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Transferred biofilms and their influence on subsequent macrofouling colonization

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\textbf{ABSTRACT}

Biofilm organisms such as diatoms are potential regulators of global macrofouling dispersal because they ubiquitously colonize submerged surfaces, resist antifouling efforts and frequently alter larval recruitment. Although ships continually deliver biofilms to foreign ports, it is unclear how transport shapes biofilm microbial structure and subsequent macrofouling colonization. This study demonstrates that different ship hull coatings and transport methods change diatom assemblage composition in transported coastal marine biofilms. Assemblages carried on the hull experienced significant cell losses and changes in composition through hydrodynamic stress, whereas those that underwent sheltered transport, even through freshwater, were largely unaltered. Coatings and their associated biofilms shaped distinct macrofouling communities and affected recruitment for one third of all species, while biofilms from different transport treatments had little effect on macrofouling colonization. These results demonstrate that transport conditions can shape diatom assemblages in biofilms carried by ships, but the properties of the underlying coatings are mainly responsible for subsequent macrofouling. The methods by which organisms colonize and are transferred by ships have implications for their distribution, establishment and invasion success.

\textbf{Introduction}

Ships’ hulls are commonly coated with biofilms that withstand the use of antifouling (AF) paints and cleaning programs designed to target macrofoulers (Cassé & Swain 2006; Tribou & Swain 2010; Hearin et al. 2016). Surveys have found that the two dominant components of marine biofilms, diatoms and bacteria, are present on all areas of ships’ hulls (Hunsucker et al. 2014), ships traveling through disparate latitudes (Leary et al. 2014) and surfaces exposed to water velocities > 18 m s\textsuperscript{-1} (Holland et al. 2004). Recruitment of macrofouling species is often driven by the presence and composition of these underlying biofilms, which serve as cues for larval settlement. Relationships between biofilm organisms and recruiting macrofoulers have been reviewed extensively (Wieczorek & Todd 1998; Hadfield & Paul 2001; Qian et al. 2007; Dobretsov et al. 2013; Salta et al. 2013) and continue to be the subject of much investigation (Lagos et al. 2016; Li et al. 2016; Shikuma et al. 2016; Watson et al. 2016; Yang et al. 2016). These studies suggest that two otherwise identical surfaces colonized by dissimilar biofilms can develop into divergent macrofouling communities based on the larvae and spores they attract. Coating type, port of origin and transport route form distinctive ship hull biofilms (Zargiel et al. 2011; Camps et al. 2014; Hunsucker et al. 2014; Leary et al. 2014). However, the mechanisms behind transport effects, and responses of macrofoulers to transport-altered biofilms, remain unknown.

Fouling communities are often modified by various disturbances that offer competitive advantages to some species while reducing fitness in others. Studies have demonstrated that benthic communities are altered by desiccation (Lenz et al. 2004; Hopkins et al. 2016), predation (Mook 1981; Swain et al. 1998) and other disturbances that expose substrata (Valdivia et al. 2005; Altman & Whitlatch 2007; Cifuentes et al. 2007) and create space for new recruits. However, these investigations have not considered transport of biological communities as a potential disturbance. Biofilms transported \textit{via} shipping are likely to encounter stresses that differentially remove species and change assemblage structure, while retaining some organisms that act as indicators of travel history and
cues for subsequent larval recruitment. A few studies have begun to establish a connection between vessel history and macrofouling composition. Floerl and Inglis (2005) found small but significant differences in macrofouling communities encrusting resident versus visiting vessels in three Australian marinas. In a separate investigation, the authors allowed macrofouling to accrue on test panels at two of these marinas (Floerl et al. 2005). Panels were then subjected to the cursory cleaning procedures common on recreational vessels, scraping off most fouling but leaving behind traces of soft tissue and shell. After panels were cleaned and reciprocally transplanted between the two marinas, subsequent macrofouling mirrored that of the original panel locations. Ralston and Swain (2014) took this experiment a step further by removing all traces of macrofouling and biofilms on panels, but still found lingering effects of panel history that lasted up to 14 months. Such studies demonstrate that recruitment can be determined by previous biological cues, which further underscores the need to consider biofilms as engineers of macrofouling transport and establishment.

Macrofouling communities that colonize hard surfaces are some of the most heavily invaded marine systems (Wasson et al. 2005), and most invasive species in these communities originate from translocation via ship hulls (Drake & Lodge 2007; Molnar et al. 2008; Hewitt et al. 2009; Chan et al. 2015). Dispersal risk is crucial in determining invasion potential (Molnar et al. 2008) and may be assessed by following persistence and survival through transport. Freshwater passages have been considered barriers to interocean transport of marine fouling organisms (Brock et al. 1999). However, introductions of at least 60 marine fouling-associated species are linked to on-hull freshwater transport through the Panama Canal alone (Cohen 2006). Diatoms in the precursor biofilms that lead to marine macrofouling (Zargiel et al. 2011; Sweat & Johnson 2013; Hunsucker et al. 2014) are often euryhaline (Wachnicka et al. 2010, 2011; Potapova 2011; Nodine & Gaiser 2014), yet no study has examined their persistence in biofilms transported through freshwater. Diatoms enduring freshwater transport are likely to survive many other routes and be broadly dispersed.

This study was conducted at the Okeechobee Waterway (OWW) in central Florida (USA), which bisects the peninsula and affords a unique opportunity to test the effects of freshwater transport on the survival of diatoms in coastal marine biofilms traveling between oceans. Over nine thousand vessels navigate the OWW each year, more than 90% of which are recreational (USACE 2016). Many of these boats travel the entire length of the waterway, potentially carrying biofilms between the Atlantic Ocean and Gulf of Mexico. Despite continuous traffic of fouled hulls, no attempts have been made to assess the potential of the OWW as a route for marine biofilm dispersal. This investigation addresses the following hypotheses: (1) mortality and assemblage composition of diatoms in transported biofilms differ among ship hull coatings and transport methods, and (2) macrofouling colonization on transported biofilms differs among ship hull coatings and transport methods.

