~~Invasive dreissenid mussels induce shifts in bacterioplankton diversity through selective feeding on high nucleic acid bacteria~~

Flow cytometric monitoring of bacterioplankton phenotypic diversity predicts high population-specific feeding rates by invasive dreissenid mussels

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Running title

Phenotypic tracking of bacterioplankton

Conflicts of interest

The authors declare that there exist no conflicts of interest.

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Originality-Significance Statement

While most global change studies in microbial ecology are focusing on the impacts of climate change and land use change, the third component of global change, i.e. species invasions, gets considerably less attention. Here, we focused on the impacts of one of the most impactful invasive species in aquatic systems, invasive dreissenid mussels (IDMs). Our knowledge about their impacts on bacterial communities remains very limited, despite potentially large implications to ecosystem functioning if bacterial communities are affected. This is in part due to the innate sensitivity of IDMs to invasive sampling strategies which are required for many molecular analyses (i.e., they halt their feeding activity), complicating the acquisition of highly resolved temporal surveys. Using a recently developed flow cytometry method, which calculates phenotypic diversity estimates, we discovered direct impacts on natural lake bacterioplankton populations, within one hour of being exposed to filter feeding pressures from ~~this~~ invasive ~~species~~quagga mussels. We also established a strong correlation between these phenotypic diversity measurements and their taxonomic counterpart calculated from 16S rRNA gene amplicon sequencing over different freshwater environments. This allowed us to predict the magnitude of actual community shifts solely based on flow cytometric measurements, facilitating a temporal resolution of this biological process that would not have been possible otherwise. We could then further attribute this predicted shift in community diversity to the removal of physiological subpopulations previously shown to contribute disproportionately to community metabolism. Thus, this new analysis pipeline led us to the hypothesis that IDMs directly ~~impact microbial elemental cycling, as they may drive~~drive bacterioplankton communities toward less diverse and potentially less productive states within short time periods.

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Summary

Species invasion is an important disturbance to ecosystems worldwide, yet knowledge about the impacts of invasive species on bacterial communities remains sparse. Using a novel approach, we simultaneously detected phenotypic and derived taxonomic change in a natural bacterioplankton community when subjected to feeding pressure by quagga mussels ~~invasive dreissenid mussels (IDMs)~~, a widespread aquatic invasive species. We detected a significant decrease in diversity within one hour of feeding, and a total diversity loss of 11.6 ± 4.1 % after 3h. This loss of ~~bacterial~~ microbial diversity was caused by the selective removal of high nucleic acid (HNA) populations (29 ± 5% after 3h). We were able to track the community diversity at high temporal resolution by calculating phenotypic diversity estimates from flow cytometry data of minute amounts of sample. Through parallel flow cytometry and 16S rRNA gene amplicon sequencing analysis of environments spanning a broad diversity range, we showed that the two approaches resulted in highly correlated diversity measures and captured the same seasonal and lake-specific patterns in community composition. Based on our results, we predict that selective feeding by IDMs directly impacts the microbial component of the carbon cycle, as it may drive bacterioplankton communities toward less diverse and potentially less productive states.

Keywords

Invasive species, flow cytometry, HNA bacteria, phenotypic diversity, disturbance ecology

Introduction

Anthropogenic disturbances can lead to rapid changes in microbial community diversity (species richness, evenness, and composition). Many studies aim to better understand feedbacks between global change and microbial communities, as changes in microbial diversity can either mitigate the predicted direct effects of disturbances on ecosystem fluxes (Singh et al., 2010; Zhou et al., 2012), or lead to major shifts in bacterially mediated fluxes (Schimel and Gulledge, 1998; Finlay et al., 2007; Levine et al., 2011). The responses of microbial communities to disturbances are often monitored by means of high-throughput molecular techniques, such as 16S rRNA gene amplicon sequencing (Shade et al., 2012). Community shifts in response to altering environmental parameters can occur within hours (Props et al., 2016b) to days (Datta et al., 2016), and demand substantial sampling effort at a preferably fixed frequency to allow accurate statistical inference (Faust et al., 2015). Current technology allows sequencing data to be generated from low-volume samples (e.g., 100 mL) of low-density environments (≤ 10^6 cells mL^{-1}), which comprise many aquatic ecosystems, but larger sample volumes (> 1L) are required in order to yield a robust census of the microbial community (Padilla et al., 2015). ~~For low-density environments (≤ 10^6 cells mL^{-1}), which comprise many aquatic ecosystems, this becomes particularly challenging as large sample volumes (> 1L) are required in order to yield sufficient DNA and provide reliable results (Padilla et al., 2015).~~ This prohibits the use of this approach in many longitudinal microcosm studies, for which repeated invasive sampling itself would act as a disturbance.

Recently a new approach has been developed that can generate phenotypic diversity metrics based on physiological information derived from flow cytometry (FCM) data (Props et al., 2016a). These diversity metrics have been shown to be highly correlated to taxonomic diversity, as derived from amplicon sequencing. Yet, their derivation avoids invasive, high volume sampling practices (≤ 1mL of sample required), and simultaneously offers information on the physiological state of the community, as well as on the absolute density of its constituent populations. Briefly,

this approach performs kernel density estimations on multiple bivariate single-cell parameter combinations (e.g., fluorescence and scatter intensity) and concatenates these into a feature vector that is called the *phenotypic fingerprint*. The phenotypic fingerprint represents the community structure in terms of physiological aspects, such as nucleic acid content and morphology. From this fingerprint, the community diversity can be calculated by means of the Hill diversity numbers (Hill, 1973), which examine both richness and evenness components of the phenotypic community structure. In parallel, this approach facilitates beta-diversity assessments through the ordination of samples by means of a dissimilarity matrix calculated between phenotypic fingerprints. The ability to simultaneously track impacts on phenotypic and taxonomic diversity offers opportunities to address gaps in our understanding of microbial disturbance ecology. Currently, this method has only been tested in one, low-complexity system, and validation across a broader range of diversities is needed to fully assess its potential ~~for broad applicability~~.

Species invasion, which is one of the main components of global change (Chapin et al., 2000), is a particularly useful system to help address knowledge gaps in microbial disturbance ecology as we can readily mimic the real-world conditions (i.e., sudden introduction) in laboratory or field experiments.. The current distribution of invasive dreissenid mussels (IDMs) across North America (>30 states) is a prime example of a successful invasion event (Higgins and Vander Zanden, 2010). Initially introduced through ballast water, IDMs display high filtration rates (Vanderploeg et al., 2002), and are able to rapidly populate benthic and littoral substrates in densities of up to 19,000 individuals per m² (Nalepa et al., 2010). With respect to their feeding ~~behaviour~~behavior, IDMs show highly selective feeding ~~behaviour~~behavior towards ~~on~~ seston and different algal and microzooplankton taxa over a broad range of size (~ 1 to 200 μm) (Tang et al., 2014). While IDMs are known to strongly impact phytoplankton and zooplankton abundance and composition (Higgins and Vander Zanden, 2010), the few studies focused on their impacts on bacterioplankton report contradicting results ~~(Denef et al, in review).~~ Several of these studies reported selective feeding on bacterial species (Silverman et al., 1995; Pires et al.,

2004; Denef et al., 2017) while a long-term environmental survey of the Hudson River prior- and post-invasion did not observe negative effects on bacterial community density and productivity (Findlay et al., 1998).

In this study, we investigated the effect of IDM grazing (with *Dreissena bugensis* as model) on the natural bacterioplankton community of Lake Michigan through (near) non-invasive tracking of the phenotypic biodiversity, as well as the density of physiological subpopulations. We first validated whether the existing correlation between taxonomic and phenotypic diversity metrics holds for the high diversity environments of ~~low productivity~~ Lake Michigan (low primary and secondary productivity) and one of its ~~high productivity~~ freshwater estuaries (high primary and secondary productivity), Muskegon Lake. We then used phenotypic alpha and beta diversity analyses to assess the impact and extent of IDM grazing on the bacterioplankton community of Lake Michigan. The observed biodiversity dynamics were further related to the dynamics of well-established physiological populations in freshwater bacterioplankton, for which the grazing rate was determined.

Results

We aimed to use flow cytometry-derived phenotypic diversity metrics as a proxy for taxonomic diversity shifts occurring during quagga mussel feeding on lake bacterioplankton. The experiment consisted of 12 L microcosms of 153 µm screened water and quagga mussels, both retrieved from Lake Michigan. The Lake Michigan bacterioplankton has previously been shown to contain both grazing-resistant and grazing-sensitive taxa allowing the study of direct grazing impacts (Tang et al., 2014; Denef et al., 2017). Prior to the onset of the experiment, we assessed whether the previously established correlation between phenotypic and taxonomic diversity metrics for low diversity environments could be extended to higher diversity aquatic environments, such as Lake Michigan.

1. Validation of phenotypic diversity as a proxy for taxonomic diversity

~~In order to interpret the flow cytometry-derived phenotypic diversity as a proxy for the taxonomic diversity, we first needed to validate whether the previously established correlation for low diversity environments was also valid for higher diversity environments, such as Lake Michigan and Muskegon Lake (**Fig. 1**).~~

Microbial communities can be classified as relatively low or high diverse communities based on their Hill diversity metrics as these are expressed in terms of *effective number of taxa*, which depict the number of equally abundant taxa required to obtain the same diversity value as the community in question (Hill, 1973). The diversity metrics derived from flow cytometric analysis are calculated in the same way as their taxonomic counterparts but they can only be interpreted in arbitrary units. In order to determine whether there was a general relationship between the phenotypic and taxonomic diversity, we included an independent dataset from a low diversity cooling water microbial community (1 – 10 effective number of taxa) (Fig. 1). This cooling water dataset contains two 40 day surveys of bacterioplankton communities present in the secondary cooling water system of a nuclear test reactor that was subjected to multiple operational phases.

The positive correlation between taxonomic and phenotypic diversity metrics of orders 1 (D_1, exponential of Shannon entropy) and 2 (D_2, Inverse Simpson index) could be extended to the Lake Michigan and Muskegon Lake communities (cross-validated $r^2 = 0.89$, Pearson's correlation $r_p = 0.94$ for both D_1 and D_2). We opted for a single regression model as opposed to individual regressions for each environment in order to avoid overfitting as well as to construct a generalizable model that provides robust inference for all three environments. Individual regression models did not significantly differ in slope and the cross-validated r^2 of the single regression model was high ($r^2 = 0.89$), thereby permitting the use of a single regression model for monitoring diversity dynamics (**Fig S1**, **Table S1**). ~~The positive correlation between taxonomic and phenotypic diversity metrics of orders 1 and 2 could be extended to the Lake Michigan and Muskegon Lake communities (adj. $r^2 = 0.89$, Pearson's correlation $r_p = 0.94$ for both D_1 and D_2).~~

The dynamic range of the regression, defined as the ratio between the largest and smallest taxonomic diversity used in its calculation, was 88.7 for D_1 and 42.5 for D_2. Goodness-of-fit analysis of the linear regression model indicated a normal distributed residual distribution with homogenous variance over the entire regression range (**Fig. S2~~1~~**). The observed richness (D_0) did not show a distinct linear correlation ($r^2 = 0.32$, $r_p = 0.54$, **Fig. S3~~2~~**). Due to the high level of correlation between the phenotypic diversity (D_1, D_2) and the taxonomic diversity, it was permissible to use the phenotypic diversity as a stand-alone metric for evaluating bacterioplankton diversity. Additionally, only D_2 was used in further analyses due to the high degree of correlation between D_1 and D_2 ($r_p = 0.99$). ~~(Hill, 1973)~~

In contrast to alpha diversity, beta diversity cannot be captured by a single metric. Therefore we compared the taxonomic and phenotypic beta diversity by their performance to detect seasonal- and lake-specific community structures in the Lake Michigan and Muskegon Lake data set (**Fig. 2**). Procrustes analysis demonstrated that both approaches were significantly correlated in terms of the patterns that they captured in the data (~~correlation strength = 0.652,~~ $p = 0.001$).

Additionally, both approaches identified season and lake type (Lake Michigan or Muskegon Lake) as significant predictors of the community structure ($p = 0.001$). The season explained the most variance in the beta diversity (i.e., 19.6% of the taxonomic beta diversity and 22.5% of the phenotypic beta diversity). Lake type still captured 17.4% of the variance in the taxonomic beta diversity, but only 7.0% in the phenotypic beta diversity. Lastly, the seasonal effect was dependent on the lake type, representing an extra 9.4% of the variance in the taxonomic beta diversity ($p = 0.001$) and 4.6% in the phenotypic beta diversity ($p = 0.018$).

2. Diversity dynamics during IDM feeding

~~Combined with the non-invasive nature of the sampling associated with the flow cytometry approach, it becomes possible to track sensitive ecological processes, such as the filter feeding by IDMs (*Dreissena bugensis* as model), in high resolution (Kryger and Riisgård, 1988).~~ The temporal trajectory of the bacterioplankton community of ~~Lake Michigan~~the microcosms was monitored for 3h at a resolution of 0.5h when subjected to the direct feeding pressure by 15 IDMs per microcosm (**Fig. 3**). This time period was sufficiently long to allow the assessment of direct feeding effects (removal of 30 to 60% of seston), but short enough to avoid indirect effects, e.g. due to trophic cascades or substantial accumulation of feces and pseudofeces (Vanderploeg et al., 2010). Importantly, all mussels were subjected to an extensive pretreatment consisting of specific handling, rinsing, and acclimatization steps in order to avoid contamination of the bacterioplankton community by external periphyton, debris, and ingested particles at the onset of the experiment (see experimental procedures section). The size distribution of the mussels was not significantly different between the microcosms (22.7 ± 2.3 mm, Kruskal-Wallis test, $p = 0.08$). The total dry weight of the mussels per microcosm was 0.24 ± 0.018 g DW.

