Terrestrial dissolved organic matter inflow drives temporal dynamics of the bacterial community of a subarctic estuary (northern Baltic Sea)

Daniela Figueroa,1,2† Eric Capo,1† Markus V. Lindh,3‡ Owen F. Rowe,4 Joanna Paczkowska,1,2‡ Jarone Pinhassi3 and Agneta Andersson1,2*  
1Department of Ecology and Environmental Science, Umeå University, Umeå, SE-901 87, Sweden.  
2Umeå Marine Sciences Centre, Hörnefors, SE-905 71, Sweden.  
3Ecology and Evolution in Microbial Model Systems, EEMiS, Linnaeus University, Kalmar, SE-391 82, Sweden.  
4Baltic Marine Environment Protection Commission HELCOM, Helsinki, FI-00160, Finland.

Summary  
Climate change is projected to cause increased inflow of terrestrial dissolved organic matter to coastal areas in northerly regions. Estuarine bacterial community will thereby receive larger loads of organic matter and inorganic nutrients available for microbial metabolism. The composition of the bacterial community and its ecological functions may thus be affected. We studied the responses of bacterial community to inflow of terrestrial dissolved organic matter in a subarctic estuary in the northern Baltic Sea, using a 16S rRNA gene metabarcoding approach. Betaproteobacteria dominated during the spring river flush, constituting ~ 60% of the bacterial community. Bacterial diversity increased as the runoff decreased during summer, when Verrucomicrobia, Betaproteobacteria, Bacteroidetes, Gammaproteobacteria and Planctomycetes dominated the community. Network analysis revealed that a larger number of associations between bacterial populations occurred during the summer than in spring. Betaproteobacteria and Bacteroidetes populations appeared to display similar correlations to environmental factors. In spring, freshly discharged organic matter favoured specialists, while in summer a mix of autochthonous and terrestrial organic matter promoted the development of generalists. Our study indicates that increased inflows of terrestrial organic matter-loaded freshwater to coastal areas would promote specialist bacteria, which in turn might enhance the transformation of terrestrial organic matter in estuarine environments.

Introduction  
Heterotrophic bacteria play a fundamental role in the cycling of organic matter and nutrients, thereby regulating fluxes of energy and matter in aquatic ecosystems (Azam and Malfatti, 2007). Bacteria utilize both autochthonous and terrestrial dissolved organic matter (DOM) as their main carbon sources (Azam and Malfatti, 2007). While autochthonous DOM is the major food source for marine bacteria in offshore areas (Sarmento and Gasol, 2012), terrestrial DOM supports an important portion of bacterial production in coastal areas (Hitchcock and Mitrovic, 2015; Figueroa et al., 2016). Differences in DOM composition may influence the distribution of bacterial populations due to their metabolic capabilities (Gómez-Consarnau et al., 2012; Logue et al., 2016). Additionally, bacterial communities are shaped by a complex interplay between bottom-up (e.g., DOM composition) and top-down factors (e.g., grazing and competition) (Azam and Malfatti, 2007; Bunse and Pinhassi, 2017; Lindh and Pinhassi, 2018).

The export of terrestrial DOM to aquatic ecosystems is an important component of the global carbon cycle (Reynolds, 2008). In subarctic coastal zones, dissolved organic carbon (DOC) concentrations are relatively high compared to offshore areas, mainly due to inflow of terrestrial DOM from rivers (Reader et al., 2014). In fact, in the semi-enclosed northern Baltic Sea as much as ~ 90% of the DOM is of terrestrial origin (Alling et al., 2008). Climate change projections indicate that precipitation in subarctic and boreal areas will increase (Meier et al., 2012), leading to an increased inflow of
Terrestrial inflow shaping coastal bacteria

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freshwater rich in DOC from land to the coastal zones (Andersson et al., 2015). In the subarctic Bothnian Bay, the terrestrial DOM discharges may increase as much as ~30%, due to increased precipitation and permafrost melting (Straat et al., 2018).

Experimental studies have examined the functional responses of certain bacteria to changes in DOM quality and concentrations. For example, members of the Roseobacter clade of Alphaproteobacteria exhibit high uptake of carbon monomers and amino acids (Alonso-Sáez and Gasol, 2007), linking them to the autochthonous DOM released during phytoplankton blooms (Pinhassi et al., 2004). Likewise, bacteria in the class Flavobacteria (Bacteroidetes), proliferate in highly productive waters (Alonso and Pernthaler, 2006; Alonso-Sáez et al., 2007). In contrast, some members of the Alphaproteobacteria genus Sphingomonas thrive at low carbon concentrations (Eiler et al., 2003). In a wide range of aquatic ecosystems terrestrial DOM has also been shown to stimulate heterotrophic bacterial activity, for example in alpine and boreal lakes, subtropical and subarctic estuaries (Ask et al., 2009; Barrera-Alba et al., 2009; Wikner and Andersson, 2012; Figueroa et al., 2016). An experimental study showed that terrestrial DOM alters the bacterial enzyme activity, which in turn affect the recycling of nutrients in the system (Traving et al., 2017). Furthermore, bacteria can alter the DOM pool by a selective processing of substances (Boyd and Osburn, 2004; Berggren and Giorgio, 2015; Rowe et al., 2018). Yet, the influence of terrestrial DOM on the ecological functioning of bacterial communities is poorly understood, particularly under natural conditions.