**Methods**

**Study area**

The OWW stretches 248 km through central Florida from the Atlantic Ocean at Stuart to the Gulf of Mexico at Fort Myers (Figure 1), providing mariners with a protected route across the Florida peninsula since its construction in 1937. A series of five locks operate on a 12-hour cycle to raise and lower vessels between sea level and the elevated Lake Okeechobee (USACE 2016). Water flows westward from the lake through the Caloosahatchee River into the Gulf of Mexico at San Carlos Bay and eastward through the St Lucie River into the Atlantic Ocean at St Lucie Inlet. Salinity in the OWW varies seasonally, but is commonly < 1 ppt throughout due to terrestrial runoff and lake discharge (Wilson et al. 2005; Qiu & Wan 2013).

In this study, Atlantic coast biofilms cultivated at Fort Pierce were transported through the OWW to the Gulf of Mexico at Fort Myers (Figure 1). Fort Pierce and Fort Myers are coastal harbors with frequent recreational and commercial vessel activity. Both study sites are situated in estuarine marinas near inlets, Fort Pierce at Elizer’s Dock in Fort Pierce Inlet (27° 27.822’ N 80° 18.333’ W) and Fort Myers at Port Sanibel Marina in San Carlos Bay (26° 29.433’ N 81° 59.447’ W). However, the Fort Myers site is unique in that the freshwater of the OWW discharges into it directly. Fort Pierce has no measurable freshwater influence from the OWW, which begins 36 km to the south in Stuart (27° 10.152’ N 80° 11.301’ W).

**Recruitment panel preparation**

Natural, diatom-rich marine biofilms were cultivated on sets of nine 10 × 20 × 0.6 cm PVC panels. Each panel set included three hull coating treatments (n = 3 panels for each coating type): (1) International Intersleek® 700 silicone, biocide-free, foul-release coating (International Paint Ltd, Gateshead, UK), (2) Sherwin-Williams SeaGuard® 5000HS inert epoxy barrier coating (Sherwin-Williams, Cleveland, OH, USA) and (3) International Intersleek™ 700 silicone, biocide-free, fouling-release coating (International Paint Ltd, Gateshead, UK). All coatings were applied per manufacturers’ specifications to five sets, for a total of 45 panels. These coatings represent three different approaches to protection and antifouling commonly used on vessel hulls.
The AF action of biocidal coatings such as Interspeed® BRA640 is determined by the leaching rates of chemical compounds from the surface (Ferry & Ketchum 1952). These rates are considerably higher immediately after immersion, preventing most fouling when the coating is new. As the coating ages in seawater, leaching rates are reduced and biofouling begins to accrue. All coatings were aged for two months prior to field deployment to replicate coating conditions characteristic of most vessels. To accomplish this, panels were divided according to coating type and suspended in laboratory tanks containing 1-μm filtered seawater. This arrangement prevented biocide exchange among coating types during the aging process. Ultraviolet light (Turbo-Twist UV sterilizer, 9-W, 200 gph, Coralife, Franklin, WI, USA) was used to sterilize laboratory seawater, and tanks were covered with blackout material to minimize potential for pre-deployment biofilm growth. Seawater was exchanged thrice weekly for two months to accelerate biocide leaching and produce panels resembling seasoned hulls (Ferry & Ketchum 1952; Ketchum 1952).

**Biofilm cultivation and transport**

Panels were deployed in the field, four sets at Fort Pierce and one set at Fort Myers, in November 2014 following the two-month laboratory aging process. Using floating racks, all panels were suspended vertically in a stratified random design about 0.5 m below the water line (Ralston & Swain 2014). The transport experiment was launched after three weeks of biofilm growth, when macrofouling recruitment was about to begin. Atlantic coast panels were randomly divided into four transport treatments (Figure 2). For the first treatment, termed stationary Atlantic (SA), panels were briefly removed to a tank with recirculating Fort Pierce water before being back-transplanted to their original location as a control treatment. These panels were held in the recirculating tank for approximately 13.5 h, equivalent to the transit time of panels from the other transport treatments. The remaining three sets were transported through the OWW using three different transport methods: (1) transport (T) in an on-board recirculating tank with water from the port of origin, (2) transport with changing water quality (TW) in an on-board flow-through tank drawing ambient water from the OWW and (3) transport with changing water quality and hydrodynamic conditions (TWH) on the vessel hull. TWH panels were attached to a plate that formed an integral part of the hull via insertion through a wet well built into the aft section of the vessel (Swain et al. 2007). Except for some macroalgal filaments that were contained in the biofilm assemblages and therefore left undisturbed, no macrofouling recruitment was detected on the test surfaces. Any macrofouling on the uncoated panel surfaces was removed prior to transport.

![Figure 1. Map of the Okeechobee Waterway spanning the central Florida peninsula. Gray dots indicate locations where water quality and flow rate data were collected during transit. Concentric circles denote biofilm cultivation sites.](image-url)
Westbound $T_W$ and $T_{WH}$ panels were first exposed to OWW water at the east entrance of the waterway in Stuart. During the brief transit from Fort Pierce to Stuart, all panels were transported in recirculating tanks with Fort Pierce water before being assigned to their official treatments prior to entering the OWW. As part of this transition, biofilms on $T_W$ and $T_{WH}$ panels were gradually acclimated to ambient water conditions. Once all panels were in place, the 13.5-h transit commenced across the OWW to the Fort Myers study site, where all panels were then submerged (0.5 m depth) to follow subsequent macrofouling recruitment on the transported biofilms. Joining the panels from the Atlantic coast were those initially deployed at Fort Myers. This panel set acted as a stationary Gulf ($S_A$) location control (Figure 2), which was back-transplanted in a way analogous to that of $S_A$ described above.

