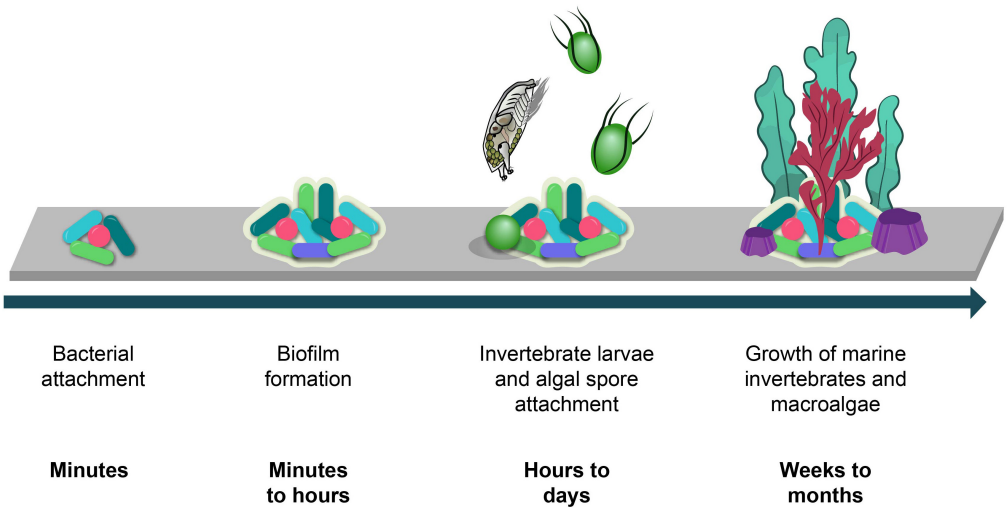


FIGURE 1 | Marine biofouling process timeline.

(Flemming, 2009). This EPS matrix allows biofilms to strongly adhere to surfaces through physicochemical interactions such as Van der Waals forces, hydrogen bonding and electrostatic forces (Mayer et al., 1999). Although these forces are generally weak, the overall combination of all these forces makes the biofilm mechanically stable. Quorum sensing (QS) is an additional mechanism which gives biofilms mechanical stability. This signaling system is used by bacteria to allow cells to communicate and aggregate for biofilm maturation and it is also the communication system behind luminescence and virulence (Miller and Bassler, 2001).

Through various settlement cues, marine biofilms encourage the settlement of marine invertebrate larvae and algal spores (Dobretsov and Rittschof, 2020) which mature and then grow in abundance. These organisms quickly colonize the entirety of subsea surfaces which is problematic for the maritime industries due to the increase in maintenance requirements and the corresponding financial losses. Antifouling (AF) coatings are commonly applied to subsea structures to prevent biofouling, however, many of the AF agents which are incorporated into these coatings have serious consequences on the marine environment highlighting the need for alternative, environmentally friendly solutions. This article highlights the negative impact biofouling has on maritime industries, the environmental challenges caused by currently used AF agents and the use of marine natural products as an alternative source of AF active agents.

EFFECTS OF BIOFOULING ON MARITIME INDUSTRIES

The accumulation of marine organisms on subsea structures is problematic for many maritime industries including the shipping, offshore oil and gas, and the offshore renewable energy sector. The effects caused by biofouling typically lead to economic losses for these industries as well as losses in structural integrity. Biofouling can also have serious ecological implications on marine biodiversity and there are ongoing debates regarding the creation of new ecosystems surrounding oil and gas structures and the subsequent destruction of these new ecosystems come the time of decommissioning.

Shipping

Biofouling of the hulls of ships by marine organisms results in an increase in drag, due to increased surface roughness (Farkas et al., 2018), which in turn leads to higher fuel costs (Schultz et al., 2011). A recent study by the International Maritime Organisation (2021) shows that even low amounts of biofouling on ships can lead to an increase in greenhouse gas emissions within the range of 20–25%. This negatively contributes to worldwide concerns surrounding climate change therefore biofouling of ships should be prevented to help reduce harmful emissions. To effectively remove persistent fouling from the surfaces of ships, they must be dry-docked and scraped which is costly and time consuming for these companies. Additionally, fouling of ships can encourage the introduction of invasive and non-native species into ecosystems. As ships travel from one location to another, organisms which foul the ship's surface are transported to, and introduced into, ecosystems in which they do not belong which can be very damaging for these environments (Molnar et al., 2008). *Didemnum vexillum* is an example of an invasive sea-squirt species in British waters which was likely introduced via ships or recreational boats. This non-native species interferes with marine habitats by rapidly growing and forming "carpets" over native species, resulting in a smothering effect and preventing the growth of other native species (Marine Scotland, 2021).

Oil and Gas/Decommissioning

Biofouling of oil and gas structures is undesirable during the working life of installations and provides additional challenges

at the time of decommissioning. Extensive build-up of marine organisms contributes to early aging and the need to replace and repair structures due to corrosion. Microbially induced corrosion (MIC), which involves anaerobic sulfate-reducing bacteria and aerobic iron-oxidizing bacteria within the biofilm layer (Li and Ning, 2019), is one of the main causes of corrosion in the offshore industry. To help prevent the build-up of biofouling, routine maintenance of subsea jackets of oil and gas rigs must be conducted which requires remote operating vehicles with cleaning attachments to carry out the work—adding additional operating costs. If biofouling is not removed, new ecosystems are formed surrounding oil and gas platforms (van Elden et al., 2019), however, this is unfavorable as these new ecosystems are quickly destroyed come the time of decommissioning when the structure is removed. Under the current Oslo-Paris Convention regulations (OSPAR Commission, 2013), oil and gas jackets must be removed during the decommissioning process and the jackets cannot be left in place as artificial reefs as per the "Rigs-to-Reefs" program which is implemented in the Gulf of Mexico, Brunei and Malaysia. Marine fouling can also add thousands of tons of additional weight to subsea structures (Hustoft and Gamblin, 1995) hence larger machinery are required to lift the structures out of place, resulting in further economic implications for these companies since they cannot be left in place to allow the new ecosystems to thrive.

Offshore Renewables

Similarly to the oil and gas industry, the offshore renewables sector is also negatively affected by biofouling. One of the main implications is that marine biofouling drastically lowers the lifespan of these systems (Yang et al., 2017). The colonization of renewable energy structures such as wind turbines and wave energy converters, impacts various engineering parameters which in turn affects the overall functionality and efficiency of these systems (Shi et al., 2012; Martinez-Luengo et al., 2017; Jahjouh, 2020; Arcigni et al., 2021). It is also essential that renewable energy structures are engineered to account for the additional weight from biofouling, however, this weight can be challenging to predict.

CURRENTLY USED ANTIFOULING AGENTS AND THEIR ENVIRONMENTAL EFFECTS

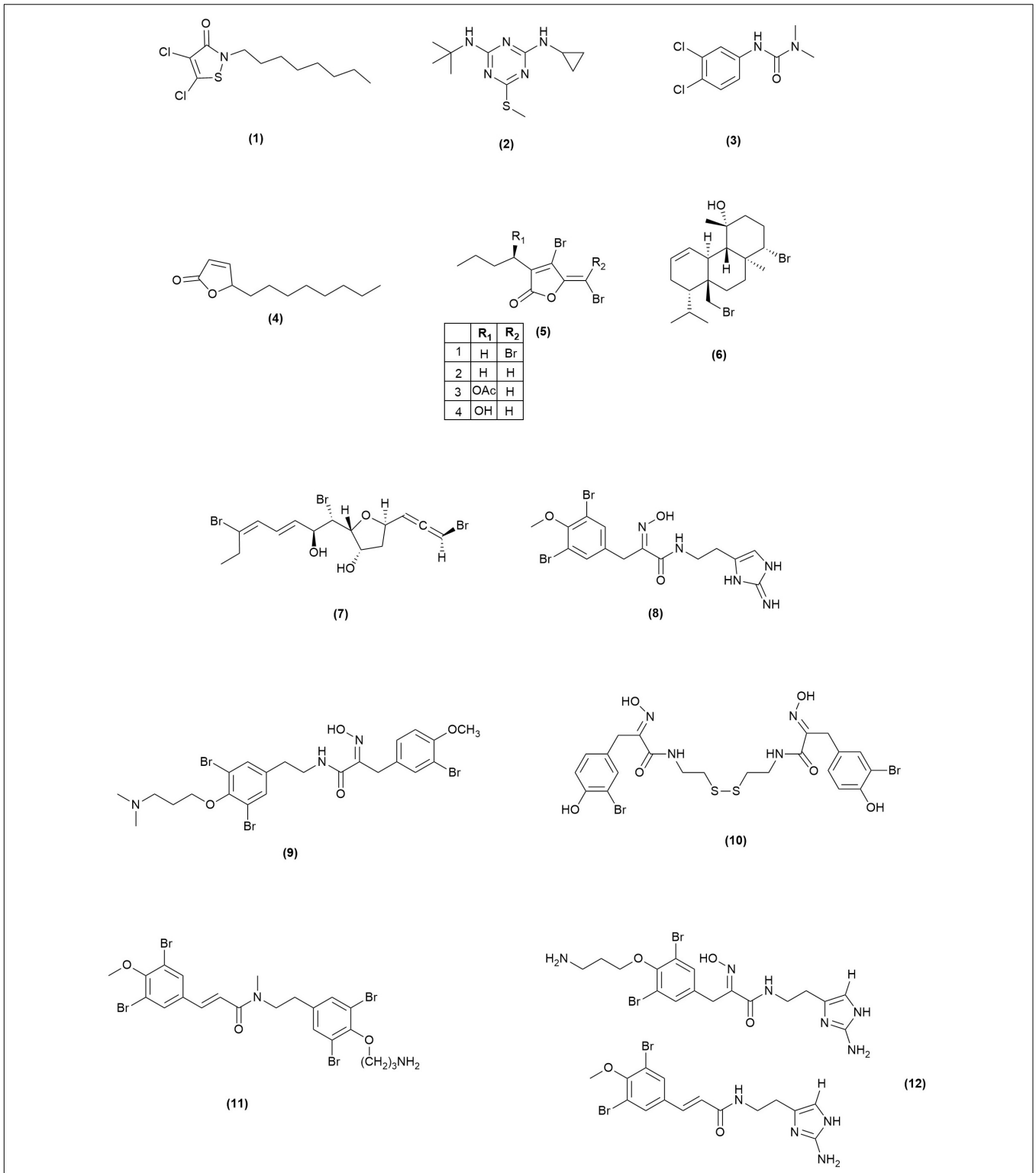
Antifouling active, chemical-based coatings have been used to prevent marine fouling since the 1800s when the first copper sulfide coating was manufactured by Bonnington Chemical Works, United Kingdom (Ronalds, 2019) to replace antifouling methods such as covering ship hulls with metal sheathing. When tributyltin (TBT)-based coatings were later introduced, they quickly became the most effective and commonly used AF solution until they were banned in 2008 by the International Maritime Organization. TBT is toxic to non-target species at low concentrations, notably the female sea snail, whose fertility is damaged when exposed to TBT (Gibbs et al., 1991). Alternative AF agents are currently used in the marine industries, but recent studies highlight that these agents are not as "green" as they were once thought to be. Antifouling agents can also have devasting effects on ecosystems and marine biodiversity if they show toxicity towards marine wildlife. According to the Biocidal Products Regulation, an antifoulant must show high activity, low toxicity effects on target and non-target organisms and must not show unacceptable effects on the environment to be authorized as a new antifouling compound for commercial use (Biocidal Products Regulation, 2012). To understand the true impacts of introducing these compounds into the marine environment, long term monitoring is required, and the effects of these compounds are often not well understood until after many years of application. Current literature supports that many of the commonly used AF agents used are no longer suitable for use and should be replaced with alternative, non-toxic solutions (**Figure 2**).