One approach to resolve complex ecological bacterial associations is to use network analysis. Although correlations do not necessarily imply causations (Carr et al., 2019a,b), networks allow to identify potential associations between microbial populations and links with abiotic factors over both spatial and temporal scales (Fuhrman and Steele, 2008; Barberán et al., 2012; Bissett et al., 2013; Lima-Mendez et al., 2015). Populations inhabiting the same ecosystem exhibit stronger or weaker connectivity based on their spatial and temporal distributions. Thus, ecological networks often show large heterogeneity in modularity (Olesen et al., 2007), where modules are defined as group of populations with strong associations. Populations having strong and numerous links with other populations, either within or between the modules, are therefore thought of as habitat specialists, while populations with weak and few connections to each other can be defined as habitat specialists (Olesen et al., 2007). By identifying bacterial consortia present in network modules and their links with specific environmental parameters, it is possible to provide new insights about the likely ecological niches of bacterial populations. Thereby it allow us to identify specialists feeding on certain substrates – e.g., terrestrial DOM – and generalists utilizing both autochthonous and terrestrial DOM.

The objective of the present study was to investigate how the bacterial community in a subarctic estuary is modified over the productive season with changes in terrestrial organic matter inflow. We expected that (i) high inflow of terrestrial DOM changes the bacterial community structure in the estuary (ii) terrestrial DOM affects the associations between bacterial populations and their ecological niches. Water samples were collected at 19 stations in a subarctic estuary in the northern Baltic Sea (Råne estuary) during the productive season – May–August 2011 – (Fig. S1). We used a 16S rRNA gene metabarcoding approach to study the spatial and temporal variation in bacterial community diversity and structure to unravel the presence of population co-occurrences. We specifically searched for links between the temporal changes in the bacterial community and modifications in environmental conditions. Using network analysis, we identified bacterial populations responding to the riverine runoff, differentiating generalist and specialist populations.

Results

Physico-chemical conditions and microbial production in Råne subarctic estuary

The ice break-up occurred in May just before our study period started, and the peak of the spring river flush coincided with our first sampling week (Fig. S2, Table 1A). The average seawater temperature was lowest in May (~7°C), peaked in July (21°C) and decreased again in August (16°C) (Table 1A). The average salinity in the estuary increased from ~0.2 in spring (May) to 1 part per thousand in summer (June–August), and the coefficient of variation was twice as high in spring than in summer (CV spring ~1.2, CV summer ~0.6), indicating less stable hydrological conditions in spring. pH showed a similar temporal pattern as salinity.

The concentrations of DOC and terrestrial DOM related variables – in this study humic substances, coloured DOM (CDOM), total nitrogen (TotN) and organic suspended particulate matter (SPM_org) – exhibited highest values in May, concomitant with the peak in spring river flush, followed by lower values in summer (Table 1A). Further, the terrestrial DOM related variables generally showed higher values in the inner estuary close to the river mouth and values decreased in seaward direction (Table S1). The concentrations of total phosphorus (TotP) and inorganic suspended particulate matter
Phytoplankton and heterotrophic bacteria constitute the lowest trophic level in the pelagic food web, and their summed production is here defined as the basal production. During spring, the average phytoplankton primary production was low (Table S1). In contrast, heterotrophic bacterial production peaked during spring when terrestrial DOM concentration was high (Table S1). Thereafter, bacterial production decreased over the study period whereas primary production increased. In spring bacterial production constituted $\sim90\%$ of the basal production, while in late summer it only constituted $\sim30\%$ of the basal production (Table S1).

Spatio-temporal variability of the bacterial community and links to environmental conditions

Betaproteobacteria was the dominant bacterial group across the study period in terms of total richness and relative abundance (Fig. 1). In May, Betaproteobacteria – largely dominated by Burkholderiales – constituted $>75\%$ of the community in the river and inner estuary stations. Bacteroidetes (i.e., Sphingobacteriales, Flavobacteriales) and Actinobacteria (i.e., Frankiales) were also notable groups that occurred abundantly throughout the study period. The relative abundance of Verrucomicrobia (vadinHA64), Planctomycetes (Planctomycetales) and Gammaproteobacteria (Oceanospirillales, Alteromonadales, Pseudomonadales) increased from spring to summer, whereas Betaproteobacteria decreased. Furthermore, in summer, Verrucomicrobia and Planctomycetes were more prominent in outer estuary stations as compared to the inner estuary (Fig. 1).

