Bacterial Community Diversity Dynamics Highlight Degrees of Nestedness and Turnover Patterns

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Additional Supporting Information may be found in the online version of this article.

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Abstract

Bacterial communities change their structure rapidly due to short generation times of their members. How bacteria assemble to certain structures provides insight into ecological mechanisms that shape a bacterial community. Microbial community flow cytometry was used to create community fingerprints based on subcommunity distributions and to visualize the dynamic variations of 10 independently grown communities under equal conditions. Inventory diversity values were recorded by α- and γ-diversity whereas the degree of subsistence of subcommunities (nestedness) and the degree of gain or loss of subcommunities (turnover) was calculated as multi-sites β-diversity terms βESGS and βESDM. Numbers of unique subcommunities of pairwise samples were determined by intra- and inter-community β-diversity values. Although all communities were exposed to niche-differentiating conditions they assembled to disparate structures. In our study, the turnover coefficients were high (> 0.6), while the nestedness coefficients were complementary low in the separate 10 bioreactors. Intra- and inter-community β-diversity values indicated fast community shifts. Microbial community flow cytometry straightforwardly identifies the dominance and subsistence of subsets of cells in a community or the degree of their replacement. The calculation of either turnover or nestedness patterns might have implications in medical, biotechnological, or environmental research.

Key terms

microbial flow cytometry; microbial community dynamics; ecological β-diversity; turnover; nestedness

Microbial communities are the main drivers of carbon and nutrient cycles on earth, are essential occupants of higher living organisms and of central importance for e.g. the renewable bioeconomy. Most of microbial community members can still not be cultivated as pure strains and, therefore, cultivation-independent methods must be employed to resolve community structures. The most widely used applications are sequencing technologies such as platforms for Miseq (Illumina, San Diego, CA), SMRT (PacBio, Menlo Park, CA), or Nanopore (Oxford Nanopore Technologies, Oxford, UK) but also flow cytometry is an accepted tool to gain knowledge on structure and function of microbial communities.(1) Bacteria have generation times between minutes to hours and thus can change their abundance in communities very fast. Microbial community flow cytometry does not only provide fast analytics and resolution comparable to the genus level but also enables for data evaluation on the sampling day by using quasi-automatic evaluation pipelines.(2–4)

This study aims for further insight into microbial community behavior by searching for subsistent identical subcommunities that do not leave a consortium over time or out of locations. We also want to explore the degree of turnover patterns of communities that indicate total restructuring processes. Coming from the macroecological background, nestedness has been defined to describe the subsistence of specific organisms in explicit localities independent on conditions that are prevailing.
Instead, turnover patterns highlight variations of community structures in localities due to selective differentiation.(5)

Here, we want to use such macroecological concepts to study microbial community behavior by flow cytometry. The composition of a community is described by inventory α-diversity values that engross numbers of species and their distribution in a community to define richness and evenness values, respectively. In flow cytometry, subsets of cells (technically gates) are counted to determine the richness of a community. How cell numbers are distributed between gates provides the evenness value.(6, 7) These differences can be calculated by the order-based Hill numbers that increasingly include evenness characteristics of communities with increasing values of the order (q = 0, q = 1, and q = 2). γ-diversity is also an inventory term and describes the diversity of all communities that are included in a study and sampled over time and space. The impact of the environment on communities or of intrinsic ecological mechanisms on community structures over time or between locations can be highlighted by the β-diversity. The term β-diversity was invented by Whittaker(8) and is still much under discussion in macroecology. Several concepts of β-diversity are available that provide useful insights in community behavior. Recently, we used intra-community β-diversity values to detect stochastic shifts in microbial communities and intercommunity β-diversity values to point to site depended dissimilarities among community structures.(9) Intercommunity β-diversity describes differences (numbers of different subcommunities) between specified microbial communities while intracommunity β-diversity describes differences (numbers of different subcommunities) of two successive samples of one community.

