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Quantification of aquatic unicellular diazotrophs by immunolabeled flow cytometry

Eyal Geisler · Hagar Siebner · Eyal Rahav · Edo Bar-Zeev

Abstract Unicellular diazotrophs are recognized as important contributors to the aquatic nitrogen cycle. Yet, quantifying the number of aquatic diazotrophs, especially the unicellular fraction, is highly challenging and relies mostly on microscopical and molecular approaches. In this study, a new flow cytometry-based method was developed to quantify unicellular diazotrophs by immunolabeling the nitrogenase enzyme. The quantification method was initially developed using a diazotrophic monoculture (*Vibrio natriegens*) and verified by different controls as well as various auxiliary approaches such as N_2 fixation and confocal laser scanning microscopy. It was evident that only 15–20% of the total number of *V. natriegens* cells synthesized the nitrogenase enzyme, even though the media was N-limited and anaerobic. This approach was further tested on field samples collected from marine and freshwater environments. Overall, the number of unicellular diazotrophs measured in various aquatic environments ranged from 1 to 6×10^7 cells L^{-1}. However, the fraction of these diazotrophs compared to total bacteria was significantly lower in the Eastern Mediterranean Sea (0.1%) than that collected along the Jordan River (4.7%). Nevertheless, N_2 fixation rates per cell were highest in the Mediterranean Sea (~90 attomole N $cell^{-1}d^{-1}$), while lower in the lake and the river measured herein (1.4 to 0.3 attomole N $cell^{-1}$ d^{-1}, respectively). Following the above, this direct quantification approach can provide new insights on the number and specific contribution of unicellular diazotrophs to total N_2 fixation in marine and freshwater environments.

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Introduction

Biological N_2 fixation is a central process in marine and freshwater environments as it supplies bio-available nitrogen and supports primary production (Gruber and Galloway 2008; Zehr and Capone 2020). Dinitrogen (N_2) fixation is carried out by a specific subgroup of bacteria and archaea known as diazotrophs. These

organisms use the nitrogenase enzyme, a two-component complex comprised Mo-Fe protein and Fe-reductase protein (Hoffman et al. 2014). Studies have indicated that aquatic diazotrophs use autotrophic (Zehr 2011), heterotrophic (Bombar et al. 2016; Riemann et al. 2010), and mixotrophic (Benavides et al. 2020; Feng et al. 2010) metabolic strategies to maintain the energetic requirements of the nitrogenase enzyme. Aquatic diazotrophs can be found in benthic mats, organized in long filamentous chains (up toa few hundred micrometers), free-living planktonic cells, or associated with aggregates (Bergman et al. 2013; Geisler et al. 2022; Riemann et al. 2022).

Despite the biochemical and ecological importance of unicellular diazotrophs, quantifying their abundance is challenging thus missing in most ecological studies. Traditionally, microscopical approaches were used to count diazotrophs, a majority of which were filamentous bacteria such as *Trichodesmium* sp. and *Anabaena* sp. (Spungin et al. 2016; Zulkefli and Hwang 2020). During the last two decades, various molecular methods were also applied to estimate the numbers of smaller, unicellular diazotrophs (Foster et al. 2007; Turk et al. 2011; Moisander et al. 2014). These molecular methods are mostly based on evaluating the number of diazotrophs by focusing on the transcription (DNA) or expression (mRNA) of *nifH* genes. In addition, diazotrophs were also visualized and counted by tagging the nifH gene via fluorescence in situ hybridization (FISH) (Krupke et al. 2013). Other studies have moved beyond the *nifH* gene to visualize aquatic diazotrophs by immunolocalization the nitrogenase enzyme (Currin et al. 1990; Geisler et al. 2019; Lin et al. 1998). Following the above, it can be surmised that investing energy in nitrogenase synthesis indicates that these diazotrophs were likely fixing N_2. Nevertheless, the advantages of nitrogenase immunolabeling were not harnessed to quantify diazotrophs.