The average number of Operational Taxonomic Units (OTUs), as well as the estimated Shannon diversity index, Chao1 richness and the Pielou’s evenness metrics, showed lower values in May (spring) than during the summer months. The average number of OTUs decreased again in August (Table S2A). Non-metric multidimensional scaling (nMDS) plots highlighted the spatial differences in bacterial community structure between inner and outer estuary stations (Fig. 2) as well as the structure modifications over the productive season (Fig. S3). PERMANOVA analysis confirmed that spatial differences in bacterial community structure were significant and explained by differences in concentrations of DOC and terrestrial DOM related variables such as humic substances, CDOM, the SPM$_{org}$ and SPM$_{inorg}$ particularly from June to August (Table 1B, Fig. 2). In contrast, non-terrestrial DOM related variables such as total phosphorous, phytoplankton production and temperature showed weaker to no significant correlations with community structure, this pattern being most notable in May (Table 1B).

### Table 1. Average values of environmental variables during different months. ANOVA test of differences between months (A). Relationship between bacterial community structure and environmental variables, analysed using permutation test (B).

| A. Environmental variable | May   | June  | July  | August | ANOVA test p-value |
|---------------------------|-------|-------|-------|--------|------------------|
| Salinity (mg l$^{-1}$)     | 0.2   | 0.5   | 0.5   | 1      | 1.47E-07***      |
| DOC (mg l$^{-1}$)          | 7.6   | 5.6   | 6.3   | 5.9    | 0.000281***      |
| Humic substances (μg l$^{-1}$) | 61.5 | 43.8  | 53.6  | 41.9   | 3.82E-06***      |
| CDOM (m$^{-1}$)            | 3     | 2.8   | 2.9   | 2.1    | 0.002**          |
| SPM$_{org}$ (g m$^{-3}$)   | 1.5   | 1.4   | 1.5   | 1.4    | 0.828            |
| SPM$_{inorg}$ (g m$^{-3}$) | 1.6   | 2     | 2     | 2.3    | 0.497            |
| Prim. Prod. (μg C l$^{-1}$ day$^{-1}$) | 13.5 | 19.9  | 34.4  | 53.6   | 5.53E-06***      |
| TotN (mg l$^{-1}$)         | 0.38  | 0.28  | 0.29  | 0.29   | 3.87E-10***      |
| TotP (μg l$^{-1}$)         | 9     | 10    | 10.1  | 10.4   | 0.128            |
| Temperature (°C)           | 6.7   | 15.7  | 21.4  | 16.5   | <2e-16***        |
| pH                        | 6.9   | 7.2   | 7.2   | 7.4    | 2.90E-10***      |
| River discharge (m$^{3}$ s$^{-1}$) | 99.3 | 33.4  | 33.1  | 24.8   |

| B. Relationship to bacterial community | DOC | Humic substances | CDOM | pH | SPM$_{org}$ | SPM$_{inorg}$ | Prim. Prod. | TotN | TotP | Temperature |
|---------------------------------------|-----|-----------------|------|----|-------------|--------------|-------------|------|------|------------|
| PERMANOVA test p-value                | 0.48* | 0.72**         | 0.87*** | 0.73** | 0.44* | 0.88*** | 0.94*** | 0.92*** | Ns   | 0.74***    | 0.41* | 0.82** |
|                                       | 0.59** | 0.70***        | 0.88*** | 0.95*** | Ns   | 0.76*** | 0.78*** | 0.93*** | Ns   | 0.56**     | 0.41* | Ns   |
|                                       | 0.53** | 0.76***        | 0.78*** | 0.93*** | Ns   | 0.74*** | 0.71*** | 0.69**  | Ns   | Ns         | 0.41* | Ns   |
|                                       | Ns   | 0.81***        | Ns    | 0.82*** | Ns   | Ns       | Ns         | Ns     | Ns   | 0.36*      | Ns    | Ns   |

* <0.05; ** <0.01; *** <0.001.
Fig 1. Number of OTUs of different bacterial groups (left) and relative number of reads of different bacterial groups (in %) at the sampling stations in the Råne estuary during May, June, July and August (right). Light brown and light blue colours correspond to inner and outer estuary, respectively. Map of sampling locations provided in Fig. S1. [Color figure can be viewed at wileyonlinelibrary.com]
Network structure, modularity, connectivity and links with environmental conditions