In this study, we want to elevate the ecological tool box in microbial community cytometry and provide a workflow on how community diversity values nestedness β_{NESS} and turnover β_{SIM} can be calculated by using further aspects of the term β-diversity. Nestedness in β-diversity flow cytometric data will describe the subsistence of subsets of subcommunities in larger communities, a process suggesting nonrandom loss of the other part of the microbial community. Turnover, on the contrary, describes replacement of subcommunities by others leading to a new composition of a microbial community. We used equations from Baselga(5) to find the contributions of nestedness and turnover on β-diversity of microbial communities, which were originally developed to describe the distribution of long-horn beetles over Europe. The dynamic changes in 10 insular communities, cultivated each up to seven days, were analyzed. α-diversity, intra- and inter-community β-diversity, as well as β_{NESS} and β_{SIM}-diversity and finally, γ-diversity values were calculated. The study gives fast indications on subsistent subcommunities as well as on community transformation.

**METHODS**

**Cultivation of the Microbial Communities**

The microbial community was obtained from an activated sludge basin of a wastewater treatment plant of the town Eilenburg, Germany (51°27’39.4”N, 12°36’17.5”E) and handled as described earlier.(9) In short, the cells of the community were precultivated in a mixture of peptone medium and synthetic wastewater (v:v = 2%:98%; 0.198 g L⁻¹ peptone (from meat), 0.2 g L⁻¹ meat extract, 0.219 g L⁻¹ yeast extract, 0.1 g L⁻¹ glucose, 0.49 g L⁻¹ Na-propionate (filtered), 0.0059 g L⁻¹ CaCl₂·2H₂O, 0.0294 g L⁻¹ KCl, 0.06 g L⁻¹ NaCl, 0.04 g L⁻¹ K₃HPO₄, 0.2156 g L⁻¹ KH₂PO₄, and 0.0196 g L⁻¹ MgSO₄·7H₂O), which were purchased from Merck KGaA (Darmstadt, Germany), SERVA Electrophoresis GmbH (Heidelberg, Germany), and Carl Roth GmbH Karlsruhe, Germany. The community was precultivated on a rotary shaker at 125 rpm at 30°C for 17 h. Following, 50 ml of the preculture was used for inoculation of 1 l bioreactors with a final volume of 800 ml, respectively. The reactors were run at 30°C (Incubator Hood TH 25, Edmund Bühler GmbH, Hechingen, Germany) and 350 rpm using a multipoint magnetic stirrer (Thermo Electron LED GmbH, Langenselbold, Germany) and stirrer bars (45 × 8 mm, Labsolulte® Th. Geyer GmbH, Renningen, Germany), and at an aeration rate of 150 ml min⁻¹ compressed sterile filtered ambient air controlled by a rotor gas flowmeter (six measuring channels, Analyt-MTC GmbH, Müllheim, Germany). Five equal bioreactors were used in parallel twice, providing data from 10 reactor runs. The dilution rate was set to 0.72 days⁻¹ by a microprocessor controlled dispensing pump IPC-N 12 (Ismatec®, Cole-Parmer GmbH, Wertheim, Germany). Samples for flow cytometry were taken after 1, 2, 3, 4, 5, and 7 days after inoculation.

**Cell Preparation and Staining**

Samples were harvested and centrifuged at 3200 g, 10 min, at 4°C. The cells were washed with phosphate buffered saline once (PBS, 6 mM Na₂HPO₄, 1.8 mM NaH₂PO₄, 145 mM NaCl, pH 7, 3,200 g, 10 min, 4°C) and stabilized by adding 2 ml paraformaldehyde solution (PFA, 2% in PBS) to the cell pellet and incubated for 30 min at room temperature. After another centrifugation step 4 ml of ethanol (70%) were added for fixation and the cell solutions were stored at −20°C.