Over the span of the ~~3h~~ experiment, the control microcosm's phenotypic diversity exhibited a minor overall positive temporal drift ($p = 0.038$). In contrast,~~-~~ the bacterioplankton phenotypic diversity underwent a clear and significant decrease ($p < 0.0001$) during filter feeding of the

IDMs, signifying the enrichment of the community by certain taxa (**Fig. 3A**). The treatment effect became significantly distinguishable from the control at the 1 hour mark (at $p < 0.05$). Using the ~~model~~ regression model, an average loss in taxonomic diversity (D_2) could be predicted of 2.6 ± 1.0 effective number of taxa, corresponding to a decrease of 11.6 ± 4.1% over the course of the experiment. Conceptually, this means that in a hypothetical community of 23 equally abundant taxa (diversity prior to mussel feeding), an average of 2.~~7~~ 6 taxa would be lost due to IDM feeding. To put these measurements in perspective, we analyzed data from a recently published mussel-feeding study that used the same experimental design, and had 16S rRNA gene amplicon data at time points 0 and 3h available (**Fig. 4**) (Denef et al., 2017). We calculated a mean loss of taxonomic diversity (D_2) of 5.32 ± 4.65 for their three independent experiments which is comparable to the taxonomic diversity loss predicted for our experiment (2.6 ± 1.0).

While the monitoring of the phenotypic alpha diversity allowed us to track the treatment effect through time, a beta diversity analysis was also conducted to evaluate the treatment and temporal effects on the complete phenotypic structure of the bacterioplankton community (**Fig. 3B**). ~~At time point 0h the~~Over the course of the experiment the bacterioplankton communities ~~were similar~~of ~~between~~ the control and treatment microcosms became more dissimilar~~, but they became more dissimilar over time~~. In agreement with the alpha diversity analysis, a time-dependent treatment effect ($r^2_{feeding} = 0.34$, $p = 0.005$) was driving the bacterioplankton structure, with the control bacterioplankton community also experiencing a minor temporal effect ($r^2_{control} = 0.08$, $p = 0.006$).

3. Bacterioplankton population dynamics during IDM feeding

Next, we investigated whether the observed diversity dynamics were caused by selective feeding on specific phenotypic populations of the bacterioplankton community. To do so, contrasts between the phenotypic fingerprints of the treatment and the control at three different time points were calculated (**Fig. 5~~4~~A**). This analysis allowed the visualization of regions in a specified

bivariate parameter space which are relatively more or less abundant in the treatment versus the control. We ~~opted for the FL1-H and FL3-H parameters~~opted for the primary fluorescence channels of the SYBR Green nucleic acid stain (i.e., FL1-H, FL3-H) ~~because these are often used in identifying~~allowing us to identify distinct physiological populations with varying nucleic acid content (Gasol et al., 1999; Hammes and Egli, 2010; Koch et al., 2014). The results demonstrate that during filter feeding the bacterioplankton community ~~becomes~~ became enriched with a low nucleic acid content population (LNA, low ~~fluorescence~~ FL1-H/FL3-H intensity), and was ~~is~~ depleted from a high nucleic acid content population (HNA, high FL1-H/FL3-H ~~fluorescence~~ intensity).

As these are relative changes, that do not necessarily reflect a direct feeding effect on the HNA population, the absolute abundances for both the HNA and LNA population were extracted from the total community according to the guidelines by (Prest et al., 2013) (**Fig. S4~~3~~**). The LNA cell densities show similar temporal ~~behaviour~~behavior for the control (coefficient of variation – CV = 5.8%) and treatment (CV = 5.2%) microcosms (**Fig. 5~~4~~B)**. This level of variation falls within the technical variation of current flow cytometry technology (CV = 5%), and is thus not indicative of a feeding effect (Hammes et al., 2008). In contrast, the HNA population was directly affected by the filter feeding (**Fig. 5~~4~~C**). The HNA population of the control microcosms displayed a similar variation to the LNA population (CV = 5.1%), while the HNA population in the treatment microcosms showed a monotonic decrease throughout the experiment (CV = 12.8%). Analogous to the diversity analyses, a significant treatment effect could be detected within 1h. Using robust linear regression, the HNA specific removal rate was estimated at 43,~~174~~ 000 ± 3,~~381~~ 000 cells mL^{-1} h^{-1} ($p < 0.0001$), while the control HNA cell density remained constant ($p = 0.98$). After 3 hours of being subjected to filter feeding, 29 ± 5% of the HNA bacterioplankton population was removed from the water column. The clearance rate on the HNA population was 4.56 ± 0.81 mL mg^{-1} DW h^{-1}.

Discussion

Our understanding of microbial disturbance ecology has been partially constrained by a lack of temporal resolution, caused by methodological limitations in either sampling, logistics, or analysis. In order to combat these bottlenecks, we further developed and validated a (near) non-invasive flow cytometry-based approach dedicated to detect changes in the phenotypic diversity of microbial communities. We validated and applied these phenotypic diversity metrics to natural, high diversity environments, and investigated the response of bacterioplankton communities to a filter feeding disturbance caused by invasive dreissenid mussels, which are highly sensitive to the invasive sampling imposed by alternative monitoring techniques (i.e., they would cease their filter feeding activity). Our experimental results highlight the sensitivity of our method to detect subtle changes in diversity over short timeframes. Based on (1) our presented relationship between phenotypic and taxonomic diversity, (2) the positive relationship that exists between ~~the diversity~~HNA bacterial populations and ~~functionality~~ bacterial productivity in many ecosystems (Zubkov et al., 2001; Servais et al., 2003), and (3) the results of our filter feeding experiment, we hypothesize that IDM feeding directly influences both microbial diversity and ecosystem functionality.

Relation between taxonomic and phenotypic diversity

The regression between the taxonomic and phenotypic diversity data is in agreement with and expands upon previous research (Props et al., 2016a) and offers further insight into the fundamental relation between these metrics. Concretely, the regression's dynamic range has been extended from 10.3 in the previous study (cooling water) to 42.5 for the diversity of order 2 (D_2). The quality of the cross-validated regression is worth highlighting as there were substantial differences in the sample treatment and data generation of the data sets, which could have led to systematic bias. First, the flow cytometry samples of Lake Michigan and Muskegon Lake were fixed with glutaraldehyde and archived at -80°C, whereas the cooling water samples were

~~analysed~~analyzed directly. The glutaraldehyde fixative used in this research has been shown to increase autofluorescence and may have increased the instrument noise (Gunther et al., 2008). Nevertheless, the bacterioplankton community could be reproducibly isolated from the raw data with one fixed denoising strategy for the entire data set (**Fig. S~~3~~4**). Secondly, the amplicon sequencing of the 16S rRNA gene targeted the V4 region for the Lake Michigan and Muskegon samples, whereas the V3-V4 region was targeted for the cooling water samples. This difference in sequenced region has been shown to potentially alter the observed diversity (Schmalenberger et al., 2001; Yu and Morrison, 2004). ~~Both potential biases would have been visible by a shift in the taxonomic diversity of the cooling water samples relative to the other data set. However, this was not observed.~~ We did not observe this bias in the taxonomic diversity, but we did observe that the phenotypic diversity was incapable of resolving the Lake Michigan and Muskegon Lake communities, even though they had distinct taxonomic diversities. The regression analysis showed that the strength of the relationship between the phenotypic and taxonomic diversity was unaffected, but that the intercept of the linear models was different ($p<0.001$). Therefore we emphasize that the absolute values of the phenotypic diversity metrics need to be compared within a single ecosystem or experimental setting. Lastly, rescaling or subsampling the community composition to an equal library size, which is a common yet debated practice in microbial community analyses (McMurdie and Holmes, 2014), did not negatively affect the regression (**Fig. S~~4~~5**). In fact, it improved the regression for all diversity metrics and in particular D_0, indicating that the phenotypic diversity ~~is~~ metrics are primarily sensitive to fluctuations in the density of abundant taxa. As such, we recommend to evaluate changes in richness (D_0) solely by means of 16S rRNA gene amplicon sequencing, for which novel statistical approaches are being developed that account for both observed and unobserved taxa (Willis and Bunge, 2015). ~~-~~It is important to note that in order to compare flow cytometry-derived diversity metrics with each other, the underlying raw data must have been generated by the same flow cytometer platform with identical detector and flow rate settings, which was the case for all data presented in this study.

The linearity on the log-scale implies that the change in phenotypic diversity required to detect a corresponding change in taxonomic diversity systematically increases (Fig. 1). For example, at low diversities, a change from 1,000 to 1,500 units in phenotypic diversity corresponds to a predicted change of 3.4 units in the taxonomic diversity, while an increase from 1,500 to 2,000 units only corresponds to a predicted change of 2.0 units in the taxonomic diversity. This is one of the limitations of relying on a ~~limited~~ fixed number of phenotypic parameters (i.e., fluorescence and scatter intensities); the available parameter space that bacterial cells can occupy ~~becomes saturated at higher diversities~~is limited, resulting in a loss of sensitivity at higher diversities. ~~It is theoretically possible to increase the number of physiological parameters (e.g., membrane permeability) through combining additional stains, but these approaches require substantial standardization, and are not always reproducible enough to be used for natural communities (Buysschaert et al., 2016).~~

The beta diversity analyses yielded similar statistical inference on the seasonal- and lake-specific effects, with both the taxonomic and phenotypic beta diversity identifying seasonality as the most important predictor of community structure. A higher degree of variance could be explained by the lake type in the taxonomic beta diversity, which suggests that the phenotypic approach was less sensitive to differences in taxon distributions between the lakes, or that additional variation based on phenotypic plasticity weakens the relationship between taxonomic and phenotypic beta diversity. This is congruent with the alpha diversity measurements where Lake Michigan and Muskegon Lake samples showed similar phenotypic diversity despite possessing distinct taxonomic diversities. Overall, Procrustes analysis confirmed that the phenotypic beta diversity was able to largely capture the same patterns in the data as the taxonomic beta diversity. As such, phenotypic beta diversity analyses constitute a valid approach for hypothesis testing in high diversity environments but they are susceptible to a higher degree of variability and thus generate potentially lower effect sizes (e.g., for the lake type in this analysis).

Diversity and population dynamics

The phenotypic diversity dynamics during the 3h filter feeding experiment were more subtle than in our previous study on the cooling water dataset (< 150 vs. > 500 units; (Props et al., 2016a)) but occurred over a much shorter time scale (3 h~~rs~~ours vs. multiple days). Importantly, the predicted loss in taxonomic diversity based on the phenotypic diversity (2.6 ± 1.0) lies well within the range of expected diversity shifts (5.32 ± 4.65) calculated from previous experiments which had start and endpoint measurements of community composition (Figure 5). Our predictions also suggest i) a general season-dependent bacterioplankton diversity with a higher diversity in summer than in fall and winter and ii) a season-dependent specific feeding effect resulting in a higher diversity loss in summer than in fall and winter. Overall, the conformity of our predictions to these previous experiments further validates the phenotypic diversity approach. ~~(Denef et al., 2017)~~

The diversity dynamics ~~further~~ suggested that *D. bugensis* was selectively feeding upon a fraction of the bacterioplankton community, thereby altering the community composition, and lowering the diversity. This was confirmed by identifying populations that were selectively enriched through contrast analysis, which demonstrated the selective feeding on bacteria with high nucleic acid content (HNA bacteria) (Fig. 4~~3~~A). The HNA clearance rate (4.56 ± 0.81 mL mg^{-1} DW h^{-1}), which can be interpreted as the water volume that is fully depleted of HNA bacteria per hour, was comparable to those previously reported for *Dreissena polymorpha* feeding on laboratory strains (3.5 – 4.8 mL mg^{-1} DW h^{-1}) ranging in size between 1 and 4 µm in length (Silverman et al., 1995). The clearance rates on laboratory strains were measured for high cell densities (> 10^7 cells mL^{-1}) relative to the natural densities in Lake Michigan in this study (~ 10^6 cells mL^{-1}), and with different IDM species at a higher temperature, thus making direct comparisons difficult. With respect to experiments on natural bacterioplankton, mixed results have been reported. For river bacterioplankton, short-term mesocosm experiments provided no evidence of a direct feeding

effect, while long-term environmental surveys suggested a doubling in bacterioplankton densities (Findlay et al., 1998). In lakes, feeding on bacteria in low nutrient systems was thought to be limited (Cotner et al., 1995), though short-term feeding experiments on natural bacterioplankton from Lake Michigan did detect significant decreases in bacterioplankton densities (Denef et al., in review).

HNA and LNA populations have been well-characterized in aquatic environments, yet considerable debate remains regarding the characteristics of each population. Initially, it was thought that the HNA population was the active fraction of the bacterial community, whereas the LNA population served as a reservoir of dormant, inactive, dead, dying, and damaged cells (Lebaron et al., 2002). Nowadays, the LNA population has been shown to be able to actively grow and to be metabolically active in the environment without adopting HNA properties, such as high nucleic acid content and increased cell size (Jochem et al., 2004; Scharek and Latasa, 2007; Wang et al., 2009). Most studies do report an elevated cell-specific activity for the HNA bacteria that can be more than an order of magnitude higher than the activity of the LNA bacteria (Lebaron et al., 2002; Servais et al., 2003). HNA bacteria are also generally larger and exhibit higher growth rates than LNA bacteria (Lebaron et al., 2002; Jochem et al., 2004; Scharek and Latasa, 2007), and this large, active fraction of the bacterioplankton is preferred by zooplankton grazing (Boenigk et al., 2004; Tadonleke et al., 2005; Garcia-Chaves et al., 2016).

HNA population densities tend to be positively correlated with heterotrophic productivity (Zubkov et al., 2001; Bouvier et al., 2007). Thus, we would expect relatively low HNA densities in Lake Michigan, which has been rendered increasingly oligotrophic (low primary and secondary productivity) since the ingress of IDMs (Evans et al., 2011). HNA population densities in Lake Michigan field samples (29.6 ± 4.2 %, n = 30) were even lower than those reported in previous surveys of freshwater lakes (40 – 42.5 %, n = 81) with similar levels of primary productivity (2 ± 1.5 µg chlorophyll *a* L^{-1} vs. 1.5 ± 1.2 µg chlorophyll *a* L^{-1} in our 2015 survey of Lake Michigan)

(Bouvier et al., 2007; Shuchman et al., 2013). We observed that IDMs predominantly feed on the HNA population; this may in part explain these lower than expected HNA densities, as Lake Michigan is characterized lake-wide by high densities of IDMs (Nalepa et al., 2010). With the HNA bacteria leveraging as much as 80 % of the community's ~~biomass-specific~~ secondary production, and mediating up to 70 % of the bacterial carbon flux in other aquatic environments, the selective feeding by IDMs may have a significant impact on elemental cycling in lake systems (Zubkov et al., 2001; Scharek and Latasa, 2007).

