2017

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Running title: Microbial diversity in ballast water

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Abstract

Aim Ballast water is a major vector of non-indigenous species introductions worldwide. Our understanding of the population dynamics of organisms entrained in the vector is largely limited to studies of zooplankton and phytoplankton. Bacteria are more numerous and diverse than zooplankton or phytoplankton, yet they remain comparatively understudied. Here we apply a metagenomics approach to characterize changes in the microbial ballast water community over the course of three voyages on one ship, and assess the effects of ballast water exchange (BWE), spring/summer sampling month, and time since voyage start.

Location Quebec City and Deception Bay, Quebec, and the coastal marine region offshore of eastern Canada

Methods We used universal primers to Ion Torrent sequence a fragment of the bacterial 16S ribosomal DNA for samples collected over three voyages of a single ship between Quebec City and Deception Bay in June, July, and August, 2015. We compared richness and diversity measures using linear mixed effects analysis and compared community composition using nonmetric multidimensional scaling and permutational multivariate analysis of variance. Initial comparisons were between months, with subsequent analyses focused on each month separately.

Results Ion Torrent sequencing returned approximately 2.9 million reads and revealed significant monthly differences in diversity and species richness, as well as in community structure in ballast water. June had significantly higher richness (total number of species in the community) and diversity (accounts for both species abundance and evenness) than either July or August, and showed most clearly the effect of BWE on the microbial community.

Main Conclusions Our results suggest that differing environmental conditions associated with different spring/summer sampling months drive differences in microbial diversity in ballast

water. This study showed that BWE removes some components of the microbial community from the starting port and replace them with other taxa. BWE also changed proportional representation of some microbes without removing them completely. However, it also appears that some taxa are resident in the ballast tanks and are not removed by BWE.

Keywords: bacteria, ballast-water exchange, biological invasion, invasive species, non-indigenous species, non-native species, ship, transport, 16S rRNA

Introduction

Ships have used ballast water for stability and trim for over 150 years (Carlton, 1985; Ruiz et al., 2000). As cargo is unloaded, water is pumped into ballast tanks to offset the weight removed from the vessel and maintain trim. This ballast water is subsequently pumped out into another port as cargo is taken aboard (Carlton, 1985). Water pumped into ballast tanks contains a diverse community of organisms present in the loading port, including microbes, plankton, nekton, and organisms living on or within the sediment (Carlton, 1985; Briski et al., 2012). It is now well established that some of these organisms are able to survive the voyage and establish populations in receiving waters (e.g. Carlton & Geller, 1993; Briski et al., 2012). Exploration into the role of ballast water in transporting organisms from one port to another increased in the early 1970s, with attention initially focused on disease-causing bacteria (International Maritime Consultative Organization, 1973; Carlton, 1985). The introduction via ballast water of a few highly-visible and damaging species, such as the zebra mussel (*Dreissena polymorpha*) to the Great Lakes (Hebert et al., 1989), focused international attention on the problem. The first measures to control the transport of organisms in ballast water were implemented in the late 1980s. Initially voluntary, mid-ocean ballast-water exchange (BWE) or tank flushing of residual water is now mandated by the governments of the United States and Canada for vessels entering the Canadian or American Exclusive Economic Zones (EEZ; Canadian Coast Guard, 1989; Canada Shipping Act SOR/2011-237; United States Coast Guard, 1993; US Code of Federal Regulations 33 CFR 151), and is recommended by the International Maritime Organization (IMO, 2008). Vessels exclusively transiting coastal waters within one country's EEZ are not required to perform BWE, but some may do so voluntarily (S. Bailey, pers. comm.), as is the case with our study vessel.

Ballast water exchange limits the transport of organisms in several ways. In cases where the source port is fresh water, BWE raises the salinity of the water in tanks above the tolerance level of freshwater organisms that may remain following discharge of the original ballast water, thereby killing them (Bailey et al., 2011). In cases where the destination port is fresh water, the oceanic organisms taken up during BWE are likewise killed by the low salinity of the new environment when they are released (MacIsaac et al., 2002; Gray et al., 2007). Irrespective of whether the source port is fresh water or marine, BWE discharges water potentially containing high concentrations of organisms in deep, high-salinity waters far from shore, where they will likely die or otherwise do little harm, and to dilute it with water containing a lower concentration of mid-ocean organisms which are less likely to survive when released into coastal waters (Bailey et al., 2015). In reality, coastal marine species may not be completely purged during BWE and are less affected by changes in salinity than freshwater species, while BWE may actually add potentially harmful oceanic species that can survive in saltwater recipient ports (Cordell et al., 2009; Roy et al., 2012; Bailey et al., 2015). Thus, since BWE partially relies on exceeding the salinity tolerance of transported organisms, mid-ocean BWE is most effective at preventing the transport of organisms between freshwater ports.

Even without BWE, many organisms in ballast tanks die during the voyage owing to changes in temperature, decreased oxygen concentration, starvation, and toxicity associated with antifouling paint in the ballast tanks, although mortality rates likely depend on voyage length (e.g. Carlton, 1985; Mimura et al., 2005). However, our knowledge of the population dynamics of organisms entrained in ballast water is based mainly on studies of eukaryotes, particularly phytoplankton (e.g. Paolucci et al., 2016; Gollasch et al., 2000; Olenin et al., 2000) and zooplankton (e.g. Gollasch et al., 2000b; Olenin et al., 2000; Ghabooli et al., 2016). Although

bacteria are numerically dominant in the aquatic environment (Drake et al., 2007), comparatively little is known about their diversity and dynamics in ballast water. This knowledge gap stems from multiple sources, including the small size and limited morphological diversity of bacteria and the difficulty (or impossibility) of culturing many species under laboratory conditions (Rappé & Giovannoni, 2003). However, available information indicates that bacterial dynamics may be complex. Bacteria concentration and/or biomass may decrease (Drake et al., 2002; Seiden et al., 2011), remain relatively consistent (Mimura et al., 2005; Burkholder et al., 2007), or even increase over time during a voyage (Seiden et al., 2010; Tomaru et al., 2014). Many studies also suggest that BWE does little to impact the concentration of bacteria in ballast water (Drake et al., 2002; Mimura et al., 2005; Burkholder et al., 2007; Seiden et al., 2011), although this research has primarily been performed on transoceanic ships that collect and discharge ballast water in coastal (marine or estuarine) rather than fresh water.

Early studies of microbial communities in ballast water were limited by difficulty in identifying species (but see Tomaru et al., 2014). The advent of high-throughput sequencing and metagenomic approaches have begun to fill this knowledge gap and inform our understanding of bacterial communities worldwide, including in ballast water (e.g. Aridgides et al., 2004; Fujimoto et al., 2014; Pagenkopp Lohan et al., 2015; Brinkmeyer, 2016). These approaches, which typically involve DNA sequencing of the bacterial 16S ribosomal RNA gene (16S rRNA) and subsequent matching of DNA sequences to known bacterial groups, provide a vastly more detailed view of the microbial community in ballast water. However, metagenomics studies on the bacterial ballast water community are still rare. The few papers that have been published to date provide detailed information on the diversity of microbes in ballast water related either to water sources (Ng et al., 2015; Brinkmeyer, 2016) or to effects of alkali treatment (Fujimoto et

al., 2014). None of the metagenomics papers published to date examine changes that occur over the course of a voyage, or that might be associated with BWE.

In this study, we utilized next-generation sequencing metagenomics to characterize the bacterial community in ballast water during repeated voyages of a single vessel, the *M/V Arctic*, from Quebec City, Quebec, Canada, to Deception Bay, Quebec during summer 2015. We sampled both source and destination ports and sampled repeatedly from ballast tanks that underwent no, early or late BWE. We used universal bacterial primers to PCR amplify the 16S rRNA gene and next-generation sequencing to characterize the diversity and relative abundance of bacteria in the ballast water, as well as the effects of BWE and ballast water age. We also compared the bacterial community in source and recipient ports with that in ballast water to identify a signature of bacterial uptake and transport, potentially indicating the movement of species from one region to another.