In this study, nitrogenase immunolabeling was coupled with flow cytometry to count unicellular diazotrophs in aquatic samples. The quantification method is based on immunolabeling the Mo-Fe subunit of the nitrogenase enzyme by two antibodies conjugated to a green fluorophore. The method was calibrated with *Vibrio natriegens* as representative unicellular diazotroph and further validated with non-diazotroph bacteria, *E. coli*, as well as co-cultures. The method was then tested *in-situ* by immunolabeling samples from

different aquatic environments with diverse microbial communities. Auxiliary approaches using confocal laser scanning microscopy and $^{15}N_2$ were also applied to further verify this approach and determine the N_2 fixation rates per cell.

Materials and methods

Culturing unicellular diazotroph and non-diazotroph bacteria

Vibrio natriegens (ATCC 14,048) is a diazotrophic, Gram negative bacteria, that was used in this study as a positive control (Urdaci et al. 1988; Currin et al. 1990). Concurrently, *Escherichia coli* (ATCC 11,303) is a non-diazotroph, facultative anaerobic bacteria that served as a negative control. Bacteria cultures (100 ml) were acclimated in gas-tight bottles containing Luria Bertani broth media (LB, Merck Millipore, BD, 0,083,370) under anaerobic conditions (~ 0.2 mg L^{-1} O_2) at 26 °C overnight. The LB media used to grow the *V. natriegens* also included 1.5% NaCl (Sigma Aldrich, 312,525). Cells were further diluted to ~ 5×10^6 cells ml^{-1} and re-grown to a mid-logarithmic phase with ~ 2×10^8 cells ml^{-1} for 1–2 h under the same conditions. Bacterial cells were centrifuged (3500 g for 6 min) to remove the LB and resuspended in artificial brackish water (1 ml) to a final cell concentration of ~ 2×10^6 cells ml^{-1} as previously described (Lee et al. 2016; Geisler et al. 2019). The chemical composition of the artificial media is detailed in the supporting information.

Monocultures of *V. natriegens* or *E. coli,* were resuspended in nitrogen-limited brackish-water, enriched with $^{15}N_2$ (99%, Cambridge Isotopes, lot #NLM-363-PK, final concentration 1% v:v). Monocultures were then incubated for 48 h under dark and anoxic conditions at 26 °C. Co-cultures (*V. natriegens* and *E. coli*, 1:1) were also tested to determine the accuracy of the new method and verify the specificity of the immunolabeling in a mixed community with a non-diazotrophic bacteria. Each type of experimental setup (i.e., *V. natriegens*, *E. coli* and co-cultures) was done in biological triplicates to statistically confirm the results. Two additional bottles from each bacterial culture were not enriched with $^{15}N_2$ media (e.g., Mohr et al. 2010)), and served as 'controls' to determine the natural isotopic ratio on nitrogen (i.e., "natural

abundance"). At the conclusion of the incubation, subsamples were analyzed for N_2 fixation rates, colony forming units (CFU), as well as total bacterial abundance (BA) and diazotrophic abundance (DA), as detailed below. In addition, diazotrophs were visualized by capturing immunolabeled subsamples via confocal laser scanning microscopy (CLSM).

Collection of natural samples for diazotrophic quantification

Surface waters were collected from three sampling locations, the Southeastern Mediterranean Sea, Jordan River, and Sea of Galilee Lake (Table S1). Water was incubated in a set of triplicate Nalgene bottles (1 L) for each sampling location. N_2 fixation rates were determined by enriching the samples with 7.5% of dissolved $^{15}N_2$ stock (Final concentration). Enriched samples were incubated for 48 h at room temperature under 12 h light/dark conditions. An additional bottle from each location was not enriched with $^{15}N_2$ to determine their natural isotopic ratio. Subsamples (1.7 ml) were collected at the end of the incubation for BA and DA analysis, as well as diazotroph microlocalization.