Seanine 211 (DCOIT)

Seanine 211 is a globally used antifouling agent containing the biocidal ingredient DCOIT **(1)** which is effective against a wide range of fouling organisms. There are, however, concerns surrounding the use of this AF agent due to its inconsistent degradation properties and its toxic nature. For example, a study by Jacobson and Willingham (2000) states the half-life of Seanine 211 in seawater to be 24 h, however, a review by Chen and Lam (2017) shows that different environmental factors affect Seanine 211 degradation, and its half-life can in fact range from <1 to 13.1 days. This shows that the degradation rates of Seanine 211 are variable and depend on many external factors. In addition, the biocidal agent, DCOIT, has been found to have comparable or higher toxicities than organotin toward marine bacterium *Vibrio fischeri*, crustacean *Daphnia magna* and freshwater green algae *Selenastrum capricornotum* (Fernández-Alba et al., 2002). It also exhibits comparable toxicity effects to triphenyltin on oyster embryos (Tsunemasa and Okamura, 2010) and is more toxic than tributyltinoxide when considering the development of sea urchins (Kobayashi and Okamura, 2002). Due to the similarities in toxicities between organotin species and DCOIT, the global use of Seanine 211 should be reconsidered and the importance of finding non-toxic alternatives is further validated.

Irgarol 1051

Irgarol 1051 **(2)** is another widely used biocide which is one of the least toxic to non-target species when compared against other antifouling agents and organotin (Fernández-Alba et al., 2002). Although it shows low toxicity effects, Irgarol 1051 is in fact classed as an environmental contaminant and has been found to persist in various marine environments all over the world (Konstantinou and Albanis, 2004). This AF agent has poor biodegradability which is reflected in its long half-lives of 100 and 200 days in seawater and freshwater, respectively (Ciba Geigy, 1995) and could accumulate in sediments according to its high octanol/water coefficient of log K_{ow} = 3.95 (Bard and Pedersen, 1992). Although the



	R_1	R_2
1	H	Br
2	H	H
3	OAc	H
4	OH	H

FIGURE 2 | Structures of currently used AF agents **(1–3)** and AF active marine natural products **(4–12)**.

degradation time of Irgarol 1051 is slow, the degradation product has been found in environmental samples and is moderately toxic toward *V. fisheri*, and four crustaceans: *D. magna*, *Daphnia pulex*, *Thamnocephalus platyurus,* and *Artemia salina*. It is also highly toxic toward green algae *S. capricornutum* (Okamura et al., 2000).

Diuron

Diuron, also known as DCMU **(3)**, is an algaecide, herbicide and PSII inhibitor. Because of its detrimental environmental effects, Bulgaria is the only EU state member which permits its use but diuron continues to be used in other parts of the world (Lewis et al., 2016). When compared with other herbicides, diuron effects the growth rate of non-target diatom *Chaetoceros muelleri* at very low concentrations (Thomas et al., 2020) and it has also been shown to be moderately toxic toward fish and slightly toxic to aquatic invertebrates (Giacomazzi and Cochet, 2004). The degradation product of diuron, 3-4-dichloroaniline, shows higher toxicity against different marine species including various fish, bacteria, and microalgae amongst others (Giacomazzi and Cochet, 2004).

ANTIFOULING ACTIVE MARINE NATURAL PRODUCTS

Marine organisms such as bacteria, fungi and invertebrates are producers of interesting and complex compounds exhibiting a wide range of bioactivities. These organisms are most commonly explored for compounds with use as therapeutic drugs against cancerous tumors, viruses, microbial infections, and other ailments. Just under 30 marine natural products (MNPs), including derivatives, are currently in clinical trials and 17 compounds have been approved for use by the FDA to date (Marine Pharmacology, 2021). With the emergence of new viruses and the growing resistance of infectious bacterial strains, it is important to keep looking to these alternative sources for new drugs.

Alongside investigating MNPs for their medicinal properties, there is a growing interest in understanding the chemical ecology of our marine ecosystems and the ways in which marine organisms interact with one another. This can be done by studying the chemical profiles of the metabolites produced by these organisms, the mechanisms by which they are biosynthesized and discovering the "whys" of their production. As a means of chemical defense, marine organisms can produce and excrete metabolites to deter predators, to compete with other organisms and to prevent fouling of their outer surfaces, in the case of marine invertebrates and macroalgae (Puglisi et al., 2019). A recent review by Liu et al. (2021) reports that 182 AF active compounds were isolated from marine organisms between August 2014 and May 2020 and in contrast to the 1,490 new MNPs reported in 2019 alone (Carroll et al., 2021), this figure is very small. This is an indicator that AF active compounds from marine sources are under-explored and that there are many more compounds with AF activity to be discovered. Following the discovery of AF active MNPs, analogs can be synthesized and tested to determine whether the activity can be increased by altering the structure of the compound. If the compounds are isolated from marine invertebrates or algae, the ideal route for mass production would be large scale synthesis. In the case of AF natural products from microbial origins, these compounds can often be sustainably produced via biotransformation processes which can also help to reduce carbon emissions.

Butenolides

The butenolides, a class of natural products containing a 2-furanone moiety, were first discovered to have potent AF activity when they were isolated from a marine *Streptomyces* strain after the bacterium showed AF activity during preliminary bioassay testing (Xu et al., 2010). The isolated compounds, alongside some previously isolated butenolides, show AF activity against the settlement of marine foulers and it was found that the 2-furanone moiety is responsible for the bioactivity. A modified butenolide **(4)** has since been synthesized which, when incorporated into a paint and tested in the field, exhibits more potent inhibition activity (lowest EC_{50} = 0.02 μg/mL) against the settlement of common fouling organisms whilst maintaining low toxicity. The butenolide compounds are a notable success story as this modified compound was patented for use as an antifouling agent (Peiyuan et al., 2017). Additionally, a study by Chen et al. (2015) found the half-life of a butenolide compound to be 0.5 days whereas DCOIT, the active compound in commonly used SeaNine 211 AF agent, had still not degraded after 4 days. Due to the quick degradation of butenolide compounds, their efficacy at low concentrations and low toxicity, they are a perfect example of marine natural product inspired AF agents with environmentally friendly potential.

Halogenated Compounds From Macroalgae

Algal extracts have been shown to exhibit antifouling activity against marine bacteria (Hellio et al., 2001) with macroalgae being the main producer of antifouling active compounds, including some interesting, halogenated compounds (Dahms and Dobretsov, 2017). Halogenated furanones from the red alga *Delisea pulchra* are an example of AF active compounds with activity against both micro and macrofoulers. *D. pulchra* is a macroalgae which does not show fouling on its outer surface and four surface furanones **(5)** have been isolated which show AF activity against ecologically relevant marine organisms (Dworjanyn et al., 2006). These compounds are an example of chemical defense compounds produced by this species to deter settling of organisms on the algal surface via quorum sensing inhibition by altering the EPS matrix produced by the bacteria hence inhibiting biofilm growth. Another red alga, *Sphaerococcus coronopifolius*, produces various brominated terpenes with AF activity, notably bromosphaerol **(6)** which shows promising results by inhibiting the settlement of the barnacle *Amphibalanus amphitrite* at an EC_{50} of 0.23 mg/L whilst showing low toxicity effects (LC_{50} > 100 mg/L) (Piazza et al., 2010). *Laurencia* sp. also produces AF active compounds such as bromoallene-containing natural products including omaezallene **(7)** and its corresponding congeners, which are also active against the settlement of *A. amphitrite* larvae (Umezawa et al., 2014).

Bromotyrosine Containing Compounds

In addition to macroalgae, marine sponges also biosynthesize halogenated compounds with antifouling properties. Amongst these compounds are bromotyrosine derivatives which have been isolated from various sponge species and are active

against both micro and macrofoulers. Ianthelline **(8)** is an example of a bromotyrosine containing compound which was originally isolated from the Caribbean sponge *Ianthella ardis* (Litaudon and Guyot, 1986) and then later isolated from an Arctic sponge and tested for its AF bioactivity. Hanssen et al. (2014) found that ianthelline shows varying degrees of AF activity against marine bacterial growth, bacterial adhesion, and in larval settlement assays using *B. improvisus* cyprids an IC_{50} of 3 μg/mL was recorded. Low toxicity was also reported at the highest concentration of 20 μg/mL. Ianthelline has been isolated from many sponge species and has been shown to be active in inhibiting bacterial attachment of bacterial species associated with the sponge *Ailochroia crassa* (Kelly et al., 2005). This could indicate the ecological role of ianthelline as a defense compound produced to prevent bacterial surface colonization, again highlighting the importance of exploring marine invertebrate chemical defense compounds as new sources of antifouling agents. Additionally, several bastadin derivatives isolated from *Ianthella basta*, aplysamine-2 **(9)** from *Pseudoceratina purpurea* and psammaplin A **(10)** isolated from *Aplysinella rhax* have been found to show larval settlement inhibition activity of *B. improvisus* at low concentrations (Ortlepp et al., 2007). In this study, it was found that the oxime moiety was an important functional group for producing the antifouling activity in addition to the bromine atoms. Aplyzanzine E **(11)** and two bromine containing 2-aminoimidazolic compounds **(12)** isolated from *Pseudoceratina* sp. show AF activity against three strains of marine bacteria, three strains of microalgae and also inhibited the quorum sensing behavior of *Vibrio harveyi* (Tintillier et al., 2020).

CONCLUDING REMARKS

Marine biofouling is a significant issue for the maritime industries due to the economic losses associated with biofouling induced structural degradation and increased fuel costs. The ecological implications caused by biofouling are also unfavorable as they can be detrimental to marine biodiversity. Although it is important that the attachment of marine organisms on subsea structures is prevented, the application of currently used antifouling agents should be reconsidered due to the high number of studies supporting their negative impact on the marine environment. With the success of butenolide and the promising AF activity and low toxicities of other classes of MNPs, it is important that research into the discovery of new AF-active natural products continues to be explored. Compounds produced by marine microbes, macroalgae, sponges, and corals which grow in different global environments, including extreme environments, should be prioritized and halogenated compounds, such as brominated compounds, should also be targeted due to the high proportion of AF active MNPs containing halogenated atoms. It is important, however, to test these compounds for their toxicity against non-target species to ensure they are safe for use as AF agents. MNPs are a very promising source of inspiration for synthesizing new and environmentally friendly AF agents and are the way forward in preventing the many environmental issues caused by the currently used AF compounds.