**Environmental data collection**

At both deployment sites and regularly during transport through the OWW (Figure 1, gray dots), data were collected for environmental variables that could affect diatom persistence and survival. A Yellow Springs Instruments Pro Plus handheld multiparameter meter (YSI Inc., Yellow Springs, OH, USA) was used to measure salinity (ppt), water temperature (°C), dissolved oxygen (mg l$^{-1}$) and pH in all treatments. During transit, vessel speed was used as the flow rate (m s$^{-1}$) experienced by $T_{WH}$ biofilms (limit of detection 0.1 kts = 0.05 m s$^{-1}$). Flow rates over $T$ and $T_W$ biofilms were calculated based on container volume and pump flow rates. Logged data from a field data recorder (Indian River Lagoon Observatory Network of Environmental Sensors, Florida Atlantic University Harbor Branch, fau.loboviz.com) located in the Fort Pierce Inlet area provided conditions for the $S_A$ treatment panels while the other panels were in transit.

**Collection and analysis of diatoms in biofilms**

Before and after the transport period, a sterile polyethylene cell lifter was used to scrape a 0.9 × 6.0 cm band of biofilm from each panel. All samples were immediately preserved in 4% formalin pending analysis in the laboratory. Aliquots (100 μl) of each sample were analyzed via light microscopy at a total magnification of 400–1000× until ≥300 cells were counted (BS EN 14407:2004 2004). Diatoms were enumerated and identified to the lowest possible taxonomic level using sources listed in Zargiel et al. (2011) and Sweat and Johnson (2013), and cell densities were normalized by volume for comparison among samples. A concurrent investigation revealed that two stains commonly used for live/dead determination, vital neutral red and mortal Evans blue, failed in most instances when applied to diatoms. Therefore, diatoms were categorized as live if chloroplasts were retained and cell integrity was uncompromised. All other cells were labeled as ‘dead’ (Knoechel & Kalff 1978; Beninger & Decottignies 2005; Beninger et al. 2008).

**Macrofouling recruitment on transported biofilms**

Some macrofoulers grow as solitary individuals, while others form sprawling colonies of clones. Percentage cover (resolution 0.7%) was used to analyze recruitment among macrofoulers with diverse growth strategies. Macrofouling
development was visibly different between the scraped and undisturbed areas of the panels. Therefore, percentage cover for all detectable macrofouling species was calculated in an undisturbed area of each panel (3.8 x 17.8 cm) by visual assessment of photographs using a modified ASTM standard (ASTM D6990-05 2005). Digital images were overlaid with 136-cell grids and the total number of cells containing the target species was counted. Through dissection and photomicroscopy of voucher specimens, each species was identified to the lowest possible taxonomic level. This process was performed at three weeks post-transport, when new recruits first became discernible, and repeated at 14 weeks post-transport.

**Statistical analyses**

All multivariate community analyses of diatoms and macrofoulers were conducted with PRIMER-E version 5 software (PRIMER-E, Auckland, New Zealand). Univariate analyses were completed with the XLSTAT Base version 2016.5 (Addinsoft, New York, NY, USA) add-on for Microsoft Excel (Microsoft, Redmond, WA, USA).

**Diatom assemblage composition**

Diatom assemblage composition was compared among (1) coating types and (2) transport treatments with a two-way multivariate analysis of similarities (ANOSIM, \( \alpha = 0.05 \)) based on Bray–Curtis similarities (Clarke 1993). These comparisons were conducted among assemblages before and after transport, and among assemblages exposed for macrofouling at the destination, which included the stationary Gulf (\( S_G \)) location controls. All data were square-root transformed and taxa comprising \( \leq 1\% \) of the total abundance across all treatments were excluded (Lavoie et al. 2009). Similarity percentage (SIMPER) analysis was used to identify key species that characterized each treatment and contributed to differences among treatments. Non-metric multidimensional scaling (NMDS) ordinations were used to visualize those differences. The stress value associated with each ordination is a measure of its goodness of fit. A value \( \leq 0.2 \) indicates that the relationships among samples were accurately depicted by the 2-D ordination (Clarke & Warwick 2001).

**Diatom abundance and mortality**

Diatom abundance and mortality were assessed among treatments via univariate analyses. Total densities of live diatoms and live densities of each key species were compared before and after transit within each coating type. Two-way ANOSIM results from pre-transport Fort Pierce biofilms revealed no significant differences among diatom assemblages within any coating type. Therefore, pre-transport samples were pooled (\( n = 12 \)) to characterize baseline diatom assemblages for each coating. Abundances of each key species from baseline assemblages were then compared to those in post-transport assemblages from each transport treatment (\( n = 3 \) for each). All before/after comparisons were analyzed on untransformed diatom densities using Student's \( t \)-tests or unequal variance \( t \)-tests (\( \alpha = 0.05 \)), as determined by Cochran's \( C \) tests for homoscedasticity. In \( T_w \) and \( T_{WH} \) treatments, where there were \textit{a priori} expectations of diatom mortality, one-tailed tests were used. Two-tailed tests were used for all other treatments. To assess the effects of coating type on total diatom abundance, one-way analyses of variance (ANOVA, \( \alpha = 0.05 \)) with Tukey's HSD \textit{post hoc} tests were used after Cochran's \( C \) tests confirmed homoscedasticity. Total densities of live diatoms were compared among coating types within each transport treatment, both before and after transport (\( n = 3 \) for all groups).

