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PROTOCOL

Flow cytometric monitoring of the bacterial phenotypic diversity in aquatic ecosystems

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Flow cytometry is a promising tool used to identify the phenotypic features of bacterial communities in aquatic ecosystems by measuring the physical and chemical properties of cells based on their light scattering behavior and fluorescence. Compared to molecular or culture-based approaches, flow cytometry is suitable for the online monitoring of microbial water quality because of its relatively simple sample preparation process, rapid analysis time, and high-resolution phenotypic data. Advanced statistical techniques (e.g., denoising and binning) can be utilized to successfully calculate phenotypic diversity by processing the scatter data obtained from flow cytometry. These phenotypic diversities were well correlated with taxonomic-based diversity computed using next-generation 16S RNA gene sequencing. The protocol provided in this paper should be a useful guide for a fast and reliable flow cytometric monitoring of bacterial phenotypic diversity in aquatic ecosystems.

Keywords: flow cytometry, phenotypic diversity, online monitoring, binning, aquatic ecosystem

Overview

Safe and effective management of water resources (e.g., groundwater, drinking water, and water supply network) requires a robust monitoring system to detect waterborne microorganisms. It is very important to characterize microorganisms accurately in water because the cell density or distribution of waterborne microorganisms can pose a fatal threat to public health. Currently, 90% of the population is not supplied with advanced purified water, and annually, approximately 7.15 million people in the United States suffer from the diseases caused by contaminated water costing the US healthcare system $3.3 billion dollars (Collier *et al.*, 2021). Evaluating the water quality by only using indicators (e.g., fecal coliforms or *Escherichia coli*) or waterborne microorganisms may miss information on other threats that cause water pollution. Thus, it is also necessary to analyze the total bacterial community to derive meaningful information (Leight *et al.*, 2018). It is necessary to provide a new perspective on water management; this could be achieved through an approach that can obtain overall information on bacterial communities in aquatic environments.

In most countries, culture-based methods, which are relatively simple and inexpensive, are used as standard methods to detect indicator species and other waterborne microorganisms (Ramírez-Castillo *et al.*, 2015). As culture-based methods are labor intensive and time consuming, they are unsuitable for online monitoring systems in the field. The practice of periodic sampling during microbiological testing is particularly problematic regarding sample representation, as most waterborne microorganisms are present in a viable but non-cultivable state in nutrient-limited water, which inevitably leads to a low-detection efficiency (Li *et al.*, 2014). Recent attempts to introduce molecular methods, including polymerase chain reaction, high-throughput sequencing, and oligo-based biosensors, have led to faster and more sensitive detection methods for waterborne microorganisms than culture-based methods (Tan *et al.*, 2015; Nurliyana *et al.*, 2018; Shrestha and Dorevitch, 2019). In addition, high-throughput sequencing with specific primer sets (e.g., 16S rRNA gene and *amoA* gene) can also provide detailed taxonomy and diversity information for aquatic bacterial communities. Despite the rapid development of these technologies, they are limited to operate in automated or online monitoring because of the interference of molecular reactions under the sampling conditions (e.g., humic acid and pH), complexity of the experimental process, and experimental costs. Thus, there is a need for a new approach that can overcome the limitations of conventional detection methods.

Flow cytometry (FCM) is an alternative technology that can measure the physical and chemical properties of cells or particles based on their light scattering behavior and fluorescence (Picot *et al.*, 2012). Over the past few decades, developments in optical technologies and detectors have improved both instrumental performance (e.g., sensitivity and particle size resolution) and system ruggedness (e.g., computational capacity and optics stability). Thus, FCM has been applied to

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obtain early diagnoses of microbial contamination in foods, as well as in clinical and water quality assessment (Buzatu *et al.*, 2014; Gillespie *et al.*, 2014; Jelinek *et al.*, 2017). FCM provides results with high precision and accuracy compared to conventional culture-based technologies and sequencing technologies, and its measurement time is fast enough to measure more than 10,000 events or cells/min (Krediet *et al.*, 2015; Props *et al.*, 2016). FCM's high measurement accuracy and large measurement scale have prompted researchers to explore its usage regarding online or automated water quality assessment in water reuse systems, wastewater treatment plants, and drinking water supply networks (Props *et al.*, 2016; Rockey *et al.*, 2019; Sadler *et al.*, 2020). Here, we describe a fast and reliable experimental and analytical procedure that can be used to evaluate the microbial properties of water samples using FCM to acquire the phenotypic information of bacterial communities.