To resolve potential associations between the bacterial OTUs, we applied network analysis using the WGCNA procedure as extensively described in the section ‘Experimental procedures’. The networks were created independently for each month (four networks) based on the differences observed in the structure of the bacterial communities over time (Fig. 3). To reduce the complexity of each network, OTUs present less than nine times per dataset were removed. Networks were composed of nodes (OTUs) linked by edges (correlations between OTU based on their relative abundance in each sample). Modules correspond to group of nodes in which each node has a minimum number of 8 edges with other nodes. Overall, networks showed a shift in co-occurrence patterns of bacterial populations over time (Fig. 3). In May, the network consisted of 222 nodes (OTUs) linked by 705 edges, and as the season progressed the number of nodes and edges increased (Table S2B). The largest number of nodes ($n = 517$) was observed in July, forming 4459 edges (Fig. 3 and Table S2A and B). Still, the largest number of edges was observed in August (5373), indicating that more OTU associations occurred later in the season (Table S2B). The diameter, i.e., the distance between the two most distant nodes, was similar in May, July and August (Table S2B), indicating similar connectivity patterns between OTUs during these months. However, in June the diameter was twice as large and the average network distance was also higher, i.e., the connection between all pairs of nodes (see average path lengths in Table S2B). This suggests that the OTU co-occurrence and the network connectivity were lower in June than in the other months. On the other hand, the degree of node clustering (clustering coefficient) was higher in June and August than in May and July, while the modularity index was high in June and low in August (Fig. 3 and Table S2B).

The relationships between OTUs gathered within modules and the environmental variables were investigated for each month independently (Fig. 4). In May, pH values were strongly correlated to several modules, positively and negatively. Humic substances, CDOM, and DOC concentrations were also correlated to certain modules.
For example, the modules ME_{May} 8 and ME_{May} 13 were positively correlated to DOC. In June, July and August most of the environmental variables were negatively correlated to modules (in total 19, 22 and 18 modules respectively). In June, the total phosphorous showed low degree of correlation to a large number of modules, whereas temperature was less related to modules in July (Fig. 4). On the other hand, temperature was negatively correlated to some modules in August, whereas ME_{August} 13, ME_{August} 14 and ME_{August} 15 did not respond to any environmental variable (Fig. 4).

**Generalist and specialist bacteria identified with the network analysis**

Generalist and specialist bacteria were identified by analysing the level of node connectivity within modules ($z_i$) and among-modules ($P_i$) in each monthly network (Olesen et al., 2007). Differences in the proportion of generalists and specialists, defined by their topological roles in networks, were observed between months (Fig. S4). ‘Super generalist’ bacteria, i.e., highly connected OTUs within and between modules, were rare throughout the season, representing at most 6% of the community nodes in July. For example, in May only one super generalist OTU occurred, the Flavobacteriales OTU 933 from the Bacteriodetes phylum. Therefore, in subsequent months they were included in the group of generalist bacteria. In May, populations consisted of 62% specialists and 38% generalists (Fig. S4). In June, the proportion of specialists decreased to 41% and the generalists increased to 59%, while in July and August the specialists and generalists constituted 31% and 69%, respectively.

Numerous bacterial populations (i.e., OTUs in the context of the current paper) shifted from being specialists to become generalists and vice versa (Table S3). For example, some Gammaproteobacteria such as...
Oceanospirillales, Alteromonadales and Pseudomonadales (OTUs 7, 10 and 92, respectively) or Bacteroidetes such as Flavobacteriales and Sphingobacteriales (OTUs 13 and 14) started as specialists in May and became generalists later in summer. Other bacterial populations maintained the same ecological behaviour during the whole season, such as Sphingobacteriales and Flavobacteriales (OTUs 40 and 233), Betaproteobacteria as Methylophilales, Burkholderiales and Nitrosomonadales (OTUs 61, 64 and 169) and the Verrucomicrobia Verrucomicrobiales and OPB35 soil group (OTUs 97, 98 and 133) that all expressed generalist behaviour throughout the season. On the other hand, the Betaproteobacteria (OTU 6: Burkholderiales), Verrucomicrobia (OTU 47: Opitutales) and Actinobacteria (OTU 176: Thermoleophilia) were consistently identified as specialist bacteria.

Discussion

Environmental factors influenced the diversity, composition and the structure of the bacterial community of a subarctic estuary over the productive season

The temporal variation in hydrography and physicochemical factors caused a distinct bacterial succession, where the community changed from low alpha diversity
in May (spring) to a more diverse community in late summer. Variables related to terrestrial DOM influenced the bacterial community structure in the estuary during the entire study period (DOC, humic substances and CDOM), while other environmental variables were important during parts of the study period (e.g., total phosphorous in July and August). Even if the terrestrial DOM concentrations were highest in May, shifts in community structure in the estuary were more correlated with the terrestrial DOM components later in the season. This was probably due to the widespread occurrence of terrestrial DOM in the estuary in May and thus lower spatial gradients than in summer. In summer, the terrestrial DOM and salinity gradients were stronger across the study region, thereby more clearly structuring the bacterial community composition. Consequently, the correlation between terrestrial DOM variables and bacterial community composition was stronger in the summer. Overall, terrestrial DOM had a major influence on the bacterial community structure, indicating that an increase in terrestrial organic matter inflow would strongly influence the bacterial community composition and thus the ecosystem function.