Analytical methods

Diazotroph immunolabeling for flow cytometric analysis. Monoculture and natural samples (1.7 ml) were fixed with 50% glutaraldehyde (final concentration, 0.2% Sigma-Aldrich, G7651) for 10 min at room temperature, flash frozen in liquid nitrogen, and stored at − 80 °C until analysis. Samples were slowly thawed at room temperature before analysis (Fig. 1A). Flow cytometry is based on counting single cells, therefore, ethylenediaminetetraacetic acid (EDTA, Sigma Aldrich) was first added (5 μM) to impair the structure and facilitate the dispersion of aggregates present in the sample (Bogler and Bar-Zeev 2018). Samples were further agitated in a bath sonicator for 6 min to disassociate cells from the aggregate matrix and one another. Subsamples (1 ml) were transferred and centrifuged for 10 min at×4000 g (a subsample was also collected for total bacterial abundance, details are provided below). The supernatant was carefully discarded to maintain the bacterial pellet. Wash solution was prepared by mixing phosphate buffer saline (PBS) and Triton X-100 (T, final concentration of

0.1%, Sigma Aldrich, X100), defined hereafter as PBST. The wash solution was added to perforate the cell envelope. Samples were centrifuged for 10 min at×4000 g while the supernatant was carefully discarded. This washings-centrifugal cycle was repeated three times to increase the efficiency of cellular perforation. Fresh anti-nitrogenase antibody (3 μg ml^{-1}, Agrisera Antibodies AS01 021A) was prepared with PBST and bovine albumin serum (BSA, filtered 0.2 μm, 1 mg ml^{-1}, Sigma Aldrich A2153) to minimize unspecific antibody binding (Fig. 1B). Samples were then incubated while slowly rotating (Benchmark Scientific Roto-Therm Plus, H2024) for one hour at room temperature to facilitate binding between the nitrogenase Mo-Fe subunit and the primary antibody. Unbonded antibodies were removed by washing the samples three times with PBST, similarly to the above. Washed samples were then incubated at room temperature in the dark for 45 min with the secondary antibody (3 μg ml^{-1}, Thermo Fisher Scientific A-11039) conjugated to a green fluorophore (Alexa Fluor™ 488) with excitation (Ex) spectra of 498 nm and emission (Em) of 520 nm (Fig. 1C). Any untagged residues of the secondary antibodies were removed by washing the samples three times with PBST as described above. Immunolabeled samples were suspended with sterile PBS (1 ml) without any additions. Few central controls were also prepared following the above procedure and tested to evaluate the specific tagging of diazotrophs: (1) negative control, namely PBST-BSA without any antibodies, to determine whether any autofluorescence could be detected; (2) no addition of the 1st antibody (PBST-BSA with the 2nd antibody only); and (3) without the addition of the 2nd antibody (PBST-BSA with the 1st antibody only) to verify if any unspecific adsorption occurred. (4) Additional negative control included the addition of two antibodies as described for *V. natriegens* to *E. coli*, thus check for any unspecific tagging by immunolabeling a non-diazotrophic bacteria. Negative control was necessary to check for any unspecific tagging by testing the immunolabeling approach on non-diazotroph (*E. coli*) bacteria. Moreover, bacterial abundance was measured after the washing stage from representative samples to evaluate the number of bacterial cell loss (Fig. S1).