Application

The fluctuation of environmental conditions causes phenotypic variations (e.g., cell size, complexity, and cellular biomolecules) in bacterial cells (Kim *et al.*, 2020). Such phenotypic features are used as important indicators for detecting target bacterial cells or tracking physiological changes using optical-based technologies (Hong *et al.*, 2021). FCM can obtain phenotypic information on cell size, cell complexity, and nucleic acid contents using forward scatter, side scatter, and fluorescence intensities, respectively. It can also provide phenotypic information at a rate of more than 10,000 events or cells/min, enabling analysis at the community level (Props *et al.*, 2016). However, phenotype analysis at the community level is not conducted in most FCM-based studies because typical FCM analyses simply count the number of events or cells above a certain threshold when measuring light scattering or fluorescence intensity.

To obtain phenotypic features at community level using the FCM, the data should be structurally reconstructed using a statistical method called binning. Binning is the process of grouping individual data from a secondary planar graph and reproducing new datasets by counting the number of data points contained in each group. Depending on the shape or size of the bin (group), the size and reliability of the data can vary. Each data point can be converted to a data format similar to the table of operational taxonomic units required for 16S rRNA amplicon sequencing analysis; this format can then be used to calculate microbial diversity indices (Props *et al.*, 2016). This diversity index, calculated using binned data, is called a phenotypic diversity; it can be used to quantitatively evaluate taxonomic diversity in environmental samples, in a comparable way to 16S rRNA gene sequencing (Props *et al.*, 2018). Although taxonomic information (e.g., name of a genus or phylum) cannot be provided at the same time as a taxonomic diversity index, the FCM-based phenotypic diversity index, which can be calculated faster and more cheaply than experimentally and analytically complex taxonomic diversity calculations, is advantageous for water quality assessment applications that require online or automated water quality monitoring.

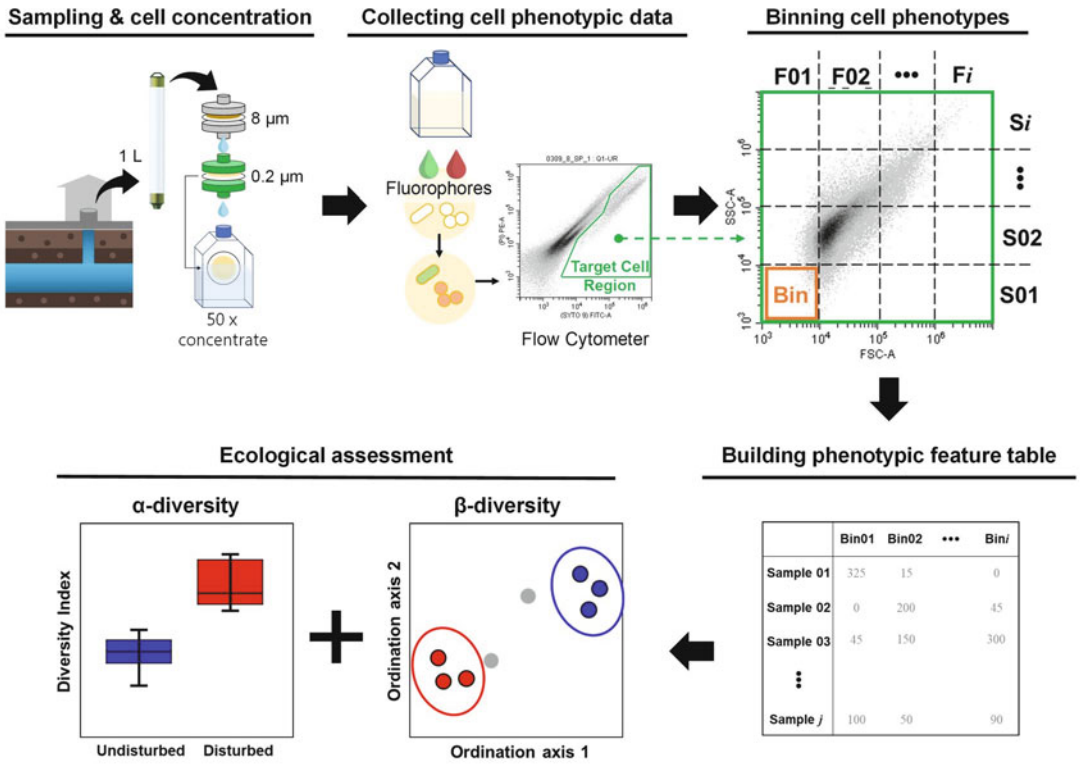

Fig. 1. **Overview of the operational workflow for investigating the phenotypic diversity of a groundwater microbiome using FCM.**

Phenotypic diversity can be measured at different scales in a similar way to species diversity measures: alpha and beta diversity, allowing for the similarities of bacterial communities in different environmental samples to be compared. Previous studies have reported that the phenotypic diversity of bacterial communities increase rapidly when organic or bacterial contamination occurs in a bioreactor (Sadler *et al.*, 2020). As water contamination or ecological disturbances can be assessed by monitoring the phenotypic diversity using FCM, it is a promising diagnostic technology for water quality assessment, as well as for food quality or clinical assessments.