Methods

Sample Collections

Samples were collected over the course of three voyages of the *M/V Arctic* between Quebec City (freshwater port) and Deception Bay (saltwater port) in June, July, and August 2015. The *M/V Arctic* is a bulk carrier with a length and a beam of 220.82 and 22.93 m, respectively, and a gross register tonnage of 20 236 t. The ship has seven ballast tanks on both port and starboard sides, and a maximum ballast water capacity of 28161 m^3. All BWE used the sequential method, which is a process by which a ballast tank is first emptied and then refilled with new ballast water to achieve at least a 95% volumetric exchange. The *M/V Arctic* operates as a Canadian domestic vessel (i.e. sailing exclusively within the Canadian Exclusive Economic Zone) and performs voluntary ballast water

exchange in coastal waters. This may limit the efficacy of reducing organism abundance as compared to mid-ocean exchange. In each voyage, three separate ballast tanks (one per treatment) were designated as control (no BWE performed), early exchange (BWE performed in Jacques Cartier Strait; mean distance from shore: 33 km), and late exchange (BWE performed in Strait of Belle Isle; mean distance from shore: 43 km) (Fig. 1). Samples were collected from each tank early in the voyage, before and after an exchange in Jacques Cartier Strait (control and early exchange tanks), before and after an exchange in Belle Isle Strait (control and late exchange tanks), and from all tanks immediately before discharge in Deception Bay. Samples were also collected from the harbors at Quebec City and Deception Bay at the start and end of each voyage. Concurrent with sampling, temperature and salinity at the sampling location (ballast tank or port) were measured with a SBE 19plus V1 CTD (Seabird Electronics). Maximum depth of each ballast tank was assessed with a weighted rope, to identify appropriate sampling depths to represent the complete water column and to avoid hitting the bottom of the ballast tank and resuspending sediment with our deepest samples. At each sampling point, a Niskin bottle was used to sample 5 L each from the surface, middepth, and bottom of the ballast tank. These samples were mixed, filtered through a 20 μm nytex sieve to remove larger planktonic organisms and organic material, and three ~250 mL samples collected and filtered through 0.2 μm polyethersulfone membrane filters (PALL Life Sciences) to collect bacterial cells. June samples were filtered and preserved immediately after pre-filtering, while July and August samples were pre-filtered through 20 μm nytex mesh, then held at ambient temperature and filtered and preserved within two hours. While this delay may have allowed for some bactivory, we consider that this should be relatively minor as overall bacterial community structure has been shown to be resilient to predation over much longer time scales (i.e. 8 days; Baltar et al., 2016). Filters

were preserved in a high-salt solution (25 mM sodium citrate, 10 mM EDTA, 5.3 M ammonium sulphate, pH 5.2) at ambient temperature prior to DNA extraction.

DNA Extraction, PCR, and Ion-Torrent Sequencing

Filters were rinsed with nanopure H_2O using a vacuum-filtering apparatus to remove salts prior to extraction. DNA was extracted from whole or half filters using either a standard phenol-chloroform extraction protocol or EZ-10 Spin Column Soil DNA Mini-Preps Kit (Bio Basic Inc., Markham, Ontario, Canada).

Bacterial 16S rDNA was PCR-amplified using primers 787f (Roesch et al., 2007, 5'-ATTAGATACCCNGGTAG-3') and B-1046R (Sogin et al., 2006, 5'-CGACAGCCATGCANCACCT-3') in 21 µL reactions containing 1.0 µL of template DNA (diluted 1:10), 1.0 µL 25mM $MgCl_2$ (Genscript, Piscataway, NJ, USA), 0.5 µL 10 mM dNTPs (Bio Basic), 0.5 µL each 10 mM primer, 2.5 µL 10X PCR buffer (Genscript), and 0.1 µL 5U/ µL *Taq* polymerase (Genscript). PCR primers were tagged with UniA (5'-ACCTGCCTGCCG-3') and UniB (5'-ACGCCACCGAGC-3') tails for next-generation sequencing. Cycling conditions for PCR were: an initial denaturation step of 95°C for 1 min, 35 cycles of 95°C for 15 sec, 55°C for 15 sec, and 72°C for 30 sec, and a final extension at 72°C for 7 min.

PCR products were cleaned with Agencourt Ampure XP (Beckman Coulter, Mississauga, ON, Canada) to remove unincorporated primers, primer dimer, and short PCR products. Samples were assigned a unique 10-12 bp IonX barcode to allow separation of sample data after next-generation sequencing. IonX barcodes and sequencing adaptors were ligated to the initial PCR products as a single oligonucleotide construct incorporating the complement to the 5' overhang sequence on the initial PCR primers, an IonX barcode, and a sequencing adaptor. Ligations occurred in a 25 µL PCR reaction that contained 10 µL of Ampure-cleaned PCR product, 2.5 µL

of 10x PCR buffer (Genscript), 1.0 μL 20 mM $MgCl_2$ (Genscript), 0.5 μL 10 mM dNTPs (Bio Basic Inc.), 0.5 μL each 10 mM second-stage primer, and 0.1 μL Taq polymerase. Thermocycler conditions for second-stage PCR consisted of an initial denaturation at 94°C for 2 min, followed by 6 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min, followed by a final extension at 72°C for 5 min. Second-stage PCR products were cleaned using Ampure XP, combined, concentrated via isopropanol precipitation, and purified via agarose gel electrophoresis and subsequent gel extraction using a Qiagen MinElute Gel Extraction Kit (Qiagen Inc.). Extracted PCR products were analyzed for DNA quantity and purity using an Agilent 2100 Bioanalyzer with a High Sensitivity DNA kit (Agilent, Santa Clara, CA, USA). The pooled DNA was diluted to 55 pM and sequenced using an Ion 318 Chip kit and Ion PGM Sequencing 400 kit on an Ion Personal Genome Machine (ThermoFisher Scientific).

Data Processing

After sequencing was completed, sequence reads were filtered within the PGM software to remove polyclonal and low quality sequences. Also, sequences that matched the PGM 3' adaptor were trimmed. Further metagenomics data processing was performed using the UPARSE algorithm (Edgar, 2013) using the default parameters. Cluster analysis was used to determine operational taxonomic units (OTUs) by clustering similar sequences based on a 97% similarity threshold and taxonomy was assigned using the Ribosomal Database Project (RDP). As the 97% similarity threshold was used to create OTU's, the relative abundance of organisms was collected using an 80% sequence match at the lowest possible taxonomic level (Hildebrand et al., 2014) to construct bacterial community composition structure. We constructed rarefaction curves (Figs S1-S3) in PAST and, in order to ensure that analysis only included samples with a good

representation of diversity, samples with fewer than 1000 sequences/sample were excluded from analysis.

Statistical Analysis

The Shannon-Wiener (H) alpha diversity estimator and Chao1 richness estimator were calculated for each replicate sample using PAST v3.12 software (Hammer et al., 2001). Linear mixed effects analysis was performed using the lme4 package in R (Bates et al., 2015; R Core Team, 2016) to examine the role of treatment (control, ballast exchange, or port), sampling month, and days from voyage start on species diversity and richness while controlling for repeated sampling of the same ballast tanks (Schank & Koehnle, 2009). We used likelihood ratio tests to assess the significance of each predictor. Here, we constructed a full model including all factors and compared it with simplified models that dropped each predictor in turn using a Chi-Square test to assess the significance of each predictor.

Random sampling at multiple research stages (field sampling, DNA extraction, PCR, and sequencing) may artificially inflate β-diversity if rare species are included (Zhan and MacIsaac, 2015). Thus, we trimmed the data set to exclude OTUs with fewer than 1000 total reads across all samples. This approach is conservative in focusing solely on the most common OTUs in our microbial community analysis. We calculated distance matrices among samples using the Bray-Curtis index and used these distances for two-dimensional nonmetric multidimensional scaling (NMDS) using the "metaMDS" function from the R package vegan (Oksanen et al., 2016). The significance of differences between NMDS communities was assessed using a Permutational Multivariate Analysis of Variance (PERMANOVA) as implemented in the "Adonis" function in the R package vegan. Differences among sample groups were assessed using similarity percentages (SIMPER) and pairwise PERMANOVA analysis in PAST. P-values for pairwise

comparisons were adjusted using a sequential Bonferroni correction for multiple comparisons. Initial analysis was performed using all replicate samples. Further analysis was performed using samples separated by month.

Results

Environmental Data

Water temperatures in the port of Quebec City at the start of the June voyage was approximately 17°C, while for the July and August voyages temperatures averaged approximately 22°C. Initial temperature within the ballast tanks was similar, ranging from approximately 15-16°C in June and from 21-23°C in both July and August. Over the course of the voyage, temperature declined gradually to lows of 1-5°C in June, 3°C in July, and 6°C in August, reflecting temperature at Deception Bay at the time of arrival.

Initial salinity in the ballast tanks for all three months was largely consistent with fresh water collected from Quebec City port (average: 0.8 psu, range: 0.13-7.57 psu). The high end of the starting salinity range was driven by one ballast tank with elevated starting salinity (7.57 psu, June late exchange tank), likely due to incomplete ballast exchange. Post-exchange ballast had salinity values approaching that of seawater, averaging 25.29 psu (range: 20.35-27.71 psu). Deception Bay salinity ranged widely, from 6.16 in June - likely due to the influence of freshwater flows and the continued presence of sea ice in the bay - to 24.67 and 27.61 in July and August, respectively, reflecting estuarine conditions in the bay during summer.

Commented [H1]: use same number of signif digits for all salinity values (i.e. 1)

Complete Genetic Results

Approximately 6.6 million Ion Torrent sequence reads were obtained for 124 samples. After quality control, slightly more than 2.9 million reads - representing 3220 OTUs - remained.

Although the distribution of reads was relatively even among sampling months, with 774,835 non-singleton reads for June, 843,287 reads for July, and 1,292,076 for August, the distribution of OTUs was very uneven. June had 2975 unique OTUs in 32 samples, July had 270 OTUs in 39 samples, and August had 415 OTUs in 44 samples. To focus our examination of the effect of ballast water treatment in different months on the most common microbiota samples at the OTU level, we further trimmed the data set to exclude OTUs with fewer than 1000 total reads.