Counting immunolabeled diazotrophs and total bacteria using flow cytometry. Diazotroph abundance (DA) was determined by detecting and counting
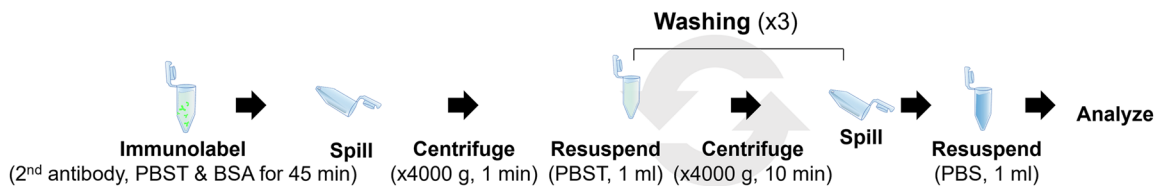
A Preparing samples
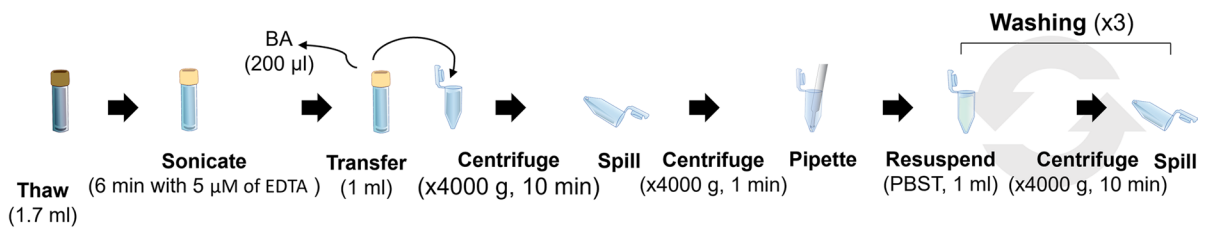
B Immunolabeling with 1st antibody
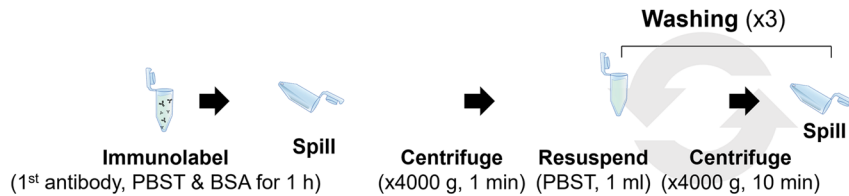
C Immunolabeling with 2nd antibody

Abbreviations: PBS – Phosphate Buffer Saline, PBST – PBS with Triton, and BSA – Bovin Albumin Serum

Fig. 1 Graphical summary of the immunolabeling stage for flow cytometric analysis. The method is divided into three main stages: sample preparation (**A**), Immunolabeling with the 1st antibody (**B**), and tagging with the 2nd antibody conjugated to the green fluorophore (**C**)

immunolabeled subsample (200 µl) using an Attune-Next acoustic flow cytometer (Applied Biosystems). Changes in the abundance of monoculture diazotrophs were determined after diluting the immunolabeled samples (1:100, 1:250, 1:500, 1:1000). Monocultures were analyzed at a flow rate of 100 µl min^{-1} while reduced to 25 µl min^{-1} for environmental samples to increase the temporal resolution and accuracy of counting immunolabeled diazotrophs. Stop condition was set to 20,000 counts for all samples. Calibration beads (1 µm, F8815, Invitrogen, Ex: 350 nm Em: 440 nm) were added (final concentration of 1.8×10^4 beads ml^{-1}) every 12 samples to evaluate the size spectrum of the sample.

Following the above, total bacterial abundance (BA) was quantified by staining non-immunolabeled subsamples (200 µl) with SYBR Green I (S7563, Invitrogen, final concentration, 1 nM) (Vaulot and Marie 1999). Samples were incubated for 15 min under dark conditions. Stained samples were measured with an Attune-Next acoustic flow cytometer. MilliQ (sterile) water samples were used to clean the system every five samples, while PBS samples were tested and subtracted as blanks. The specified lasers, dyes, and filters are detailed in Table 1.

Quantifying cells using colony forming units (CFU) counts. Samples from each individual experiment (n = 3) were collected from the incubation bottles and serially diluted in parallel to the immunolabeled samples (1:100, 1:250, 1:500, 1:1000). Subsamples (25 µl) were plated on an agar plate (1.5%, Bacto Agar, DF0140) with an N limited

Table 1 Dedicated setup of the flow cytometer to detect and count the immunolabeled diazotrophs

Parameter	Laser (nm)	Excitation (nm)	Emission (nm)	Detector (nm)
Side scattering (SSC)	450 (Blue)	450	–	488 ± 10
SYBR GREEN I	450 (Blue)	497	520 (Green)	520 ± 30
Nitrogenase Immunolocalization	450 (Blue)	500	520 (Green)	520 ± 30

brackish water media (the recipe is detailed in the supporting information). CFU samples were incubated under anaerobic conditions for 48 h at 26 °C. CFU was determined at the end of the incubation by counting plates with 30–300 colonies.