AUTHOR CONTRIBUTIONS

JG-B developed the concept for the review and wrote all sections.

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Ship Biofouling as a Vector for Non-indigenous Aquatic Species to Canadian Arctic Coastal Ecosystems: A Survey and Modeling-Based Assessment

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Ship biofouling is a major vector for the introduction and spread of harmful marine species globally; however, its importance in Arctic coastal ecosystems is understudied. The objective of this study was to provide insight regarding the extent of biofouling (i.e., percent cover, abundance, and species richness) on commercial ships operating in the Canadian Arctic. A questionnaire was used to collect information on travel history, antifouling practices, and self-reported estimates of biofouling extent from ships operating in the region during 2015–2016. Twenty-five percent of ships operating in the region during the study period completed the questionnaire (n = 50). Regression trees were developed to infer the percent cover of biofouling, total abundance of fouling invertebrates, and fouling species richness on respondent ships based on previous underwater wetted surface assessments of commercial ships in Canada. Age of antifouling coating system was the only significant predictor of percent cover and total abundance of biofouling invertebrates, while the number of biogeographic realms previously visited and port residence time were significant predictors for fouling species richness. Comparison of relevant travel history features reported through the questionnaire to the regression tree models revealed that 41.9% of 43 respondent ships had antifouling coating systems older than 630 days and are therefore inferred to have relatively high (> 9.3%) biofouling percent cover. More than half of respondent ships (62.8%) had antifouling coating systems older than 354 days and are therefore inferred to have a relatively high total abundance (over 6,500 individuals) of fouling invertebrates. Nearly half of respondent ships (45.9% of 37 ships) had visited at least three biogeographic realms during their last 10 ports-of-call and are therefore inferred to have relatively high fouling species richness (mean 42 taxa). Self-reported estimates of biofouling cover were unreliable, being much lower than model inferences. Although

the regression tree models have relatively low predictive power, explaining only 15–33% of the variance in biofouling extent, this study indicates that commercial ships are an active pathway for the transportation of non-indigenous aquatic species to Canadian Arctic coastal ecosystems via biofouling.

Keywords: Arctic, biofouling, biological invasions, hull fouling, invasive species, non-indigenous species, regression trees, shipping

INTRODUCTION

Fouling by aquatic organisms on wetted surfaces of ships is an important means of transferring species to new regions, especially in marine and coastal ecosystems (Hewitt et al., 2009; Bailey et al., 2020). Such attached organisms can fragment, escape, become dislodged, or reproduce while ships are in port and, if they become established in new locations, could be detrimental to the environment, human health, property, and resources (Bailey et al., 2020). Ship biofouling is typically managed using antifouling systems, which includes applying antifouling coating systems (e.g., biocidal coatings and/or fouling-release coatings) and operating marine growth prevention systems to exposed wetted surfaces (IMO, 2011; Arndt et al., 2021). Biocidal coatings are the most common type of antifouling coating used, which prevent organisms from accumulating on surfaces through low-level continuous release of a biocidal agent that kills or deters settling organisms (Dafforn et al., 2011). Conversely, fouling-release coatings reduce the adherence of organisms to wetted surfaces so that they more easily detach while ships are underway (Dafforn et al., 2011).

Biofouling on ships is typically concentrated in niche areas (e.g., sea chests, water cooling systems, and propellers), rather than on flat exterior surfaces on the main hull (Coutts and Dodgshun, 2007; Davidson et al., 2009; Chan et al., 2015). This is because fouling on the hull is typically well managed by ship owners, since fouling can increase drag and, in turn, increase the fuel consumption of a ship (Schultz et al., 2010; Hakim et al., 2019). Furthermore, antifouling coating systems are generally more effective on the hull when exposed to higher water velocities experienced during sailing (Coutts and Taylor, 2004). Conversely, niche areas tend to become more heavily fouled with organisms since the antifouling coating systems cannot be applied (e.g., sacrificial anodes) or they are typically less effective in these physically complex areas (Coutts and Taylor, 2004; Davidson et al., 2009; Frey et al., 2014). Marine growth prevention systems (e.g., anodic copper dosing and electrolysis) deliver antifouling agents to reduce fouling in recessed or internal niche areas, such as sea chests and water cooling systems (Coutts and Dodgshun, 2007; Grandison et al., 2011).

Several variables related to the size and operational profile of ships have been used to estimate biofouling in the absence of direct measurements of biofouling assemblages on ships. For example, it has been hypothesized that larger ships have a higher likelihood of non-indigenous species introduction than smaller ships as they have more wetted surface area to accumulate a larger number of fouling organisms (Lo et al., 2012; Moser et al., 2016; Miller et al., 2018). However, the wetted surface area of a ship may not directly indicate the abundance of fouling organisms, since fouling organisms are typically concentrated in niche areas, which make up between 7 and 27% of a ship's total wetted surface area and do not normally increase proportionally with wetted surface area (Frey et al., 2014; Chan et al., 2015; Moser et al., 2017). The operational profile of a ship includes time between dry-docking visits, sailing speed, residence time in ports, and travel history. Time since the last dry-docking typically indicates the age of the antifouling coating system as coatings are applied when the ship is in dry-dock for maintenance or repairs. Antifouling coating systems generally become less effective with age, such that ships with older antifouling coatings tend to accumulate more fouling organisms than those with newer coatings (Davidson et al., 2009; Sylvester et al., 2011). The typical sailing speed of a ship may influence biofouling extent since fouling organisms are more likely to dislodge from ship hulls (and other unprotected wetted surfaces) at higher sailing speeds (Coutts et al., 2010; Davidson et al., 2020). The residence time of a ship at previous ports may serve as an indicator of biofouling extent since longer port stays increase the exposure time to propagules in the port environment and antifouling coating systems may be less effective while stationary due to low flow conditions (Davidson et al., 2009, 2020; Sylvester and MacIsaac, 2010). Travel history has been used as an indicator of the diversity and abundance of fouling communities on ships, since fouling organisms accumulate over time and ships that visit more regions are likely to be exposed to a greater variety of species (Sylvester et al., 2011). Furthermore, ships operating at lower latitudes may be exposed to a larger number of fouling organisms, given that the productivity of biological communities is typically greater in warmer climates (Sylvester et al., 2011).

The introduction and establishment of non-indigenous marine species in the Arctic are predicted to increase with the growing demand for shipping in support of resource development, tourism, and fisheries (Miller and Ruiz, 2014; Ware et al., 2014; Ricciardi et al., 2017; Goldsmit et al., 2020). Furthermore, the decline in sea ice cover due to climate warming is lengthening the Arctic shipping season and increasing the viability of northern shipping routes (e.g., the Northwest Passage and the Northeast Passage; Melia et al., 2016; Pizzolato et al., 2016). Increased shipping activity will result in a greater abundance of non-indigenous species transported to the Arctic by ships. Although many non-indigenous species are expected to have low survivorship in Arctic ecosystems, some may survive and become established in recipient ports (Chan et al., 2016, 2019; Goldsmit et al., 2019).

Globally, shipping is the main vector of marine non-indigenous species translocations through the biofouling and ballast water pathways (Bailey et al., 2020). It has been reported that a greater diversity and abundance of non-indigenous

organisms may be transported to the Canadian Arctic via biofouling compared to ballast water (Chan et al., 2015); however, the introduction of non-indigenous species by biofouling to this region remains understudied. The objective of this study was to provide insight regarding the extent of biofouling (i.e., percent cover, abundance, and species richness) on commercial ships operating in the Canadian Arctic. A questionnaire survey was conducted to collect data on variables related to biofouling from a sample of ships operating in the Canadian Arctic. Then, regression trees were developed for predicting the extent of biofouling using the best available data (i.e., direct measures of fouling on wetted surfaces of—mainly international—ships in Canada from previous studies). Finally, the results of the regression tree models were compared to the questionnaire survey data to infer the extent of biofouling on the sampled population of ships operating in Canadian Arctic coastal ecosystems.

MATERIALS AND METHODS

Ship Biofouling Questionnaire Survey

A questionnaire survey was conducted to characterize the operational profile and antifouling practices of ships operating in the Canadian Arctic during the summers of 2015 and 2016 (**Supplementary Material 1**). The questionnaire was distributed electronically to all commercial ships entering the Northern Canada Vessel Traffic Services zone by the Canadian Coast Guard as part of the standard entry clearance communications, though participation in the survey was voluntary. The questionnaire was developed based on the voluntary ship biofouling management plan detailed in the International Maritime Organization's *Guidelines for the Control and Management of Ships' Biofouling to Minimize the Transfer of Invasive Aquatic Species* (IMO, 2011).

Information was collected on ships' operational profile (travel history, typical sailing speed, port residence time, and time at sea), information on antifouling systems installed, general characteristics (ship type, year built, gross tonnage, length, and wetted surface area), and self-reported estimates of percent cover of biofouling. The travel history of ships was requested for the last 10 ports-of-call preceding arrival at a Canadian Arctic port as well as the planned stops in the Arctic. Survey data were supplemented with information collected through mandatory ballast water reporting forms, where necessary. The last 10 ports-of-call were categorized into biogeographic realms following the largest spatial units in the Marine Ecoregions of the World (MEOW) system (Spalding et al., 2007); the Laurentian Great Lakes was included as an additional biogeographic realm.

Self-reported estimates of percent cover of biofouling were obtained to determine if it is feasible to consider these as alternatives to direct measurements of biofouling in lieu of scientific underwater biofouling assessments, which are costly (particularly for remote Arctic ports) and are therefore usually limited by small sample size. Self-reported estimates of percent cover of biofouling were based on the Captains' general knowledge of the ship (i.e., best guess) and results from the latest underwater inspection. The term "inspection" is used here to refer to opportunistic observations on biofouling from the most recent underwater inspection as part of regular cleaning, repair, and/or maintenance of the hull conducted by ship owners. While there is additional information that may provide insight into the extent of biofouling on ships (e.g., travel history beyond the last 10 ports-of-call and details of antifouling systems onboard), only those cited in scientific literature as predictors of biofouling and readily available to ship crews were requested to keep the questionnaire to a reasonable length.