When analyzing all samples collectively, both sampling month ($\chi^2 = 128.11$, $p < 0.0001$) and days from voyage start ($\chi^2 = 7.06$, $p = 0.008$) significantly affected Chao1 species richness. There was no significant difference in Chao1 between treatments (port samples or ballast tanks before or after the exchange, $\chi^2 = 8.53$, $p = 0.073$). Chao1 values in June were 351 ± 35 (SE) higher than those in July and 333 ± 27 higher than August (Fig. 2). Values in July and August were very similar, with an average difference of 16 ± 30. Overall, richness declined with time. During a voyage, each additional day resulted in a mean decline in Chao1 richness of ~8 ± 3. Sampling month also significantly altered Shannon H diversity ($\chi^2 = 125.54$, $p < 0.0001$). There was no significant effect of either days from voyage start ($\chi^2 = 2.02$, $p = 0.16$) or treatment (ballast water exchange/port sampling, $\chi^2 = 3.91$, $p = 0.42$) on Shannon H diversity. As with Chao1 richness, Shannon H values in June were 2.50 ± 0.24 higher than in July and 1.91 ± 0.18 higher than in August (Fig. 2). Shannon H values were significantly lower in July than in August with a mean decrease of 0.60 ± 0.19.

Bacterial communities were significantly different between months (PERMANOVA, $F = 11.33$, $p = 0.001$). Mean dissimilarity was of 67.3% in our SIMPER analysis, with differences most pronounced between June and later months. These differences were also apparent in the NMDS analysis (Fig. 3).

June Results

Analyzing June separately, neither Chao1 nor Shannon H varied significantly with either days from voyage start (Chao1 $\chi^2 = 3.67$, $p = 0.055$, Shannon H $\chi^2 = 3.63$, $p = 0.057$) or port vs. ballast tank treatment (Chao1 $\chi^2 = 5.51$, $p = 0.24$, Shannon H $\chi^2 = 3.36$, $p = 0.50$). However, the composition of the bacterial community differed significantly between treatments (PERMANOVA, $F = 3.50$, $p = 0.001$). We identified significant community differences between most treatment pairs (SIMPER & PERMANOVA, Table 1), except for Deception Bay versus post-BWE ($p = 0.414$), Quebec City versus control tanks ($p = 0.214$), and Quebec City versus Deception Bay ($p = 0.099$). Except for the lack of differences between Quebec City and Deception Bay samples, NMDS results support these findings, with considerable overlap between Deception Bay (DPort) and post-exchange (Post-Exch), and Quebec City (QPort) and control groups (Fig. 4). The lack of differences between Quebec City and Deception Bay may best be explained by the low power of the statistical test owing to low sample number, as SIMPER analysis revealed an overall average dissimilarity of 78.9%, and the groups did not overlap in the NMDS plot (Fig. 4).

Regarding the changes associated with BWE in June, the SIMPER analysis included 75 OTUs with > 50 total reads across both treatments. Of these 75 OTUs, 21 increased and 54 decreased their number of reads. 16S rRNA gene amplicon sequence data analysis revealed that microbial community structure characterized at the genus level was altered by ballast water exchange during June (Figure S4). Microbial communities of control tanks and exchange tanks were similar to the composition of samples from Quebec City, with *Actinobacter* (Actinobacteria), *Trabulsiella* and *Enterobacter* (Gammaproteobacteria), *Acidovorax*, *Curvibacter*, and *Bordetella* (Betaproteobacteria), and *Sphingobacteria* being particularly

prominent; averaging 50% of the relative abundance across all samples. In one exchange tank, *Flavobacteria* was a major component of the microbial community pre-exchange, but declined markedly thereafter.

July Results

For July samples, Chao1 did not differ significantly with either days from voyage start (χ^2 = 2.76, p = 0.10) or treatment (χ^2 = 3.13, p = 0.54) Shannon H increased significantly with days from voyage start (χ^2 = 31.43, p < 0.0001) but did not differ significantly among treatments (χ^2 = 8.14, p = 0.09). For each additional day of the voyage, Shannon H increased by 0.13 ± 0.02 . However, the bacterial community did not differ significantly between treatments in July (PERMANOVA, F = 1.84, p = 0.067). This is reflected in the considerable overlap between groups in the NMDS (Fig. 4). SIMPER analysis identified 11 OTUs with >50 total reads, all of which increased after BWE. Similar to June, *Trabulsiella* and *Enterobacter* were common both before and after BWE in July, although both increased in an absolute number of reads after BWE (Fig. S5). In July samples *Vibrio* (Gammaproteobacteria), and *Mycoplasma* (Mollicutes), increased in abundance in both treated and control tanks such that they were a significant component of the microbial community in all tanks late in the voyage.

August Results

For August, neither Chao1 nor Shannon H varied significantly in relation to voyage length (Chao1 χ^2 = 0.04, p = 0.85, Shannon H χ^2 = 2.16, p = 0.14). However, we identified near-significant differences between treatments for Chao1 (χ^2 = 9.31, p = 0.054) and significant treatment differences for Shannon H (χ^2 = 13.98, p = 0.007). Here, differences appeared to be driven by higher diversity in Deception Bay relative to the other samples, whereas all the remaining treatments are relatively similar to one another (Fig. S6). Similar to July, the bacterial

community did not differ significantly between treatments in August (PERMANOVA F = 2.01, p = 0.053), and groups overlapped significantly in the NMDS plot (Fig. 4). During August, 24 OTUs with > 50 total reads differed across BWE in our SIMPER analysis, of which all but one increased after BWE. As in previous months, *Trabulsiella* and *Enterobacter* were common in both treatments. *Vibrio* and *Mycoplasma* were also common before and after BWE during August, with the four genera together accounting for 90% of the community composition. No significant variations were noticed between the treatments.

Discussion

In the present study, we utilized Ion Torrent next-generation sequencing to characterize changes in the microbial community over the course of three voyages of the *M/V Arctic* between Quebec City and Deception Bay in summer of 2015. As reflected in the substantially lower water temperature in June (~16°C) versus July or August (~23°C), environmental factors associated with spring vs. summer sampling months were the strongest drivers of ballast water microbial diversity. Our June samples had by far the highest richness and diversity, with an order of magnitude greater number of OTUs present versus July or August, even though sequence reads were similar across all months. This finding is consistent with other studies that demonstrated seasonal peaks in diversity and abundance of aquatic microbes (e.g. Pernthaler et al., 1998; Salcher et al., 2011; Gilbert et al., 2012). However, additional sampling throughout the year will be necessary to draw firm conclusions about the specific drivers of microbial biodiversity in the system.

Two of the OTUs we identified are numerically dominant and occur in high numbers across all samples. These two OTUs correspond to members of the genera *Trabulsiella* and

351 *Enterobacter*, respectively. Both genera are facultative anaerobes (Holt & Krieg, 1994; Brenner
352 et al., 2005), and may be resident in the ballast tanks. Because bacteria are present in biofilms
353 and sediments in the ballast tank (Drake et al., 2005, 2007; Mimura et al., 2005), in addition to
354 the ballast water itself, these may represent a potential reservoir of hardy species that can quickly
355 repopulate newly-exchanged ballast water. It is plausible that this occurred during August (not
356 shown), where the number of reads was relatively high immediately pre-exchange, then dropped
357 to low numbers immediately post-exchange, before increasing gradually to the end of the
358 voyage. Tomaru et al., (2014) observed a similar pattern in which abundance of culturable
359 bacteria initially decreased after BWE and then increased over time, potentially as a result of
360 dilution of cell numbers followed by population growth. These results may also be consistent
361 with previous reports that demonstrated the consistency of bacterial abundances with or without
362 BWE (Drake et al., 2002; Mimura et al., 2005; Burkholder et al., 2007; Seiden et al., 2011).
363 Because earlier studies were unable to differentiate most of the bacterial diversity in the
364 ballast tanks, differences between a resident community repopulating the ballast tank versus a
365 diverse new oceanic community replacing a diverse coastal community was difficult to
366 distinguish (Drake et al., 2002). Our results, based on powerful new next-generation sequencing
367 techniques, suggest that both situations might occur at different times of the year. In June, many
368 members of the community initially collected from Quebec City appear to have been eliminated
369 by BWE and replaced by different species. In contrast, the community appeared to be much
370 more stable during July and August, possibly owing to repopulation of resident species via
371 resting stages or in biofilms. Our overall results also suggest that species richness declined
372 gradually over the course of the voyage. This may be consistent with previous research that
373 showed declines in bacterial abundance measures with increasing voyage length (Drake et al.,

2002; Seiden et al., 2011). However, it isn't clear how comparable the two types of results may be, as next-generation sequence read number has been shown to be an unreliable way of quantifying organism abundance in other organisms (i.e. zooplankton; Sun et al., 2015)

Because invasion risk is in part related to the number of species introduced (e.g. MacIsaac & Johansson, 2016), our results suggest that the risk of successful invasion by new microbial species varies widely over time, with greater threats corresponding with periods of diversity blooms in source ports. However, while the potential risk of invasion may vary seasonally for bacteria, it is likely that it is usually high, given the vastly higher concentrations of bacteria compared with other taxonomic groups in ballast water (i.e. 6-8 orders of magnitude; Carlton & Geller, 1993; Ruiz et al., 2000; Drake et al., 2007). High propagule pressure (species' introduction effort) and high colonization pressure (number of species introduced) would seem to predispose bacteria to a pattern of invasiveness when transported. Our data reveal distinctly mixed messages for the effectiveness of BWE for preventing the spread of microbes between ports. While it is clear in the June data that the microbial community changes significantly as a result of BWE and that abundances of many OTUs are sharply reduced, results from all three months - but especially from July and August - suggest that some hardy species are able to persist in ballast tanks. This result is in spite of the fact that salinities increased markedly as a result of ballast water exchange (although not to the salinity of full seawater). The presence of these same few hardy species in our ballast tanks and in the port at Deception Bay raises the possibility that some species may have been transported there via ballast water. Whether these species were introduced or are native and common remains an open question. However, these results suggest that ballast water treatment will be necessary to limit the spread of microbes via ballast water. As these treatments are already proposed and are due to become mandated in 2017

for newly-built vessels traveling outside of any single nation's exclusive economic zone (IMO, 2008), one question that remains is how effective these treatment approaches will be. This is already an active area of research (e.g. Fujimoto et al., 2014), and one where next-generation sequencing approaches, such as those used here, will play a major role. For vessels like the *M/V Arctic* that exclusively travel within the coastal waters of a single nation, and which will be exempt from new ballast water treatment requirements, the risk of transporting microbes via ballast water will likely remain high. Further research is needed to understand the magnitude of this risk.