Methods

Collecting the groundwater microbiome

***Groundwater sampling*:** Prior to sampling, the top layer (~3 L) of groundwater was discarded and 2 L of groundwater was collected using a biobailer (Biobailers). The samples were stored in ice during transportation and were analyzed immediately after arrival at the laboratory. For diagnostic purposes, background (undisturbed, G01-10) and test (disturbed, T01-05) groundwater samples were collected.

***Cell concentration*:** Groundwater samples were filtered using a two-step process (Fig. 1). First, coarse abiotic particles in groundwater were filtered out using 8-μm alpha-cellulose pulp filters (Whatman). Then, cells in 1 L of prefiltered groundwater were harvested onto 0.22-μm mixed cellulose ester (MCE) filters (Millipore). All filtration processes were conducted using a vacuum pump system. Cells on the MCE filters were detached into 20 ml of filtered groundwater in 50-ml conical centrifuge tubes by vortexing them at the maximum speed for five minutes. Finally, the 50× concentrated groundwater microbiome was obtained.

Investigation of phenotype data

***Cell staining and flow cytometry*:** Each microbiome sample (100 μl) was diluted with 900 μl of phosphate-buffered saline (PBS) buffer in an amber colored microcentrifuge tube. Cells in the diluted sample were stained using 5 μM SYTO9 (excitation/emission at 482/501 nm) and 30 μM propidium iodide (PI; excitation/emission at 493/636 nm) provided in the LIVE/DEAD BacLight Bacterial viability kit (Invitrogen; Fig. 1). Then, the samples were incubated at room temperature for 15 min in the dark. Stained cells were analyzed using a CytoFLEX flow cytometer (CytoFLEX V0-B3-R2, Beckman coulter). The sheath buffer (Beckman coulter) was used as carrier fluid for FCM; the threshold was set to 8,500 on the forward scatter channel. Events were collected for 1 min at the flow rate of 10 μl/min; the signals of green (fluorescein isothiocyanate [FITC], 525/40 nm) and red (phycoerythrin [PE], 585/42 nm) fluorescence and forward (FSC) and side (SSC) optical scattering were recorded.

***Denoising*:** The FCM results, which are outputted in the ".fcs" format, were converted into ".csv" files using the R packages "flowCore" and "biobase." Events with intensity values satisfying the criteria of: "$\{(FSC > 10^3) \cap (SSC > 10^3) \cap (FITC > 10^3) \cap (PE > 10^3)\}$" were collected, and subsequently, these filtered data were subjected to a denoising process (Fig. 2). Noise signals can be derived from various circumstances, especially when analyzing environmental samples. Particles containing attached debris fragments, cell aggregates, or self-fluorescent generating bacteria are typical sources of noise (Frossard *et al.*, 2016). To solely collect true cell data, it is necessary to exclude the events of noise signals from the dataset. Here, negative control samples were prepared in two ways: heat-killed (autoclaved at 121°C for 15 min) and unstained. On the FCM biplots, which were generated using the CytExpert program (ver. 2.4.0.28, Beckman coulter), the area on the FITC–PE biplot where the events of negative control were observed was specified as the "noise-area." Scatter plots were generated with FITC and PE intensity values on the x- and y-axes, respectively, avoiding the noise-area defined above. The region where the cellular events were observed was identified and gated using the R package "alphahull" (Fig. 2). For SYTO9 and PI fluorescence, live cells were identified accord-