**Macrofouling recruitment**

The effects of coating type and transport treatment on subsequent macrofouling recruitment were tested using (1) multivariate analyses to detect community-level differences and (2) univariate analyses to detect species-level differences (Ralston & Swain 2014). Variations in recruitment at the species level may not be dramatic enough to affect overall community structure, especially for new recruits and small organisms such as spirobolid polychaetes, but they are nevertheless important for evaluating the colonization of individual taxa. Therefore, both methods were selected \textit{a priori} to provide a complete account of macrofouling colonization among the treatments. Macrofouling percentage cover data were converted to proportions and arcsine-transformed prior to all analyses.

**Community-level differences**

A two-way ANOSIM was employed to compare overall macrofouling community composition among (1) coating types and (2) transport treatments. NMDS ordinations were used to visualize differences among communities, and SIMPER analysis was used to identify key species that contributed to those differences.

**Species-level differences**

Univariate analyses were used to detect differences in the percentage cover of each species observed in the communities. Following Cochran's \( C \) tests, one-way ANOVAs (\( \alpha = 0.05 \)) with Tukey's HSD \textit{post hoc} tests were utilized for sample groups with equal variance. For groups with unequal variance, Kruskal-Wallis ANOVAs (\( \alpha = 0.05 \)) with Dunn's \textit{post hoc} tests were used. To compare percentage cover to other treatments where that species was absent, 95\% confidence intervals were applied (Cumming & Finch 2005; Sweat & Johnson 2013). Macrofoulers were
structure in biofilms (Wachnicka et al. 2010; Hunsucker et al. 2014; Nodine & Gaiser 2014; Zargiel & Swain 2014). $S_A$ biofilms (Figure 3a) were subjected to relatively small fluctuations in salinity (31.5–35.6 ppt) and flow (0.1–0.6 m s$^{-1}$) caused by tidal exchange at the Atlantic coast study site. $T$ biofilms (Figure 3b) were transported in coastal water (35.6 ppt salinity) with very low flow ($3.5 \times 10^{-4}$ m s$^{-1}$). $T_W$ biofilms (Figure 3c) experienced low flow ($3.4 \times 10^{-3}$ m s$^{-1}$), but widely fluctuating salinities (0.18 to 36.5 ppt) from the OWW. Finally, $T_{WH}$ biofilms (Figure 3d) experienced the same variable salinities as $T_W$ but with the widely fluctuating flow rates ($< 0.05$ to 10.8 m s$^{-1}$) encountered on the hull. Temperature (16.4–22.4°C), dissolved oxygen (3.5–8.6 mg l$^{-1}$) and pH (7.3–8.6) also varied during transport, but ranges stayed within those experienced by Florida coastal diatoms (Badylak & Philips 2004; Wachnicka et al. 2010, 2011; Zargiel et al. 2011).

Diatom recruitment and portability in biofilms

A total of 96 diatom species (83 pennate and 13 centric) were identified across all treatments, 74 of which were found in two or more treatment groups (Figure 4). The remaining rare taxa were present on only one transport treatment from the following coatings: *Achnanthes manifera*, *Cocconeis* sp. 2, *Lyrella* sp. 1, *Rhopalodia* sp. and an unknown centric species from copper; *Amphora* sp. 1, *Cyclophora* sp., *Licmophora ehrenbergii*, *Nitzschia longissima*, *Nitzschia* sp. 3, *Nitzschia* sp. 5, *Plagiogrammopsis vanheurckii*, *Staurosirellaa/Staurosira* sp., *Biddulphia tridentis, Eunotogramma leave, Eunotogramma* sp. 1 and *Triceratium bicone* from epoxy; and *Glyphodesmis* sp., *Licmophora remulus*, *Lyrella clavata* and Unknown pen- nate sp. 1 from silicone. Coating type was the major driver of diatom assemblage composition before and after transport (Table 1, Figure 5a and c). The dominant baseline species on each coating type remained dominant following transport, including: *Amphora cf. montgomeryi, Navicula* sp. 2 and *Nitzschia fonticola* on copper; *Navicula* sp. 4 and *N. fonticola* on epoxy; and *Cylindrotheca closterium* on silicone. Although total live diatom densities were initially higher on epoxy than both other coating types ($p << 0.001$), this pattern was not maintained following transport. Regardless of transport method, final live diatom densities did not differ among coatings.

Transport method altered diatom survivorship and was a secondary factor affecting assemblage composition (Table 1, Figure 5b and d). Live diatoms were reduced by $T_{WH}$ transport on all three coating types ($p \leq 0.045$) (Figure 6a, c and e). Reductions were also observed in $T$ and $T_W$ epoxy ($p \leq 0.023$) and in $S_A$ copper biofilms ($p = 0.023$). However, live diatoms persisted on all panels regardless of coating type or transport method, ranging

categorized as having positive or negative selectivity for a given treatment based on a significantly higher or lower percentage coverage, respectively, than one or more other treatments in the analyzed group.

Results

Environmental conditions

The four transport treatments were defined mainly by different salinities and flow rates (Figure 3), which are also two of the most probable factors affecting diatom assemblage
Figure 4. Quantitative inventory of 74 live diatom species before and after transport on copper, epoxy and silicone coatings distributed among four transport treatments: stationary Atlantic (SA), transport (T), transport with changing water quality (TW), and transport with changing water quality and hydrodynamic conditions (TWH). Final diatom densities on stationary gulf (SG) panels were also included. Densities are categorized as absent (0 cells cm⁻², white), low (<100 cells cm⁻², light gray), moderate (100–1,000 cells cm⁻², dark gray) and high (>1,000 cells cm⁻², black). Asterisks indicate key species contributing to assemblage composition.
Table 1. Results of two-way ANOSIM analyses of coating and transport effects on the composition of diatom assemblages transported from Fort Pierce, diatom assemblages exposed for macrofouling at Fort Myers, and the subsequent macrofouling communities developed at Fort Myers.