Acknowledgements

The authors would like to thank C. Venney for valuable assistance in the molecular lab. Funding was provided by NSERC Discovery grants to DDH and HJM, and a Canada Research Chair to HJM. The manuscript was substantially improved by two anonymous reviewers and editor Aibin Zhan.

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Biosketches

The MacIsaac Lab is interested in a broad variety of questions pertaining to invasion biology, particularly with respect to aquatic ecosystems. Our questions range from fundamental questions such as what determinants affect species invasion patterns in general, to more applied topics such as how to prevent ship-mediated invasions of the Great Lakes or spread of species to inland lakes. Author contributions: M.L.J. and S.R.C. designed the study, performed genetic lab work, and wrote the manuscript, N.S. advised on project design, performed field work, and edited the manuscript, K.H., G.W., and A.R. advised on project design and edited the manuscript, F.L. and P.T. performed field work and edited the manuscript, D.D.H. advised on genetic lab work and edited the manuscript, and H.J.M designed the study and cowrote the manuscript.

569 Table 1. Similarity percentage (SIMPER) results (above the diagonal) and pairwise
570 Permutational Multivariate Analysis of Variance (PERMANOVA) probabilities (below the
571 diagonal). Qport: Quebec City port; Pre: Pre-exchange; Post: Post-exchange; Dport: Deception
572 Bay port

June

	Control	Dport	Post	Pre	Qport
Control		74.73	70.74	65.74	44.91
Dport	0.0118		69.21	78.22	78.93
Post	0.0027	0.414		76.1	74.94
Pre	0.0034	0.0331	0.0093		65.18
Qport	0.214	0.0988	0.0109	0.0466	

July

	Control	Dport	Post	Pre	Qport
Control		59.37	59.4	54.7	63.87
Dport	0.233		52.52	64.73	85.39
Post	0.14	0.0978		59.28	72.59
Pre	0.0443	0.5613	0.0322		45.46
Qport	0.0331	0.3235	0.095	0.7577	

August

	Qport	Pre	Control	Post	Dport
Qport		36.3	41.42	43.71	61.92
Pre	0.431		49.68	52.17	65.33
Control	0.2287	0.0286		46.75	59.38
Post	0.2086	0.0326	0.8445		60.29
Dport	0.3095	0.0296	0.1992	0.4634	

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Figure Legends

Figure 1. Map of the *M/V Arctic's* approximate route (~3100 km). Ballast water exchange areas are indicated by dark patches over the dotted line (Blue : Jacques Cartier Strait, Red : Strait of Belle Isle). Approximate ballast water sampling points are indicated by stars.

Figure 2. Box and Whisker plots of bacterial species richness (left) and diversity (right) over all three months based upon operational taxonomic units detected in ballast water. Thick bar is median, boxes represent the middle 50% of the data, whiskers represent the upper and lower quartiles (i.e. the largest 25% and smallest 25% of the data).

Figure 3. Two-dimensional nonmetric multidimensional scaling (NMDS) plot for all months.

Figure 4. Two-dimensional nonmetric multidimensional scaling (NMDS) plots for June, July, and August separately. Qport: Quebec City port; Pre-exch: Pre-exchange; Post-exch: Post-exchange; Dport: Deception Bay port.

Figure S1. Rarefaction curve for June Ion Torrent sequence data. Each line represents one sample.

Figure S2. Rarefaction curve for July sequence data. Each line represents one sample.

Figure S3. Rarefaction curve for August sequence data. Each line represents one sample.

Figure S4a. Taxonomy bar chart for early and late voyage June samples. Quebec City and Deception Bay are sampled from port water. Control, Exchange 1, and Exchange 2 are samples from ballast tanks early and late in the voyage. Arrows connect early and late samples from the same ballast tanks.

Figure S4b. Taxonomic legend for Figure S4a.

Figure S5a. Taxonomy bar chart for early and late voyage July samples. Quebec City and Deception Bay are sampled from port water. Control, Exchange 1, and Exchange 2 are samples

598 from ballast tanks early and late in the voyage. Arrows connect early and late samples from the
599 same ballast tanks.
600 Figure S5b. Taxonomic legend for Figure S5a.
601 Figure S6b. Taxonomy bar chart for early and late voyage August samples. Quebec City and
602 Deception Bay are sampled from port water. Control, Exchange 1, and Exchange 2 are samples
603 from ballast tanks early and late in the voyage. Arrows connect early and late samples from the
604 same ballast tanks.
605 Figure S6b. Taxonomic legend for Figure S6a.
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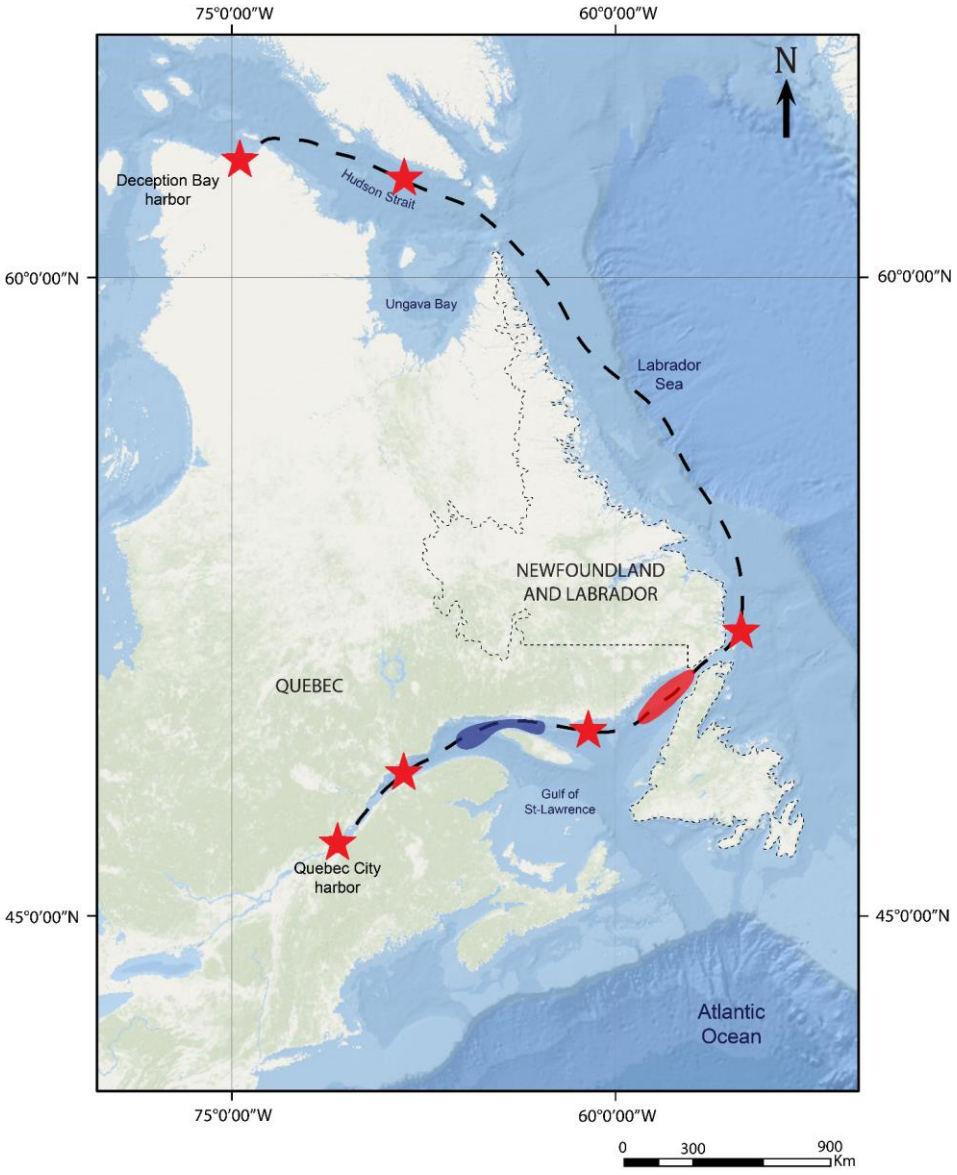