Microlocalization of diazotrophs using confocal laser scanning microscopy (CLSM). Immunolabeled samples (50 µl) were stained with 250 µg ml^{-1} of 40,6-Diamidino-2- Phenylindole (DAPI, Ex 360 nm and Em 460 nm, Thermo Fisher, D1306) or (SYBR Green I, Ex 497 nm, and Em 520 nm) and incubated for 20 min under dark conditions. Samples were drop-casted on a glass microscope slide and sealed by a cover slide and nail polish to minimize dehydration. Immunolabeled nitrogenase enzyme was visualized by a CLSM 900 equipped with a 488 nm laser (Power, 0.2% digital gain, ~500 V, pinhole, 32 µm). Stained bacteria were visualized with a 405 nm laser (Power, 0.9%, digital gain, ~730 V and pinhole, 37 µm). Samples were observed under an ×63 lens (63x/1.4 Oil DIC M27, respectively). Non stained samples were used before to identify and subtract autofluorescence. Images were processed using Zeiss ZEN Blue edition (3.5, lite, Germany).

Measuring N$_2$ fixation rates. Triplicate samples were filtered on a pre-combusted glass microfiber filter (GF/F, Cytivia, 1,825,025, 450 °C, 4.5 h) after 48 h incubation in ^{15}N$_2$ enriched media. Different volumes of samples (25 ml for lab cultures and 1L for environmental microcosms) were filtered to ensure sufficient biomass (resulting in a minimum amplitude of ~1000 mv, translated to ~10 µg, see details below) on the filter as their source varied from monocultures to different natural environments. Samples were dried in the oven overnight (60 °C) and stored in the desiccator until measurements. Filters were carefully packed in tin capsules, with clean, pre-combusted GF/F used as blanks. The samples were then analyzed using an elemental analyzer (EA; Thermo Scientific, Flash 2000 HT) coupled with an isotope ratio mass spectrophotometer (IRMS; Thermo Scientific, Delta V Plus).

Working with filters and environmental samples over a wide range of concentrations requires caution during the isotopic measurement. The quality control measures taken during the measurement are detailed in the supporting information. Briefly: Three standards (Glutamic Acid USGS 40, Glycine USGS 64, and Caffeine USGS 62) were chosen for calibration, bracketing the expected range for δ^{15}N of the enriched, as well as natural abundance samples and ensuring accuracy (Fig. S2A). Acetanilide (Thermo Scientific, BN240741) was used for the linearity test over the measured range (Fig. S2B) and for quantitative calibration of peak amplitude vs. µg N in the sample's biomass (Fig. S2D). A working range of 10 to 55 µg N per sample was determined to assure precision and avoid the linearity effect. Similar ranges of nitrogen (>10 µg N per filter) were previously determined (White et al. 2020). No drift was measured (slope = 0.01), and good precision was found (± 0.3‰) throughout the analysis (Fig. S2C). The natural abundance of ^{15}N, reflected by the ratio of ^{14}N/^{15}N in each culture or environmental sample, was subtracted from that of the corresponding enriched sample to calculate N$_2$ fixation rates, according to previous reports (Montoya et al. 1996).

Statistical analyses

Statistical tests were run using XLSTAT (2022.2, New York). Before analyses, normal distribution of the data was validated using the Shapiro Wilk test. A Pearson correlation test evaluated the links between BA, DA, CFU and N$_2$ fixation. For comparing N$_2$ fixation and BA/DA between different lab cultures (*i.e., V. natriegens, E. coli,* and both) or natural samples (Jordan River, Sea of Galilee, and Mediterranean Sea), Analysis of Variance (ANOVA) was used with post hoc Tukey test. All the tests were run under the confidence level of 95% ($\alpha = 0.05$).