Regression Tree Analyses

Since previous quantitative underwater assessments of biofouling on commercial ships operating in the Canadian Arctic are very limited, the biofouling extent models were developed using data from underwater assessments conducted across Canadian marine temperate and Arctic ports. The dataset includes underwater biofouling assessments from 53 commercial ships arriving at Halifax, Nova Scotia (20 international ships), Vancouver, British Columbia (20 international ships) during 2007–2009, and Churchill, Manitoba (11 international and 2 domestic ships) in 2010–2011. The data were collected and analyzed using comparable methodologies, and the methods for sample collection, enumeration, and taxonomic identification are detailed in Sylvester et al. (2011) and Chan et al. (2015). It is possible that there have been changes in shipping patterns (e.g., changes in dominant trade routes or hull maintenance activities) during the 4–9-year gap between the underwater biofouling assessments and the questionnaire survey that may alter the observed trends in biofouling; however, these data are considered best-available to support the present study as there have been no more recent biofouling studies in Canada.

A series of regression tree analyses were performed on ship variables to identify predictors of: (i) percent cover of biofouling (algae and invertebrates), (ii) total abundance of fouling invertebrates, and (iii) richness of fouling species (invertebrates). Percent cover of biofouling was determined using underwater video footage. Percent cover for the entire ship was determined by calculating the weighted average of percent cover of biofouling of wetted surface sections (e.g., bulbous bow, hull, sea-chest gratings, stern tube, rope guard, propeller, and rudders), based on their contribution to the total wetted surface area (Sylvester and MacIsaac, 2010; Chan et al., 2015). The total abundance of invertebrates on each ship was estimated by summing abundance estimates for each wetted surface section based on biological quadrat sampling, percent cover information, and surface area of each location (Sylvester et al., 2011); the total abundance values were $\log10(x + 1)$ transformed before analysis to meet assumptions of normality. The richness of fouling species represents the Chao-2 species richness estimates based on the total number of fouling invertebrate species identified in biological samples obtained from each ship (Chan et al., 2015). The ship variables assessed included ship size (maximum length, wetted surface area, and gross tonnage), operational profile (typical sailing speed, port residence time, and age of antifouling coating system), and travel history (number of biogeographic realms visited and average, minimum, and maximum port

latitude; **Table 1**). Variables related to travel history were based on the last 10 ports-of-call visited by each ship.

The data exploration protocol of Zuur et al. (2010) was used to detect multicollinearity in the dataset. As variables related to travel history (i.e., the number of biogeographic realms visited and average, minimum, and maximum port latitude) were correlated with each other, only the number of biogeographic realms visited and maximum port latitude were used in the regression tree analyses. These variables were selected because they are expected to account for the potential diversity of source locations of biofouling organisms. Variables related to ship size (maximum length, wetted surface area, and gross tonnage) and typical sailing speed were also correlated with each other, therefore, only gross tonnage was used in the analyses.

Regression trees were constructed using the number of biogeographic realms visited, maximum port latitude, gross tonnage, port residence time, and age of the antifouling coating system (**Table 1**). A "region" variable was also included in the analyses to account for the potential effects of the sampling region (East Coast, West Coast, and Arctic) on biofouling extent on ships. For each analysis, the most parsimonious regression tree was selected by pruning the tree to the size where the optimal complexity parameter minimized the cross-validation error. The data were partitioned into a training set (70%) and a test set (30%). A 10-fold cross-validation procedure was used to train and validate the models. In addition, the percent variation (R^2) explained by each regression tree was calculated using the following equation, R^2 = 1–relative error (Sharma et al., 2012). The mean absolute error and root-mean-square error were calculated to measure the prediction error of the models. The data were also analyzed using random forest and boosted tree models, but since the predictions were no better than the simpler method, only the results of the regression tree analysis are presented. All regression trees were developed using the "rpart" package in R (Therneau et al., 2019).

The validated tree-based models were compared to the questionnaire survey data to infer the biofouling extent on respondent ships that visited the Canadian Arctic during 2015 and 2016. Agresti-Coull binomial confidence intervals (CIs, 95%) were calculated for these inferences, assuming the regression tree models were true. As ships often made multiple trips to the Arctic and, in some cases, made multiple stops in the Arctic during a single trip, only data from each unique ship's first visit to the Canadian Arctic during the study period were used in the analysis to avoid bias. Finally, the self-reported percent cover of biofouling estimates was compared to the inferred values generated by the model to determine the reliability of these estimates.

RESULTS

Ship Biofouling Questionnaire Survey

A total of 86 questionnaire surveys were returned by 50 unique ships, a response rate of ∼25% of ships operating in the Canadian Arctic during 2015 and 2016 (J. P. Lehnert, Canadian Coast Guard, personal communication). More than one survey was returned by 15 ships that made multiple trips to the Canadian Arctic. Thirty-two ships submitted incomplete surveys, primarily missing information for the same survey questions (i.e., wetted surface area of the ship and remaining service life of antifouling systems installed). Bulk carriers (36%) were the most common respondent ship type, followed by general cargo ships (26%), tankers (18%), passenger ships (8%), tugs and supply ships (8%), and other ship types (4%), which roughly corresponds to the breakdown of ship types in the greater population of ships operating in the Canadian Arctic (Chan et al., 2012; Dawson et al., 2018). Gross tonnage of ships ranged from 119 to 68,870 tons (mean 20,178 tons; SD 16,459 tons), wetted surface area was between 380 and 25,000 m^2 (mean 6,840 m^2; SD 5,189 m^2), and typical sailing speed ranged from 6 to 15 knots (mean 12 knots; SD 2 knots). The newest ship was 1 year old, whereas the oldest ship was 47 years old (mean 15 years; SD 13 years). The age of the antifouling coating system ranged from 17 to 2,014 days (mean 614 days; SD 516 days). Ships spent between 54 and 335 days (mean 218 days; SD 72 days) per year at sea and between 30 and 277 days (mean 133 days; SD 65 days) per year at port.

Of the 46 ships that provided information on their antifouling systems, 18 had both antifouling coatings and marine growth prevention systems installed. All 38 ships with antifouling coatings used biocidal coatings, with two of these ships

TABLE 1 | Variables assessed in the regression tree analyses to develop tree-based models for predicting biofouling extent on ships.

Variable type	
Variables included	Total residence time (days) in port over the last 10 voyages prior to sampling
	Time (days) since last dry-docking (i.e., age of antifouling coating system)
	Number of Marine Ecoregions of the World realms visited over the last 10 voyages prior to sampling
	Maximum port latitude over the last 10 voyages prior to sampling
	Gross tonnage (t) of ship
	Ship biofouling sampling region in Canada (East Coast, West Coast, and Arctic)
Variables excluded	Typical sailing speed (knots) of ship
	Average port latitude over the last 10 voyages prior to sampling
	Minimum port latitude over the last 10 voyages prior to sampling
	Maximum length (m) of ship
	Wetted surface area (m^2)

Redundant variables were excluded from the regression tree analyses.

using biocidal coatings in combination with fouling-release coatings. Twenty-seven ships were fitted with marine growth prevention systems. Four ships did not have any antifouling systems installed.

Thirty-two ships provided self-reported estimates of percent cover of biofouling (mean 2% cover; SD 6%). While the majority (63%) of these ships claimed to have no biofouling (0% cover), one ship estimated 30% biofouling cover. The time since the last inspection ranged from 0 to 24 months (median four months) based on 14 ships that provided the date of their most recent inspection.

The 50 ships surveyed made 462 arrivals at 73 Canadian Artic ports during 2015 and 2016 (**Figure 1**); 32 ships made numerous stops during a single trip to the Arctic. Deception Bay, Québec, was the most visited port (30 visits per year), followed by Milne Inlet, Nunavut (21 visits per year), and Edward's Cove, Newfoundland and Labrador (10 visits per year). Ports of departure (most recent port-of-call) were concentrated in the Arctic and Temperate Northern Atlantic (Eastern North America and Western Europe; **Supplementary Figure 1**). The Canadian Arctic is connected by ship voyages to the Great Lakes and at least 9 of the 12 MEOW biogeographic realms (Arctic, Temperate Northern Atlantic, Tropical Atlantic, Temperate Northern Pacific, Tropical Eastern Pacific, Temperate South America, Temperate Southern Africa, Western Indo-Pacific, and Central Indo-Pacific) when considering the full list of the last 10 ports-of-call preceding arrival at a Canadian Arctic port (**Figure 2**).

Regression Tree Analyses

Regression tree analysis indicates that the age of the antifouling coating system was the only measured predictor for percent cover of biofouling on commercial ships in Canada (R^2 = 33.0%). Ships tended to have a higher percent cover of biofouling (mean 9.3% cover) when the antifouling coating system was older than 630 days (hereafter referred to as the percent cover model; **Figure 3A**). The mean absolute error and root mean square error for the percent cover model were 5.4 and 6.6, respectively. Age of antifouling coating system was also the only measured predictor for the total abundance of fouling invertebrates (R^2 = 19.1%). Ships were more likely to have a higher total abundance of fouling invertebrates (mean $10^{3.8256}$ -1 or 6,692 individuals) when the antifouling coating system was older than 354 days (hereafter referred to as the abundance model; **Figure 3B**). For the abundance model, the mean absolute error was 1.8 and the root-mean-square error was 2.3 (note that these values are logarithmically transformed). The number of biogeographic realms visited and port residence time were predictors for the richness of fouling species on ships (R^2 = 15.5%). Ships that visited at least three biogeographic realms or had port residence

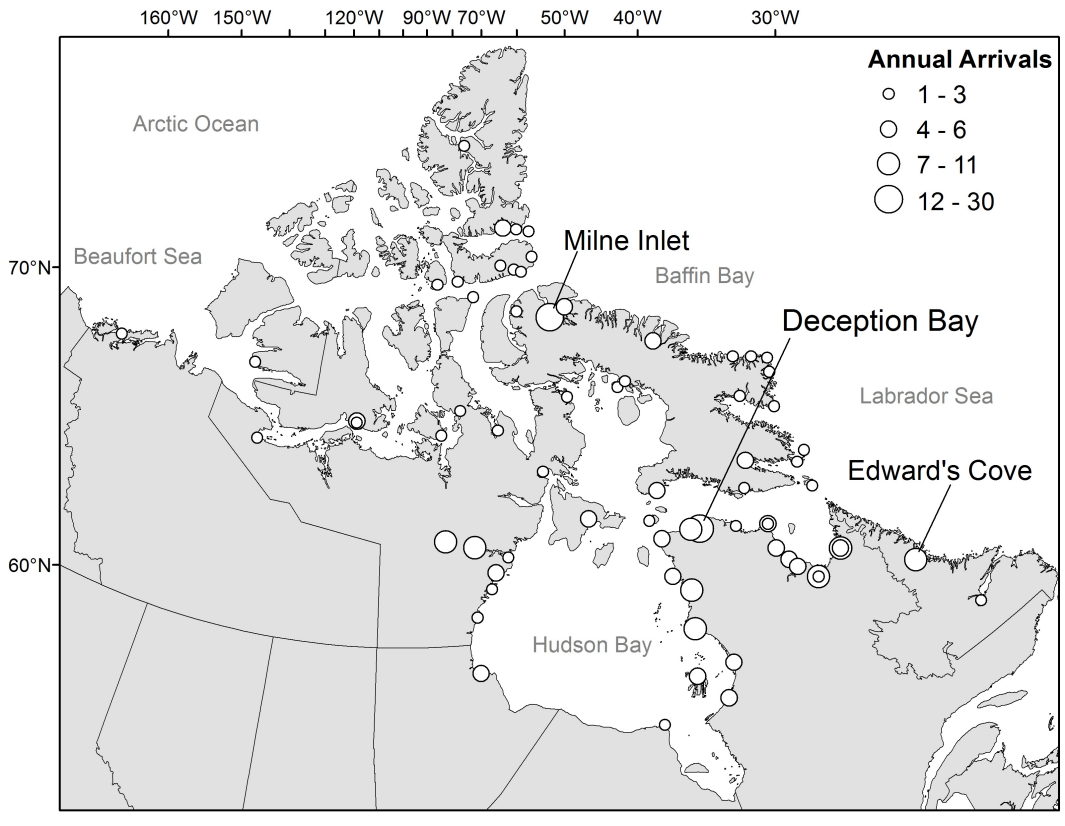

FIGURE 1 | Number of annual arrivals at 73 Canadian Arctic ports during 2015–2016, based on data collected from the questionnaire survey. The three most visited ports by respondent ships were Deception Bay, QC, Milne Inlet, NU, and Edward's Cove, NL.