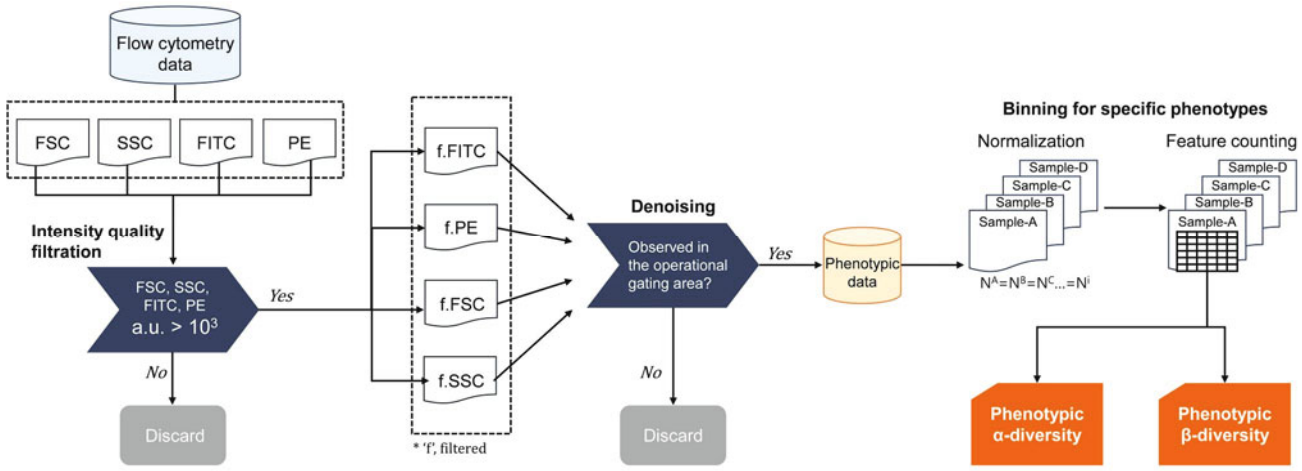

Fig. 2. Data analysis pipeline for deducing phenotypic diversity from FCM results. The entire process comprises "denoising," "cellular data extraction," "normalization," and "binning."

ing to the fluorescence intensity ratio; hence, the intensity values of the events observed in the gated area were extracted as a live cell dataset.

Measurement of phenotypic diversity

***Data normalization and binning*:** Cells harboring different phenotypes were observed from different positions on the FCM scatter plot. The distribution pattern of FCM events on a scatter plot can be a proxy for the phenotype fingerprint. Using a fixed-bin approach, the FSC–SSC scatter plot was partitioned into 10 × 10 equally sized bins, and the frequency of events in each bin was counted and summarized as a feature table (Fig. 2). All the data analysis processes were conducted in R (ver. 4.0.2, http://www.R-project.org); the 'FCM-based phenotypic diversity' script is available at GitHub repository (https://github.com/ecobiolab/FCM-based-phenotypic-diversity.git).

***Ecological assessment based on the phenotypic alpha and beta diversity*:** The Shannon's diversity index was calculated using the feature table generated from the binning process and the R package "vegan" (Oksanen *et al.*, 2020). The significance of the difference in the alpha diversity between undisturbed and disturbed groups was evaluated using the Wilcoxon signed rank test, which was calculated using the R package "ggpubr". The interrelationships of samples were investigated and visualized using principal component analysis (PCA) plots, which were constructed with R package the "stats". The significance of each clustered group on each PCA plot was tested using the permutational multivariate analysis of variance (PERMANOVA) in the "vegan" package. Ecological disturbance was evaluated based on the distance to the test samples from the centroid of the cluster of the undisturbed samples on each PCA plot.

Materials

Reagents

- SYTO 9 (3.34 mM in dimethylsulfoxide [DMSO]) and PI (20 mM in DMSO) from the LIVE/DEAD BacLight bacterial viability kit (Invitrogen; CAS 25535-16-4)
- PBS buffer adjusted to pH 7.2–7.4 (0.13 M NaCl, 0.01 M NaH_2PO_4, and 0.01 M Na_2HPO_4)
- Sheath fluid (Beckman coulter, B51503)
- FlowClean cleaning agent (Beckman coulter, A64669)

Materials

- Clear polyvinyl chloride BioBailer (4.1 cm × 9.0 cm; 1 L; BioBailers, BB-154)
- Grade 2 filter paper (alpha-cellulose pulp; 8-μm pore size; 110-mm diameter; Whatman, 1002-25)
- Membrane filter (MCE; 0.22-μm; 47-mm diameter; Millipore, G SWP04700)
- Pipette tips (20, 200, and 1,000 μl; Axygen)
- Amber color microcentrifuge tube (1.5 ml; SPL, 62015)
- Conical centrifuge tube (50 ml; SPL, 50050)

Equipment

- CytoFLEX flow cytometer equipped with two optical detectors and five fluorescent detectors (Beckman coulter, CytoFLEX V0-B3-R2)
- Vacuum pump (Air capacity, 32 L/min; maximum 60 psi; GAST, DOA-P704-AC)
- A 2-L filtering flask (Vissal, JF-3000-002)
- Filtration assemblies (500-ml glass funnel; 47-mm diameter vacuum filtration fritted glass base; clamp; Vissal, JF-3000-002)
- Pipette set (Eppendorf)
- Vortex mixer Genie2 (maximum 3,200 rpm; Scientific Industries, SI-0256)
- Vertical 50-ml tube holder for vortex mixer (six tubes; Scientific Industries, SI-V506)