<table>
<thead>
<tr>
<th>Corresponding NMDS</th>
<th>Pre-transport diatoms</th>
<th>Post-transport diatoms</th>
<th>Diatoms exposed for macrofouling</th>
<th>Macrofouling at week 3</th>
<th>Macrofouling at week 14</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Figure 5a and b</td>
<td>Figure 5c and d</td>
<td>Figure 7a and b</td>
<td>Figure 7c and d</td>
<td>Figure 7e and f</td>
</tr>
<tr>
<td>Coating Global</td>
<td>0.790 0.001</td>
<td>0.587 0.001</td>
<td>0.600 0.001</td>
<td>0.371 0.001</td>
<td>0.625 0.001</td>
</tr>
<tr>
<td>C vs E</td>
<td>0.859 0.001</td>
<td>0.550 0.001</td>
<td>0.682 0.001</td>
<td>0.644 0.002</td>
<td>0.751 0.001</td>
</tr>
<tr>
<td>C vs S</td>
<td>0.861 0.002</td>
<td>0.639 0.001</td>
<td>0.713 0.001</td>
<td>0.032 0.292</td>
<td>0.468 0.005</td>
</tr>
<tr>
<td>T vs TWH</td>
<td>0.702 0.001</td>
<td>0.757 0.001</td>
<td>0.555 0.002</td>
<td>0.516 0.005</td>
<td>0.586 0.004</td>
</tr>
<tr>
<td>Transport Global</td>
<td>0.141 0.068</td>
<td>0.353 0.002</td>
<td>0.403 0.001</td>
<td>– 0.019</td>
<td>0.581 0.096</td>
</tr>
<tr>
<td>*S&lt;sub&gt;A&lt;/sub&gt; vs T</td>
<td>– –</td>
<td>0.319 0.074</td>
<td>0.494 0.021</td>
<td>– –</td>
<td>– –</td>
</tr>
<tr>
<td>*S&lt;sub&gt;A&lt;/sub&gt; vs T&lt;sub&gt;W&lt;/sub&gt;</td>
<td>– –</td>
<td>0.272 0.068</td>
<td>0.407 0.009</td>
<td>– –</td>
<td>– –</td>
</tr>
<tr>
<td>*S&lt;sub&gt;A&lt;/sub&gt; vs T&lt;sub&gt;WH&lt;/sub&gt;</td>
<td>– –</td>
<td>0.580 0.009</td>
<td>0.679 0.002</td>
<td>– –</td>
<td>– –</td>
</tr>
<tr>
<td>T vs T&lt;sub&gt;W&lt;/sub&gt;</td>
<td>– –</td>
<td>0.096 0.241</td>
<td>0.096 0.217</td>
<td>– –</td>
<td>– –</td>
</tr>
<tr>
<td>T vs T&lt;sub&gt;WH&lt;/sub&gt;</td>
<td>– –</td>
<td>0.541 0.016</td>
<td>0.541 0.017</td>
<td>– –</td>
<td>– –</td>
</tr>
<tr>
<td>T&lt;sub&gt;WH&lt;/sub&gt; vs T&lt;sub&gt;WH&lt;/sub&gt;</td>
<td>– –</td>
<td>0.531 0.016</td>
<td>0.531 0.010</td>
<td>– –</td>
<td>– –</td>
</tr>
</tbody>
</table>

Note: Table 1: Pairwise results are listed for copper (C), epoxy (E), and silicone (S) coatings and for the following transport treatments: stationary Atlantic (S<sub>A</sub>), stationary Gulf (S<sub>G</sub>), transport (T), transport with changing water quality (T<sub>W</sub>), and transport with changing water quality and hydrodynamic conditions (T<sub>WH</sub>). Bold text highlights global values and dashes indicate where pairwise comparisons were inappropriate due to non-significant (p > 0.05) global values. *<sub>S</sub> samples were used in pre- and post-transport diatom analyses. <sub>S</sub><sub>G</sub> samples were used in analyses of diatoms exposed for macrofouling, and macrofouling at three and 14 weeks.

Figure 5. 2-D NMDS ordination plots of diatom assemblages before and after transport on copper, epoxy and silicone coatings (a and c) distributed among four transport treatments (b and d): stationary Atlantic (S<sub>A</sub>), transport (T), transport with changing water quality (T<sub>W</sub>), and transport with changing water quality and hydrodynamic conditions (T<sub>WH</sub>). Stress is a dimensionless parameter.

from 9 cells cm<sup>-2</sup> on T<sub>WH</sub> copper to 24,640 cells cm<sup>-2</sup> on S<sub>G</sub> epoxy. Significant reductions (p < 0.05) were detected in the abundance of eight key diatom species: *Cylindrotheca closterium*, *Cocconeis* sp. 1, *Navicula* sp. 2, *Navicula* sp. 4, *Nitzschia fonticola*, *Nitzschia* sp. 1 and *Parlibellus delognei* (Figure 6b, d and f). Declines in these species were detected in T<sub>WH</sub> biofilms for all three coatings, S<sub>G</sub> copper, T<sub>W</sub> epoxy, and S<sub>G</sub> and T<sub>W</sub> silicone. Pairwise comparisons of post-transport diatom assemblages revealed that cell losses in T<sub>WH</sub> made those assemblages distinct from all other treatments (Table 1 and Figure 5d). No differences in assemblage composition were detected in the S<sub>A</sub> / T<sub>A</sub> / T<sub>W</sub> or T / T<sub>W</sub> comparisons. These results demonstrate that neither of the sheltered treatments, transport (T) and transport under osmotic stress (T<sub>W</sub>), had significant impacts on diatom assemblage structure, and that changes in T<sub>WH</sub> diatom assemblages were the result of hydrodynamic stress from on-hull transport.