For flow cytometric measurement the samples were washed twice with PBS, and the cell solutions were adjusted to an OD of 0.035 (d₅₀₀nm = 5 mm) with PBS. Two milliliter of this solution were centrifuged again and the pellet resuspended in one milliliter solution A (0.11 M citric acid and 4.1 mM Tween 20, with distilled water) and incubated at room temperature for 10 min in an ultrasonication bath (Merck Eurolab, Darmstadt, Germany) and 10 min without any further treatment. After another centrifugation step the cells were stained with solution B (0.24 μM DAPI (4’,6-diamidino-2-phenylindole) in phosphate buffer (289 mM Na₂HPO₄ and 128 mM NaH₂PO₄ in distilled water)) overnight at room temperature.

**Standards**

A MoFlo cell sorter (DakoCytomation, Santa Clara, CA) was daily calibrated in the linear range by using 1 μm blue fluorescent FluoSpheres (Molecular Probes, F-8815, Eugene, OR) and 2 μm yellow-green fluorescent FluoSpheres (ThermoFisher Scientific, F8827, Waltham, MA) and in the logarithmic range...
by using 0.5 μm and 1.0 μm UV Fluoresbrite Microspheres (both from Polysciences, Cat. No. are 18339 and 17458, respectively, Warrington, PA). In addition, all samples were spiked with the same bead combination. Furthermore, to ensure the reliability of the cell fixation and staining procedures a microbial cytometric mock community (mCMC) was employed consisting of three different bacterial species harvested from LB medium plates (Stenotrophomonas rhizophila DSM 14405, Kocuria rhizophila DSM 348, and Paenibacillus polymyxa DSM 36). The mCMC was mixed at proportions 1%: 19%: 80%, respectively, before analysis. The cells of the mCMC were handled identically to the protocol above to safeguard reliable and comparable procedures and guaranty highly resolved cytometric patterns of the bioreactor samples. This comparability of data is mandatory for the evaluation of the cytometric community patterns by highly sensitive and nearly automatically working bioinformatics tools (flowCHIC, flowCyBar, see later). Two hundred thousand cells were measured per sample and cell gate, which excluded beads and noise during each measurement.

Instrumental Settings
A MoFlo Legacy cell sorter (DakoCytomation, Santa Clara, CA) equipped with a 488 nm argon laser (400 mW, Coherent, Santa Clara, CA) and a UV laser (355 nm, 150 mW, Xcyte CY-355-150, Lumentum, Milpitas, CA) was used for the cytometric measurements. Forward scatter (FSC, neutral density filter 1.9, 488/10 nm band pass) and side scatter (SSC, trigger signal, neutral density filter 1.9, 488/10 nm band pass) were used for the analysis of intrinsic cell parameters. The UV excitation was used to measure DAPI fluorescence (450/65 nm band pass). Sheath pressure was constant at 56.0 psi, sample pressure was adjusted within the range of 55.9–56.0 psi to equal the event rate to about 3000 events per second. The PMTs were adjusted by fitting the calibrations beads into the bead calibration template. Measured. fsc files can be downloaded from the FlowRepository, with reference No.: FR-FCM-Z28S.

Bioinformatics Tools
2D-plots were visualized and gated with FlowJo™ (V10.6.1, Becton, Dickinson and Company, Franklin Lakes, NJ). The resulting fcs files were gated and evaluated with the gate based analyzing tool flowCyBar (http://bioconductor.org/packages/flowCyBar/) in R (V3.6.1).(10) The creation of the gate template is a routine application in microbial community flow cytometry. How a gate template is created was first described by Koch et al.(10) In short, we mark present subcommunities by a gate and add gates when new subcommunities are appearing in successive 2D-plots. The gates should not overlap and cover 80% of the measured 200,000 cells. For the evaluation of the diversity values, α-diversity, intra- and inter-community β-diversity were quantified with a previous published R-script (https://github.com/LiuZishu/MCFlowCytoAnalysis).(9) β-diversity βFES and βSIM were evaluated with the R package “beatapart.”(11)

RESULTS

Dissimilarities in Community Structures
Fifty samples of microbial communities from 10 identically run insular bioreactors, taken over seven days, were analyzed by flow cytometry. The resulting community patterns, in addition to those from the mCMC and the inoculated preculture, are shown in Supporting Information Fig. S1. A gate template was created to enable the comparison of all microbial community data and to determine the number of subcommunities emerging in all 10 reactors, the position of the subcommunities within the 2D-plots, and the numbers of cells per subcommunity. All in all 46 subcommunities were defined (Supporting Information Fig. S2). These data were used to profile the dissimilarity of the community structures between all bioreactors and their respective progress over time, based on cells abundance per subcommunity. The dissimilarity of the community structures was calculated by using the Bray-Curtis index and the data were visualized as NMDS plot (Fig. 1).