Community characterization of HNA and LNA populations has shown that there can be significant differences in community composition, with few shared taxa between the populations (Schattenhofer et al., 2011; Vila-Costa et al., 2012). As a consequence, selective removal of a single population (e.g., HNA) will alter the community diversity, which was observed during this filter feeding experiment. Other studies using basic molecular fingerprinting techniques did not observe distinct community structures (Servais et al., 2003; Longnecker et al., 2005). Hence, several scenarios have been developed to explain the dichotomy between HNA and LNA bacterioplankton populations (Bouvier et al., 2007). Our results only allow us to support the scenario in which each population has a separate community structure, since considerable repopulation of the HNA population through growth or potential recruitment from the LNA population can take several days (Gasol et al., 1999; Sintes and del Giorgio, 2014; Baltar et al., 2016).

While few investigations into the impacts of IDM on bacterial community composition have been performed, our observations are congruent with studies that have shown altered composition in the sediment (Frischer et al., 2000; Lohner et al., 2007; Lee et al., 2015) and water column (Denef et al., 2017) ~~(Denef et al., in review)~~ following IDM introduction. In these studies specific taxonomic groups were shown to become relatively enriched within the microbial community. Among others, taxa of the phylum Actinobacteria and the genus *Polynucleobacter*,

which are known to possess LNA-type characteristics such as small cell sizes (Wang et al., 2009), increased in relative abundance during short-term microcosm experiments ~~(Denef et al, in review~~ (Denef et al., 2017)~~)~~. Other taxa (e.g. Chloroflexi) became enriched despite their larger cell size, suggesting that multiple phenotypic traits beyond mere cell size determine the feeding success on bacterial taxa. Relative enrichments do need to be interpreted with care as these can provide biased interpretations of the actual taxon abundance dynamics (Nayfach and Pollard, 2016; Props et al., 2016b; Stammler et al., 2016).

In conclusion, we have shown that advanced data analysis of flow cytometry data can lead to robust predictions of taxonomic diversity within a large dynamic range. We further demonstrated that the diversity of natural bacterioplankton communities can be reliably tracked during sensitive ecological processes in a fast, non-invasive manner. Using this approach we were able to detect subtle shifts in biodiversity emerging within one hour of feeding by invasive dreissenid mussels. The selective removal of HNA bacteria was shown to be underlying cause of the loss of biodiversity, suggesting size-selective feeding behavio~~u~~r in the ~~micrometre~~micrometer range. As a result, ~~the direct effects of~~ IDMs are ~~likely~~ capable ~~to~~ of locally reducing the ~~lead to an overall less productive~~ diversity and ~~less diverse~~productivity of the bacterioplankton community ~~that mediates a smaller carbon flux~~during feeding. The approach presented here can be readily applied to help address a broad range of questions in marine and freshwater systems, for which new analytical and computational tools are needed (Labbate et al., 2016). Flow cytometry is now also increasingly being developed for other environments such as soils, sediments, and sludges, opening new possibilities for these systems as well (Frossard et al., 2016).

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Experimental Procedures

16S rRNA gene amplicon sequencing analysis

We used a combination of a previously published data set from 2013 and newly generated V4 16S rRNA gene amplicon sequences from 2014 and 2015 lake surveys (see data availability section). V4 amplicon sequencing data from Lake Michigan (2015 survey) and Muskegon Lake (2014, 2015 surveys) were generated exactly as previously described (Schmidt et al., 2016). Samples were taken in September (Fall), April (Spring), and July (Summer). The DNA was extracted according to a previously optimized protocol (McCarthy et al., 2015) and submitted for sequencing of the V4 hypervariable region (515F/806R) by Illumina MiSeq with v2 chemistry (2x250bp). All raw sequencing reads from these surveys were processed together, after which the samples with matching flow cytometry data were extracted.

Contigs were created by merging paired-end reads based on the Phred quality score heuristic (Kozich et al 2013) in MOTHUR (v.1.38, seed = 777) (Schloss et al., 2009). Contigs were aligned to the Silva database (v123), and filtered from those with (i) ambiguous bases, (ii) more than 8 homopolymers, (iii) a length outside of the 240 – 275 nt range, and (iv) those not corresponding to the V4 region. The aligned sequences were filtered and dereplicated, and sequencing errors were removed using the *pre.cluster* command. Chimera removal was performed by UCHIME. Sequences were clustered into operational taxonomic units (OTUs) at 97 % similarity with the *cluster.split* command (average neighbour algorithm). Sequences were then classified using the TaxAss pipeline (https://github.com/McMahonLab/TaxAss) which classifies sequences according to both a small, manually curated freshwater taxonomy database (Newton et al., 2011) and a large, general database (SILVA v123). The complete workflow is available at https://github.com/rprops/Mothur_oligo_batch and was run in batch mode. For comparison to the flow cytometry data, only the samples comprising the bacterioplankton fraction (0.22 – 3 μm

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fraction) were used in further analyses, as this fraction was the most directly comparable to the measurements taken by the flow cytometer.

The cooling water reference data contain publicly available V3-V4 16S rRNA gene amplicon sequences, and are available from the NCBI Sequence Read Archive (SRA) under accession number SRP066190. We utilized the OTU-table from a previous publication as basis for the diversity calculations (Props et al., 2016b). This OTU-table was generated according to the same pipeline as described above.

Flow cytometry analysis

1 mL of unfiltered water samples were fixed with 5 µL glutaraldehyde (20 % (v/v) stock), incubated for 10 minutes in the dark, and flash frozen in liquid nitrogen (storage at -80°C). Prior to flow cytometry analysis, batches of eight samples were sequentially defrosted, acclimated to room temperature, diluted twofold in triplicate and stained with SYBR Green I (10,000x in DMSO, Invitrogen) to a final concentration of 1x SYBR Green I. Samples were then incubated at 37 °C for 20 min in the dark, and analyzed directly on a BD Accuri C6 cytometer (BD Biosciences) in fixed volume mode (50 µL) (Props et al., 2016a). This resulted in a multiparametric description of each microbial cell by four fluorescence parameters (FL1: 533/30 nm, FL2: 585/40 nm, FL3: > 670 nm long pass, FL4: 675/25 nm) and two scatter parameters (FSC, SSC). Instrument performance was verified daily using eight peak rainbow particles (Spherotech, Lake Forest, IL, USA).

Phenotypic diversity analysis

The alpha diversities for both the flow cytometry and sequencing data were assessed by the Hill diversity numbers, which incorporate both richness and evenness components (Hill, 1973). We followed the previously published protocol available here: https://github.com/rprops/Phenoflow_package/wiki/Phenotypic-diversity-analysis (Props et

al., 2016a). Raw flow cytometer data were exported in FCS format and imported into R (v3.3.0), using functions from the *flowCore* package (v1.38.2). The data were denoised from (in)organic noise based on previous experience with freshwater communities and according to published guidelines for robust denoising (Prest et al., 2013) (as described in **Fig. S43**). The denoising strategy remained the same for all samples. Samples with less than 10,000 cells were discarded since sample sizes larger than this threshold were required for the robust estimation of the diversity (**Fig. S56**). All single-cell parameters were normalized based on the maximum signal height (-H) of the FL1 parameter. The *Diversity* function from the *Phenoflow* package (v1.0, https://github.com/rprops/Phenoflow_package) was then used to calculate the phenotypic alpha diversities of the four primary parameters (FL1-H, FL3-H, FSC-H, SSC-H). Errors on the diversities were generated after 100 bootstraps and propagated to the mean diversity over the three technical replicates. The kernel density estimations were performed with a bandwidth of 0.01, a grid size of 128x128 and a rounding factor of 3. The alpha diversity was evaluated through the first three Hill numbers: D_0, D_1 and D_2, which correspond to the observed richness, the exponential of Shannon entropy, and the inverse Simpson index, respectively. Beta diversity analyses were performed by principal coordinate analysis (PCoA) of the phenotypic fingerprints (*flowBasis* function, d = 3, bw = 0.01) using the Bray-Curtis dissimilarity metric (*beta_div_fcm* function, default settings). Contrasts between the phenotypic fingerprints of the control and treatment groups were made by the *fp_contrasts* function (see tutorial here: https://github.com/rprops/Phenoflow_package/wiki/Making-contrasts).

Taxonomic diversity analysis

For calculating the taxonomic alpha diversity, we used the *Diversity_16S* function from the *Phenoflow* package because this allowed a direct comparison between the taxonomic and phenotypic diversity metrics. The community data were not rarefied because our hypothesis was that the taxonomic diversity was correlated with an unrelated variable, the phenotypic diversity.

Subsampling to the lowest sample size would result in the poorest estimate of the taxonomic diversity for all samples, thereby potentially obscuring the true relationship between these variables. Instead, we selected only samples which had a sample size larger than 10,000 reads (n = 138), generated 100 bootstrap samples for each sample, and took the average diversity as the sample representative diversity. Parallel results of our analyses for the rescaled data to 10,000 reads are available in supplementary information (**Fig. S54**). For beta diversity analysis the ~~taxon~~ OTU abundances were rescaled by calculating their proportions and multiplying them by the minimum sample size present in the data set (McMurdie and Holmes, 2014). The beta diversity was then assessed by PCoA of the Bray-Curtis dissimilarity matrix, which was calculated based on the taxon proportions instead of the read counts in order to be directly comparable to the phenotypic beta diversity approach.

Feeding experiment

Quagga mussels and lake water (5 m above lake floor) were collected at 45m deep from Lake Michigan (43° 12' N, 86° 27' W). Mussels were rinsed of adhering sediment and were transported (< 8h) submerged in lake water at 5-7°C. The standard handling and experimental design of (Vanderploeg et al., 2010) was followed and is briefly outlined as follows. In the lab, the mussels were cleaned of debris and placed in a tank filled with 90 L of 153 µm-screened Lake Michigan water in order to remove grazing mesozooplankton in an environmental room set to ambient temperature of the lake water (9.7 °C). The next morning the mussels were transferred to a 40 L aquarium with 153 µm screened Lake Michigan water for 2 h. The mussel cleaning and ~ 14 h re-acclimation period allowed the removal of external periphyton and debris, cleared the mussel guts of sediment ingested during capture, and gave mussels time to reach digestive equilibrium with their natural food source. All materials were washed with bleach and rinsed with deionized water to minimize bacterial contamination. Seven 19 L HDPE cylindrical containers were filled with 12 L of 153 µm-screened lake water each. Forty-five adult mussels were spread

evenly across three containers (15 mussels per container) and two containers remained mussel-free. ~~The size distribution of the mussels was not significantly different between the containers, hereafter described as microcosms (22.7 ± 2.3 µm, Kruskal-Wallis test, p = 0.08). The total dry weight of the mussels per microcosm was 0.24 ± 0.018 g DW.~~ Gentle mixing was provided by bubbling air through a pipette, and all experiments were carried out under dim light (~ 8 µmol quanta m^{-2} s^{-1}) . Water samples were taken before the addition of the mussels and every 0.5 h after the mussels showed signs of active feeding (after approx. 15 minutes). The number of mussels added and the experiment duration were chosen to allow healthy mussels to clear 30 – 60 % of preferred seston. As shown in a previous study (Denef et al., 2017), our procedure ensures that mussel-associated bacteria do not significantly impact observed shifts in bacterial community composition over the duration of the experiment. One mL water samples from the top water layer were taken every 30 minutes throughout and at the end of the 3h experiment. The samples were fixed with 5 µL glutaraldehyde (20 % (v/v)), incubated for 10 minutes in the dark, and flash frozen in liquid nitrogen (storage at -80°C).

Statistical analysis

All statistical analyses were performed in the R statistical environment (v3.3.0) (R Core Team, 2015), using functions from the *vegan* (v2.4-1), *sandwich* (v2.3-4), *MASS* (v7.3-45), *car* (v2.1-3), *phyloseq* (v1.16.2), *lmtest* (v0.9-34), and *caret* (v6.0-73) packages. Errors on all summary statistics represent standard deviations on the mean and were calculated by propagating individual standard deviations as randomly distributed, independent errors. Ordinary least squares regression was used to relate the phenotypic diversity to the taxonomic diversity (both $\log_2$ transformed). Model assumptions (i.e., normality and homoscedasticity) were evaluated through analysis of the residuals (**Fig. S2~~1~~**). Goodness-of-fit statistics were calculated through tenfold cross validation with 100 repeats. Inference on the temporal treatment effect on the phenotypic diversity (D_2) was performed by spline regression. We opted for natural splines because these

provide more stable estimates at the boundaries (James et al., 2014). Splines were given 3 degrees of freedom, allowing two knots to occur at the 33.3% and 66.6% quantiles (i.e., at time points 1h and 2h). Parameter estimation was performed by the robust ordinary least squares method. Robust linear regression is a variation on the traditional ordinary least squares (ols) regression that provides more correct inference when assumptions for ols regression are invalid (i.e. less sensitive to outliers). Due to the presence of moderate temporal autocorrelation in the model residuals (**Fig. S8~~7~~**), robust parameter errors, calculated from the autocorrelation adjusted covariance matrix (*vcovHAC* function), were used in the statistical inference (Wald test). Differences between groups in the beta diversity analysis were evaluated by means of permutational multivariate ANOVA (PERMANOVA, *adonis* function, 999 permutations) of the Bray-Curtis dissimilarity matrix, after confirmation of the homogeneity of the variance in the groups (*betadisper* function). Similarity between beta diversity analyses was evaluated through Procrustes analysis (*protest* function, 999 permutations). Temporal trends in the HNA cell density, as well as the feeding rate were determined through robust ordinary least squares linear regression. Statistical inference on the model parameters was performed with the Wald test. The clearance rate (CR) was determined based on the robust linear regression of the HNA cell dynamics:

$$CR = \frac{V}{n} * \frac{a}{b}$$

V is the water volume of the container (mL), n is the average dry weight of the mussels (mg), a is the slope of the regression (cells mL^{-1} h^{-1}), and b is the intercept of the regression (cells mL^{-1}).