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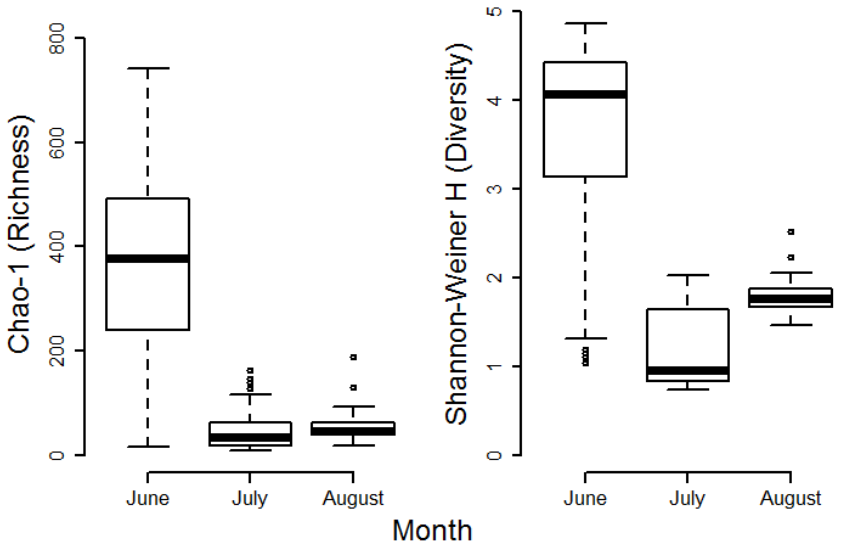

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611 Figure 2.

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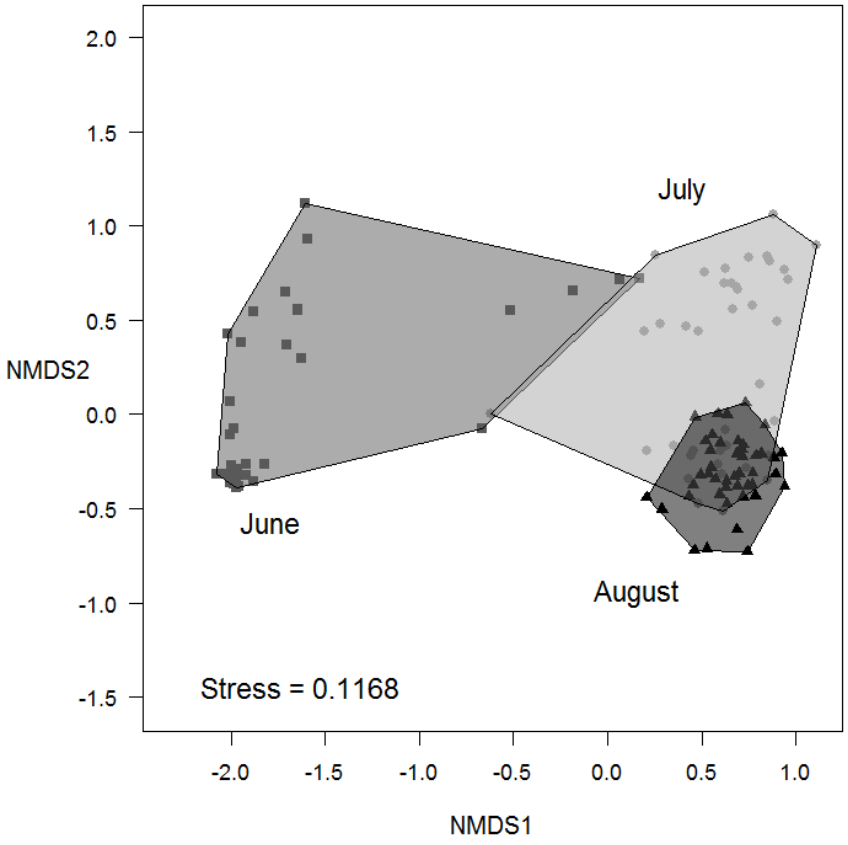

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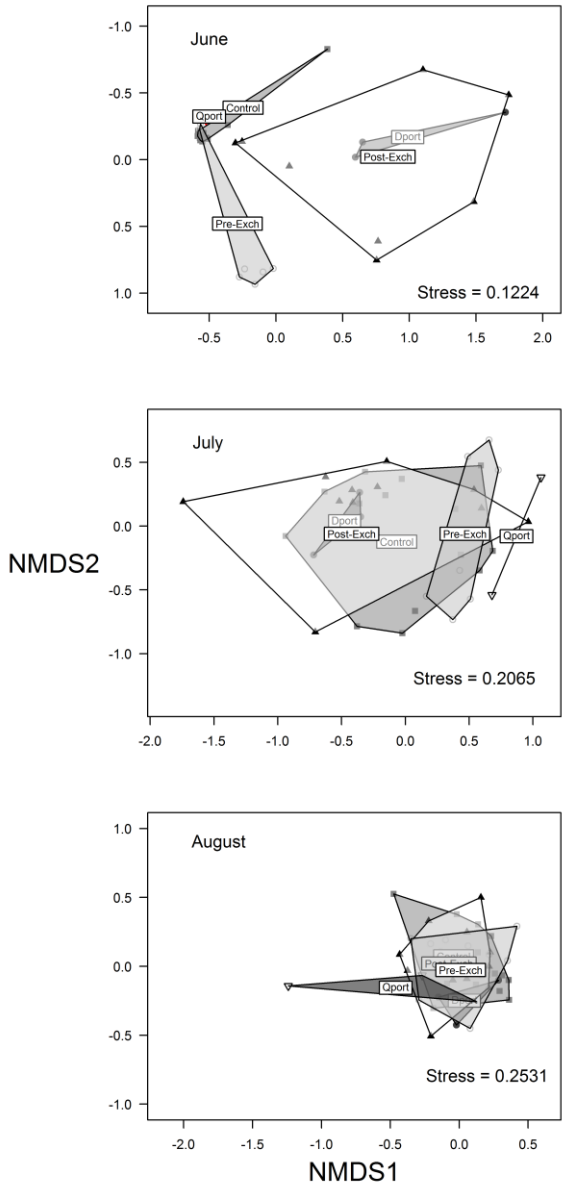

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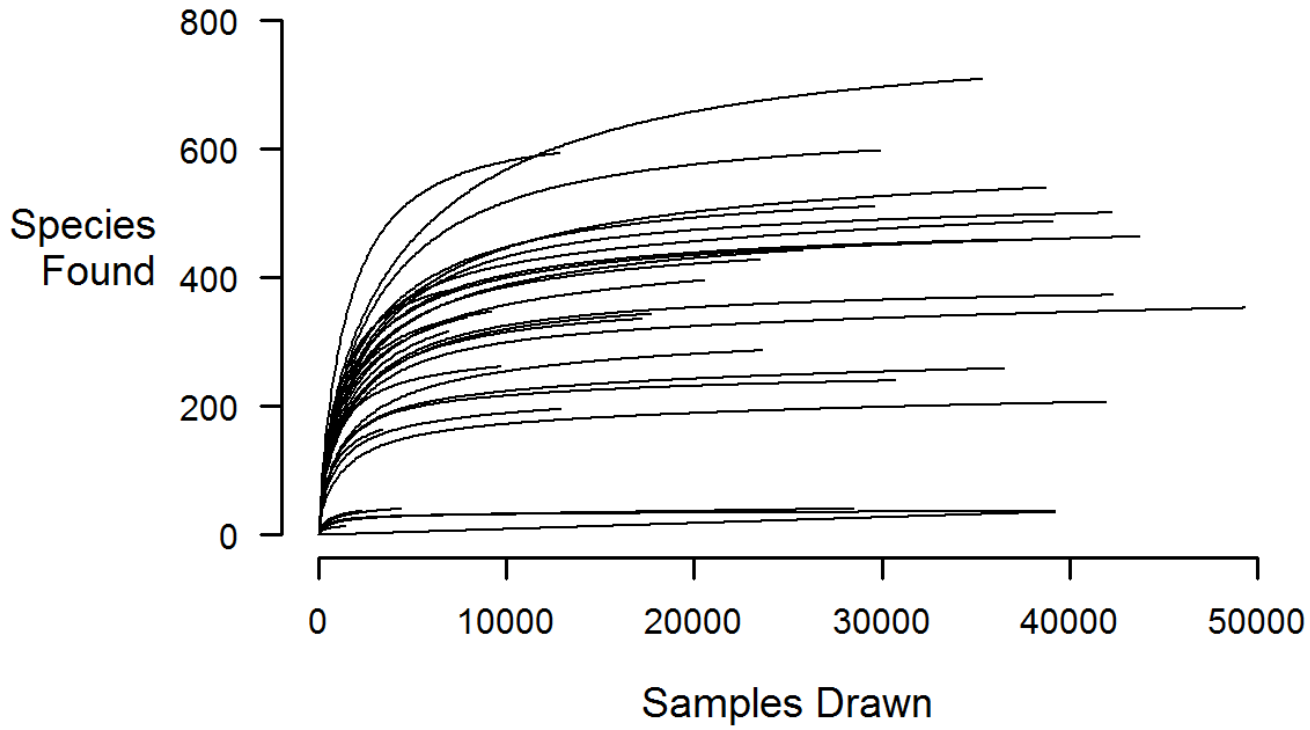
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Samples Drawn

Figure S1.