The samples from the two precultivated inocula (IN) and the 10 reactors (R01–R10) are represented by colored dots with increasing sizes for increasing sampling times. The crossing of lines indicates the affiliation of samples to the respective sampling time (one to seven days). The data show that the bioreactor communities (1) are clearly different from the precultivated inocula, (2) are more similar between reactors R01 – R10 at the first two sampling points but progress into increasingly dissimilar structures, and (3) are maximum dissimilar after seven days between R01 – R10 but also within each of the identically run bioreactors. This dissimilarity

![Figure 1. Visualization of community dynamics. Analysis of community dynamics based on cells abundance per subcommunity. Dissimilarities (Bray-Curtis index) of 50 samples from 10 reactor parallels (R0-R10) and two inocula (IN) were calculated and the assembly process of communities from their inoculation to seven days is visualized as a NMDS plot.](https://example.com/figure1.png)
analysis on the bioreactor community dynamics indicates an increasingly disparate development of community structures over time and per location.

α- and γ-Diversity Values
To find indications for causes of these remarkable variations in community structures we recorded the changes in diversity more closely. Values of α-diversity and γ-diversity provide inventory overviews of communities at fixed time points or time periods. The γ-diversity value was determined by the gate template and accounts for 46 subcommunities. The mean cell abundance value per subcommunity was 2.17% and subcommunities with cell numbers above this value were regarded as dominant. All in all, 38 dominant subcommunities were found in the dataset. Presuming that mainly the dominant subcommunities were responsible for richness changes, we assessed the dominant subcommunities per day and reactor using Hill’s Number $q = 0$. The α-diversity $q = 0$ values were comparatively similar on days 1 and 2 with between 12 and 17 dominant subcommunities for all 10 bioreactor communities (Fig. 2). Following, this richness was lost to a quantity that one bioreactor community (R05, five days) was dominated by only four subcommunities out of the 38 dominant ones found for all samples. However, the downsfalls of the richness values were not subsistent. The richness was reestablished with finally 9 to 17 dominant subcommunities in all bioreactors (day 7).

On how even the cells were distributed among the gates can be calculated using the term evenness. Evenness is a part of the α-diversity definition and can be determined by means of the Hill Numbers $q = 1$ and $q = 2$. In this calculation all 46 subcommunities (γ-diversity) should be included. Using this condition, Hill Numbers $q = 0$ describes richness that is nearly the same for all samples of the 10 bioreactors because almost all gates contained cells over time (Fig. S3). Hill Numbers $q = 1$ and $q = 2$ increasingly weight diversity with regard to evenness. Abundances of cells per subcommunity became more important and highlighted subcommunities that showed α-diversity $q = 2$ values as high as 27.4 (R07, day 3) but also as low as 3.9 (R05, day 5) out of 46 subcommunities. The α-diversity $q = 2$ values recuperated for a few reactors (R06, R08, day 7), but not all. The data suggest a faster retrieval of richness values (α-diversity $q = 0$) compared to α-diversity $q = 1, 2$ values (Fig. S3).

β-Diversities
β-diversity is not an inventory term such as α-diversity and γ-diversity values. Using β-diversity parameters provide information on population changes and even dynamics. For community flow cytometry β-diversity parameters are already established as intra- or inter-community β-diversities. Intracommunity β-diversity describes changes inside of a community by pairwise comparison of samples at successive time points, counting loss or gain of unique dominant subcommunities. Intercommunity β-diversity counts numbers of unique dominant subcommunity between communities, also after pairwise comparison of samples, at different locations.