Results and discussion

Detecting and quantifying a unicellular diazotrophic monoculture

Immunolabeled *V. natriegens* formed a distinct cluster after analyzing the samples by flow cytometry using a green detector over side scatter (Fig. 2A). In contrast, only a few unlabeled cells (< 1000 events) were captured in the same region (Fig. 2B). Similarly, no *V. natriegens* cells were detected after tagging with the first or the second antibodies only (Fig. 1C-D). Following the above, only the conjunction of the two antibodies led to positive detection of *V. natriegens* by flow cytometry, excluding negligible autofluorescence or unspecific adsorption of the stain to the cells. Moreover, no immunolabeling by non-diazotroph *E. coli* cells were detected in the region of interest by the flow cytometer (Fig. 2E). This negative control highlights that only cells that synthesized the nitrogenase enzyme could be tagged by the antibodies and detected, as previously reported in other studies (Chelius and Triplett 2000; Geisler et al. 2019). Total bacterial abundance was counted by an independent test after tagging a subsample with only a nucleic acid stain (SYBR green). Tagging bacteria with SYBR green resulted in a distinct cluster that was identified in the same region of interest as described above (Fig. 2F). Complimentary visualization of *V. natriegens* and *E. coli* subsamples by CLSM confirmed the results detected by the flow cytometry (Fig. 2, circles), similarly to previous studies (e.g., Geisler et al. 2019).

Linear and significant correlation was detected between the number of immunolabeled *V. natriegens* and the total number of cells tagged by SYBR green from the same monoculture (Pearson, r = 0.999, p = 0.001, Fig. 2G). That trendline indicated that between 15 to 20% of all *V. natriegens* bacteria were specifically tagged by immunolabeling, namely the cells that synthesized the nitrogenase enzyme. Correspondingly, a linear correlation was also found between immunolabeled cells and CFU counts that grew on limited nitrogen agar plates under anaerobic conditions for 48 h (Fig. 2H). It should be noted that the number of immunolabeled cells detected by flow cytometry was 20–25 times higher than those counted on the agar plates. In addition, a linear relationship was found between the number of *V. natriegens* that synthesized the nitrogenase enzyme and N_2 fixation rates, resulting in an averaged N_2 fixation rates per cell of 1.3 ± 0.3 attomole N $cell^{-1}$ (Fig. 2I). Interestingly, previous study that used halogen in-situ hybridization-secondary ion mass-spectroscopy to measure N_2 fixation per cell also reported on rates between 0.2 and 20 attomole N $cell^{-1}$ d^{-1} (Halm et al. 2009), further supporting the results herein.

A lower percentage of free-living diazotrophs that synthesized the nitrogenase enzyme compared to the total cell count may indicate that heterotrophic N_2 fixation was partly suppressed even under anaerobic conditions and limited concentrations of inorganic nitrogen. Although the scope of the study was to develop a new quantification method for unicellular diazotrophs, it could be surmised that other constraints that were not measured here, such as pH (Luo et al. 2019) and/or carbon availability (Benavides et al. 2020; Rahav et al. 2016) impaired the N_2 fixation rates by heterotrophic bacteria.

Counting diazotrophic and non-diazotrophic mixed cultures

Two monocultures that included unicellular diazotrophic (*V. natriegens*) and a non-N_2 fixing (*E. coli*) bacteria were mixed to test the differentiation capacity and accuracy of the new immunolabeled—flow cytometry-based approach with mixed cultures. Staining the DNA of subsamples with SYBR green for total bacterial count formed a distinct cluster (Fig. 2F). Immunolabeling diazotrophic monoculture, as well as a mixture of *V. natriegens* and *E. coli* bacteria, resulted in a clear cluster over the conjugated nitrogenase (green) threshold (Fig. 3A).