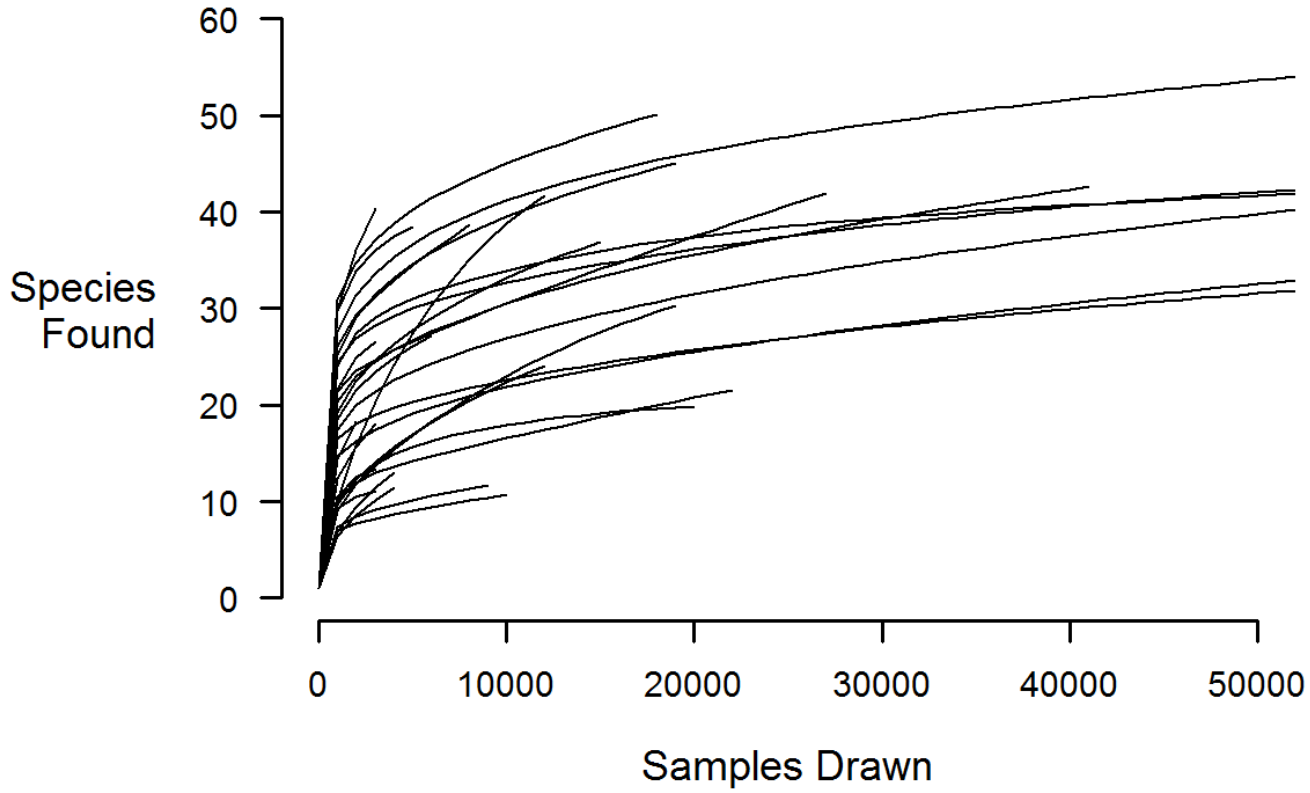
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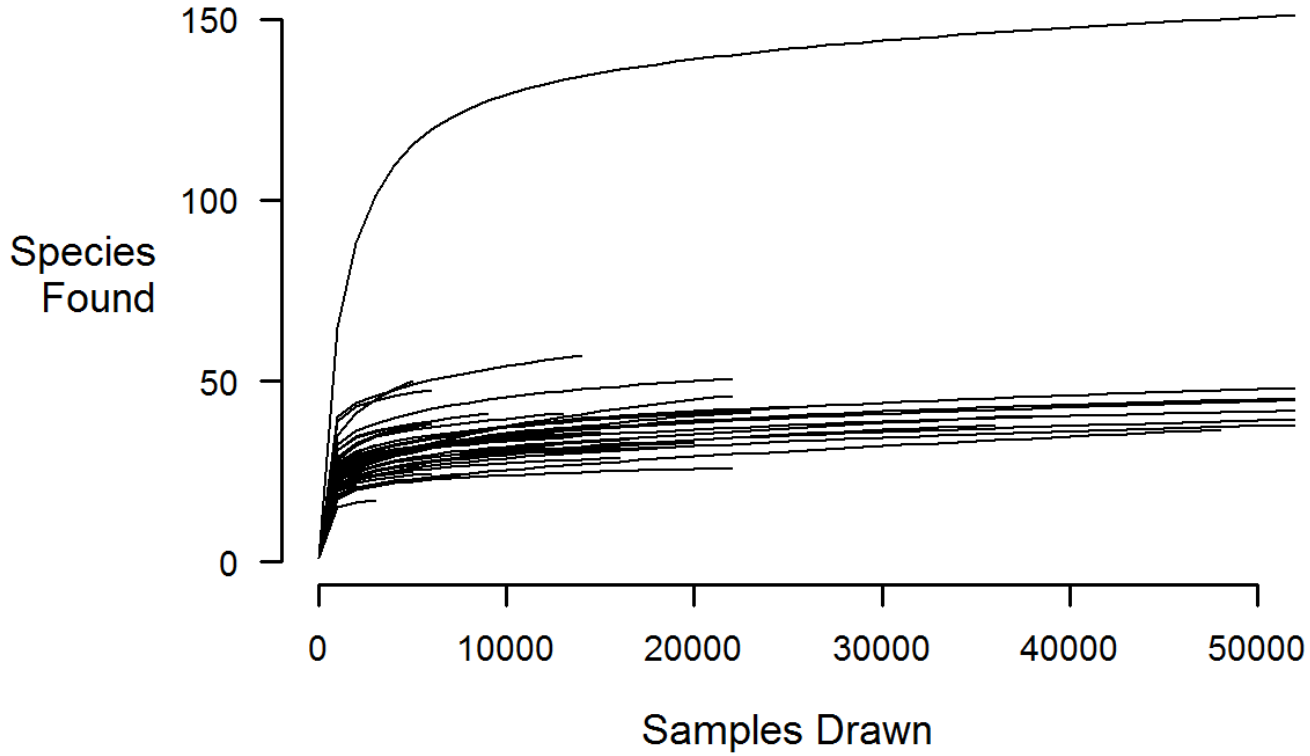
Figure S2.



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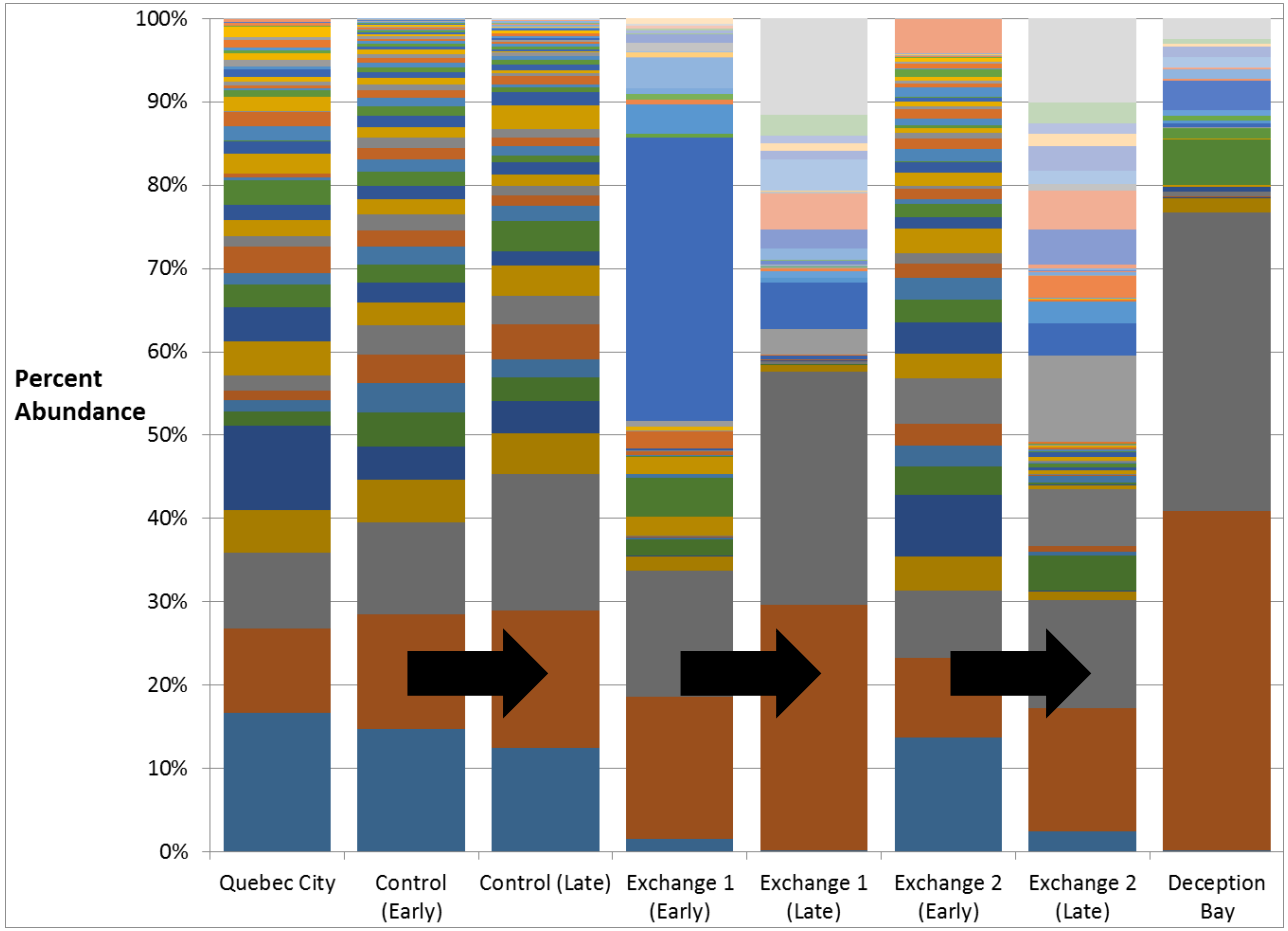