To determine the intra- and inter-community β-diversity values in this study, the whole cytometric data set of 50 samples based on the dominant 38 subcommunities was involved. The intracommunity β-diversity values revealed that the singular 10 reactor communities disclosed exclusive and contrasting

Figure 2. Calculation of α-diversity $q = 0$ values. The α-diversity $q = 0$ values to determine community richness on the basis of dominant subcommunities. Data of 10 bioreactors, run in parallel for seven days, were used and visualized in a violin plot. Three horizontal lines indicate 75%, 50%, and 25% quantiles of these values from top to bottom. The colors mark the α-diversity $q = 0$ of the respective bioreactor over time. α-diversity $q = 0$ values significantly varied on different days, which was proven by the analysis of variation (ANOVA, $P$-value <0.05, see Table S3-1, Supplementary Information 3).
alterations over time. Unique subcommunity numbers between 2 and 20 revealed almost no as well as huge variations in community structure after pairwise comparison of samples at successive time points per reactor. The highest variation between pairs of samples was found in R04 and R05 with 19 and 20 unique subcommunities, respectively (Fig. 3A). Furthermore, intercommunity β-diversity values were determined after pairwise comparison of samples between reactors. While the numbers of unique dominant subcommunities between the 10 reactors were low at the first two days of cultivation, this value increased (with exception of day 4) until day 7. The highest numbers of unique subcommunities were found at days 3 and 7, pointing to increasingly different community structures between the 10 reactors (Fig. 3B). Therefore, intra- and intercommunity β-diversity values highlighted the unequal development of the singular community structures per reactor and, at the same time, the increasing disparity of the 10 communities between the reactors.

Additional measures of β-diversity can describe the likelihood that given subcommunities will subsist or be replaced in a community over time and at different locations. Besides determining variations in unique dominant subcommunity numbers of successive samples (i.e. intra- and inter-community β-diversities) we want to identify the subsistence as well as gain or loss of specific subcommunities. The degree of subsistence of subcommunities (nestedness) is described as β-diversity term βNES, while the degree of gain or loss of subcommunities (turnover) is defined by βSIM.(5) These values are determined between 0 and 1 and a higher value stands for higher turnover or nestedness events, respectively. We used the R package "betapart"(11) to determine β-diversities βNES and βSIM on the basis of presence/absence data of dominant subcommunities per sample (38 subcommunities in the whole sample set, a positive control by using the mCMC is provided in Supporting information 5). We calculated β-diversities for 10 locations, respectively, over time (R01 to R10, Fig. 4A) and from start to end of cultivation comparing the 10 locations per day (Fig. 4B). We used a multiple-sites comparison for the calculation of β-diversities βNES and βSIM,(11) applying the Sørensen dissimilarity index as was recommended by Baselga(12) and determined the turnover and nestedness coefficients. The data suggest that nestedness events were low in the 10 reactor comparison while turnover events dominated. The highest value for the multiple-sites comparison βSIM was found at day 7 (0.678, Fig. 4B) suggesting that at this day the lowest fraction of subcommunities was shared between the 10 reactors. The β-diversity βNES did not play a substantial role. Within 50 community samples, all classified by the 38 dominant subcommunities and their cell numbers, none of those subcommunities was always present (i.e., having an abundance above 2.17%). Only one

![Figure 3](image-url)

**Figure 3.** Determination of intra- and inter-community β-diversity values. (A) Flow cytometric intra-community β-diversity was counted as the number of unique dominant subcommunities (SC) at successive time points per each reactor (R01 - R10, size of points increase with time, e.g. the smallest point per reactor indicate the intra-community β-diversity between the first two sampling days). (B) intercommunity β-diversity was counted as the number of unique dominant subcommunities (SC) for each pair of two reactors and counted numbers are summarized as a boxplot for each single day (day 1 to day 7).