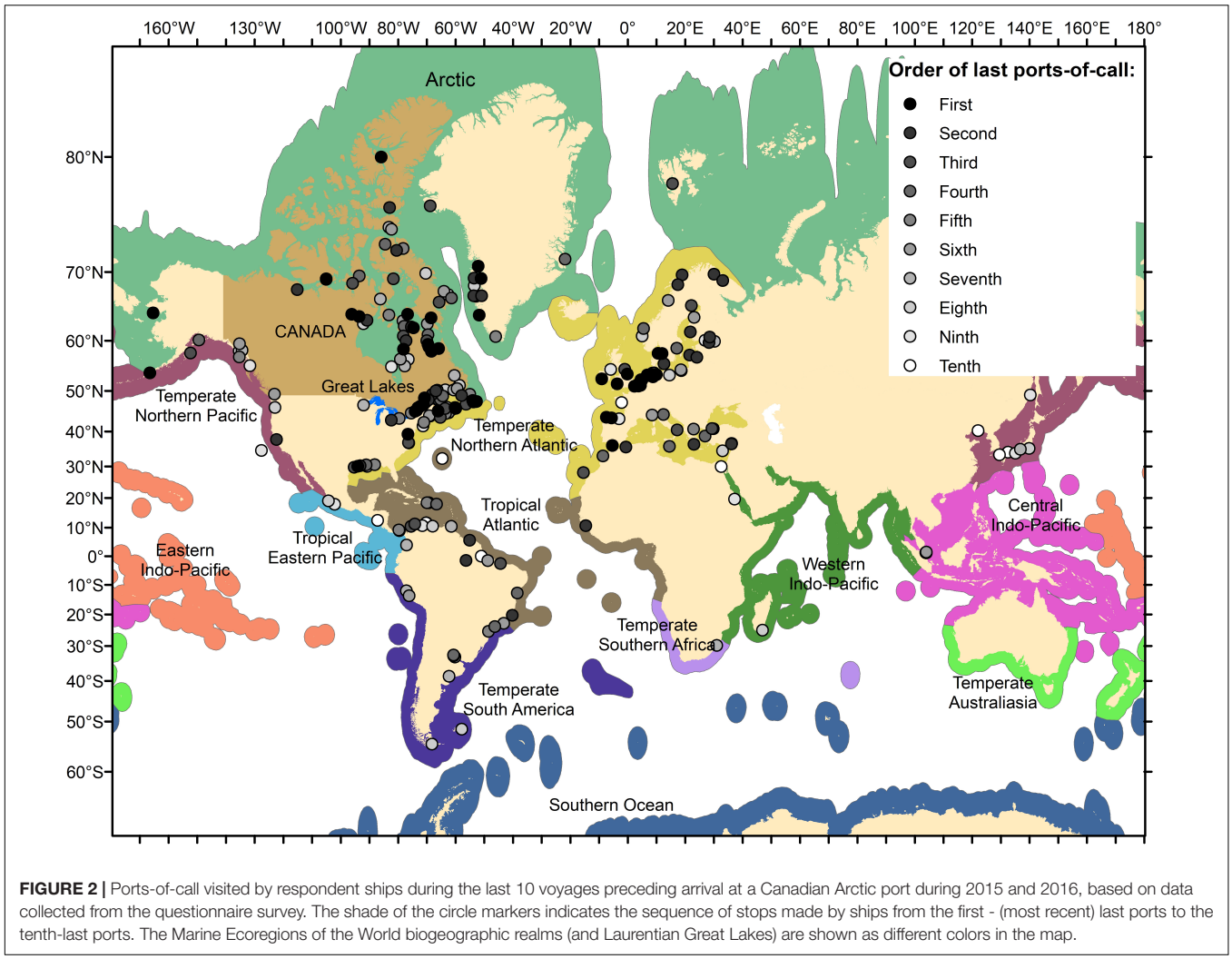

FIGURE 2 | Ports-of-call visited by respondent ships during the last 10 voyages preceding arrival at a Canadian Arctic port during 2015 and 2016, based on data collected from the questionnaire survey. The shade of the circle markers indicates the sequence of stops made by ships from the first - (most recent) last ports to the tenth-last ports. The Marine Ecoregions of the World biogeographic realms (and Laurentian Great Lakes) are shown as different colors in the map.

times ≥ 20 days tended to have higher fouling species richness (mean 42 taxa and mean 30 taxa, respectively; hereafter referred to as the species richness model; **Figure 3C**). The mean absolute error and root mean square error for the species richness model were 28.8 and 36.2, respectively.

Comparison of the relevant travel history features reported by ships via the questionnaire to the regression tree models provides insight on the biofouling extent of ships visiting the Canadian Arctic during 2015 and 2016. A large proportion of respondent ships (41.9% of 43 ships) had antifouling coating systems older than 630 days and are therefore inferred to have relatively high (> 9.3%) percent cover of biofouling according to the percent cover model (95% CIs: 28.4–56.7% of ships; **Figure 3A**). In contrast, self-reported estimates of percent cover of biofouling from 32 ships indicated that only 6.3% of them had > 9.3% biofouling cover. More than half of respondent ships (62.8% of 43 ships) had antifouling coating systems older than 354 days and are therefore inferred to have a relatively high total abundance of fouling invertebrates (> 6,692 individuals) according to the abundance model (95% CIs: 47.8–75.7% of ships; **Figure 3B**). Nearly half of respondent ships (45.9% of 37 ships) had visited at least three biogeographic realms during their last 10 ports-of-call, and are therefore inferred to have relatively high fouling species richness (mean 42 taxa) according to the species richness model (95% CIs: 31.0–61.6% of ships), while the remaining respondent ships had port residence times longer than 20 days and are inferred to have medium fouling species richness (mean 30 taxa; 95% CIs: 38.4–69.0% of ships; **Figure 3C**).

DISCUSSION

Our tree-based models of biofouling extent (coverage, abundance, and richness) suggest that ships are an active vector for the transportation of non-indigenous aquatic species to Canadian Arctic coastal ecosystems via biofouling. The probability of introducing non-indigenous species to the Canadian Arctic via biofouling is expected to be higher at ports that receive greater numbers of ship arrivals, such as Milne Inlet, Deception Bay, and Edward's Cove (see also Chan et al., 2013; Goldsmit et al., 2019).

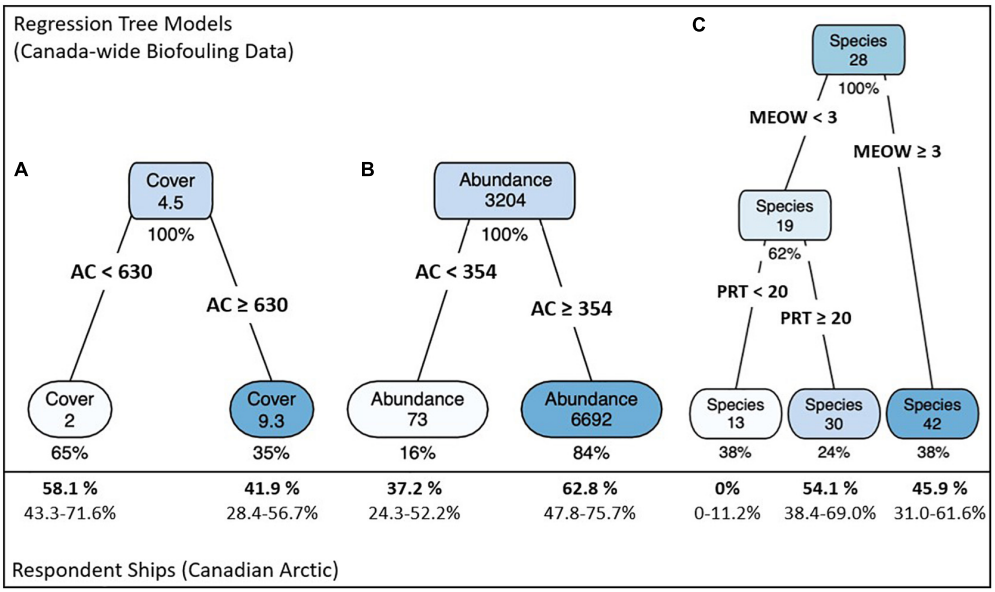

FIGURE 3 | Regression trees for predicting the percent cover of biofouling **(A)**, total abundance of fouling invertebrates [log 10 (x + 1) transformed] **(B)**, and fouling species richness **(C)** on commercial ships, based on 53 commercial ships arriving at Halifax, NS, Vancouver, BC, and Churchill, MB. The measured predictors of biofouling extent were age (days) of antifouling coating system (AC), total port residence time over the last 10 ports-of-call (PRT), and number of Marine Ecoregions of the World biogeographic realms (MEOW) visited over the last 10 ports-of-call. The proportion of respondent Canadian Arctic ships that fall under each terminal node of the regression tree models, based on questionnaire data, are shown in the lower box in bold, with 95% Agresti-Coull binomial confidence intervals.