Data availability

The entire data-analysis pipeline is available as an R Markdown document at https://github.com/rprops/Mussel_feeding. Raw flow cytometry data is available on FlowRepository under accession IDs FR-FCM-ZZNA (cooling water), FR-FCM-ZYZA (mussel

feeding experiment), FR-FCM-ZYZN (Lake Michigan and Muskegon Lake survey). Newly generated V4 16S rRNA sequences from lake Michigan and Muskegon Lake were deposited on the NCBI SRA under accession number XXXXX. For Lake Michigan, the 2013 data set is publicly available on the Joint Genome Institute's genome data portal (http://genome.jgi.doe.gov/; Project IDs 1041195 and 1041198).

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Contributions

R.P. and V.J.D. wrote the paper, R.P. performed laboratory work, and performed all statistical analyses. H.A.V. contributed in the laboratory work. J.H. performed the flow cytometry data acquisition. M.L.S. generated sequencing data. V.J.D., H.A.V., N.B. and R.P. designed the study. All authors reviewed and approved the manuscript.

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Figure legends

Figure 1: Validation for the use of the phenotypic diversity (derived from FCM) across environments with varying degrees of taxonomic diversity (derived from 16S rRNA gene amplicon sequencing, n = 138). The cooling water samples represent bacterioplankton communities sampled throughout two 40-day temporal surveys of a cooling water system of a nuclear test reactor (Props et al., 2016a). Lake Michigan and Muskegon lakes samples represent bacterioplankton communities sampled over a productivity gradient, at various depths (110m, 45m, 15m), and throughout three seasons (Fall, Spring, Summer). Fall, Spring and Summer denote samples taken in September, April and July respectively. The shaded area represents the 95% confidence interval around the ordinary least squares regression model. Both diversities are depicted on a $\log_2$ scale. In addition to the average variance explained (~~adj.~~ r^2) after tenfold cross validation with 100 repeats, Pearson's correlation coefficient (r_p) is also provided. Bootstrap error intervals fell within the label size and were not displayed.

Figure 2: Application of the taxonomic (A) and phenotypic (B) beta-diversity (PCoA of Bray-Curtis dissimilarity matrix) to investigate season- and lake-specific effects on the community structure of Lake Michigan and Muskegon Lake (n = 87). Fall, Spring and Summer denote samples taken in September, April and July respectively. The variance explained by the lake and season variables, as well as the interaction effect between the lake and season variable is provided (PERMANOVA). All effects were significant at the p = 0.001 level with the exception of the interaction effect for the phenotypic beta diversity (p = 0.018). Procrustes analysis confirmed the high degree of correlation between both beta-diversity analyses (correlation strength = 0.65, p = 0.001, 999 permutations). Permutations for PERMANOVA and Procrustes analyses were constrained within each survey year.

Figure 3: Feeding effect on the phenotypic alpha diversity (A), and phenotypic beta diversity (B) of the bacterioplankton community. Bootstrap error intervals on the phenotypic diversity were calculated on three technical replicates for each microcosm but fell within the label size and ~~were~~ are therefore not displayed. Shaded areas indicate 95 % confidence intervals on the robust smoothing spline regressions. Label radius of the data points in the beta-diversity analysis is proportional to the time into the experiment. The variance explained by the overall temporal and feeding effect, as well as the interaction effect between the feeding and experiment time is provided (PERMANOVA). All effects were significant at the $p = 0.01$ level.

Figure 4: Measured or predicted taxonomic alpha diversity of Lake Michigan bacterioplankton communities after a three hour exposure to invasive quagga mussels. Measured taxonomic diversity data is publicly available from (Denef et al., 2017). The measured data comes from feeding experiments that were carried out with Lake Michigan bacterioplankton communities retrieved over a two year period and under the identical experimental design as described in this manuscript. Predicted alpha diversity values were calculated based on the phenotypic diversity data generated in this study.

Figure 5: Dynamics of high nucleic acid (HNA) and low nucleic acid (LNA) populations. A) Contrasts between the flow cytometric fingerprints of the control samples and the feeding samples after 0h, 1.5h and 3h. Red contours indicate an increase in the LNA population density during feeding, while blue contours indicate a decrease in the HNA population density during feeding, both of which are relative to the control samples at the specified time point. Only contrasts with densities > |0.04|were visualized. B) Absolute cell density of the LNA population over the course of the feeding experiment. Error bars indicate standard deviation across technical replicates (n = 3). C) Absolute cell density of the HNA population over the course of the feeding experiment. Error bars indicate standard deviations across technical replicates (n = 3). Shaded areas indicate 95 % confidence intervals on the robust linear regression models.

Flow cytometric monitoring of bacterioplankton phenotypic diversity predicts high population-specific feeding rates by invasive dreissenid mussels

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Running title

Phenotypic tracking of bacterioplankton

Conflicts of interest

The authors declare that there exist no conflicts of interest.

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Originality-Significance Statement

While most global change studies in microbial ecology are focusing on the impacts of climate change and land use change, the third component of global change, i.e. species invasions, gets considerably less attention. Here, we focused on the impacts of one of the most impactful invasive species in aquatic systems, invasive dreissenid mussels (IDMs). Our knowledge about their impacts on bacterial communities remains very limited, despite potentially large implications to ecosystem functioning if bacterial communities are affected. This is in part due to the innate sensitivity of IDMs to invasive sampling strategies which are required for many molecular analyses (i.e., they halt their feeding activity), complicating the acquisition of highly resolved temporal surveys. Using a recently developed flow cytometry method, which calculates phenotypic diversity estimates, we discovered direct impacts on natural lake bacterioplankton populations, within one hour of being exposed to filter feeding pressures from invasive quagga mussels. We also established a strong correlation between these phenotypic diversity measurements and their taxonomic counterpart calculated from 16S rRNA gene amplicon sequencing over different freshwater environments. This allowed us to predict the magnitude of actual community shifts solely based on flow cytometric measurements, facilitating a temporal resolution of this biological process that would not have been possible otherwise. We could then further attribute this predicted shift in community diversity to the removal of physiological subpopulations previously shown to contribute disproportionately to community metabolism. Thus, this new analysis pipeline led us to the hypothesis that IDMs directly drive bacterioplankton communities toward less diverse and potentially less productive states within short time periods.

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Summary

Species invasion is an important disturbance to ecosystems worldwide, yet knowledge about the impacts of invasive species on bacterial communities remains sparse. Using a novel approach, we simultaneously detected phenotypic and derived taxonomic change in a natural bacterioplankton community when subjected to feeding pressure by quagga mussels, a widespread aquatic invasive species. We detected a significant decrease in diversity within one hour of feeding, and a total diversity loss of 11.6 ± 4.1 % after 3h. This loss of microbial diversity was caused by the selective removal of high nucleic acid (HNA) populations (29 ± 5% after 3h). We were able to track the community diversity at high temporal resolution by calculating phenotypic diversity estimates from flow cytometry data of minute amounts of sample. Through parallel flow cytometry and 16S rRNA gene amplicon sequencing analysis of environments spanning a broad diversity range, we showed that the two approaches resulted in highly correlated diversity measures and captured the same seasonal and lake-specific patterns in community composition. Based on our results, we predict that selective feeding by IDMs directly impacts the microbial component of the carbon cycle, as it may drive bacterioplankton communities toward less diverse and potentially less productive states.

Keywords

Invasive species, flow cytometry, HNA bacteria, phenotypic diversity, disturbance ecology

Introduction

Anthropogenic disturbances can lead to rapid changes in microbial community diversity (species richness, evenness, and composition). Many studies aim to better understand feedbacks between global change and microbial communities, as changes in microbial diversity can either mitigate the predicted direct effects of disturbances on ecosystem fluxes (Singh et al., 2010; Zhou et al., 2012), or lead to major shifts in bacterially mediated fluxes (Schimel and Gulledge, 1998; Finlay et al., 2007; Levine et al., 2011). The responses of microbial communities to disturbances are often monitored by means of high-throughput molecular techniques, such as 16S rRNA gene amplicon sequencing (Shade et al., 2012). Community shifts in response to altering environmental parameters can occur within hours (Props et al., 2016b) to days (Datta et al., 2016), and demand substantial sampling effort at a preferably fixed frequency to allow accurate statistical inference (Faust et al., 2015). Current technology allows sequencing data to be generated from low-volume samples (e.g., 100 mL) of low-density environments ($\leq 10^6$ cells mL^{-1}), which comprise many aquatic ecosystems, but larger sample volumes (> 1L) are required in order to yield a robust census of the microbial community (Padilla et al., 2015). This prohibits the use of this approach in many longitudinal microcosm studies, for which repeated invasive sampling itself would act as a disturbance.

Recently a new approach has been developed that can generate phenotypic diversity metrics based on physiological information derived from flow cytometry (FCM) data (Props et al., 2016a). These diversity metrics have been shown to be highly correlated to taxonomic diversity, as derived from amplicon sequencing. Yet, their derivation avoids invasive, high volume sampling practices (≤ 1mL of sample required), and simultaneously offers information on the physiological state of the community, as well as on the absolute density of its constituent populations. Briefly, this approach performs kernel density estimations on multiple bivariate single-cell parameter combinations (e.g., fluorescence and scatter intensity) and concatenates these into a feature

vector that is called the *phenotypic fingerprint*. The phenotypic fingerprint represents the community structure in terms of physiological aspects, such as nucleic acid content and morphology. From this fingerprint, the community diversity can be calculated by means of the Hill diversity numbers (Hill, 1973), which examine both richness and evenness components of the phenotypic community structure. In parallel, this approach facilitates beta-diversity assessments through the ordination of samples by means of a dissimilarity matrix calculated between phenotypic fingerprints. The ability to simultaneously track impacts on phenotypic and taxonomic diversity offers opportunities to address gaps in our understanding of microbial disturbance ecology. Currently, this method has only been tested in one, low-complexity system, and validation across a broader range of diversities is needed to fully assess its potential.

Species invasion, which is one of the main components of global change (Chapin et al., 2000), is a particularly useful system to help address knowledge gaps in microbial disturbance ecology as we can readily mimic the real-world conditions (i.e., sudden introduction) in laboratory or field experiments.. The current distribution of invasive dreissenid mussels (IDMs) across North America (>30 states) is a prime example of a successful invasion event (Higgins and Vander Zanden, 2010). Initially introduced through ballast water, IDMs display high filtration rates (Vanderploeg et al., 2002), and are able to rapidly populate benthic and littoral substrates in densities of up to 19,000 individuals per m^2 (Nalepa et al., 2010). With respect to their feeding behavior, IDMs show highly selective feeding behavior towards seston and different algal and microzooplankton taxa over a broad range of size (~ 1 to 200 μm) (Tang et al., 2014). While IDMs are known to strongly impact phytoplankton and zooplankton abundance and composition (Higgins and Vander Zanden, 2010), the few studies focused on their impacts on bacterioplankton report contradicting results Several of these studies reported selective feeding on bacterial species (Silverman et al., 1995; Pires et al., 2004; Denef et al., 2017) while a long-term environmental survey of the Hudson River prior- and post-invasion did not observe negative effects on bacterial community density and productivity (Findlay et al., 1998).

In this study, we investigated the effect of IDM grazing (with *Dreissena bugensis* as model) on the natural bacterioplankton community of Lake Michigan through (near) non-invasive tracking of the phenotypic biodiversity, as well as the density of physiological subpopulations. We first validated whether the existing correlation between taxonomic and phenotypic diversity metrics holds for the high diversity environments of Lake Michigan (low primary and secondary productivity) and one of its freshwater estuaries (high primary and secondary productivity), Muskegon Lake. We then used phenotypic alpha and beta diversity analyses to assess the impact and extent of IDM grazing on the bacterioplankton community of Lake Michigan. The observed biodiversity dynamics were further related to the dynamics of well-established physiological populations in freshwater bacterioplankton, for which the grazing rate was determined.

Results

We aimed to use flow cytometry-derived phenotypic diversity metrics as a proxy for taxonomic diversity shifts occurring during quagga mussel feeding on lake bacterioplankton. The experiment consisted of 12 L microcosms of 153 μm screened water and quagga mussels, both retrieved from Lake Michigan. The Lake Michigan bacterioplankton has previously been shown to contain both grazing-resistant and grazing-sensitive taxa allowing the study of direct grazing impacts (Tang et al., 2014; Denef et al., 2017). Prior to the onset of the experiment, we assessed whether the previously established correlation between phenotypic and taxonomic diversity metrics for low diversity environments could be extended to higher diversity aquatic environments, such as Lake Michigan.

1. Validation of phenotypic diversity as a proxy for taxonomic diversity

Microbial communities can be classified as relatively low or high diverse communities based on their Hill diversity metrics as these are expressed in terms of *effective number of taxa*, which depict the number of equally abundant taxa required to obtain the same diversity value as the community in question (Hill, 1973). The diversity metrics derived from flow cytometric analysis are calculated in the same way as their taxonomic counterparts but they can only be interpreted in arbitrary units. In order to determine whether there was a general relationship between the phenotypic and taxonomic diversity, we included an independent dataset from a low diversity cooling water microbial community (1 – 10 effective number of taxa) (Fig. 1). This cooling water dataset contains two 40 day surveys of bacterioplankton communities present in the secondary cooling water system of a nuclear test reactor that was subjected to multiple operational phases. The positive correlation between taxonomic and phenotypic diversity metrics of orders 1 (D_1, exponential of Shannon entropy) and 2 (D_2, Inverse Simpson index) could be extended to the Lake Michigan and Muskegon Lake communities (cross-validated $r^2 = 0.89$, Pearson's correlation

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r_p = 0.94 for both D_1 and D_2). We opted for a single regression model as opposed to individual regressions for each environment in order to avoid overfitting as well as to construct a generalizable model that provides robust inference for all three environments. Individual regression models did not significantly differ in slope and the cross-validated r^2 of the single regression model was high (r^2 = 0.89), thereby permitting the use of a single regression model for monitoring diversity dynamics (**Fig S1**, **Table S1**).