The succession of bacterial populations likely reflects the abilities and preferences of the bacteria to consume different substrates (Cottrell and Kirchman, 2000; Gómez-Consarnau et al., 2012). In line with the findings of earlier studies (Riemann et al., 2008; Newton et al., 2011), we found that Betaproteobacteria and Bacteroidetes dominated the community early in the season (May and June). These groups occur abundantly in estuaries with a large supply of organic carbon and can degrade humic matter (Teira et al., 2009; Hutalle-Schmelzer et al., 2010; Perez-Pantoja et al., 2012). Several Betaproteobacteria occurring in coastal areas have been suggested to originate from freshwater or terrestrial ecosystems (Rappé et al., 2000; Ruiz-González et al., 2015a). A previously published study in the estuary indicate that in May and June, the bacterial community was actively processing the available carbon as the highest bacterial production was observed (Table S1). (Figueroa et al., 2016). Furthermore, pH significantly explained a part of the variation in bacterial community structure, probably due to a change in DOM composition or by directly affecting the cell physiology of some bacteria (Fierer et al., 2007; Niño-García et al., 2016).

In accordance with the results by Landa and colleagues (2014), we found that the environmental conditions in summer, with reduced riverine discharge, higher temperature and increased autochthonous production, allowed richer and more diverse communities to develop. The proportion of Verrucomicrobia and Planctomycetes increased (Fig. 1), coinciding with the maximum in phytoplankton primary production (Figueroa et al., 2016). These groups are known to efficiently utilize phytoplankton exudates (Newton et al., 2011), and to dominate the bacterial community in the Baltic Sea during summer (Herlemann et al., 2014; Lindh et al., 2015). Autochthonously produced compounds might promote growth of specific groups within these phyla (Alonso-Sáez and Gasol, 2007), leading to the new community structure observed. In fact, the increase of Verrucomicrobia during summer was mainly due to the proliferation of member of the vadinHA64 order (class Opitutae) and other populations, such as representatives of OPB35 soil group, which became abundant at the river mouth.

Throughout the study period, Gammaproteobacteria occurrence was scarce, reaching a maximum abundance in summer, mainly due to increases of Oceanospirillales and Alteromonadales. Gammaproteobacteria are known to be opportunistic, able to degrade a wide range of substances, including autochthonous and terrestrial DOM (Mou et al., 2008), and can rapidly adapt to new growing conditions (Lauro et al., 2009). The increase of Gammaproteobacteria when river discharge decreased supports the idea that Gammaproteobacteria grow well in stable water conditions and when the supply of autochtonous DOM is high (Teira et al., 2009).

Different responses were observed for OTUs within different genera, showing adaptability through different strategies between closely related bacteria, as has also been observed by Teira and colleagues (2009) and Lindh and colleagues (2015). For example, associations between Betaproteobacteria and Bacteroidetes were often observed in different modules in the estuarine community, independently of the environmental factor influencing the module. This frequent relationship could be the result of an evolutionary process at the local environmental scale. Further analyses on resource utilization and metabolic activity are necessary to understand the ecological role of Betaproteobacteria and Bacteroidetes in the Råne estuary and similar aquatic ecosystems. However, taken together, our results highlight the strong influence of varying environmental conditions on the composition of coastal bacterial communities.

**Modifications of co-occurrence patterns in the bacterial community linked with changes of terrestrial DOM inflow over the productive season**

The bacterial community networks showed a more homogeneous pattern in spring than in summer, where they showed a patchier distribution. During spring, only 11 bacterial network modules were observed, and four of these...
were regulated by terrestrial DOM related variables; pH, DOC, CDOM and humic substances. The low connectivity level between the bacterial OTUs in spring was likely due to specialization of the bacterial community to the environmental conditions, i.e., a dominance of terrestrial DOM and large freshwater discharges. The influence of freshwater in the estuary was very high in May during the spring flush. In fact, > 90% of the estuarine water consisted of freshwater (Table S4). The salinity in the estuary was markedly lowered the DOC concentration distinctly elevated (Table S4), and it is likely there was a concurrent and extensive introduction of freshwater bacteria. The environmental conditions, with high terrestrial DOM concentrations, sorted for species or groups of bacteria that had a metabolic capability to utilize terrestrial DOM as their food source. As shown in other studies (Langenheder and Szekely, 2010), the mass effect and species sorting were probably significant during this initial phase of the productive season.

In summer, the bacterial community established a net of complex interactions, combining the bacterial assemblages into ~ 20 modules. The water retention time in estuaries typically increases as the river runoff decreases, reducing the dispersal and more strongly exposing bacteria to local environmental conditions other than terrestrial DOM. Thus, several different environmental factors became important for sorting the bacterial community. In summer, 18 modules of the bacterial community were influenced by terrestrial DOM related variables as well as nutrient concentrations (total nitrogen and total phosphorous), suspended particulate matter and temperature.