Differences in diatom composition were detected among assemblages that were exposed for post-transport macrofouling (Table 1, Figure 7a and b), which included all biofilms transported from Fort Pierce (T, T<sub>W</sub> and T<sub>WH</sub>) and those developed in Fort Myers (S<sub>G</sub>). Of the diatom species that were found in at least two treatment groups, six taxa were exclusive to S<sub>G</sub> biofilms: *Navicula* sp. 9, *Navicula* sp. 10, *Navicula* sp. 11, *Nitzschia* sp. 4, *Synedra* sp. 1 and *Synedra* sp. 2 (Figure 4). However, it was differences in the relative abundance of shared taxa (*Amphora cf. montgomeryi*, *Cocconeis* sp. 1, *Nitzschia fonticola* and *Nitzschia* sp. 1) that distinguished S<sub>G</sub> from other treatments, especially T<sub>WH</sub>.

Macrofouling recruitment on transported biofilms

A total of 24 macrofoulers from 10 phyla were identified (Figure 8). Coatings and their associated biofilms were the major contributors to differences in macrofouling community composition at both three and 14 weeks (Table 1, Figure 7c–f). At the species level, selectivity was exhibited among coating types by eight macrofouling taxa (p < 0.05, Figure 9). Six of the eight species selected epoxy over one or both other coatings, while the hydroid *Obelia*...
Figure 6. Diatom survivorship and assemblage change in stationary and transported biofilms originating in Fort Pierce. Total densities of live diatoms (a, c, e) and relative abundances of key diatom species (b, d, f) on copper, epoxy, and silicone coatings before and after transport. Combined pre-transport (Before) results are compared with the following post-transport (After) treatments: stationary Atlantic (SA), controlled transport (T), transport with changing water quality (TW), and transport with changing water quality and hydrodynamic conditions (TWH). Black dots indicate significant reductions in total live diatoms and assemblages with reductions of key species. Key diatom species are as follows: Cylindrotheca closterium (C. closte), Cocconeis sp. 1 (Cocco 1), Navicula sp. 2 (navic 2), Navicula sp. 4 (navic 4), Nitzschia fonticola (n. fonti), Nitzschia sp. 1 (Nitzs 1) and Parlibellus delognei (P. delog). Gray represents the total contribution of all other diatoms.
Briand et al. 2012; Camps et al. 2014; Hunsucker et al. 2014; Zargiel & Swain 2014). Coating type was the major driver of diatom assemblage composition, which parallels several studies documenting microbial uniqueness among coatings (Chen et al. 2013; Muthukrishnan et al. 2014; Watson et al. 2015; Yang et al. 2016). In most instances, abundant species on copper and silicone were also found in high numbers on epoxy. An exception is Amphora cf. montgomeryi, which dominated only copper biofilms and is from a genus known to contain other copper-tolerant species (Daniel & Chamberlain 1981; French & Evans 1988; Pelletier et al. 2009). While present in all treatments, Cylindrotheca closterium was dominant on silicone compared to other species, and remained present after transport despite significant cell loss. In both pre-transport and post-transport assemblages, C. closterium contributed >12% to the similarity among silicone samples, the highest contribution of all diatom taxa. Cylindrotheca species are common fouling diatoms, and have been documented as abundant and sometimes dominant colonizers of fouling-release coatings in other studies (Molino et al. 2009; Dobretsov & Thomason 2011; Briand et al. 2012; Zargiel & Swain 2014). Although overall diatom colonization was initially greater on inert epoxy surfaces, the lack of differences among coatings post-transport suggests that diatom removal was also higher on epoxy. These cell losses were probably a result of loosely attached diatoms sloughing from the outer layers of the thick biofilms. Conversely, copper and silicone panels were coated with visually thinner biofilms wherein most diatoms likely contacted the coating surface directly for better adhesion. Coating type varies greatly among transiting vessels and on different areas of the hull. Therefore, the routes and conditions under which diatoms are transported may largely be based on their successful colonization of particular coating types.

All transport methods reduced the total abundance of live diatoms on at least one of the three coating types. Cell loss resulted in changes to the composition of the TWH assemblages that made them distinct from all other treatments, providing evidence that on-hull transport significantly alters biofilm diatom composition through hydrodynamic stress. The lack of differences among other treatments suggests that diatom removal was also higher on epoxy. These cell losses were probably a result of loosely attached diatoms sloughing from the outer layers of the thick biofilms. Conversely, copper and silicone panels were coated with visually thinner biofilms wherein most diatoms likely contacted the coating surface directly for better adhesion. Coating type varies greatly among transiting vessels and on different areas of the hull. Therefore, the routes and conditions under which diatoms are transported may largely be based on their successful colonization of particular coating types.