Quantifying total bacteria indicated that the numbers of *V. natriegens* culture or a mixture with *E. coli* were statistically similar (0.75×10^7 cells ml^{-1}) after 48 h of anaerobic incubation in a nitrogen-limited media (Fig. 3B). Counting the immunolabeled cells indicated that the number of N_2 fixing diazotrophs, namely *V. natriegens* that synthesized the nitrogenase enzyme, constitute 18% of the total *V. natriegens* cells. Similar findings were previously captured by epifluorescent microscopy using the same media, suggesting that not all *V. natriegens* cells were fixing dinitrogen (Geisler et al. 2019). Note that no immunolabeled *E. coli* cells were detected by the flow
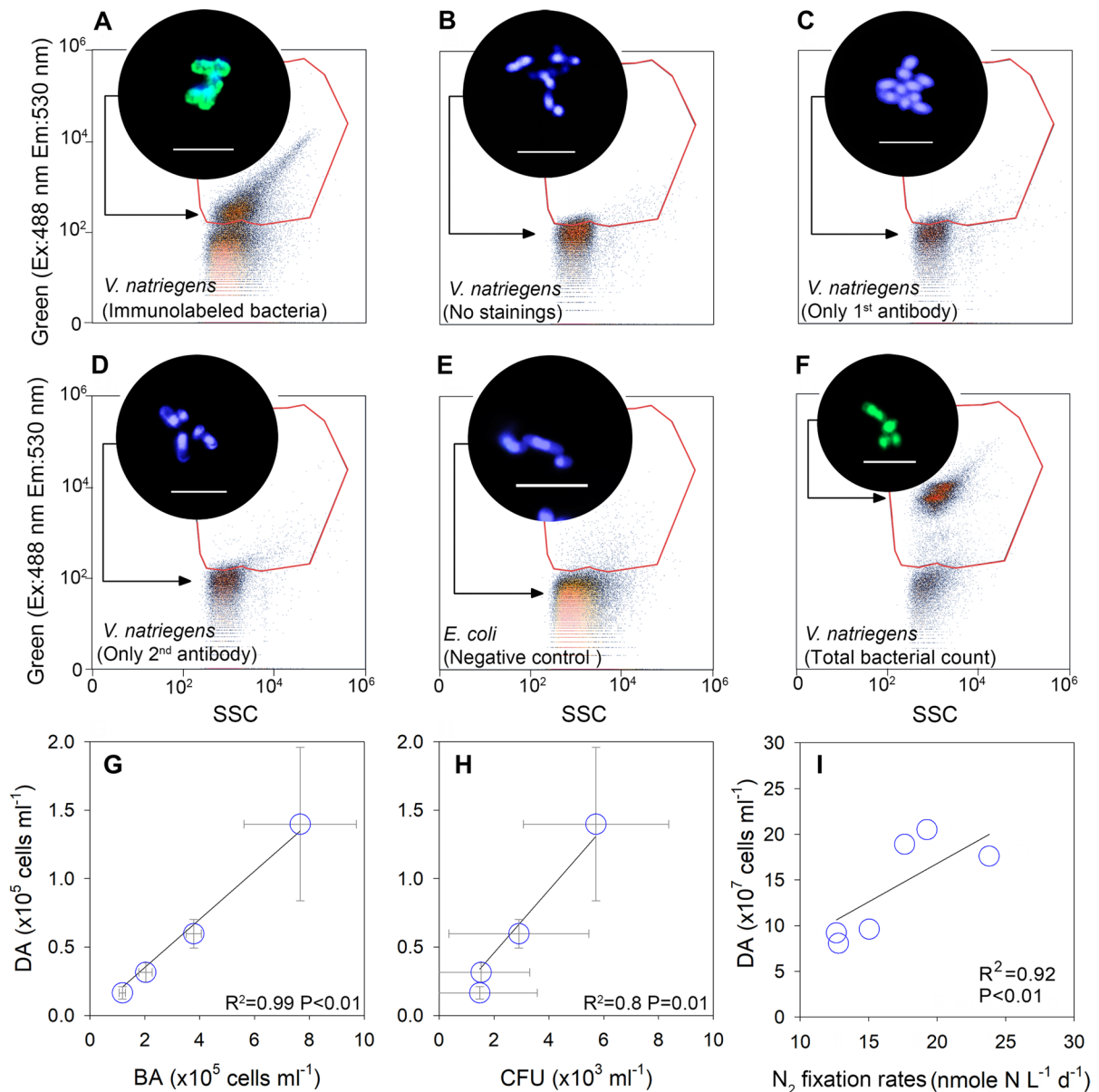
Fig. 2 Representative density plots of the main verification tests (**A-F**) and correlation charts of the corresponding quantification results (**G-I**). The region of interest for immunolabeled *V. natriegens* diazotrophs, as well as total bacterial count (individually tested after nucleic staining) was determined according to green fluorescence over side scatter (SSC). Diazotrophic abundance (**DA**) was correlated to total bacterial abundance (**BA**) (**G**) as well as colony-forming units (CFU) (**H**) and N_2 fixation rates (**I**). Top circles of each plot (**A-F**) capture subsample images using a confocal laser scanning microscope with a scale bar of 5 μm. Diazotrophs were identified by nitrogenase immunolabeling (Green), while all the cells were detected by DAPI (Blue) or SYBR green (Green). Additional images are provided in supporting information (Fig. S3A-E)