![Figure 4](image-url)

**Figure 4.** Calculation of β-diversities βNES and βSIM values. β-diversity nestedness (βNES, black) and turnover (βSIM, gray) were calculated with R-package "betapart"(11) on the basis of dominant subcommunities for (A) multiple samples per reactor over time and (B) multiple samples from 10 reactors per day.
subcommunity was constantly detectable in 40 out of 50 samples (G32) and only seven subcommunities, each, were permanently present in at least 30 out of 50 samples (G24, G04, G06, G12, G25, G19, and G43; Supporting Information Fig. S4). The same data set was compared but per reactor (Fig. 4A) and showed similar results. The highest value for the multiple-sites comparison $\beta_{\text{SIM}}$ was calculated for reactor R04 (0.678, Fig. 4A) but also values for R05 and R09 were high (0.605 and 0.626, respectively).

The four different $\beta$-diversities values calculated (1) numbers of unique subcommunities over time as well as per and between locations, (2) values for the degree of subsistent subcommunities, and (3) values for the degree of subcommunities’ turnover. $\beta$-diversities $\beta_{\text{NES}}$ and $\beta_{\text{SIM}}$ estimates provide complementary and normalized values for community turnover. While intra- and inter-communities $\beta$-diversity values provide an accurate number of unique subcommunities between successive samples, $\beta$-diversities $\beta_{\text{NES}}$ and $\beta_{\text{SIM}}$ values indicate the relative roles of subsistence as well as gain and loss of subcommunities, respectively. $\beta_{\text{SIM}}$-diversity values were high in our study indicating that the majority of subcommunities was exchanged. Complementary $\beta_{\text{NES}}$-diversity values remained low.

**DISCUSSION**

Natural microbial communities can consist of hundreds of species and can comprise more than $10^{10}$ bacteria per gram dry soil.(13) How microbial communities are organized in natural or managed environments is one of the foremost objectives when microbial communities are characterized. Frequently, 16S rDNA sequencing data are used to unravel the taxonomic affiliations of community members. In addition, microbial community flow cytometry advanced as potent and fast means to classify community structures using cell physiological data such as morphology (cell size, cell density and surface characteristics) and growth states (i.e., cellular chromosome numbers).(6,14) Cells with similar physiological characteristics are clustered in cell subsets and their presence/absence and the amount and distributions of cells within these subsets can be quantified to mark differences among microbial communities.

One of the most widely used properties to describe communities in biology is the determination of diversity. Diversity regards the presence of all organisms of a study ($\gamma$-diversity), and reports on richness and evenness ($\alpha$-diversity) of communities at definite time points or locations. Apart from such inventory terms, $\beta$-diversity in microecology or sequencing approaches from microbiology can comprise huge disparate sample numbers. $\beta$-diversity $\beta_{\text{SIM}}$ and $\beta_{\text{NES}}$ values are calculated as normalized coefficients that allow their comparison with those obtained from data of unalike origins. Also, huge variations in richness between locations and successive time points can influence turnover values. Yet, in microbial community flow cytometry the $\gamma$-diversity is restricted to the number of gates in the gate template which restricts richness values between samples of a study. $\gamma$-Diversity values are also dependent on cytometer resolution and operator decisions on the gate template. Gate
TECHNICAL NOTE

templates can also be created automatically on the basis of 2D-plots, though dynamic approaches are currently not yet available. However, in ecological studies, not only the absolute values of richness are interesting but also the dynamic changes in microbial community structures over time. Moreover, the use of the framework of Baselga(5) that was applied in this study allowed for sample richness independent calculations.

Calculating the degree of subsistent subcommunities in varying communities conveys attractive implications. On the one hand subsistent subcommunities might be interesting for the detection of fully adapted and settled organisms. On the other hand subsistent subcommunities might form the core community and stabilize a community in highly fluctuating environments. Such findings may contribute to strategies in community microbiology that aim for loss of nonproducers (e.g., in biotechnology) or of organisms with unwanted characteristics such as pathogens (e.g., in medical applications).

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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