Previous studies have shown that river discharge continuously transport bacteria from terrestrial systems to estuaries (Ruiz-González et al., 2015a). In agreement with this, we noted that the occurrence of freshwater bacteria (e.g., Betaproteobacteria), were more pronounced at nearshore than more seaward locations. Furthermore, specific Verrucomicrobia showed a similar spatial trend, for example the group OPB35 (Table S3), and the Betaproteobacteria Oxalabacteraceae and Comamonadaceae were also common in the inner parts of the estuary. These populations were present in low densities in the outer parts of the estuary but showed higher abundances at stations closer to the river mouth. However, when the river inflow was low and the autochthonous primary production high, Verrucomicrobia occurrence increased in the entire estuary. The succession of bacterial populations in summer suggests that the terrestrial system seeds the estuary with species that can feed on diverse substrates and in doing so appear capable of survival in the marine realm. Our results may therefore mirror the connectivity between the terrestrial landscape and the sea (Ruiz-González et al., 2015b), and reflect the important role of coastal environments in processing terrestrial inputs.

Reduced terrestrial DOM inflow in summer in Råne estuary caused a shift in the bacterial community from specialists to generalists

Large terrestrial discharges modified the environmental conditions in the estuary in spring, exposing free niches and allowing some bacterial species to establish. The large amount of terrestrial DOM promoted specialist bacteria, comprising 62% of the community in May (Fig. S4). For example, within the Flavobacteria specialists were more common than generalists, possibly reflecting a capability to consume complex terrestrial DOM compounds. Even if this bacterial population was not abundant in May, they might contribute to the processing of terrestrial DOC as bacterial production peaked and highest availability of DOC was observed during spring (Figueroa et al., 2016). This assumption is supported by previous studies, which have shown that some bacterial taxa can disproportionally contribute to the biogeochemical processing of carbon (Alonso-Sáez and Gasol, 2007; Elifantz et al., 2007).

Reduced river freshwater inputs in summer and increased primary production caused a more diverse DOM composition in the estuary, consisting of terrestrial material and newly produced high quality autochthonous material. Such changes favoured the establishment of generalist bacteria, which accounted for almost 70% of the total community in late summer. Similarly, the dominance of generalists has been reported in other coastal communities (Mou et al., 2008; Gómez-Consarnau et al., 2012; Lindh et al., 2015), where DOM of a varied type and quality promoted the development of a diverse community dominated by generalist bacteria (Mou et al., 2008).

Conclusions

Because of complex environmental and biological interactions, the bacterial community of the Råne estuary exhibited both temporal and spatial variation. We found that terrestrial DOM regulated the bacterial community composition and structure across the productive season in this subarctic estuary. By employing network analysis, we discovered non-random associations between bacterial populations that were influenced by environmental factors. Furthermore, a recurrent co-occurrence pattern between Betaproteobacteria, Bacteroidetes and Actinobacteria was observed. Functional roles of OTUs,
defined by their topological position within networks, confirmed the key roles of specialist bacteria in utilizing DOM dominated by terrestrial compounds, while generalist bacteria emerged within the community at later successional stages when DOM was shaped by a combination of autochthonous and terrestrial sources. Hydrological processes and the supply of DOM of differing composition sorted specialists and generalist bacteria within the estuarine community. This implies that the processing of terrestrial DOM will be affected by climate change, since the hydrological processes will be altered by increasing rainfall and the resulting elevated discharges of terrestrial matter into coastal areas. We infer that under such hydrological conditions, the consumption of terrestrial DOM in subarctic coastal estuaries will increase, likely via specialist bacteria, and could lead to restructuring of basal communities and altered carbon and energy flows.

Experimental procedures

Field sampling

The study was performed in the northern part of the Baltic Sea in a sub-arctic coastal estuary (Råne estuary). The Råne river is an unregulated river, running through a forested catchment and its coastal estuary is highly influenced by the river discharge. Monthly measurements of biological and physicochemical parameters were performed at 1 m depth at 19 stations in Råne estuary (Fig. S1), between May and August 2011 as described in Figueroa and colleagues (2016). One station was situated in the river mouth and 18 stations were distributed in the estuary corresponding to an area of 16 km$^2$. In the inner estuary station 1–11 were sampled, while in the outer estuary station 12–19 were sampled. Each sampling event spanned 3 days. The first sampling was initiated just after the ice break-up during the spring river flush and the last sampling was performed at the end of the productive season in August (Fig. S2). Water was collected in acid-washed Milli-Q rinsed Polycarbonate bottles with a Ruttner sampler and preserved or analysed within 4 h.

Physicochemical and biological variables

Humic substances (HS), coloured DOM (CDOM) and dissolved organic carbon (DOC) were used as descriptors of terrestrial DOM. Humic substances were measured in unfiltered water using a Perkin Elmer LS 30 fluorometer (350 nm excitation/450 nm emission wavelength), calibrated with quinine dihydrogen sulphate dehydrate in 0.05 M sulphuric acid (Wedborg et al., 1994; Hoge et al., 1995). Water was filtered through 0.2 µm acid washed membrane filters and CDOM was measured spectrophotometrically (300–800 nm) as described by Kratzer and colleagues (2008). Filtered (0.2 µm Supor Membrane Syringe Filter, non-pyrogenic; Acrodisc$^\text{™}$ and acidified (0.1 ml of 1.2 M HCl) water samples were sparged and analysed to measure DOC using a Shimadzu TOC-5000. The total nitrogen (TotN) and total phosphorous (TotP) was analysed using a Bran & Luebbe TRAACS 800 autoanalyser according to Grasshoff and colleagues (1983). Temperature was measured in situ. pH and conductivity were measured in the laboratory at 25°C (Mettler Toledo). Salinity was calculated using the conductivity values at in situ temperature according to Fofonoff and Millard (1983).