The dynamic range of the regression, defined as the ratio between the largest and smallest taxonomic diversity used in its calculation, was 88.7 for D_1 and 42.5 for D_2. Goodness-of-fit analysis of the linear regression model indicated a normal distributed residual distribution with homogenous variance over the entire regression range (**Fig. S2**). The observed richness (D_0) did not show a distinct linear correlation (r^2 = 0.32, r_p = 0.54, **Fig. S3**). Due to the high level of correlation between the phenotypic diversity (D_1, D_2) and the taxonomic diversity, it was permissible to use the phenotypic diversity as a stand-alone metric for evaluating bacterioplankton diversity. Additionally, only D_2 was used in further analyses due to the high degree of correlation between D_1 and D_2 (r_p = 0.99).

In contrast to alpha diversity, beta diversity cannot be captured by a single metric. Therefore we compared the taxonomic and phenotypic beta diversity by their performance to detect seasonal- and lake-specific community structures in the Lake Michigan and Muskegon Lake data set (**Fig. 2**). Procrustes analysis demonstrated that both approaches were significantly correlated in terms of the patterns that they captured in the data (p = 0.001). Additionally, both approaches identified season and lake type (Lake Michigan or Muskegon Lake) as significant predictors of the community structure (p = 0.001). The season explained the most variance in the beta diversity (i.e., 19.6% of the taxonomic beta diversity and 22.5% of the phenotypic beta diversity). Lake type still captured 17.4% of the variance in the taxonomic beta diversity, but only 7.0% in the phenotypic beta diversity. Lastly, the seasonal effect was dependent on the lake type, representing

an extra 9.4% of the variance in the taxonomic beta diversity ($p = 0.001$) and 4.6% in the phenotypic beta diversity ($p = 0.018$).

2. Diversity dynamics during IDM feeding

The temporal trajectory of the bacterioplankton community of the microcosms was monitored for 3h at a resolution of 0.5h when subjected to the direct feeding pressure by 15 IDMs per microcosm (**Fig. 3**). This time period was sufficiently long to allow the assessment of direct feeding effects (removal of 30 to 60% of seston), but short enough to avoid indirect effects, e.g. due to trophic cascades or substantial accumulation of feces and pseudofeces (Vanderploeg et al., 2010). Importantly, all mussels were subjected to an extensive pretreatment consisting of specific handling, rinsing, and acclimatization steps in order to avoid contamination of the bacterioplankton community by external periphyton, debris, and ingested particles at the onset of the experiment (see experimental procedures section). The size distribution of the mussels was not significantly different between the microcosms (22.7 ± 2.3 mm, Kruskal-Wallis test, $p = 0.08$). The total dry weight of the mussels per microcosm was 0.24 ± 0.018 g DW.

Over the span of the experiment, the control microcosm's phenotypic diversity exhibited a minor overall positive temporal drift ($p = 0.038$). In contrast, the bacterioplankton phenotypic diversity underwent a clear and significant decrease ($p < 0.0001$) during filter feeding of the IDMs, signifying the enrichment of the community by certain taxa (**Fig. 3A**). The treatment effect became significantly distinguishable from the control at the 1 hour mark (at $p < 0.05$). Using the regression model, an average loss in taxonomic diversity (D_2) could be predicted of 2.6 ± 1.0 effective number of taxa, corresponding to a decrease of $11.6 \pm 4.1\%$ over the course of the experiment. Conceptually, this means that in a hypothetical community of 23 equally abundant taxa (diversity prior to mussel feeding), an average of 2.6 taxa would be lost due to IDM feeding. To put these measurements in perspective, we analyzed data from a recently published mussel-feeding study that used the same experimental design, and had 16S rRNA gene amplicon data at

time points 0 and 3h available (**Fig. 4**) (Denef et al., 2017). We calculated a mean loss of taxonomic diversity (D_2) of 5.32 ± 4.65 for their three independent experiments which is comparable to the taxonomic diversity loss predicted for our experiment (2.6 ± 1.0).

While the monitoring of the phenotypic alpha diversity allowed us to track the treatment effect through time, a beta diversity analysis was also conducted to evaluate the treatment and temporal effects on the complete phenotypic structure of the bacterioplankton community (**Fig. 3B**). Over the course of the experiment the bacterioplankton communities of the control and treatment microcosms became more dissimilar. In agreement with the alpha diversity analysis, a time-dependent treatment effect ($r^2_{feeding}$ = 0.34, p = 0.005) was driving the bacterioplankton structure, with the control bacterioplankton community also experiencing a minor temporal effect ($r^2_{control}$ = 0.08, p = 0.006).

3. Bacterioplankton population dynamics during IDM feeding

Next, we investigated whether the observed diversity dynamics were caused by selective feeding on specific phenotypic populations of the bacterioplankton community. To do so, contrasts between the phenotypic fingerprints of the treatment and the control at three different time points were calculated (**Fig. 5A**). This analysis allowed the visualization of regions in a specified bivariate parameter space which are relatively more or less abundant in the treatment versus the control. We opted for the primary fluorescence channels of the SYBR Green nucleic acid stain (i.e., FL1-H, FL3-H) allowing us to identify distinct physiological populations with varying nucleic acid content (Gasol et al., 1999; Hammes and Egli, 2010; Koch et al., 2014). The results demonstrate that during filter feeding the bacterioplankton community became enriched with a low nucleic acid content population (LNA, low FL1-H/FL3-H intensity), and was depleted from a high nucleic acid content population (HNA, high FL1-H/FL3-H intensity).

As these are relative changes, that do not necessarily reflect a direct feeding effect on the HNA population, the absolute abundances for both the HNA and LNA population were extracted

from the total community according to the guidelines by (Prest et al., 2013) (**Fig. S4**). The LNA cell densities show similar temporal behavior for the control (coefficient of variation – CV = 5.8%) and treatment (CV = 5.2%) microcosms (**Fig. 5B)**. This level of variation falls within the technical variation of current flow cytometry technology (CV = 5%), and is thus not indicative of a feeding effect (Hammes et al., 2008). In contrast, the HNA population was directly affected by the filter feeding (**Fig. 5C**). The HNA population of the control microcosms displayed a similar variation to the LNA population (CV = 5.1%), while the HNA population in the treatment microcosms showed a monotonic decrease throughout the experiment (CV = 12.8%). Analogous to the diversity analyses, a significant treatment effect could be detected within 1h. Using robust linear regression, the HNA specific removal rate was estimated at 43,000 ± 3,000 cells mL^{-1} h^{-1} ($p < 0.0001$), while the control HNA cell density remained constant ($p = 0.98$). After 3 hours of being subjected to filter feeding, 29 ± 5% of the HNA bacterioplankton population was removed from the water column. The clearance rate on the HNA population was 4.56 ± 0.81 mL mg^{-1} DW h^{-1}.

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Discussion

Our understanding of microbial disturbance ecology has been partially constrained by a lack of temporal resolution, caused by methodological limitations in either sampling, logistics, or analysis. In order to combat these bottlenecks, we further developed and validated a (near) non-invasive flow cytometry-based approach dedicated to detect changes in the phenotypic diversity of microbial communities. We validated and applied these phenotypic diversity metrics to natural, high diversity environments, and investigated the response of bacterioplankton communities to a filter feeding disturbance caused by invasive dreissenid mussels, which are highly sensitive to the invasive sampling imposed by alternative monitoring techniques (i.e., they would cease their filter feeding activity). Our experimental results highlight the sensitivity of our method to detect subtle changes in diversity over short timeframes. Based on (1) our presented relationship between phenotypic and taxonomic diversity, (2) the positive relationship that exists between HNA bacterial populations and bacterial productivity in many ecosystems (Zubkov et al., 2001; Servais et al., 2003), and (3) the results of our filter feeding experiment, we hypothesize that IDM feeding directly influences both microbial diversity and ecosystem functionality.

Relation between taxonomic and phenotypic diversity

The regression between the taxonomic and phenotypic diversity data is in agreement with and expands upon previous research (Props et al., 2016a) and offers further insight into the fundamental relation between these metrics. Concretely, the regression's dynamic range has been extended from 10.3 in the previous study (cooling water) to 42.5 for the diversity of order 2 (D_2). The quality of the cross-validated regression is worth highlighting as there were substantial differences in the sample treatment and data generation of the data sets, which could have led to systematic bias. First, the flow cytometry samples of Lake Michigan and Muskegon Lake were fixed with glutaraldehyde and archived at -80°C, whereas the cooling water samples were analyzed directly. The glutaraldehyde fixative used in this research has been shown to increase

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autofluorescence and may have increased the instrument noise (Gunther et al., 2008). Nevertheless, the bacterioplankton community could be reproducibly isolated from the raw data with one fixed denoising strategy for the entire data set (**Fig. S4**). Secondly, the amplicon sequencing of the 16S rRNA gene targeted the V4 region for the Lake Michigan and Muskegon samples, whereas the V3-V4 region was targeted for the cooling water samples. This difference in sequenced region has been shown to potentially alter the observed diversity (Schmalenberger et al., 2001; Yu and Morrison, 2004). We did not observe this bias in the taxonomic diversity, but we did observe that the phenotypic diversity was incapable of resolving the Lake Michigan and Muskegon Lake communities, even though they had distinct taxonomic diversities. The regression analysis showed that the strength of the relationship between the phenotypic and taxonomic diversity was unaffected, but that the intercept of the linear models was different ($p<0.001$). Therefore we emphasize that the absolute values of the phenotypic diversity metrics need to be compared within a single ecosystem or experimental setting. Lastly, rescaling or subsampling the community composition to an equal library size, which is a common yet debated practice in microbial community analyses (McMurdie and Holmes, 2014), did not negatively affect the regression (**Fig. S5**). In fact, it improved the regression for all diversity metrics and in particular D_0, indicating that the phenotypic diversity metrics are primarily sensitive to fluctuations in the density of abundant taxa. As such, we recommend to evaluate changes in richness (D_0) solely by means of 16S rRNA gene amplicon sequencing, for which novel statistical approaches are being developed that account for both observed and unobserved taxa (Willis and Bunge, 2015). It is important to note that in order to compare flow cytometry-derived diversity metrics with each other, the underlying raw data must have been generated by the same flow cytometer platform with identical detector and flow rate settings, which was the case for all data presented in this study.

The linearity on the log-scale implies that the change in phenotypic diversity required to detect a corresponding change in taxonomic diversity systematically increases (Fig. 1). For example, at low

diversities, a change from 1,000 to 1,500 units in phenotypic diversity corresponds to a predicted change of 3.4 units in the taxonomic diversity, while an increase from 1,500 to 2,000 units only corresponds to a predicted change of 2.0 units in the taxonomic diversity. This is one of the limitations of relying on a fixed number of phenotypic parameters (i.e., fluorescence and scatter intensities); the available parameter space that bacterial cells can occupy is limited, resulting in a loss of sensitivity at higher diversities.

The beta diversity analyses yielded similar statistical inference on the seasonal- and lake-specific effects, with both the taxonomic and phenotypic beta diversity identifying seasonality as the most important predictor of community structure. A higher degree of variance could be explained by the lake type in the taxonomic beta diversity, which suggests that the phenotypic approach was less sensitive to differences in taxon distributions between the lakes, or that additional variation based on phenotypic plasticity weakens the relationship between taxonomic and phenotypic beta diversity. This is congruent with the alpha diversity measurements where Lake Michigan and Muskegon Lake samples showed similar phenotypic diversity despite possessing distinct taxonomic diversities. Overall, Procrustes analysis confirmed that the phenotypic beta diversity was able to largely capture the same patterns in the data as the taxonomic beta diversity. As such, phenotypic beta diversity analyses constitute a valid approach for hypothesis testing in high diversity environments but they are susceptible to a higher degree of variability and thus generate potentially lower effect sizes (e.g., for the lake type in this analysis).

Diversity and population dynamics

The phenotypic diversity dynamics during the 3h filter feeding experiment were more subtle than in our previous study on the cooling water dataset (< 150 vs. > 500 units; (Props et al., 2016a)) but occurred over a much shorter time scale (3 hours vs. multiple days). Importantly, the predicted loss in taxonomic diversity based on the phenotypic diversity (2.6 ± 1.0) lies well within the range of expected diversity shifts (5.32 ± 4.65) calculated from previous experiments which

had start and endpoint measurements of community composition (Figure 5). Our predictions also suggest i) a general season-dependent bacterioplankton diversity with a higher diversity in summer than in fall and winter and ii) a season-dependent specific feeding effect resulting in a higher diversity loss in summer than in fall and winter. Overall, the conformity of our predictions to these previous experiments further validates the phenotypic diversity approach.

The diversity dynamics suggested that *D. bugensis* was selectively feeding upon a fraction of the bacterioplankton community, thereby altering the community composition, and lowering the diversity. This was confirmed by identifying populations that were selectively enriched through contrast analysis, which demonstrated the selective feeding on bacteria with high nucleic acid content (HNA bacteria) (Fig. 4A). The HNA clearance rate (4.56 ± 0.81 mL mg^{-1} DW h^{-1}), which can be interpreted as the water volume that is fully depleted of HNA bacteria per hour, was comparable to those previously reported for *Dreissena polymorpha* feeding on laboratory strains (3.5 – 4.8 mL mg^{-1} DW h^{-1}) ranging in size between 1 and 4 μm in length (Silverman et al., 1995). The clearance rates on laboratory strains were measured for high cell densities (> 10^7 cells mL^{-1}) relative to the natural densities in Lake Michigan in this study (~ 10^6 cells mL^{-1}), and with different IDM species at a higher temperature, thus making direct comparisons difficult. With respect to experiments on natural bacterioplankton, mixed results have been reported. For river bacterioplankton, short-term mesocosm experiments provided no evidence of a direct feeding effect, while long-term environmental surveys suggested a doubling in bacterioplankton densities (Findlay et al., 1998). In lakes, feeding on bacteria in low nutrient systems was thought to be limited (Cotner et al., 1995), though short-term feeding experiments on natural bacterioplankton from Lake Michigan did detect significant decreases in bacterioplankton densities (Denef et al., in review).