Mapping of the last 10 ports-of-call prior to visiting the Canadian Arctic reveals that the region is highly connected to the rest of the world via shipping activity. As fouling species accumulate on ship wetted surfaces over time, any of these ports (and ports beyond the last 10 ports-of-call) could be sources of fouling species if they survive transit to the Arctic. Repeated measures of fouling communities on ships traveling to and from the Canadian Arctic have demonstrated the potential for fouling species to survive transits from temperate to Arctic ports (Chan et al., 2016). Additionally, Canadian Arctic ports are connected to numerous ports in regions (e.g., Arctic, Temperate Northern Atlantic, and Temperate Northern Pacific) that have similar environmental conditions (Chan et al., 2012). Biofouling organisms originating from these ports would have higher probability of survival if introduced to Canadian Arctic ports, especially for Arctic ports that have relatively milder environmental conditions, such as those in Hudson Bay (Chan et al., 2012; Goldsmit et al., 2020, 2021).

The questionnaire also provided insight into the antifouling practices of ships operating in the Canadian Arctic. At least four respondent ships did not have any antifouling system installed; this value may be an underestimation as some respondent ships did not provide information on their antifouling systems, and those that did may not be representative of the population of vessels (i.e., those willing to respond to a voluntary questionnaire may exhibit different management behaviors than those that did not respond). While the questionnaire survey was an inexpensive method to collect general data from a relatively large number of ships, this study demonstrates that self-reported estimates of percent cover of biofouling are unreliable since they were typically much lower in comparison to the regression tree analysis. Therefore, self-reported estimates should not be considered as reliable proxies for direct measurements of biofouling. Furthermore, even for the collection of factual data on operational profile and antifouling management practices, many ships submitted incomplete surveys, and some ships that submitted multiple surveys provided different information on each survey. The reliability and degree of questionnaire survey completion could be improved if ships used a vessel-specific biofouling management plan and biofouling record book to standardize and organize information on biofouling management measures undertaken on the ship (IMO, 2011; Scianni et al., 2021). In addition, the level of biofouling could be more accurately estimated with regular underwater inspections specific to this purpose (e.g., Georgiades and Kluza, 2020).

The tree-based models indicate that age of antifouling coating system, among a suite of variables known to influence biofouling extent on ships, was the only significant predictor of percent cover of biofouling and total abundance of invertebrates on commercial ships operating in Canada (i.e., including any additional variables did not improve the model). For richness of fouling species on ships, the number of biogeographic realms visited and port residence time were identified as significant predictors. Other variables known to influence biofouling, such as sailing speed (Coutts et al., 2010; Arndt et al., 2021), were not significant predictors of biofouling in the present study. However, caution must be taken when interpreting these model results since they have relatively low predictive power, explaining only 15–33% of the variance in biofouling extent. This indicates that the sample size was too low to elucidate relationships among

the numerous variables known to influence biofouling or that there could be additional variables, not measured here, that are important determinants of biofouling extent. For example, the quality of the application of antifouling coating systems varies based on price, experience, and condition of shipyards (e.g., preparation of hull surface), potentially affecting the performance of antifouling coating systems at preventing the accumulation of fouling organisms (Swain et al., 2007). Furthermore, the optimal antifouling coating system varies among ships based on their operational conditions (e.g., sailing speed, time at sea/port, and water conditions; Yebra et al., 2004; Swain et al., 2007). Thus, there is the potential to install sub-optimal antifouling coating systems on ships, which would reduce their efficacy (Swain et al., 2007). In addition, the number, size, and configuration of niche areas on ships can influence the accumulation of fouling organisms (Arndt et al., 2021), but these were not examined in the present study.

This study demonstrates that commercial ships are arriving to the Canadian Arctic after visiting a diverse array of global ports. As a relatively large proportion of these ships also have antifouling coating systems at least 630 days old (or none at all), the potential risk for the introduction of non-indigenous species is evident. Determining the actual introduction probability attributed to biofouling would require understanding the number of propagules released from ships' wetted surfaces, their survivorship in recipient Arctic environments, and the relationship between propagule pressure and probability of establishment. Despite being costly and resource-intensive, additional underwater biofouling assessments are warranted to better understand changes in trade patterns, developments in antifouling technologies, and other biofouling management practices. Larger, region-specific biofouling datasets, combined with paired survey information (i.e., operational profile and antifouling practices), are essential to improve training, testing, and ground-truthing of predictive models such as those used in this study.

DATA AVAILABILITY STATEMENT

The original contributions presented in this study are publicly available at https://doi.org/10.5061/dryad.5x69p8d4q and https://doi.org/10.5061/dryad.hdr7sqvkb.

ETHICS STATEMENT

Ethical review and approval were not required for the questionnaire portion of this study in accordance with local legislation and institutional requirements, as the individuals responding to the questionnaire were not themselves the focus of the research.

AUTHOR CONTRIBUTIONS

FTC designed the study and led data collection and analysis under the direction of SAB. DO and SAB led the drafting and revision of the manuscript. All authors contributed to writing the manuscript and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.808055/full#supplementary-material

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The Effect of Grooming on Five Commercial Antifouling Coatings

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The majority of ships are coated with antifouling paint. These coatings can fail to completely protect from fouling due to mismatches between paint type and duty cycle, the presence of biocide tolerant fouling organisms, improperly applied, old or damaged paint, etc. Grooming antifouling coatings can provide a solution. Five commercially available antifouling coatings were applied to panels. Half of the replicates were groomed weekly, the other half were immersed and allowed to freely foul, undisturbed. Photographs were taken and panels were visually assessed monthly. Over the period of two years, all the undisturbed panels became fouled with a diverse community of macrofouling organisms including encrusting and arborescent bryozoans, barnacles, tube worms, oysters, tunicates and more. The groomed panels remained clean of macrofouling for an extended period of time, up to two years depending on the coating. Cover of biofilm was also low on groomed panels. Grooming was effective at maintaining different antifouling paints clear of macrofouling and decreasing cover of biofilms for up to two years of immersion.

Keywords: grooming, antifouling, copper, copper-free, non-indigenous species

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INTRODUCTION

Grooming is the gentle, proactive, habitual, mechanical maintenance of the submerged portion of ship hulls to maintain them free of the majority of fouling and debris (Tribou & Swain, 2010; Hearin et al., 2015; Hunsucker et al., 2018a; Hunsucker et al., 2018b; Hunsucker et al., 2019; Swain et al., 2022). Fouling increases drag, fuel costs, maintenance costs and wear and tear on ships, greenhouse gas emissions and increases the risk of entrainment and transfer of non-indigenous species (NIS) (Dafforn et al., 2011; Schultz et al., 2011; McClay et al., 2015; Davidson et al., 2016; Hunsucker et al., 2018b; Hunsucker et al., 2019). Typically fouling on ships is controlled using coatings, the majority of which contain antifouling biocides (Martin and Ingle, 2009; Dafforn et al., 2011; Tribou and Swain, 2017). Antifouling coatings can fail to completely protect from fouling due to mismatches between paint type and duty cycle, the presence of biocide tolerant fouling organisms, improperly applied, old or damaged paint and many others (Davidson et al., 2009; Dafforn et al., 2011; IMO, 2011; Sylvester et al., 2011; Davidson et al., 2016).

Between dry docking, the solution to a fouled ship hull has been to clean the hull. Hull cleaning is reactive, requires a diver operated device, which introduces health and safety risks, and can damage coatings and introduce non-indigenous species (NIS; IMO, 2011; McClay et al., 2015; Hearin et al., 2016; Hunsucker et al., 2018b; Scianni and Georgiades, 2019). Despite these drawbacks, cleaning can increase the life of the hull coating and return ships to near clean conditions (Schultz et al., 2011;

McClay et al., 2015; Georgiades et al., 2018; Hunsucker et al., 2018b; Hunsucker et al., 2019). Grooming has been proven to proactively provide the benefits of cleaning, without the risk of coating damage. Additionally, the ship is maintained free of fouling and does not show loss of performance associated with fouling (Hearin et al., 2015; Hearin et al., 2016; Tribou and Swain, 2017; Hunsucker et al., 2018b; Scianni and Georgiades, 2019).

Most ships have coatings containing copper (Martin and Ingle, 2009; Dafforn et al., 2011; Tribou and Swain, 2017). Due to concerns with copper accumulation in many ports, new biocides have been introduced to the market. Different biocides are more effective against some organisms, as organisms can become tolerant to biocides. Some examples include *Amphibalanus amphitrite*, *Watersipora subtorquata* and *Bugula neritina* which have all been shown to be tolerant to copper biocides (Weiss, 1947; Johnston and Keough, 2002; Piola and Johnston, 2006a; Piola and Johnston, 2006b; McKenzie et al., 2011; McElroy et al., 2017). Biocidal antifouling coatings use different modes of action to release their biocides. Ablative coatings work by a dissolution of the matrix, which slowly releases the biocide at a decreasing level over time as a leached layer builds up. Self polishing coatings work by hydrolysis of the paint matrix, resulting in a slow consistent release of biocide as long as there is sufficient movement of water over the hull (Lewis, 1998; Chambers et al., 2006; Almeida et al., 2007; Dafforn et al., 2011; Georgiades et al., 2018). Grooming may make antifouling coatings more efficient by minimizing the leached layer and maintaining high efficiency of biocide release (Tribou and Swain, 2017).

The International Maritime Organization has started working on a framework to decrease the transfer of invasive species on ship hulls. The framework highlights how practices that control and manage biofouling can reduce the risk of transferring non-indigenous species, improve hydrodynamic performance and may enhance energy efficiency and reduce ship emissions (IMO, 2011). Many countries are beginning to develop laws concerning hull condition of ships entering their territorial waters. The best practice is maintaining the ship hull as free of fouling as is practical (IMO, 2011; McClay et al., 2015; Scianni and Georgiades, 2019; Georgiades et al., 2020). Grooming has been shown to maintain a standard ablative copper antifouling coating free of macrofouling for up to 12 months and to decrease the amount of biofilm (Hearin et al., 2015; Hearin et al., 2016; Tribou and Swain, 2017; Hunsucker et al., 2018a; Hunsucker et al., 2019). The purpose of this study was to determine the ability of grooming to maintain five different commercial antifouling coatings free of fouling over long periods of time.

METHODS

All coatings were applied according to manufacturer's instructions as a complete system. Coatings consisted of one ablative copper (ACA), two self-polishing copper formulations (SPC1 & SPC2) which were supplied by Naval Surface Warfare Center – Carderock Division (NSWC – Carderock) as part of the intersite calibration project. The intersite calibration project is a regularly repeated test, performed at various test sites around the world (regularly Florida, California, Hawaii, Singapore, etc.). The same set of coatings undergo static immersion and are compared in terms of biofouling accumulation, community structure and fouling adhesion (Swain et al., 2000). Also included were two copper free formulations (CF1 & CF2) which were coated at Florida Institute of Technology (FIT). All coatings had three replicates that were immersed at the FIT static immersion test platform at Port Canaveral, Fl. A set of epoxy panels was included as a negative control. An additional set of three replicates of the antifouling coatings were randomized and placed on a backing plate which was installed on the FIT grooming test platform adjacent to the static immersion test platform. These coatings were groomed weekly using the robotic grooming robot described in (Hearin et al., 2015; Hearin et al., 2016; Hunsucker et al., 2018b; Hunsucker et al., 2019). No epoxy was included in the grooming because it has been shown to foul too quickly for grooming to be effective (Tribou and Swain, 2010) and could damage the grooming tool/brushes. The ACA coating is a standard which has been extensively tested (Tribou and Swain, 2015; Hearin et al., 2015; Hearin et al., 2016; Hunsucker et al., 2019).