Suspended particulate matter (SPM) was measured using the gravimetric method described by Strickland and Parsons (1972). Triplicate 1 l water samples were filtrated through pre-combusted (450°C) and pre-weighed ($W_0$) Whatman GF/F filters. Post-sampling, filters were dried for 24 h at 60°C and reweighed ($W_1$). The total concentration of SPM was calculated as the average of triplicates ($W_1 - W_0$). Filters weight was measured again after combustion at 450°C for 5 h ($W_3$). The SPM$_{inorg}$ fraction was calculated as the difference between the combusted filters weight and the tara weight ($W_2 - W_0$) while SPM$_{org}$ as the difference between the total concentration of SPM and the inorganic fraction of SPM. Bacterial production (BP) was measured with the $^3$H-thymidine incorporation technique and primary production (PP) was measured in situ using the $^{14}$C technique, as described in Figueroa and colleagues (2016). The bacterial biomass for DNA extraction was collected by filtering (0.2 µm 47 mm Supor filter, PALL Life Science) 150–350 ml of water using a peristaltic pump. Filters were preserved in TE buffer and frozen at −80°C until extraction.

To identify differences over time in the values of the different environmental parameters measured, we used separate one-way ANOVAs using R function from R software (version 3.6.0).

DNA extraction, PCR amplifications and sequencing

DNA extraction was done using the phenol-chloroform protocol as described in Riemann and colleagues (2008), with some modifications by using lysozyme (Sigma-Aldrich; Final concentration: 50 mg ml$^{-1}$) at 37°C for 30 min and overnight protease K digestion (Fermentas; Final concentration: 20 mg ml$^{-1}$) at 55°C. The resuspended DNA was quantified using the fluorometric method Qubit (Invitrogen). PCR amplification of 16S rRNA gene fragments was performed with HPLC purified bacterial primers 341F and 805R (Eurofins) as described in Hugert and colleagues (2014), slightly modified since each sample was amplified in duplicates, using 58 °C as
annealing temperature and 30 cycles in the first PCR. In the second step, we used 16 cycles and after each PCR step the PCR products were purified using spin columns (EZNA Cycle Pure Kit). The purified PCR products were sequenced on the Illumina Miseq (Illumina, USA) platform using the 2 × 300 bp paired-end setting at the Science for Life Laboratory, Sweden (www.scilifelab.se).

Processing and analysis of molecular data
The raw Illumina Miseq sequences were quality filtered and then processed using the UPARSE pipeline (Edgar, 2013). Stations that showed low quality sequences, were excluded from further analyses: 10 samples in total. The DNA sequences were clustered at 95% 16S rRNA gene identity, which commonly has been used to identify bacteria at approximately the level of genera (Yarza et al., 2014). Clustering at the 97% level was not selected because of occurrence of many singletons, which were removed and thus considerably reduced the dataset. After the singletons removal, the 95% level analysis resulted in a more robust dataset containing 5855 operational taxonomic units (OTUs) in total from 2,926,632 reads. Taxonomy was determined using the SINA/SILVA database (SILVA 115) (Quast et al., 2013). DNA sequences are deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (Raw number of reads: 16074286 reads. SRA accession: PRJNA633654 (https://www.ncbi.nlm.nih.gov/sra/PRJNA633654).

Community analyses and network topology
To standardize the OTUs for further analyses the number of reads was subsampled to 5500 by rarefaction, considering 66 samples after the quality processing. Using the subsampled dataset, we performed comparisons between the bacterial communities from different stations at each month. The shifts in community composition were visualized applying non-metric multidimensional scaling (nMDS), based on Bray-Curtis distance matrices calculated from OTU relative abundances. The environmental factors (both physical and biological factors) were included in the ordination method, where relationships between environmental conditions and shifts in community structure were performed in R using permutational analyses with 1000 permutations using the function permanova from in the package R ‘vegan’ (Oksanen, 2016). OTU richness (Chao1), diversity (Shannon) and evenness (Pielou) was calculated in R using the packages ‘fossil’ and ‘vegan’ (Vavrek, 2011; Oksanen, 2016). The analyses described above were performed using the R software (version 3.6.0).