HNA and LNA populations have been well-characterized in aquatic environments, yet considerable debate remains regarding the characteristics of each population. Initially, it was

thought that the HNA population was the active fraction of the bacterial community, whereas the LNA population served as a reservoir of dormant, inactive, dead, dying, and damaged cells (Lebaron et al., 2002). Nowadays, the LNA population has been shown to be able to actively grow and to be metabolically active in the environment without adopting HNA properties, such as high nucleic acid content and increased cell size (Jochem et al., 2004; Scharek and Latasa, 2007; Wang et al., 2009). Most studies do report an elevated cell-specific activity for the HNA bacteria that can be more than an order of magnitude higher than the activity of the LNA bacteria (Lebaron et al., 2002; Servais et al., 2003). HNA bacteria are also generally larger and exhibit higher growth rates than LNA bacteria (Lebaron et al., 2002; Jochem et al., 2004; Scharek and Latasa, 2007), and this large, active fraction of the bacterioplankton is preferred by zooplankton grazing (Boenigk et al., 2004; Tadonleke et al., 2005; Garcia-Chaves et al., 2016).

HNA population densities tend to be positively correlated with heterotrophic productivity (Zubkov et al., 2001; Bouvier et al., 2007). Thus, we would expect relatively low HNA densities in Lake Michigan, which has been rendered increasingly oligotrophic (low primary and secondary productivity) since the ingress of IDMs (Evans et al., 2011). HNA population densities in Lake Michigan field samples (29.6 ± 4.2 %, n = 30) were even lower than those reported in previous surveys of freshwater lakes (40 – 42.5 %, n = 81) with similar levels of primary productivity (2 ± 1.5 μg chlorophyll *a* L^{-1} vs. 1.5 ± 1.2 μg chlorophyll *a* L^{-1} in our 2015 survey of Lake Michigan) (Bouvier et al., 2007; Shuchman et al., 2013). We observed that IDMs predominantly feed on the HNA population; this may in part explain these lower than expected HNA densities, as Lake Michigan is characterized lake-wide by high densities of IDMs (Nalepa et al., 2010). With the HNA bacteria leveraging as much as 80 % of the community's secondary production, and mediating up to 70 % of the bacterial carbon flux in other aquatic environments, the selective feeding by IDMs may have a significant impact on elemental cycling in lake systems (Zubkov et al., 2001; Scharek and Latasa, 2007).

Community characterization of HNA and LNA populations has shown that there can be significant differences in community composition, with few shared taxa between the populations (Schattenhofer et al., 2011; Vila-Costa et al., 2012). As a consequence, selective removal of a single population (e.g., HNA) will alter the community diversity, which was observed during this filter feeding experiment. Other studies using basic molecular fingerprinting techniques did not observe distinct community structures (Servais et al., 2003; Longnecker et al., 2005). Hence, several scenarios have been developed to explain the dichotomy between HNA and LNA bacterioplankton populations (Bouvier et al., 2007). Our results only allow us to support the scenario in which each population has a separate community structure, since considerable repopulation of the HNA population through growth or potential recruitment from the LNA population can take several days (Gasol et al., 1999; Sintes and del Giorgio, 2014; Baltar et al., 2016).

While few investigations into the impacts of IDM on bacterial community composition have been performed, our observations are congruent with studies that have shown altered composition in the sediment (Frischer et al., 2000; Lohner et al., 2007; Lee et al., 2015) and water column (Denef et al., 2017) following IDM introduction. In these studies specific taxonomic groups were shown to become relatively enriched within the microbial community. Among others, taxa of the phylum Actinobacteria and the genus *Polynucleobacter*, which are known to possess LNA-type characteristics such as small cell sizes (Wang et al., 2009), increased in relative abundance during short-term microcosm experiments (Denef et al., 2017). Other taxa (e.g. Chloroflexi) became enriched despite their larger cell size, suggesting that multiple phenotypic traits beyond mere cell size determine the feeding success on bacterial taxa. Relative enrichments do need to be interpreted with care as these can provide biased interpretations of the actual taxon abundance dynamics (Nayfach and Pollard, 2016; Props et al., 2016b; Stammler et al., 2016).

In conclusion, we have shown that advanced data analysis of flow cytometry data can lead to robust predictions of taxonomic diversity within a large dynamic range. We further demonstrated that the diversity of natural bacterioplankton communities can be reliably tracked during sensitive ecological processes in a fast, non-invasive manner. Using this approach we were able to detect subtle shifts in biodiversity emerging within one hour of feeding by invasive dreissenid mussels. The selective removal of HNA bacteria was shown to be underlying cause of the loss of biodiversity, suggesting size-selective feeding behavior in the micrometer range. As a result, IDMs are capable of locally reducing the diversity and productivity of the bacterioplankton community during feeding. The approach presented here can be readily applied to help address a broad range of questions in marine and freshwater systems, for which new analytical and computational tools are needed (Labbate et al., 2016). Flow cytometry is now also increasingly being developed for other environments such as soils, sediments, and sludges, opening new possibilities for these systems as well (Frossard et al., 2016).

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Experimental Procedures

16S rRNA gene amplicon sequencing analysis

We used a combination of a previously published data set from 2013 and newly generated V4 16S rRNA gene amplicon sequences from 2014 and 2015 lake surveys (see data availability section). V4 amplicon sequencing data from Lake Michigan (2015 survey) and Muskegon Lake (2014, 2015 surveys) were generated exactly as previously described (Schmidt et al., 2016). Samples were taken in September (Fall), April (Spring), and July (Summer). The DNA was extracted according to a previously optimized protocol (McCarthy et al., 2015) and submitted for sequencing of the V4 hypervariable region (515F/806R) by Illumina MiSeq with v2 chemistry (2x250bp). All raw sequencing reads from these surveys were processed together, after which the samples with matching flow cytometry data were extracted.

Contigs were created by merging paired-end reads based on the Phred quality score heuristic (Kozich et al 2013) in MOTHUR (v.1.38, seed = 777) (Schloss et al., 2009). Contigs were aligned to the Silva database (v123), and filtered from those with (i) ambiguous bases, (ii) more than 8 homopolymers, (iii) a length outside of the 240 – 275 nt range, and (iv) those not corresponding to the V4 region. The aligned sequences were filtered and dereplicated, and sequencing errors were removed using the *pre.cluster* command. Chimera removal was performed by UCHIME. Sequences were clustered into operational taxonomic units (OTUs) at 97 % similarity with the *cluster.split* command (average neighbour algorithm). Sequences were then classified using the TaxAss pipeline (https://github.com/McMahonLab/TaxAss) which classifies sequences according to both a small, manually curated freshwater taxonomy database (Newton et al., 2011) and a large, general database (SILVA v123). The complete workflow is available at https://github.com/rprops/Mothur_oligo_batch and was run in batch mode. For comparison to the flow cytometry data, only the samples comprising the bacterioplankton fraction (0.22 – 3 μm

fraction) were used in further analyses, as this fraction was the most directly comparable to the measurements taken by the flow cytometer.

The cooling water reference data contain publicly available V3-V4 16S rRNA gene amplicon sequences, and are available from the NCBI Sequence Read Archive (SRA) under accession number SRP066190. We utilized the OTU-table from a previous publication as basis for the diversity calculations (Props et al., 2016b). This OTU-table was generated according to the same pipeline as described above.

Flow cytometry analysis

1 mL of unfiltered water samples were fixed with 5 µL glutaraldehyde (20 % (v/v) stock), incubated for 10 minutes in the dark, and flash frozen in liquid nitrogen (storage at -80°C). Prior to flow cytometry analysis, batches of eight samples were sequentially defrosted, acclimated to room temperature, diluted twofold in triplicate and stained with SYBR Green I (10,000x in DMSO, Invitrogen) to a final concentration of 1x SYBR Green I. Samples were then incubated at 37 °C for 20 min in the dark, and analyzed directly on a BD Accuri C6 cytometer (BD Biosciences) in fixed volume mode (50 µL) (Props et al., 2016a). This resulted in a multiparametric description of each microbial cell by four fluorescence parameters (FL1: 533/30 nm, FL2: 585/40 nm, FL3: > 670 nm long pass, FL4: 675/25 nm) and two scatter parameters (FSC, SSC). Instrument performance was verified daily using eight peak rainbow particles (Spherotech, Lake Forest, IL, USA).

Phenotypic diversity analysis

The alpha diversities for both the flow cytometry and sequencing data were assessed by the Hill diversity numbers, which incorporate both richness and evenness components (Hill, 1973). We followed the previously published protocol available here: https://github.com/rprops/Phenoflow_package/wiki/Phenotypic-diversity-analysis (Props et

al., 2016a). Raw flow cytometer data were exported in FCS format and imported into R (v3.3.0), using functions from the *flowCore* package (v1.38.2). The data were denoised from (in)organic noise based on previous experience with freshwater communities and according to published guidelines for robust denoising (Prest et al., 2013) (as described in **Fig. S4**). The denoising strategy remained the same for all samples. Samples with less than 10,000 cells were discarded since sample sizes larger than this threshold were required for the robust estimation of the diversity (**Fig. S6**). All single-cell parameters were normalized based on the maximum signal height (-H) of the FL1 parameter. The *Diversity* function from the *Phenoflow* package (v1.0, https://github.com/rprops/Phenoflow_package) was then used to calculate the phenotypic alpha diversities of the four primary parameters (FL1-H, FL3-H, FSC-H, SSC-H). Errors on the diversities were generated after 100 bootstraps and propagated to the mean diversity over the three technical replicates. The kernel density estimations were performed with a bandwidth of 0.01, a grid size of 128x128 and a rounding factor of 3. The alpha diversity was evaluated through the first three Hill numbers: D_0, D_1 and D_2, which correspond to the observed richness, the exponential of Shannon entropy, and the inverse Simpson index, respectively. Beta diversity analyses were performed by principal coordinate analysis (PCoA) of the phenotypic fingerprints (*flowBasis* function, d = 3, bw = 0.01) using the Bray-Curtis dissimilarity metric (*beta_div_fcm* function, default settings). Contrasts between the phenotypic fingerprints of the control and treatment groups were made by the *fp_contrasts* function (see tutorial here: https://github.com/rprops/Phenoflow_package/wiki/Making-contrasts).

Taxonomic diversity analysis

For calculating the taxonomic alpha diversity, we used the *Diversity_16S* function from the *Phenoflow* package because this allowed a direct comparison between the taxonomic and phenotypic diversity metrics. The community data were not rarefied because our hypothesis was that the taxonomic diversity was correlated with an unrelated variable, the phenotypic diversity.

Subsampling to the lowest sample size would result in the poorest estimate of the taxonomic diversity for all samples, thereby potentially obscuring the true relationship between these variables. Instead, we selected only samples which had a sample size larger than 10,000 reads (n = 138), generated 100 bootstrap samples for each sample, and took the average diversity as the sample representative diversity. Parallel results of our analyses for the rescaled data to 10,000 reads are available in supplementary information (**Fig. S5**). For beta diversity analysis the OTU abundances were rescaled by calculating their proportions and multiplying them by the minimum sample size present in the data set (McMurdie and Holmes, 2014). The beta diversity was then assessed by PCoA of the Bray-Curtis dissimilarity matrix, which was calculated based on the taxon proportions instead of the read counts in order to be directly comparable to the phenotypic beta diversity approach.

Feeding experiment

Quagga mussels and lake water (5 m above lake floor) were collected at 45m deep from Lake Michigan (43° 12' N, 86° 27' W). Mussels were rinsed of adhering sediment and were transported (< 8h) submerged in lake water at 5-7°C. The standard handling and experimental design of (Vanderploeg et al., 2010) was followed and is briefly outlined as follows. In the lab, the mussels were cleaned of debris and placed in a tank filled with 90 L of 153 μm-screened Lake Michigan water in order to remove grazing mesozooplankton in an environmental room set to ambient temperature of the lake water (9.7 °C). The next morning the mussels were transferred to a 40 L aquarium with 153 μm screened Lake Michigan water for 2 h. The mussel cleaning and ~ 14 h re-acclimation period allowed the removal of external periphyton and debris, cleared the mussel guts of sediment ingested during capture, and gave mussels time to reach digestive equilibrium with their natural food source. All materials were washed with bleach and rinsed with deionized water to minimize bacterial contamination. Seven 19 L HDPE cylindrical containers were filled with 12 L of 153 μm-screened lake water each. Forty-five adult mussels were spread evenly across three

containers (15 mussels per container) and two containers remained mussel-free. Gentle mixing was provided by bubbling air through a pipette, and all experiments were carried out under dim light (~ 8 µmol quanta m^{-2} s^{-1}) . Water samples were taken before the addition of the mussels and every 0.5 h after the mussels showed signs of active feeding (after approx. 15 minutes). The number of mussels added and the experiment duration were chosen to allow healthy mussels to clear 30 – 60 % of preferred seston. As shown in a previous study (Denef et al., 2017), our procedure ensures that mussel-associated bacteria do not significantly impact observed shifts in bacterial community composition over the duration of the experiment. One mL water samples from the top water layer were taken every 30 minutes throughout and at the end of the 3h experiment. The samples were fixed with 5 µL glutaraldehyde (20 % (v/v)), incubated for 10 minutes in the dark, and flash frozen in liquid nitrogen (storage at -80°C).

Statistical analysis

All statistical analyses were performed in the R statistical environment (v3.3.0) (R Core Team, 2015), using functions from the *vegan* (v2.4-1), *sandwich* (v2.3-4), *MASS* (v7.3-45), *car* (v2.1-3), *phyloseq* (v1.16.2), *lmtest* (v0.9-34), and *caret* (v6.0-73) packages. Errors on all summary statistics represent standard deviations on the mean and were calculated by propagating individual standard deviations as randomly distributed, independent errors. Ordinary least squares regression was used to relate the phenotypic diversity to the taxonomic diversity (both $\log_2$ transformed). Model assumptions (i.e., normality and homoscedasticity) were evaluated through analysis of the residuals (**Fig. S2**). Goodness-of-fit statistics were calculated through tenfold cross validation with 100 repeats. Inference on the temporal treatment effect on the phenotypic diversity (D_2) was performed by spline regression. We opted for natural splines because these provide more stable estimates at the boundaries (James et al., 2014). Splines were given 3 degrees of freedom, allowing two knots to occur at the 33.3% and 66.6% quantiles (i.e., at time points 1h and 2h). Parameter estimation was performed by the robust ordinary least squares method.