Panels were assessed monthly over the span of two years. The static panels were removed from the water, photographed and visually assessed for percent cover (ASTM D6990, 2020). There are two data gaps (8/15 & 1/16) for the ungroomed CF coatings and the EPX where data was not able to be collected. The grooming panels were photographed *in situ* by a diver with a camera fitted with a water box. This enabled clear pictures regardless of water clarity and maintained the pictures at a set standoff distance and image size. Photographs were visually assessed in the lab following the same method as the static panels. Results are reported in terms of macrofouling cover and cover of specific taxonomic groups (i.e.: hydroid, encrusting bryozoan, barnacle, etc.). To compare fouling at the community level, a principal component analysis was run on the monthly average cover data. A series of one way ANOVAs was used to compare macrofouling cover and fouling cover by specific taxonomic groups.

RESULTS

Macrofouling cover was significantly higher on the ungroomed than the groomed surfaces (One-way Anova, $p<0.05$; **Figure 1**). Groomed surfaces did not accumulate macrofouling until nearly two years immersion. Ungroomed surfaces began to accumulate macrofouling after only a few months, despite biocides. Epoxy was typically 95% covered with macrofouling and greater, showing that fouling pressure was diverse and high throughout the experiment. The only exception was CF2. This copper-free coating began to accumulate macrofouling on the groomed surfaces after 11 months. Upon closer examination, the groomed replicates had begun to wear through, as shown by the appearance of light colored epoxy barrier coat. Despite this,

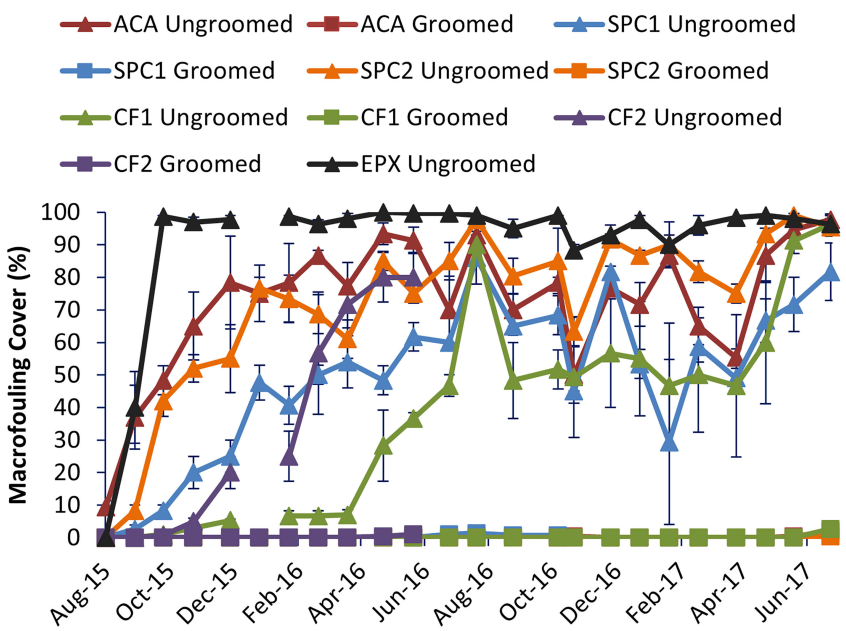

FIGURE 1 | Comparison of macrofouling cover among the different coatings. Error bars represent one standard error, n=3 replicate panels for each coating and grooming condition.

the groomed surfaces were able to be maintained in a clean condition at least 6 months longer than the ungroomed surfaces.

Biofilm cover was uniformly low to absent on groomed panels after grooming (**Figure 2**). On ungroomed surfaces, the entire surface not covered with macrofouling was typically covered with biofilm. That biofilm was usually thick and silty. On groomed surfaces, the biofilms that remained after grooming were thin and lacked surface debris. The biofilm on groomed

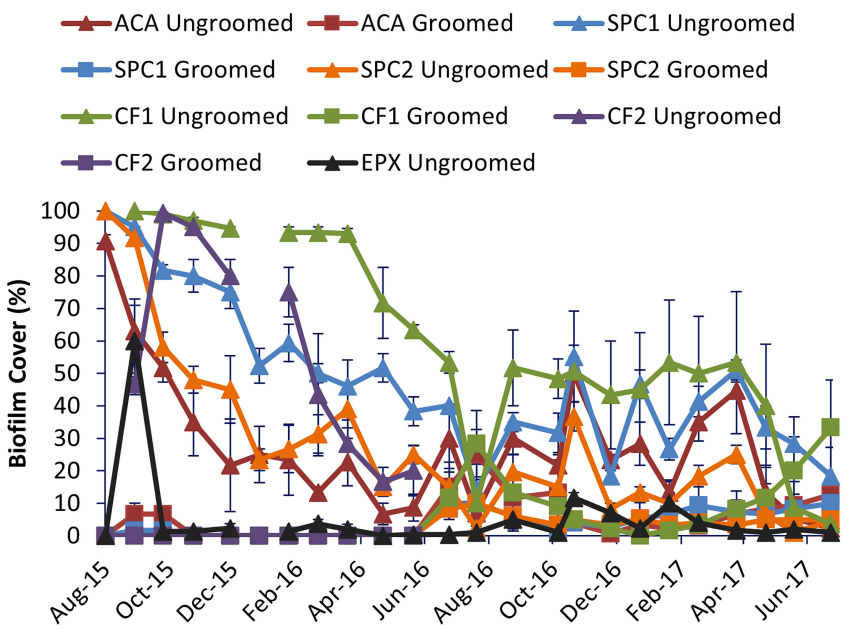

FIGURE 2 | Comparison of biofilm cover among the different coatings. Error bars represent one standard error, n = 3 replicate panels for each coating and grooming condition.

surfaces often resembled a surface stain, with no visible thickness (**Figure 5**).

Coatings differed in terms of macrofouling composition (One-way Anova, $p<0.05$; **Figures 3**–**5**). On ungroomed copper coatings, dominant macrofouling primarily consisted of encrusting bryozoans in the genus *Watersipora* and the barnacle *Amphibalanus amphitrite*. On the copper free coatings, dominant macrofouling primarily consisted of the arborescent bryozoan *Bugula neritina* and tube worms in the genus *Hydroides*. Composition on epoxy consisted of hydroids, encrusting and arborescent bryozoans, barnacles, tube worms, oysters, sponges, tunicates and sea anemones (**Figures 3**, **5**). The PCA (**Figure 4**) shows that most of the groomed surfaces cluster relatively closely together and are distinct from the ungroomed surfaces. The ungroomed surfaces are less closely grouped, but still make up one group, distinct from the groomed surfaces. The differences between groomed and ungroomed surfaces are primarily due to the cover of macrofouling and biofilms, with a lesser contribution from barnacles and tube worms. The first Principal Component is Macrofouling and accounted for 60% of the variability. The second split was Biofouling (24.6%) and the final split (8.8%) consisted of barnacles and tube worms combined.

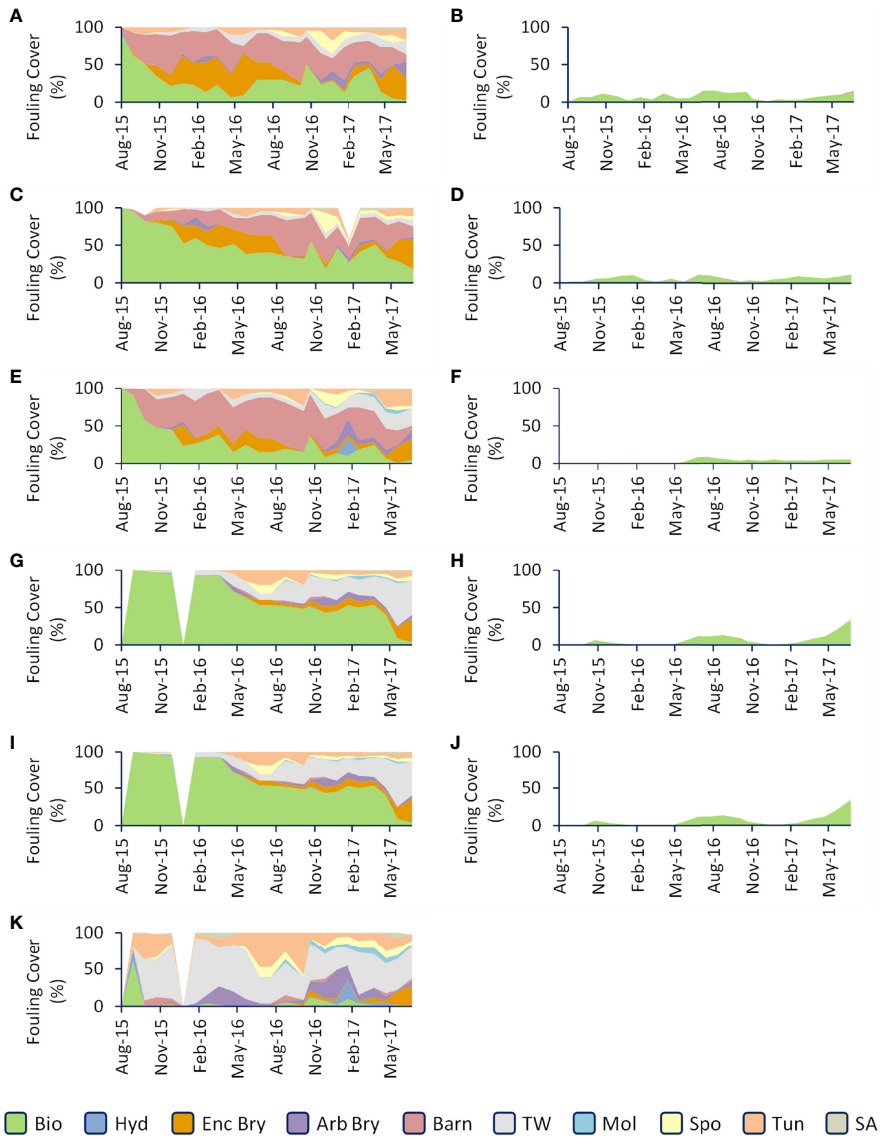

FIGURE 3 | Comparison of fouling composition among the coatings. The left graphs **(A, C, E, G, I, K)** represent ungroomed surfaces, the right graphs **(B, D, F, H, J)** represent groomed surfaces. Coating order is as follows: ACA **(A, B)**, SPC1 **(C, D)**, SPC2 **(E, F)**, CF1 **(G, H)**, CF2 **(I, J)** and EPX **(K)**. Bio, Biofilm; Hyd, Hydroid; Enc Bry, Encrusting Bryozoan; Arb Bry, Arborescent Bryozoan; Barn, Barnacle; TW, Tube Worm; Mol, Mollusc; Spo, Sponge; Tun, Tunicate; SA, Sea Anemone.