Programs

- CytExpert (ver. 2.4.0.28, Beckman coulter)
- R program (ver. 4.0.2, http://www.R-project.org)
- "flowCore" package (Bioconductor ver. 3.13, https://bioconductor. org/packages/flowCore/)
- "Biobase" package (Bioconductor ver. 3.13, https://bioconductor.org/packages/Biobase/)
- "alphahull" package (ver. 2.2, https://CRAN.R-project.org/package=alphahull)
- "vegan" package (ver. 2.5-7, https://github.com/vegandevs/vegan/issues)
- "ggpubr" package (ver. 0.4.0, https://rpkgs.datanovia.com/ggpubr)
- "stats" package (ver. 4.0.2, https://stat.ethz.ch/R-manual/R-devel/library/stats)

Protocols

Construction of groundwater microbiome library

A. Groundwater sampling

1. Wash bailer and sample container with distilled water (DW) and then with groundwater.
2. Discard 3 L of top layer and collet 2 L of groundwater using bailer.
3. Store the groundwater samples in ice until further use.
   Note: experimental procedures must be conducted at most 24 h after sampling.

B. Cell concentration

1. Pre-filter 2 L of groundwater using 8-μm filter paper and vacuum pump system.
2. Filter 1 L of prefiltered groundwater with 0.22-μm membrane filter using vacuum pump pressure.
3. Put the membrane filter and 20 ml of filtered groundwater (both from step 2) into a 50 ml tube and vortex at ~3,200 rpm for 5 min to detach the cells.
4. Discard the membrane filter from the 50× concentrated groundwater microbiome sample.
   Note: concentrated groundwater microbiome samples have to be subjected to next experimental step immediately.

FCM for the microbiome sample

A. Cell staining and flow cytometry
1. Take 100 μl of the microbiome sample and mix with 900 μl of PBS buffer in an amber color microcentrifuge tube.
   Note: at the FCM step, if the observed events per second exceed 1,000, increase the dilution ratio.
2. Add 1.5 μl of each of SYTO9 and PI solution and incubate at room temperature for 15 min in the dark.
3. Keep the stained sample on ice until the next step.
   Note: as the fluorescent intensities of both fluorophores decrease drastically, FCM should be performed at most 2 h after staining.

B. FCM
1. Clean the probe and tubes of the flow cytometer with specific cleaning solution and DW before analysis.
2. Load the stained sample into the microtube rack and run the flow cytometer.
3. Set the acquisition settings, such as threshold and gain values, for each detecting channel.
   Note: the intensity threshold and signal amplification units (also called gain) are the general acquisition settings; these can be set for each detection channel. The acquisition settings used in this study were as follows: intensity threshold for FSC channel, 8,500; gain value for FSC, 124; SSC, 300; FITC, 210; and PE, 370. However, the acquisition settings for the flow cytometer must be decided according to the operational conditions, such as the machinery specifications and the target cell type. When using the flow cytometer for bacterial cells for the first time, it is recommended to first determine the acquisition settings using cells from an axenic culture of model organisms, such as *E. coli*, prior to the experiment.
4. Record the observations of cells from samples, including background, test, and negative controls, for 1 min with a flow rate of 10 μl/min.

Phenotypic data analysis

A. Extract cellular events from FCM data
1. Define the "noise-area" on the FITC–PE scatter plot generated from negative control samples using the CytExpert program.
   Note: to clarify the noise signal, it is essential to use the FCM plot constructed using the fluorescent signal variables (SYTO9 and PI in this study).
2. Convert the FCM data obtained from the groundwater microbiome from ".fcs" format into ".csv" format using the R packages "flowCore" and "biobase."
3. Filter the events based on the intensity values according to the criteria of "$\{(FSC > 10^3) \cap (SSC > 10^3) \cap (FITC > 10^3) \cap (PE > 10^3)\}$."
4. Transform the filtered intensity data onto a logarithmic scale.
5. Create the FITC–PE scatter plot and make gating of the "cell-area," avoiding the "noise-area" using the R package "alphahull."
6. Extract the data of events observed from the "cell-area."

B. Normalization and binning
1. Transform the four variables (FSC, SSC, FITC, and PE) to a standardized 0–1 range.
2. Resample the dataset using the smallest data size among the samples.
3. Choose two phenotypic variables and plot the values on the scatter plot.
   Note: according to which combination of variables are used for this step, different phenotypes can be investigated. With FSC–SSC, the physiological features of cells can be considered (such as size and complexity), whereas FITC–SSC can reflect nucleic acid contents and cell complexity.
4. Divide the scatter plot into a square grid (n × n) with equally sized bins.
   Note: in this study, the plots were divided into 10 × 10 bins.
5. Count the events in each bin and summarize them into a table (defined as a "feature table" in this study).