**Discussion**

**Diatom recruitment and portability in biofilms**

All diatoms identified in this study are known inhabitants of marine biofilms, and many have been previously documented on ship hull coatings (Zargiel et al. 2011; Briand et al. 2012; Camps et al. 2014; Hunsucker et al. 2014; Zargiel & Swain 2014). Coating type was the major driver of diatom assemblage composition, which parallels several studies documenting microbial uniqueness among coatings (Chen et al. 2013; Muthukrishnan et al. 2014; Watson et al. 2015; Yang et al. 2016). In most instances, abundant species on copper and silicone were also found in high numbers on epoxy. An exception is Amphora cf. montgomeryi, which dominated only copper biofilms and is from a genus known to contain other copper-tolerant species (Daniel & Chamberlain 1981; French & Evans 1988; Pelletier et al. 2009). While present in all treatments, Cylindrotheca closterium was dominant on silicone compared to other species, and remained present after transport despite significant cell loss. In both pre-transport and post-transport assemblages, C. closterium contributed >12% to the similarity among silicone samples, the highest contribution of all diatom taxa. Cylindrotheca species are common fouling diatoms, and have been documented as abundant and sometimes dominant colonizers of fouling-release coatings in other studies (Molino et al. 2009; Dobretsov & Thomason 2011; Briand et al. 2012; Zargiel & Swain 2014). Although overall diatom colonization was initially greater on inert epoxy surfaces, the lack of differences among coatings post-transport suggests that diatom removal was also higher on epoxy. These cell losses were probably a result of loosely attached diatoms sloughing from the outer layers of the thick biofilms. Conversely, copper and silicone panels were coated with visually thinner biofilms wherein most diatoms likely contacted the coating surface directly for better adhesion. Coating type varies greatly among transiting vessels and on different areas of the hull. Therefore, the routes and conditions under which diatoms are transported may largely be based on their successful colonization of particular coating types.
The results presented here establish that transport, especially on ship hulls, significantly reduces diatoms in biofilms. However, some living cells persisted even through freshwater, high flow, and other environmental conditions common to shipping transit. Surviving assemblages, differing in composition based on coating type and transport method, will function as seed populations for biofilm development at the destination. Biofilms arising from these resilient and dissimilar assemblages will be encountered and evaluated by settling larvae. Therefore, shipping may play a vital and previously unacknowledged role in shaping biofilms for macrofouler recruitment.

Coating type was the major contributor to differences in macrofouling community composition and individual species recruitment. Most macrofoulers selected epoxy sheltered niche areas (e.g., rudders, thrusters, bilge keels and sea chests) where flow is reduced. In a survey of diatoms on active cruise ships, Hunsucker et al. (2014) found that diatom assemblages from niche areas were similar to those developed under static immersion. Stalked diatoms of the genus Licmophora have weak adhesion strength (Woods & Fletcher 1991) and seem to thrive in these protected areas (Woods et al. 1986; Hunsucker et al. 2014). Three species of Licmophora initially colonized TWH panels across coating types: L. abbreviata, L. flabellata and L. grandis. These diatoms were absent post-transport in all seven instances where they were previously present and sometimes abundant. However, Licmophora spp. cells frequently remained in post-transport biofilms from other treatments, including TWH. Different ship environments present diverse vectors for the dispersal of biofilm organisms, and variations in diatom assemblages from this study illustrate that transport method is important.

The results presented here establish that transport, especially on ship hulls, significantly reduces diatoms in biofilms. However, some living cells persisted even through freshwater, high flow, and other environmental conditions common to shipping transit. Surviving assemblages, differing in composition based on coating type and transport method, will function as seed populations for biofilm development at the destination. Biofilms arising from these resilient and dissimilar assemblages will be encountered and evaluated by settling larvae. Therefore, shipping may play a vital and previously unacknowledged role in shaping biofilms for macrofouler recruitment.

**Macrofouling recruitment on transported biofilms**

Coating type was the major contributor to differences in macrofouling community composition and individual species recruitment. Most macrofoulers selected epoxy...
coatings, with a few exceptions. Coverage of the sponge *Halichondria bowerbanki* was widespread on silicone in addition to epoxy, while the hydroid *Obelia dichotoma* and the encrusting bryozoan *Watersipora subtorquata* were most abundant on copper. These results are not surprising, as both *W. subtorquata* and other species of *Obelia* are known to be copper-tolerant (Floerl et al. 2004; Piola & Johnston 2006; Crooks et al. 2011). Selectivity exhibited by the peritrich ciliate *Vorticella* sp. in this investigation corresponds to results seen by Watson et al. (2015). In both studies, *Vorticella* initially selected inert control surfaces but eventually began to colonize antifouling coatings as well. While coatings have distinct chemical and physical properties that alter macrofouling recruitment, biofilms covering the coating surface may mask these properties (Faimali et al. 2004; Scardino & de Nys 2011) and emit their own cues for settlement. Diatom assemblage composition in this study varied among coating types, and recruiting macrofoulers likely encountered unique sets of cues from both the diatoms and the underlying coatings.

Biofilms altered by transport method had no effect on macrofouling colonization at the community level, but were subtle secondary contributors to differences in recruitment for four individual species. Among the four species, selectivity was split between resident $S_T$ (corophiid amphipods and *Vorticella* sp.) and imported $T_{WH}$ (*W. subtorquata* and *A. amphitrite*, formerly *T WH*). Positive selectivity for a given treatment is denoted as greater (black) percentage cover than one or more other coating (rectangles) or transport (circles) treatments. Gray denotes negative selectivity and white indicates no significant difference. Transport selectivity is positioned in line with the coating on which that difference occurred. ‘Week detected’ indicates if differences were seen during macrofouling assessments at week 3 and/or 14. The maximum percentage cover per sample is listed for each species.

### Figure 9.
Coating and transport-influenced selectivity among macrofoulers on copper, epoxy and silicone coatings from the following transport treatments: stationary Gulf (S), transport (T), transport with changing water quality (T_W) and transport with changing water quality and hydrodynamic conditions (T_WH). Positive selectivity for a given treatment is denoted as greater (black) percentage cover than one or more other coating (rectangles) or transport (circles) treatments. Gray denotes negative selectivity and white indicates no significant difference. Transport selectivity is positioned in line with the coating on which that difference occurred. ‘Week detected’ indicates if differences were seen during macrofouling assessments at week 3 and/or 14. The maximum percentage cover per sample is listed for each species.