To study bacterial associations and interactions through different seasons, network analysis was performed using relative OTU abundances during each month of sampling. In order to reduce the complexity of each dataset for the analysis, rare OTUs present less than 9 times per dataset were removed to avoid poorly represented OTUs (approximately 0.01% of the total sequences). Network construction was realized for each dataset independently – one network per monthly dataset – following the workflow suggested by Langfelder and Horvath (2008) through their WGCNA R package. First, soft thresholding power values were chosen based on the criterion of approximate scale-free topology using the pickSoftThreshold function. For the four datasets, nine soft thresholds were chosen. Pairs of nodes were connected using the adjacency function. To minimize effects of noise and spurious associations, the adjacency matrix was transformed into Topological Overlap Matrix using the TOMsimilarity function. OTUs were clustered into modules with a minimum number of OTUs per module set at eight using the cuttreeDynamic function. In the network display, each OTU was represented by a node, where positive or negative interactions between OTUs were represented by edges. Some nodes clustered together, representing densely connected bacteria, forming modules in the distribution (Barberán et al., 2012). Each module was shaped by a minimum of eight nodes per module and the modularity structure was supported when the modularity index showed values higher than 0.4 (Newman, 2006). The visualization of networks was done using the software ‘Gephi’ (Bastian et al., 2009). It included the edges with Pearson’s correlation coefficient equal to or higher than 0.1, retaining in the network visualization all OTUs (nodes) which showed at least 10% of covariation in relative abundance. Further, an eigenvalue analysis for each module was performed, where each module was assigned a singular value decomposition (SVD) of abundance called module eigenvalue. These eigenvalues were correlated with environmental factors, through a modularity-detection algorithm, to reveal potential effects of the environmental conditions on the modular structure (Deng et al., 2012). This enabled possible dynamic adjustment of bacterial populations to changes in the environmental conditions to be addressed.

Prevalence of specialists and generalist bacteria were displayed using an algorithm to determine topological roles (Olesen et al., 2007). Two parameters were calculated: (i) the standardized number of links to other OTUs in the same module, called within-module connectivity ($z_i$), and (ii) the standardized number of links among the modules, i.e. the among-module connectivity ($P_i$). The topological roles of individual nodes were defined...
following the classification of Olesen and colleagues (2007) used in pollination networks. Then, OTUs with few connections, within and between modules, were defined as peripheral taxa, which showed a low degree of interaction thus reflecting possible specialist behaviour. Largely connected OTUs within the corresponding modules were considered as hubs modules, meanwhile OTUs with lower connectivity within the own module but highly connected with other modules were considered connector hubs. The hub modules and connector hubs are defined as generalist OTUs. Highly connected OTUs within and between modules shaped the network hubs corresponding to super generalists (Deng et al., 2012). Hub modules together with connector and network hubs were enveloped in the generalist groups.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

Fig. S1 Sampling stations in the Råne river and estuary, Bothnian Bay (northern Baltic Sea). From Figuerola et al. (2016).

Fig. S2 Daily freshwater discharge from the Råne River during 2011. Vertical lines indicate sampling occasions performed in this study. From Paczkowska et al. 2019.

Fig. S3 Non-metric multidimensional scaling (nMDS) analysis showing differences in the bacterial community structure at different stations in the estuary during May (yellow dots), June (green dots), July (blue dots) and August (red dots). The analysis is based on Bray–Curtis dissimilarity values based on relative abundance of OTUs.

Fig. S4 Module functional distribution of the nodes, where each node represents an OTU. Functional roles were defined by the following metrics: (i) within-module connectivity (z), i.e., the standardized number of links to other OTUs in the same module (ii) the among-module connectivity (P) i.e., the standardized number of links among the modules. The topological roles of each OTU in the network analysis indicate different ecological groups: Module hubs and connectors hubs (CH) representing the generalists, peripherals taxa representing the specialists and network hubs representing the super generalists. The colours of each OTU correspond to taxonomic identification (see Fig. 2).

Table S1 Data on the environmental variables measured at 1 m depth at each sampling occasion; Station 01–19 (St_01–19), May–August 2011. The parameters described are Salinity, Dissolved organic carbon (DOC), humic substances (Hum. sub.), coloured dissolved organic matter (CDOM), suspended organic particulate matter (SPM org), suspended inorganic particulate matter (SPM inorg), primary production (PP), total nitrogen (TotN), total phosphorus (ToP), and temperature (Temp). The data come from Figuerola et al. (2016).

Table S2 Characterization of bacterial community composition in the Råne estuary during May, June, July and August. (A) diversity indexes and (B) network topological metrics (considering Pearson correlation coefficients between OTUs superior to 0.1).

Table S3 This table shows (i) the functional role of each OTU in the four networks (ii) the name of the modules in which they belong (iii) their taxonomy at the phylum, order, and genus level (when affiliated). The functional roles were obtained using network analysis data applying Olesen (2007) algorithm, and divided bacteria in habitat specialists (S), generalists (G) and super generalist (SG). At empty squares, the OTUs were not present in the corresponding networks.

Table S4 Influence of freshwater on the salinity and DOC concentrations in the Råne estuary during different sampling months. The salinity and DOC concentrations in the river (station 1), estuary (average of station 2–18) and at an offshore monitoring station (A5), are also provided.