Robust linear regression is a variation on the traditional ordinary least squares (ols) regression that provides more correct inference when assumptions for ols regression are invalid (i.e. less sensitive to outliers). Due to the presence of moderate temporal autocorrelation in the model residuals (**Fig. S8**), robust parameter errors, calculated from the autocorrelation adjusted covariance matrix (*vcovHAC* function), were used in the statistical inference (Wald test). Differences between groups in the beta diversity analysis were evaluated by means of permutational multivariate ANOVA (PERMANOVA, *adonis* function, 999 permutations) of the Bray-Curtis dissimilarity matrix, after confirmation of the homogeneity of the variance in the groups (*betadisper* function). Similarity between beta diversity analyses was evaluated through Procrustes analysis (*protest* function, 999 permutations). Temporal trends in the HNA cell density, as well as the feeding rate were determined through robust ordinary least squares linear regression. Statistical inference on the model parameters was performed with the Wald test. The clearance rate (CR) was determined based on the robust linear regression of the HNA cell dynamics:

$$CR = \frac{V}{n} * \frac{a}{b}$$

V is the water volume of the container (mL), n is the average dry weight of the mussels (mg), a is the slope of the regression (cells mL^{-1} h^{-1}), and b is the intercept of the regression (cells mL^{-1}).

Data availability

The entire data-analysis pipeline is available as an R Markdown document at https://github.com/rprops/Mussel_feeding. Raw flow cytometry data is available on FlowRepository under accession IDs FR-FCM-ZZNA (cooling water), FR-FCM-ZYZA (mussel feeding experiment), FR-FCM-ZYZN (Lake Michigan and Muskegon Lake survey). Newly generated V4 16S rRNA sequences from lake Michigan and Muskegon Lake were deposited on the NCBI SRA under accession number XXXXX. For Lake Michigan, the 2013 data set is

publicly available on the Joint Genome Institute's genome data portal (http://genome.jgi.doe.gov/; Project IDs 1041195 and 1041198).

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Contributions

R.P. and V.J.D. wrote the paper, R.P. performed laboratory work, and performed all statistical analyses. H.A.V. contributed in the laboratory work. J.H. performed the flow cytometry data acquisition. M.L.S. generated sequencing data. V.J.D., H.A.V., N.B. and R.P. designed the study. All authors reviewed and approved the manuscript.

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Figure legends

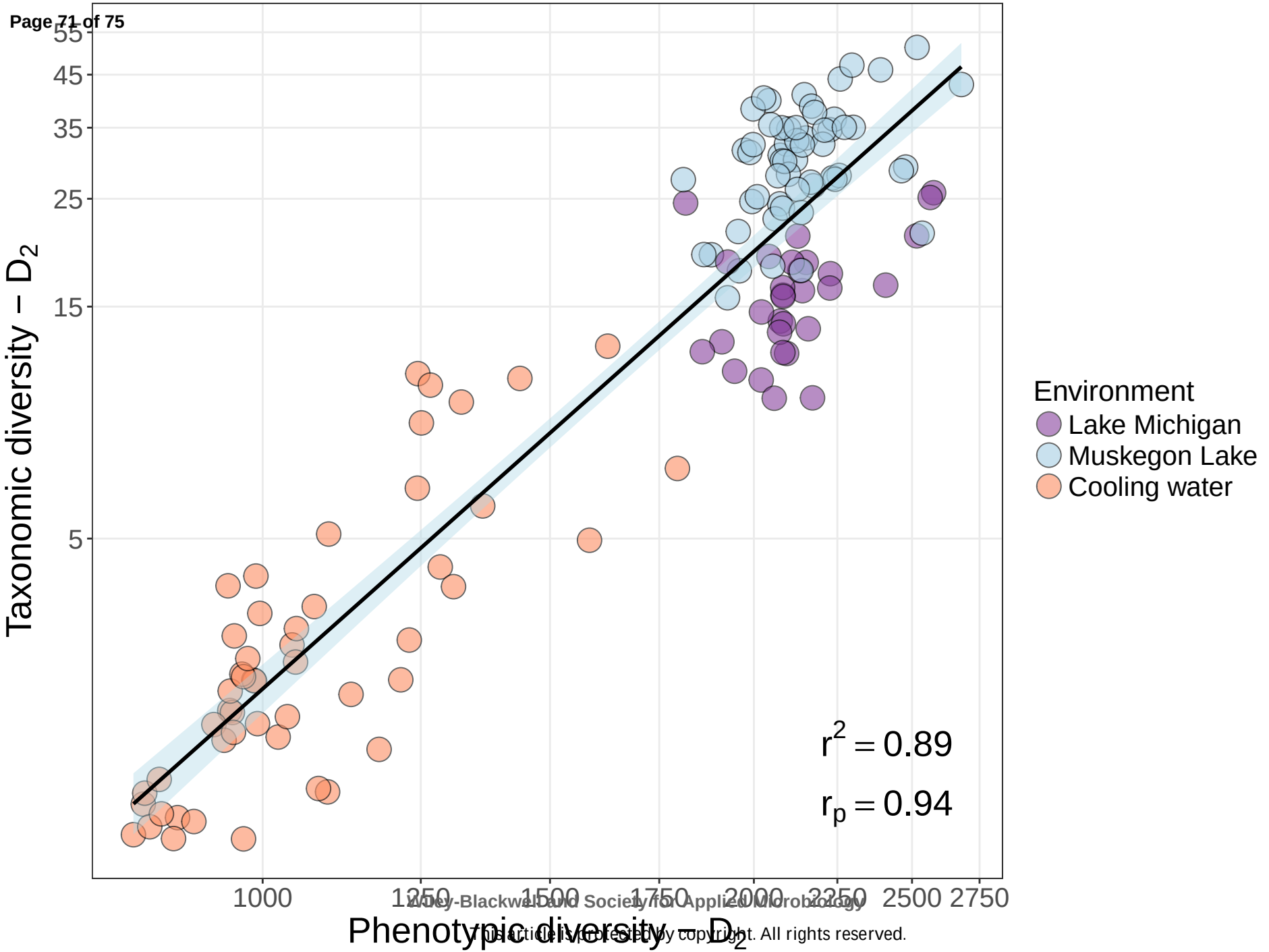
Figure 1: Validation for the use of the phenotypic diversity (derived from FCM) across environments with varying degrees of taxonomic diversity (derived from 16S rRNA gene amplicon sequencing, n = 138). The cooling water samples represent bacterioplankton communities sampled throughout two 40-day temporal surveys of a cooling water system of a nuclear test reactor (Props et al., 2016a). Lake Michigan and Muskegon lakes samples represent bacterioplankton communities sampled over a productivity gradient, at various depths (110m, 45m, 15m), and throughout three seasons (Fall, Spring, Summer). Fall, Spring and Summer denote samples taken in September, April and July respectively. The shaded area represents the 95% confidence interval around the ordinary least squares regression model. Both diversities are depicted on a $\log_2$ scale. In addition to the average variance explained (r^2) after tenfold cross validation with 100 repeats, Pearson's correlation coefficient (r_p) is also provided. Bootstrap error intervals fell within the label size and were not displayed.

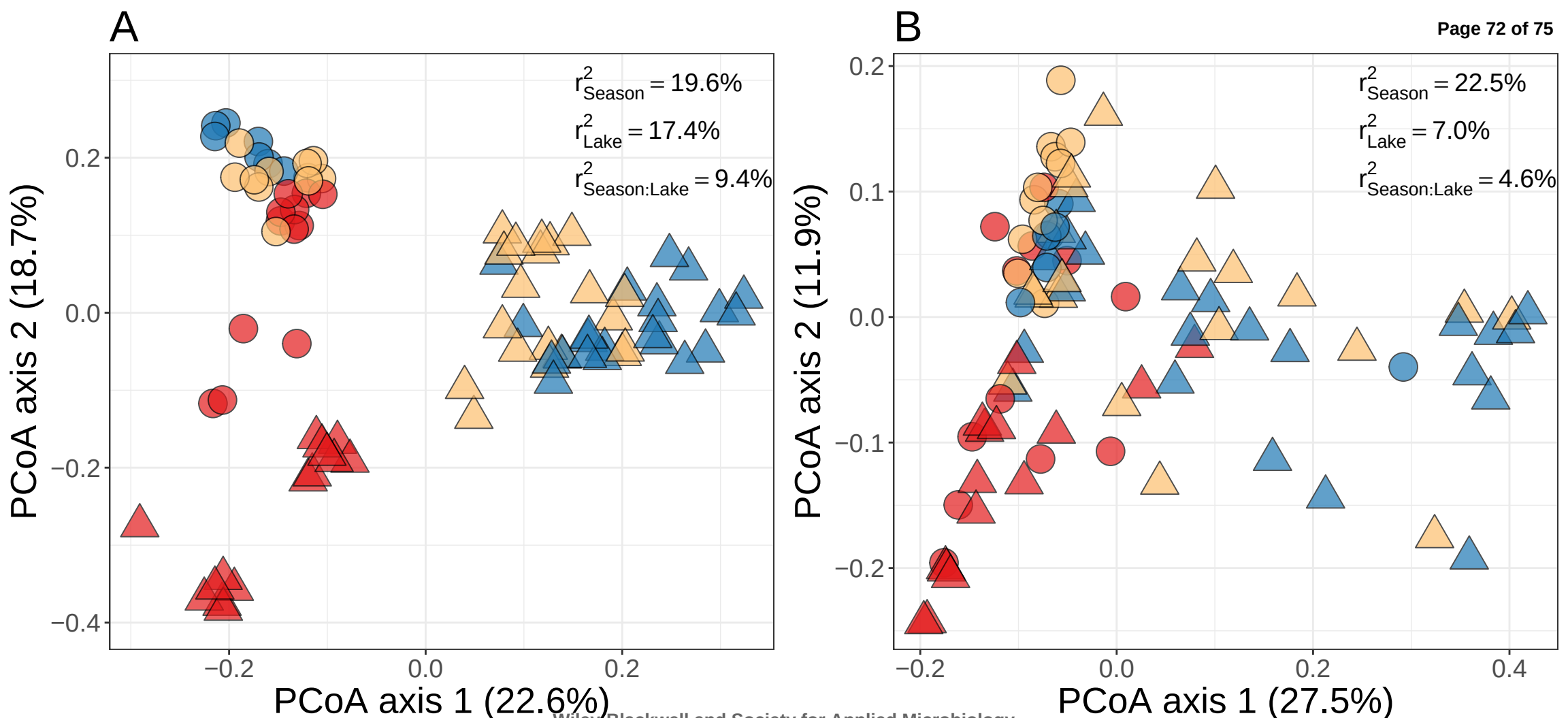
Figure 2: Application of the taxonomic (A) and phenotypic (B) beta-diversity (PCoA of Bray-Curtis dissimilarity matrix) to investigate season- and lake-specific effects on the community structure of Lake Michigan and Muskegon Lake (n = 87). Fall, Spring and Summer denote samples taken in September, April and July respectively. The variance explained by the lake and season variables, as well as the interaction effect between the lake and season variable is provided (PERMANOVA). All effects were significant at the p = 0.001 level with the exception of the interaction effect for the phenotypic beta diversity (p = 0.018). Procrustes analysis confirmed the high degree of correlation between both beta-diversity analyses (correlation strength = 0.65, p = 0.001, 999 permutations). Permutations for PERMANOVA and Procrustes analyses were constrained within each survey year.

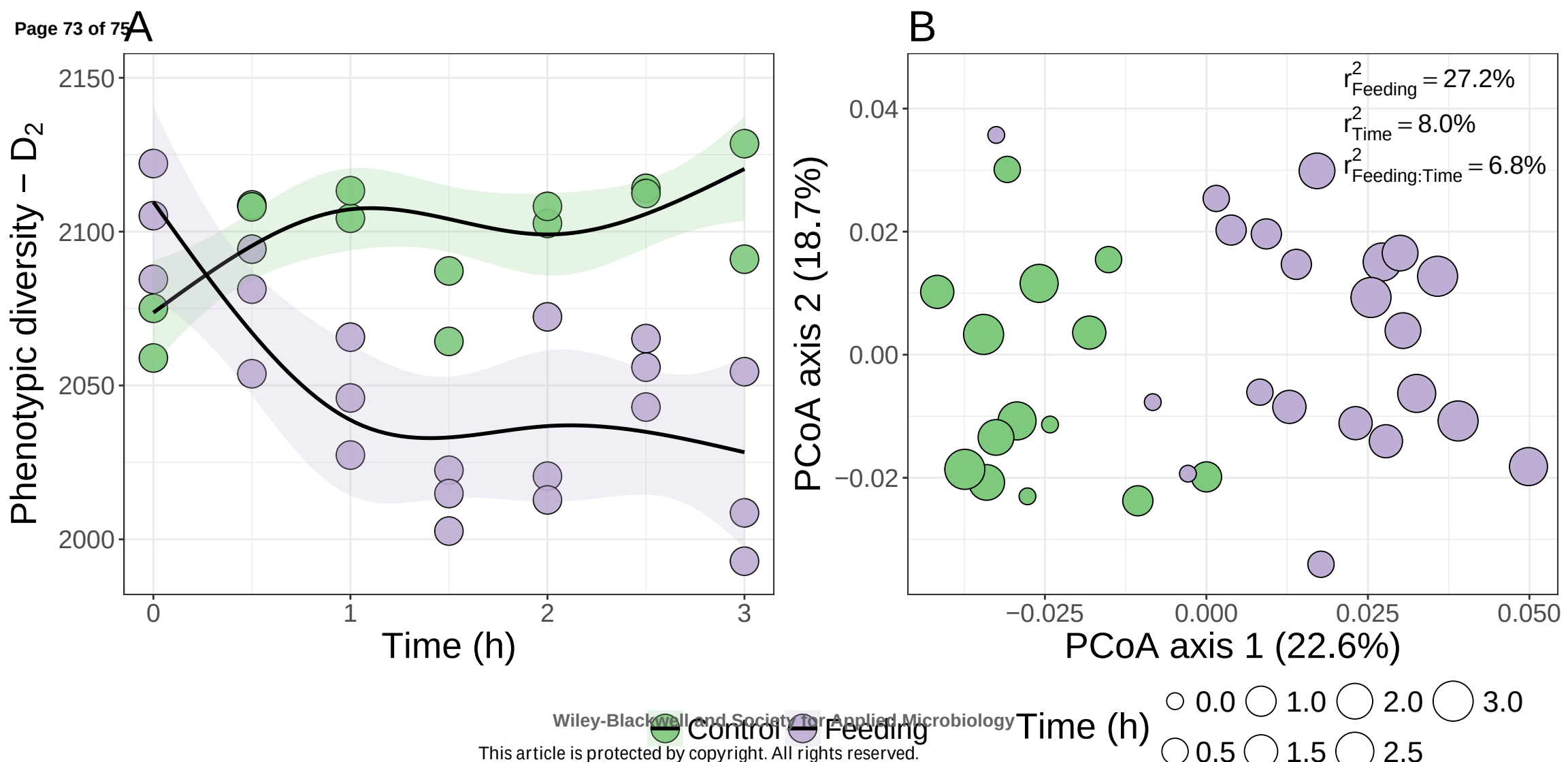
Figure 3: Feeding effect on the phenotypic alpha diversity (A), and phenotypic beta diversity (B) of the bacterioplankton community. Bootstrap error intervals on the phenotypic diversity were calculated on three technical replicates for each microcosm but fell within the label size and are therefore not displayed. Shaded areas indicate 95 % confidence intervals on the robust smoothing spline regressions. Label radius of the data points in the beta-diversity analysis is proportional to the time into the experiment. The variance explained by the overall temporal and feeding effect, as well as the interaction effect between the feeding and experiment time is provided (PERMANOVA). All effects were significant at the $p = 0.01$ level.