<table>
<thead>
<tr>
<th>Coating Influenced</th>
<th>Transport Influenced Within Coating</th>
<th>Week Detected</th>
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<tr>
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<tr>
<td>Aglaophamnion halliace</td>
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<tr>
<td>Max % cover: 35.4 ± 2.7</td>
<td>Copper</td>
<td>Epoxy</td>
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<tr>
<td>Copepium tenuisimum</td>
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<td>Max % cover: 65.4 ± 7.7</td>
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<td>Epoxy</td>
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<tr>
<td>Corophid amphipod</td>
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<tr>
<td>Max % cover: 54.2 ± 9.5</td>
<td>Copper</td>
<td>Epoxy</td>
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<tr>
<td>Halichondria bowerbanki</td>
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<td>Max % cover: 25.8 ± 10.5</td>
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<td>Epoxy</td>
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<td>Obelia dichotoma</td>
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<td>Max % cover: 59.6 ± 21.3</td>
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<td>Epoxy</td>
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<tr>
<td>Ulva flexuosa</td>
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<tr>
<td>Max % cover: 56.9 ± 28.0</td>
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<td>Epoxy</td>
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<tr>
<td>Vorticella sp.</td>
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<tr>
<td>Max % cover: 43.3 ± 7.0</td>
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<td>Epoxy</td>
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<tr>
<td>Watersipora subtorquata</td>
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<tr>
<td>Max % cover: 0.7 ± 0.0</td>
<td>Copper</td>
<td>Epoxy</td>
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with macrofouling. Selectivity during this recruitment period probably arose indirectly from the coatings and/or biofilms through intermediary macrofouling. Regardless of the proximal driver, a few significant differences persisted in macrofouling colonization until at least 14 weeks post-transport. However, biofilm selectivity was not detected for most macrofouling species. Biofilms associated with different coatings were more frequently involved in recruitment differences, suggesting that chemical and physical properties of the underlying coating were mainly responsible.

Coatings, biofilms and biological invasions

Invasions are initiated by the transport of organisms outside of their natural biogeographical ranges. Therefore, human-mediated biofilm transport is intrinsically linked to bioinvasion ecology. Biofilm organisms may themselves become invasive through ship transport (Drake et al. 2005, 2007; Costas et al. 2013; Amalfitano et al. 2015), or they may mediate secondary dispersal and establishment of invasive macrofoulers. Shipping provides over 80% of global trade (UNCTAD/RMT 2015). Routes are expanding to keep pace with population growth (Seebens et al. 2013; Muirhead et al. 2015) and climate change is opening new passages through previously impassible seas (Smith & Stephenson 2013). Global connectivity and the potential for biofilm transport will increase to record levels with anthropogenic changes to oceans, offering new sets of cues to recruiting larvae.

Results presented herein demonstrate that biofilm diatoms can withstand transport on different ship hull coatings. Despite some cell losses associated with all modes of transport, the overall composition (and presumably the associated cues) of the diatom assemblages were largely conserved under sheltered transport conditions with low hydrodynamic stress. Some diatoms, such as *Amphora* and *Navicula*, even survived transport on the open hull. These tenacious species and those transported in niche areas are therefore more likely to be distributed among ports of call, establish themselves in new environments and become invasive. Microbes with affinities to or competitive advantages on certain coatings may enjoy greater transport on hulls treated with those particular formulas. For example, *Amphora cf. montgomeryi* that dominated the copper treatment in this study could be carried primarily by ships using copper biocide antifouling paints. Coating selectivity that affects the routes and frequency with which biofilm organisms are transported will potentially lead to differences in invasion success.

Subsequent macrofouling on ships arriving in ports appears to be mostly affected by different hull coatings and their associated microbes, including distinct diatom assemblages. Like diatoms, macrofoulers that can tolerate widely used antifoulants such as copper will be more likely to become globally distributed. The connection between copper-tolerance and invasion success in the bryozoan *Watersipora subtorquata* has been documented previously (Floerl et al. 2004; Piola & Johnston 2006; Crooks et al. 2011), and recruitment results for *W. subtorquata* in this study support those earlier findings. Conversely, in < 20% of cases did macrofoulers exhibit selectivity for biofilm assemblages structured solely by transport. These results suggest that macrofouling larvae are resourceful and that many species can readily recruit to and colonize a variety of natural biofilms when other confounding factors, such as variability in the underlying substrata, are absent. A generalist response to biofilms may help explain why many macrofouling taxa have successfully invaded marine systems worldwide. However, a deeper understanding of the connection between biofilms and macrofoulers is needed. Advances in the detection and identification of microbes have led to a better grasp of their global biogeography (Pommier et al. 2007; Cermeño et al. 2010; Zinger et al. 2011; Ghiglione et al. 2012). Still, little is known about the heterogeneity of biofilm organisms among vessels and ports of call. To fully comprehend the microbial role in macrofouling dispersal and establishment, it is important to measure the degree of variation among biofilms to which macrofoulers are exposed. In addition to diatoms, future investigations should include analyses of other biofilm dwellers such as bacteria, archaea and ciliates. The distributions of these microbes often vary in time and space (Webster et al. 2004; Muthukrishnan et al. 2014; Watson et al. 2015; Briand et al. 2017), which may alter subsequent larval recruitment. Likewise, it is essential to further investigate how biofilm selectivity varies across macrofouling species because invasion success is highly variable. The Florida invasive barnacle *Amphibalanus amphitrite* is among a group of species detected in this study that are globally invasive. Other species, such as the Florida native alga *Rosenvingea intricata*, currently have no records of introduction outside of their native ranges. While these records depend on human detection and are therefore biased, most fouling communities likely contain species that exhibit different levels of invasibility. Invasion success among port organisms depends on their arrival and subsequent ability to thrive. Biofilms have the capacity to affect both.

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**Disclosure statement**

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