Phenotypic diversity calculation and ecological assessment

A. Calculation of alpha and beta diversity
1. Calculate the Shannon's diversity index using the feature table with the R package "vegan."
2. Test the normality of the Shannon's diversity values of the samples using the Shapiro–Wilk test in R.
3. Test the significances of the differences in the Shannon's diversity between the disturbed and undisturbed groups using the Wilcoxon signed rank test provided in the R package "ggpubr."
4. Create the PCA plot with the feature table using the R package "stats."
   Note: various distance matrices and ordination algorithms can be used for this step. Here, a PCA plot based on Euclidean distances was used.
5. Test the significance of the group (disturbed) to group (undisturbed) distance based on ANOSIM using the R package "vegan."

B. Assessment of ecological disturbance in the microbiome
1. Calculate the centroid coordinate of the polygon formed by undisturbed samples on the ordination plot.
2. Compute the distance of each test sample point from the group centroid of the undisturbed samples.
3. Elicit the disturbed samples among the test samples based on this distance.
   Note: the median, mean, and other statistics of the distance to the undisturbed samples from the centroid can be applied as cutoffs to determine the disturbance.

Expected Results

The most frequently detected contaminant from groundwater ecosystems is volatile organic carbon (e.g., trichloroethylene, perchloroethylene, or trichloroethane; [Kret *et al.*, 2015]; [Stroo and Ward, 2010]). Organic carbon and its intermediate molecules can be source of a carbon for the growth of some species. The changes in bacterial phenotypes following exposure to organic contaminants vary according to the metabolic capacity of the cells composing the microbiome. The expected physiological states and phenotypic characteristics of bacterial cells in a microbiome, with consideration of the organic contaminants, are as follows: i) cells that can assimilate the organic contaminant in question will show larger cell sizes and higher complexities; ii) cells susceptible to the

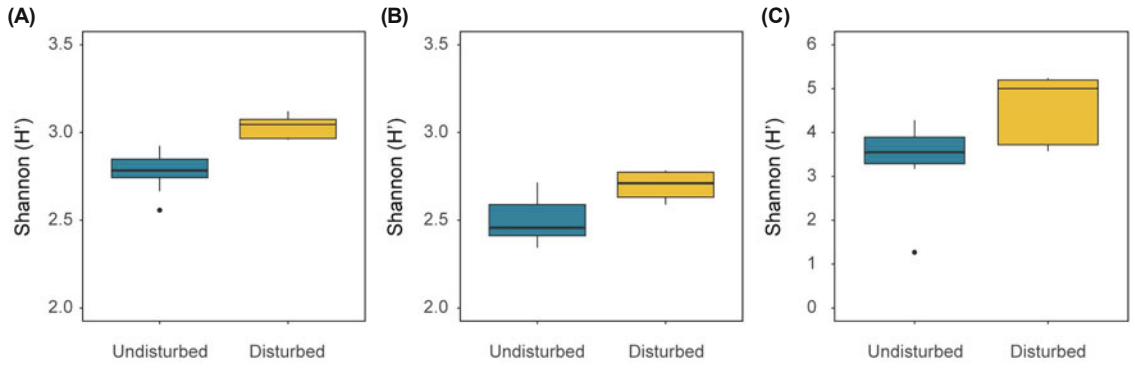

Fig. 3. Alpha diversity calculated from the phenotypic feature table retrieved from the FCM results. The cellular areas in the FCM biplots of (A) FSC–SSC and (B) FITC–PE combinations were partitioned into 100 bins (10 × 10) and the Shannon's diversity index was calculated. Shannon's diversity index calculated with (C) taxonomic features using 16S rRNA gene sequencing.

given organic contaminant will display smaller cell sizes and less complex cellular contents; iii) cells that do not interact with organic contaminants will exhibit marginal changes in their phenotypes. Therefore, at the community level, disturbances in the microbiome will cause high intra-heterogeneous phenotypes, resulting in high phenotypic diversity. The frequency of the bins can reflect this diversified phenotypic fingerprint, enabling the comparison of microbiome-based phenotypes obtained from various environments.

For this study, a total of 15 groundwater samples (ten undisturbed samples and five disturbed samples) were collected and compared to verify if the phenotypes of their groundwater microbiomes changed with or without disturbance (e.g., organic contamination). The disturbed samples were obtained from groundwater contaminated with the tetrachloroethylene and trichloroethylene. Using FCM, phenotypic information of at least 3,383 cells per sample were investigated, and a feature table was generated from the binning process using the datasets of FSC–SSC and FITC–PE combinations, respectively.