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624 Figure S4a.


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Litorimicrobium(91.7)
Gammaproteobacteria(94.0)
Gaetbulibacter(91.7)
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Clostridiales(86.7)
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Bacteria(100.0)
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Bacillales(82.5)
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Rhodococcus(93.2)
Ralstonia(98.3)
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Mycoplasma(97.5)
Planctomycetacia(83.2)
Aquaspirillum(95.4)
Bacteriovoracaceae(82.6)
Afipia
Fluviicola(84.7)
Burkholderia(99.2)
Pseudomonas(99.5)
Peredibacter(90.8)
Firmicutes(84.2)
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Acidimicrobiaceae
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Flavobacteria(85.4)
Acidimicrobiales
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Enterobacter
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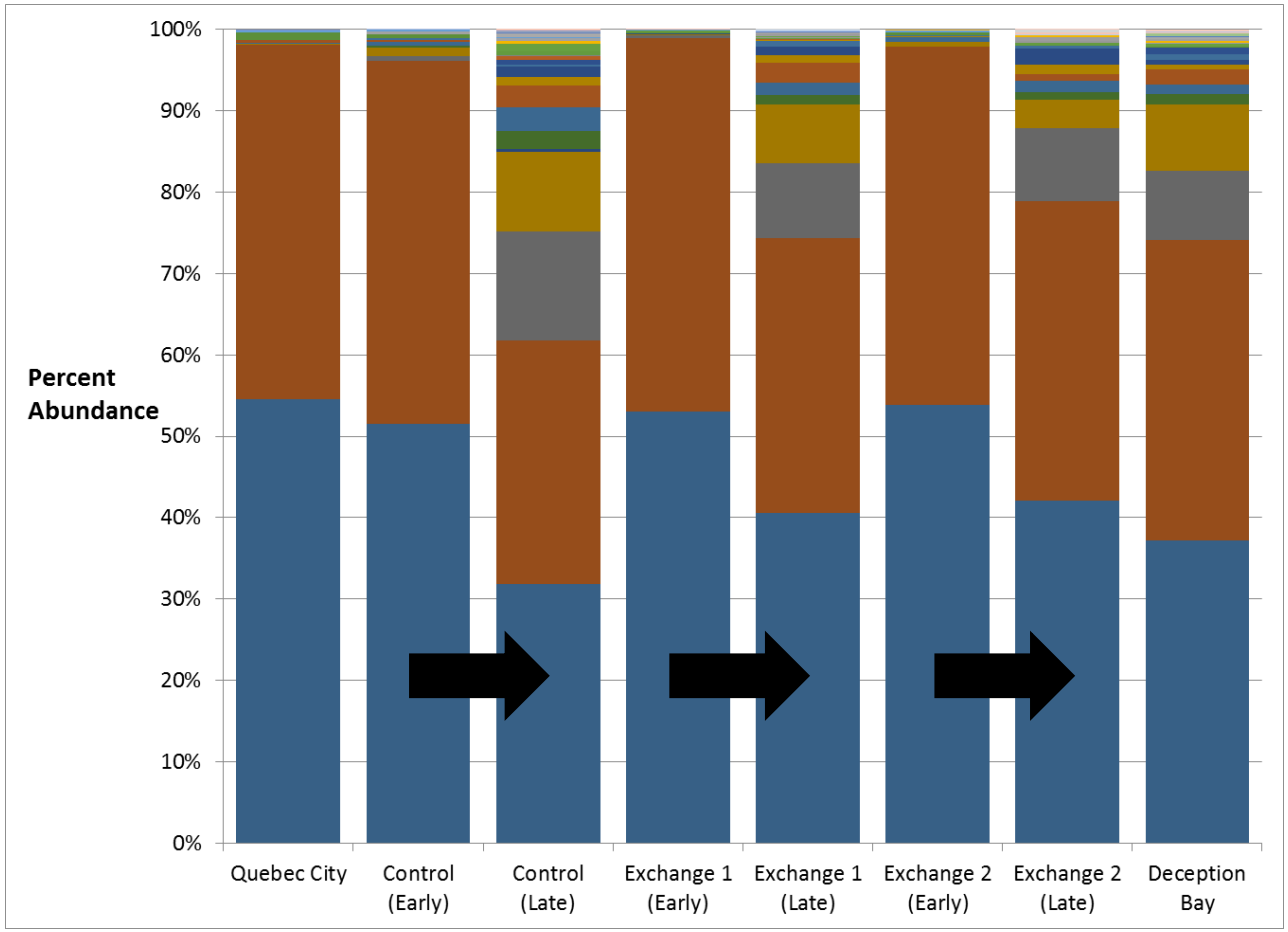
Actinobacteria

Figure S4b.