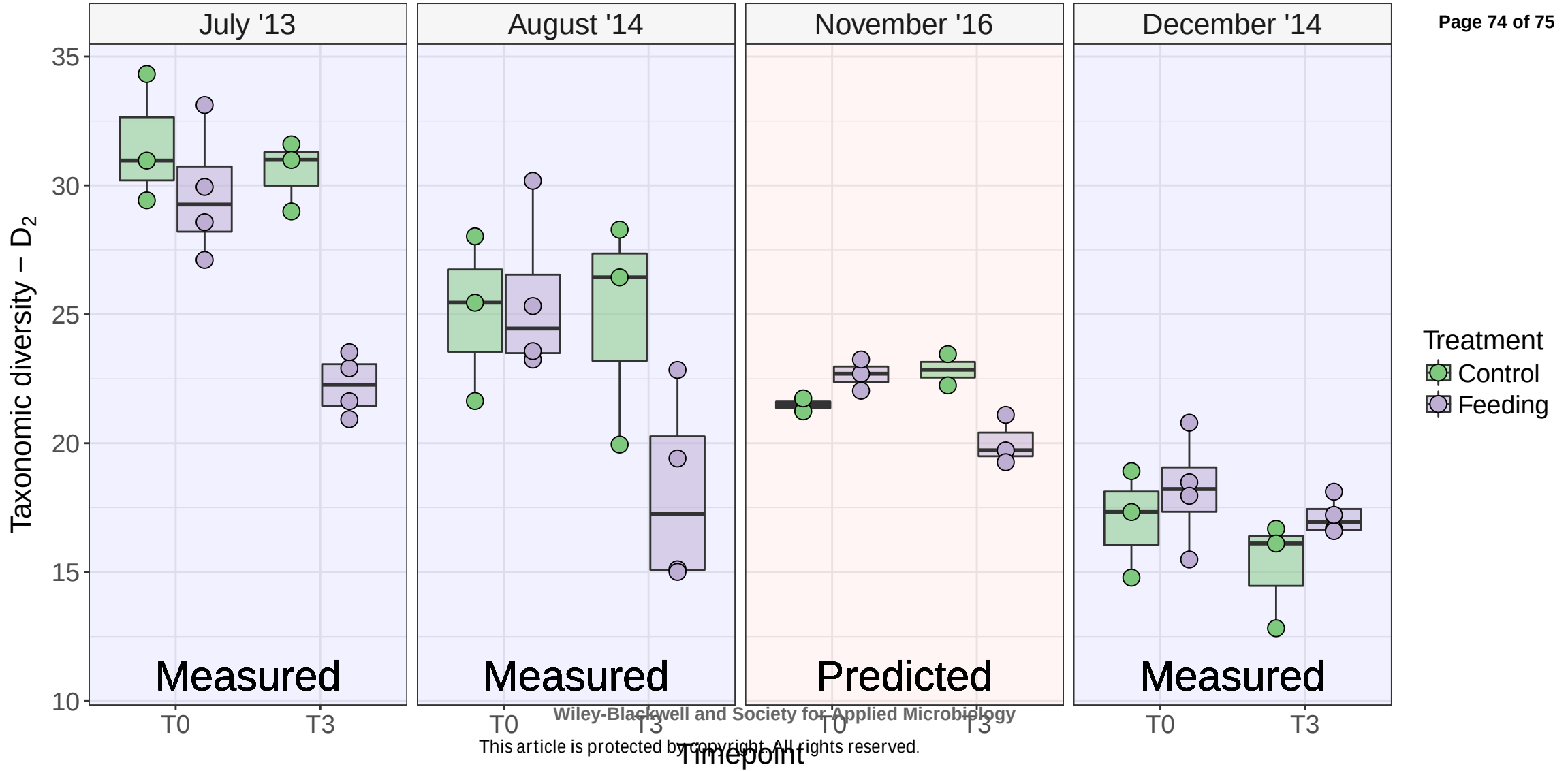
Figure 4: Measured or predicted taxonomic alpha diversity of Lake Michigan bacterioplankton communities after a three hour exposure to invasive quagga mussels. Measured taxonomic diversity data is publicly available from (Denef et al., 2017). The measured data comes from feeding experiments that were carried out with Lake Michigan bacterioplankton communities retrieved over a two year period and under the identical experimental design as described in this manuscript.

Figure 5: Dynamics of high nucleic acid (HNA) and low nucleic acid (LNA) populations. A) Contrasts between the flow cytometric fingerprints of the control samples and the feeding samples after 0h, 1.5h and 3h. Red contours indicate an increase in the LNA population density during feeding, while blue contours indicate a decrease in the HNA population density during feeding, both of which are relative to the control samples at the specified time point. Only contrasts with densities > |0.04|were visualized. B) Absolute cell density of the LNA population over the course of the feeding experiment. Error bars indicate standard deviation across technical replicates ($n = 3$). C) Absolute cell density of the HNA population over the course of the feeding experiment. Error bars indicate standard deviations across technical replicates ($n = 3$). Shaded areas indicate 95 % confidence intervals on the robust linear regression models.









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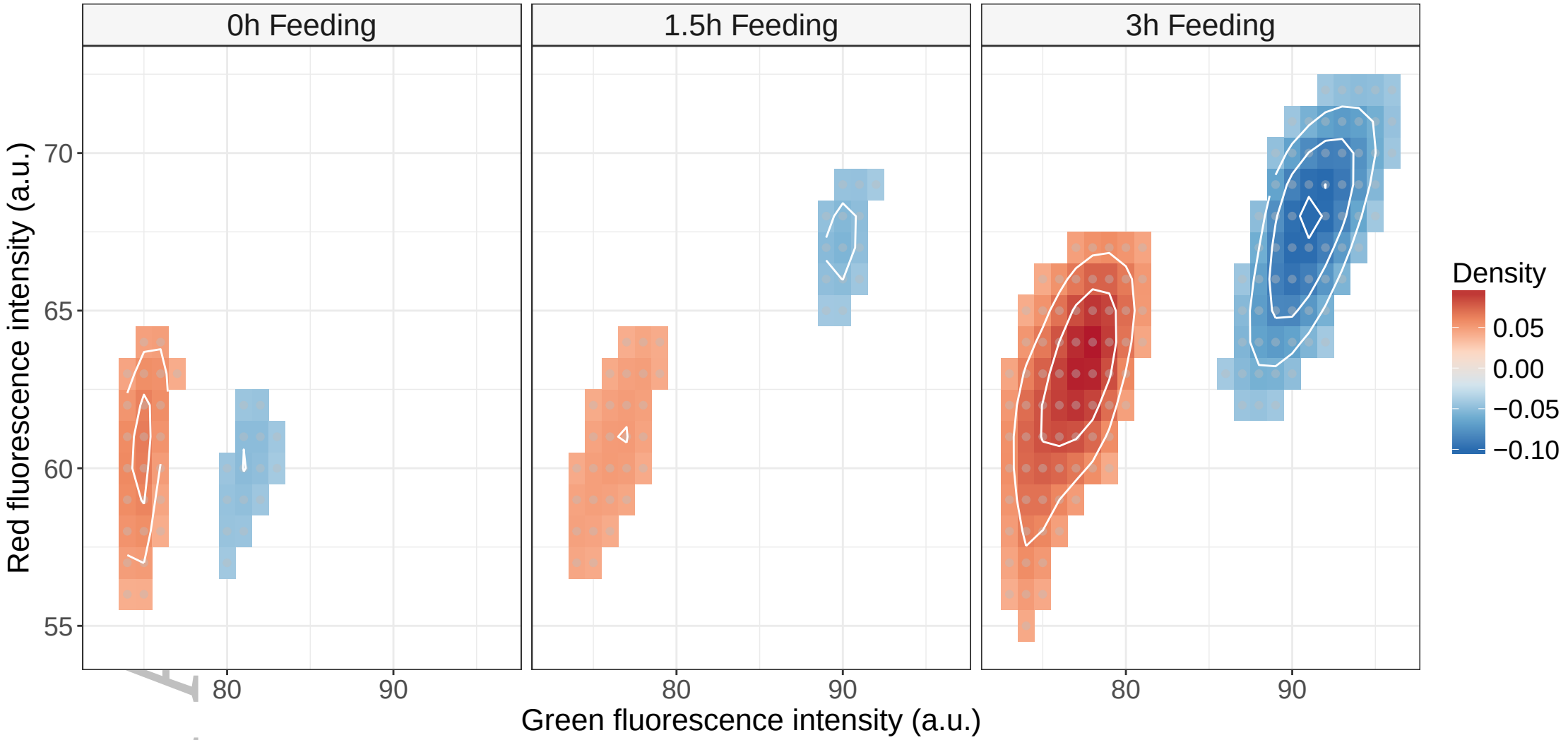

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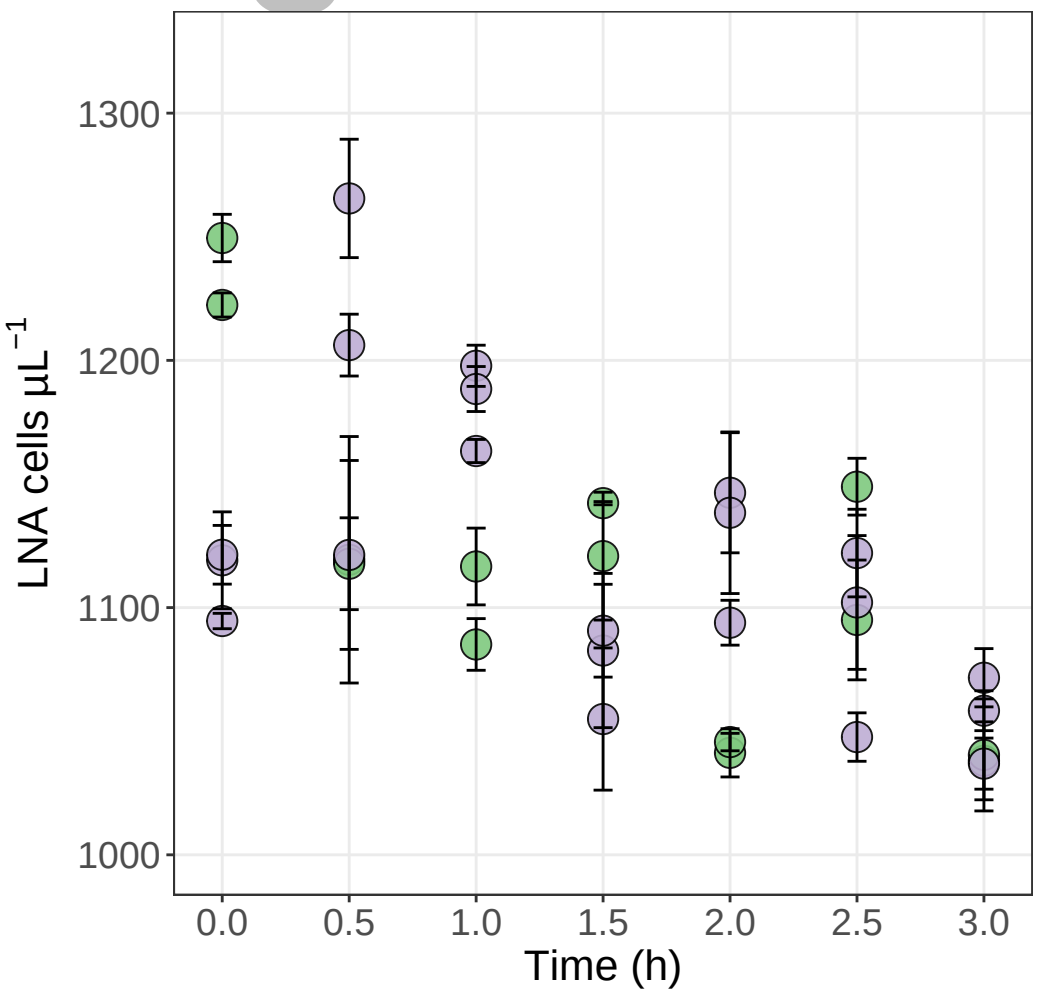

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