The phenotypic diversity of the disturbed samples was significantly higher than that of the undisturbed samples in both datasets, as expected (Wilcoxon signed rank test, $P < 0.05$) (Fig. 3A and B). To validate that the phenotypic diversity can reflect the results of conventional taxonomic diversity, bacterial 16S rRNA gene amplicon sequencing was performed. Taxonomic diversity was also significantly higher in the disturbed samples than the undisturbed samples (Wilcoxon signed rank tests $P < 0.05$)(Fig. 3C). The Shannon's diversity index calculated from the FSC-SSC feature table showed strong correlation (Spearman's rank correlation, rho = 0.76; P = 0.002) with the taxonomic diversity, whereas the correlation between the FITC-PE phenotypic diversity and taxonomic diversity was insignificant. When the phenotype feature tables were calculated for beta diversity, the disturbed samples were distantly clustered from the undisturbed samples along the PC1 axis. Furthermore, the distances between cluster of undisturbed and disturbed samples were significant (PERMANOVA, $P < 0.01$ in Fig. 4A and B). In contrast, with using 16S rRNA gene OTUs table, samples were undistinguishable on the PCA plot (PERMANOVA, $P = 0.547$ in Fig. 4C). This finding indicates that disturbance can be recognized based on the phenotypic fingerprint of microbiomes. Moreover, the distance between environmental and the background samples on a PCA plot could potentially be utilized as a metric to quantify the disturbance level of a microbiome (Fig. 4B).

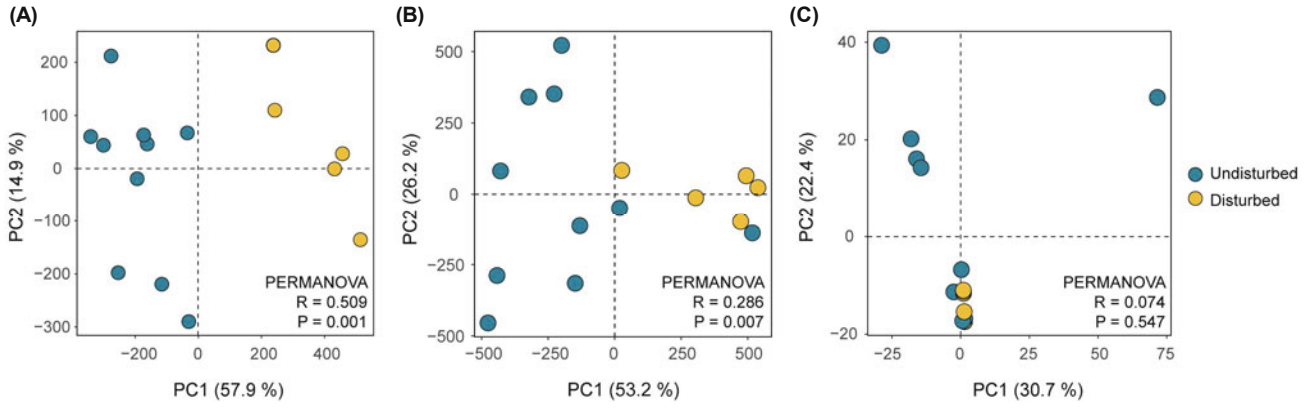

Fig. 4. Principle component analyses plot based on phenotypic features with (A) FSC–SSC and (B) FITC–PE, and (C) taxonomic features using 16S rRNA genes.

Acknowledgements

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Conflict of Interest

The authors declare no conflict of interest.

References

Buzatu, D.A., Moskal, T.J., Williams, A.J., Cooper, W.M., Mattes, W.B., and Wilkes, J.G. 2014. An integrated flow cytometry-based system for real-time, high sensitivity bacterial detection and identification. *PLoS ONE* **9**, e94254.

Collier, S.A., Deng, L., Adam, E.A., Benedict, K.M., Beshearse, E.M., Blackstock, A.J., Bruce, B.B., Derado, G., Edens, C., Fullerton, K.E., *et al.* 2021. Estimate of burden and direct healthcare cost of infectious waterborne disease in the United States. *Emerg. Infect. Dis.* **27**, 140–149.

Frossard, A., Hammes, F., and Gessner, M.O. 2016. Flow cytometric assessment of bacterial abundance in soils, sediments and sludge. *Front. Microbiol.* **7**, 903.