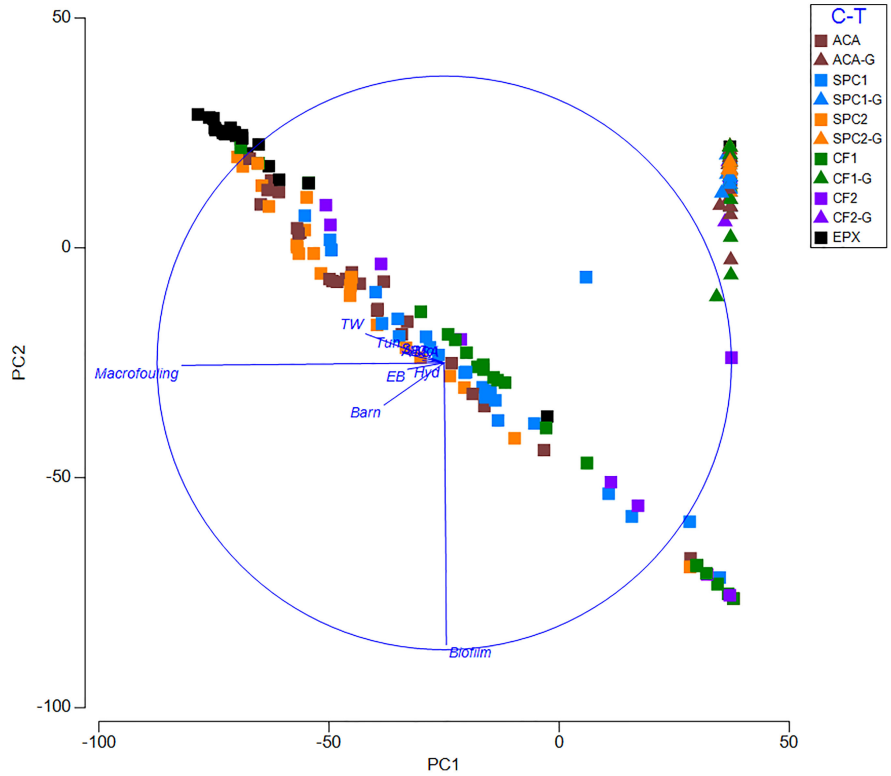

FIGURE 4 | PCA comparing the fouling communities on groomed (triangles) and ungroomed (squares) coatings.

DISCUSSION

Grooming has proven time and again to be an effective way to maintain ship hull coatings free of macrofouling. Previous experiments have focused on an ablative copper coating found on 90% of US Navy Ships, and have successfully kept macrofouling off the surfaces for up to 12 months (Martin and Ingle, 2009; Hearin et al., 2015; Tribou and Swain, 2017). This experiment looked at the efficacy of grooming on five commercially available, biocide containing, antifouling coatings over two years. These coatings included the previously tested ablative copper coating, two self-polishing copper coatings and two copper-free formulations. All coatings were able to be maintained free of macrofouling for 11 to 24 months using weekly grooming. Ungroomed coatings began to accumulate macrofouling after one to two months immersion, with greater than 50% cover in as little as four and as much as 12 months.

Organisms may have different tolerance to biocides. This is suggested by the different dominant organisms on the copper and copper-free coatings. Ungroomed copper coatings were dominated by *Amphibalanus amphitrite* and the encrusting bryozoan *Watersipora* species complex. Both taxa are reported to be tolerant of copper and both are NIS at this site (Weiss, 1947; Piola and Johnston, 2006a; McKenzie et al., 2011; McElroy et al., 2017; Tribou and Swain, 2017). Ungroomed copper-free coatings were dominated by *Hydroides* tube worms and *Bugula neritina* (NIS). There are no reports in the literature of organism tolerance to these alternative biocides, however, *B neritina* and serpulid polychaetes have been reported to be copper and pollution tolerant (Johnston and Keough, 2002; Piola and Johnston, 2006a; Piola and Johnston, 2006b). Grooming was effective at preventing macrofouling accumulation, regardless of growth form. Groomed surfaces were free of low growing forms (tube worms and encrusting bryozoans), higher growth forms (barnacles) and softer growth forms (tunicates and arborescent bryozoans).

Biofilms that remain after grooming were thin, patchy and lacked adhered sediment. On ungroomed surfaces, biofilms tended to cover all open space, were thick and had a fluffy appearance due to attached sediment and flocculated material. This was previously reported and quantified by Hunsucker et al. (2018a) who found that groomed biofilms were significantly thinner, with a different composition and had lower drag than ungroomed ones. The thin, adherent biofilm left behind after grooming has been labeled tenacious biofilm, to differentiate it from standard biofilms accumulated during static immersion (Hearin et al., 2015; Hearin et al., 2016; Hunsucker et al., 2018a).

The length of effect of grooming varied among the coatings. The copper coatings remained macrofouling free and with very low biofilm cover, consisting primarily of low-form, tenacious biofilms for the full two years. SPC2 was especially effective, with only low cover of biofilm over the entire experiment. The

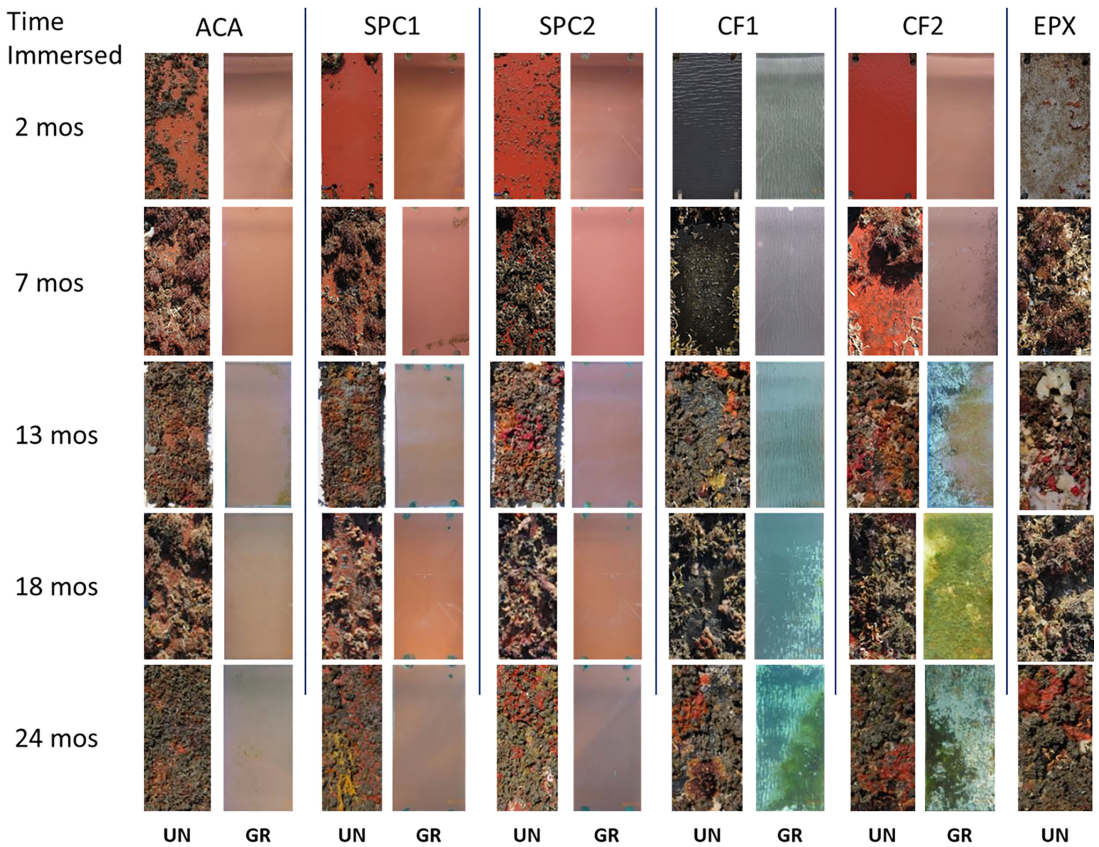

FIGURE 5 | Representative images of the panels over the duration of the experiment. UN is ungroomed, GR is groomed. Panels measured 15x30.5 cm (6x12").

copper-free coatings were more variable. CF2 was ended early because of excessive coating loss, as observed in the wear through of the coating. By the end of the experiment, CF1 was also starting to show coating loss. No estimate of coating loss on the ungroomed surfaces could be made because of the high cover of macrofouling hiding the surface of the coatings. The copper coatings did not have visible coating loss and maintained high efficacy. This has been shown in previous experiments, where coating loss was similar among groomed and ungroomed ablative copper coatings over a six year study (Tribou and Swain, 2017). Coating thickness could not be measured in this study because base panel material was not metallic and destructive methods could not be performed on the static panels, which were part of a larger experiment. However, observationally, copper coatings did not show evidence of the loss of even the top layer of coating. Additionally, no plume of paint was observed during grooming as is often observed during cleaning.

The push to replace highly toxic tributyl tin (TBT) led to an increase in copper based ship hull coatings and increased interest in developing alternative biocides. Recently, attention has turned to controlling invasive species transport on ship hulls. IMO developed guidelines to manage biofouling to manage introduction of non indigenous species (IMO, 2011). This standard recommends that ship hulls be maintained as free of fouling as is practical (IMO, 2011). In response many countries, including New Zealand, Australia and the United States, have begun to develop or have developed hull biofouling standards for ships to enter territorial waters with a clean hull (Cunningham et al., 2019; Scianni and Georgiades, 2019; Georgiades et al., 2020). Grooming was shown, in this experiment, to maintain five antifouling coatings clean of macrofouling for extended periods of time. A ship could fulfill "clean hull" requirements in a hull management plan through the use of hull grooming.

CONCLUSIONS

Grooming provides long term control of macrofouling on five different antifouling coatings. All copper coatings remained essentially free of macrofouling and with reduced biofilms for two years. The copper-free coatings worked well with grooming, until the coatings began to wear through. This provides many potential benefits including lower fuel costs and less wear and tear on engines due to reduced drag, less green house gas emissions and a reduced chance of transporting invasive species due to reduction of macrofouling on the hull.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available. Requests to access the datasets should be directed to eralston@fit.edu.

AUTHOR CONTRIBUTIONS

ER was the primary author. KH was lead researcher on the static immersion panels. HG operated the grooming robot. GS is the director of the research group. All authors contributed to earlier drafts of the manuscript. All authors contributed to the article and approved the submitted version.

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