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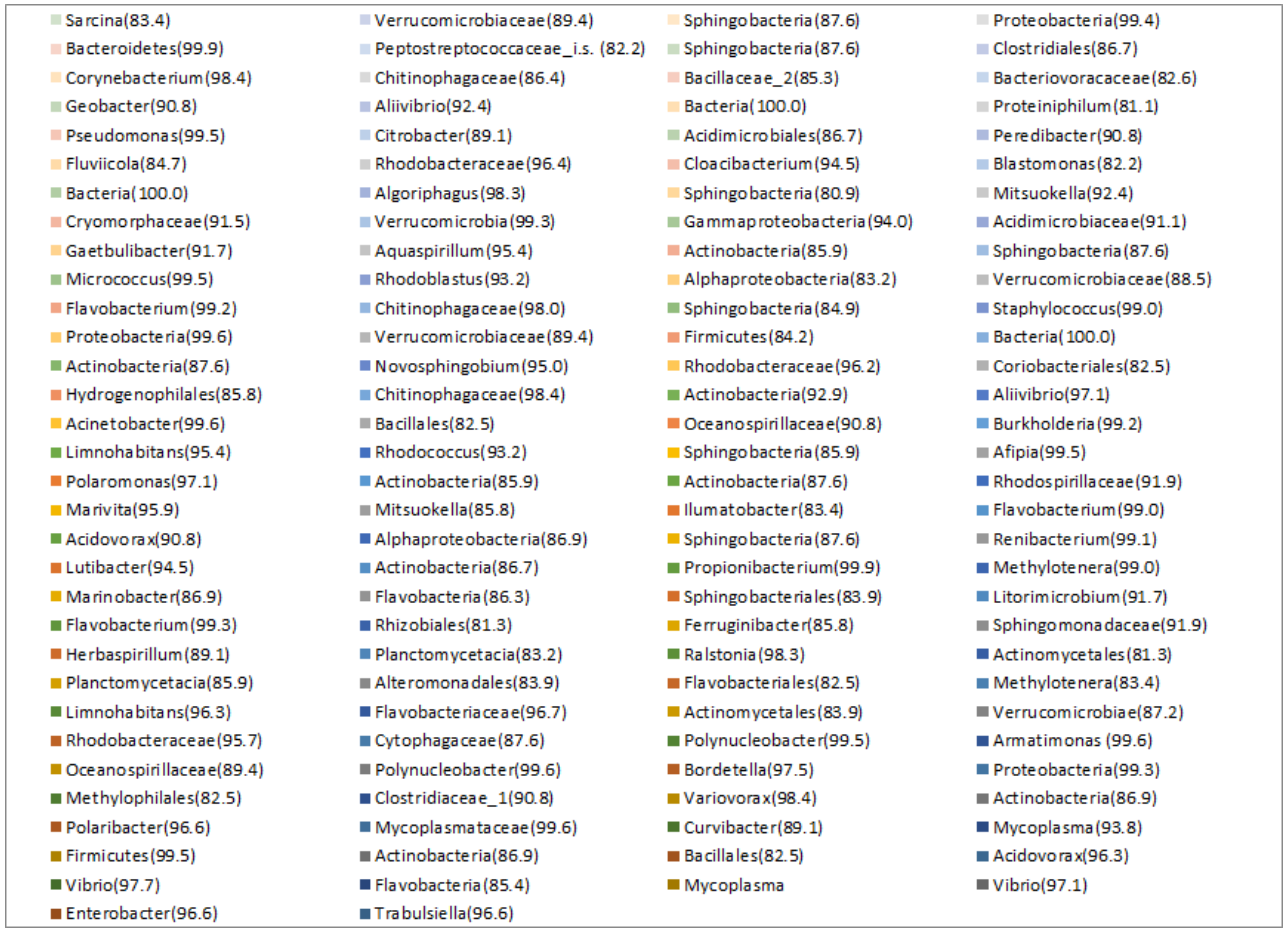
628 Figure S5a.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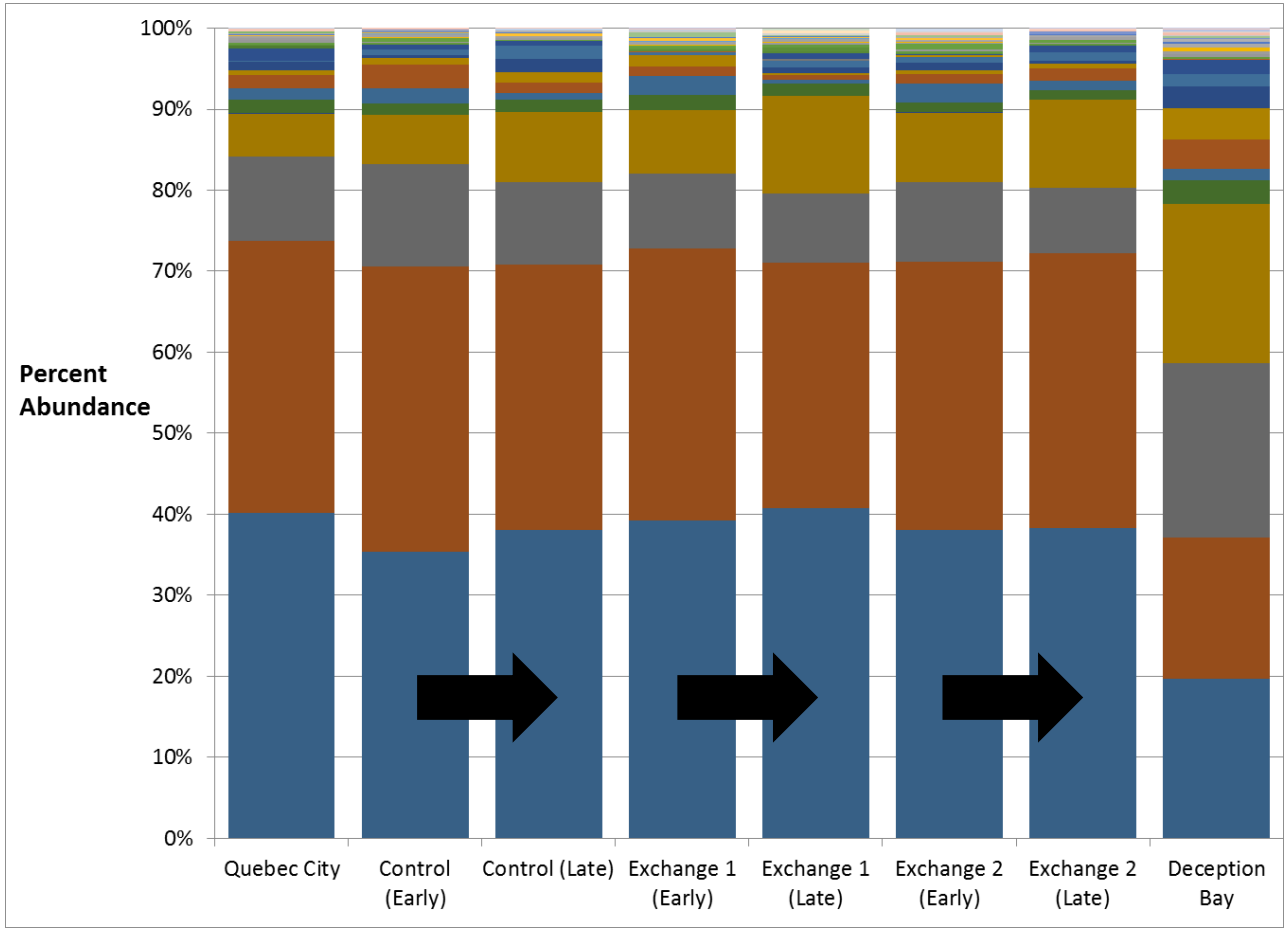
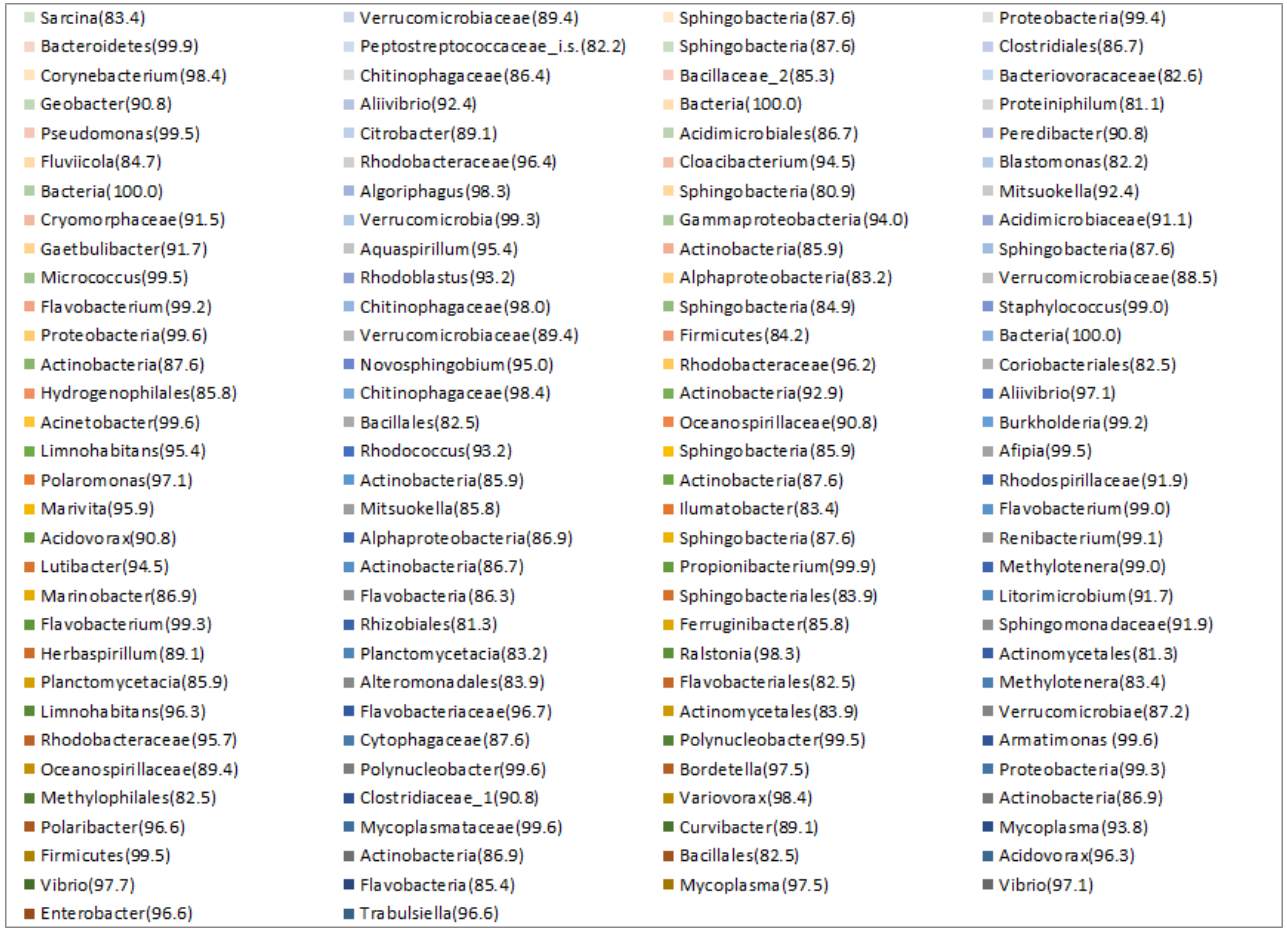
Actinomycetales(81.3)
Methylotenera(83.4)
Verrucomicrobiae(87.2)
Armatimonas (99.6)
Proteobacteria(99.3)
Actinobacteria(86.9)
Mycoplasma(93.8)
Acidovorax(96.3)
Vibrio(97.1)

Figure S5b.



631

632 Figure S6a.



633

634 Figure S6b.