Gillespie, S., Lipphaus, P., Green, J., Parsons, S., Weir, P., Juskowiak, K., Jefferson, B., Jarvis, P., and Nocker, A. 2014. Assessing microbiological water quality in drinking water distribution systems with disinfectant residual using flow cytometry. *Water Res.* **65**, 224–234.

Hong, J.K., Kim, S.B., Lyou, E.S., and Lee, T.K. 2021. Microbial phenomics linking the phenotype to function: The potential of Raman spectroscopy. *J. Microbiol.* **59**, 249–258.

Jelinek, T., Bezdekova, R., Zatopkova, M., Burgos, L., Simicek, M., Sevcikova, T., Paiva, B., and Hajek, R. 2017. Current applications of multiparameter flow cytometry in plasma cell disorders. *Blood Cancer J.* **7**, e617.

Kim, J., Darlington, A., Salvador, M., Utrilla, J., and Jiménez, J.I. 2020. Trade-offs between gene expression, growth and phenotypic diversity in microbial populations. *Curr. Opin. Biotechnol.* **62**, 29–37.

Krediet, C.J., DeNofrio, J.C., Caruso, C., Burriesci, M.S., Cella, K., and Pringle, J.R. 2015. Rapid, precise, and accurate counts of *Symbiodinium* cells using the guava flow cytometer, and a comparison to other methods. *PLoS ONE* **10**, e0135725.

Kret, E., Kiecak, A., Malina, G., Nijenhuis, I., and Postawa, A. 2015. Identification of TCE and PCE sorption and biodegradation parameters in a sandy aquifer for fate and transport modelling: batch and column studies. *Environ. Sci. Pollut. Res.* **22**, 9877–9888.

Leight, A.K., Crump, B.C., and Hood, R.R. 2018. Assessment of fecal indicator *Bacteria* and potential pathogen co-occurrence at a shellfish growing area. *Front. Microbiol.* **9**, 384.

Li, L., Mendis, N., Trigui, H., Oliver, J.D., and Faucher, S.P. 2014. The importance of the viable but non-culturable state in human bacterial pathogens. *Front. Microbiol.* **5**, 258.

Nurliyana, M.R., Sahdan, M.Z., Wibowo, K.M., Muslihati, A., Saim, H., Ahmad, S.A., Sari, Y., and Mansor, Z. 2018. The detection method of *Escherichia coli* in water resources: a review. *J. Phys. Conf. Ser.* **995**, 012065.

Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., *et al.* 2020. vegan: Community Ecology Package. R package version 2.5-6. 2019. https://CRAN.R-project.org/package=vegan.

Picot, J., Guerin, C.L., Le Van Kim, C., and Boulanger, C.M. 2012. Flow cytometry: retrospective, fundamentals and recent instrumentation. *Cytotechnology* **64**, 109–130.

Props, R., Monsieurs, P., Mysara, M., Clement, L., and Boon, N. 2016. Measuring the biodiversity of microbial communities by flow cytometry. *Methods Ecol. Evol.* **7**, 1376–1385.

Props, R., Schmidt, M.L., Heyse, J., Vanderploeg, H.A., Boon, N., and Denef, V.J. 2018. Flow cytometric monitoring of bacterioplankton phenotypic diversity predicts high population-specific feeding rates by invasive dreissenid mussels. *Environ. Microbiol.* **20**, 521–534.

Ramírez-Castillo, F.Y., Loera-Muro, A., Jacques, M., Garneau, P., Avelar-González, F.J., Harel, J., and Guerrero-Barrera, A.L. 2015. Waterborne pathogens: detection methods and challenges. *Pathogens* **4**, 307–334.

Rockey, N., Bischel, H.N., Kohn, T., Pecson, B., and Wigginton, K.R. 2019. The utility of flow cytometry for potable reuse. *Curr. Opin. Biotechnol.* **57**, 42–49.

Sadler, M.C., Senouillet, J., Kuenzi, S., Grasso, L., and Watson, D.C. 2020. Computational surveillance of microbial water quality with online flow cytometry. *Front. Water* **2**, 45.

Shrestha, A. and Dorevitch, S. 2019. Evaluation of rapid qPCR method for quantification of *E. coli* at non-point source impacted Lake Michigan beaches. *Water Res.* **156**, 395–403.

Stroo, H.F. and Ward, C.H. 2010 *In situ* Remediation of Chlorinated Solvent Plumes. Springer, New York, USA.

Tan, B., Ng, C., Nshimyimana, J.P., Loh, L.L., Gin, K.Y., and Thompson, J.R. 2015. Next-generation sequencing (NGS) for assessment of microbial water quality: current progress, challenges, and future opportunities. *Front. Microbiol.* **6**, 1027.