cytometer (Fig. 3B), ruling out any unspecific links or adsorption of the fluorophore. Corresponding N_2 fixation rates were found to be significantly higher (1.3 times) in the mixed culture than in the *V. natriegens* monoculture (Fig. 3C). That difference was even greater (2.5 times) when comparing N_2 fixation rates per cell in the mixed culture to those measured by *V. natriegens* only (3.3 ± 1.6 and 1.3 ± 0.3 attomole N cell^{-1}, respectively).
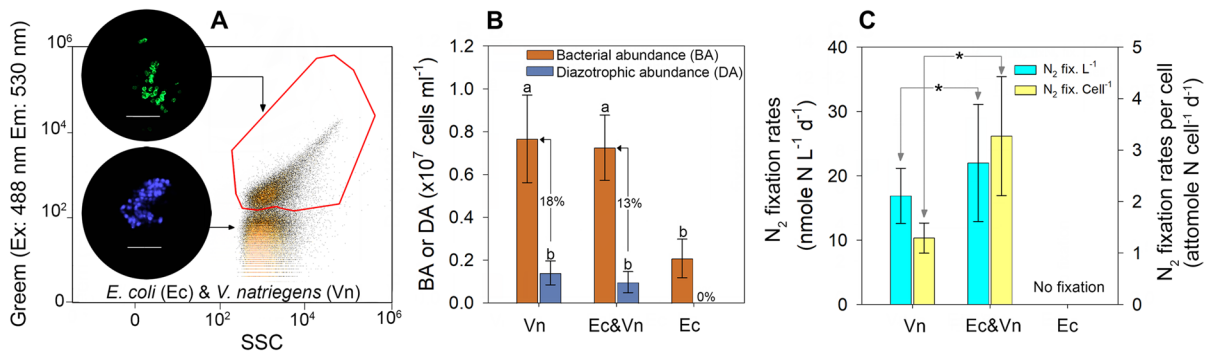
Fig. 3 Representative flow cytometry plot of *E. coli* (Ec) and *V. natriegens* (Vn) mixed culture (**A**). The corresponding CLSM micrographs capture immunolabeled diazotrophs (5 μm scale bar). An additional image is provided in supporting information (Fig. S3F). Abundance of non (*E. coli*) and diazotrophic bacteria (*V. natriegens*) in mono and mixed cultures was quantified after DNA staining and nitrogenase immunolabeling by flow cytometry (**B**). Corresponding N_2 fixation rates were determined from all the cultures (**C**). N_2 fixation rates per cell were calculated by normalizing the measured rates to the number of immunolabeled diazotrophs counted in the same culture. Values represent the mean and standard deviation from six independent replicates. The letters above the bars refer to ANOVA analysis followed by a Tukey post hoc test (**B**). The asterisks in panel C signifies statistical differences between treatments (t-test, $p < 0.05$)

Altogether, it appears that mixing *V. natriegens* with a non-diazotrophic heterotrophic bacteria such as *E. coli* did not affect the growth of diazotrophs as the number of cells synthesizing the nitrogenase remained similar. However, it was evident that in these co-cultures diazotrophs were fixing N_2 at higher rates and even more so per cell. It is plausible that in co-cultures, heterotrophic diazotrophs utilize organic matter (i.e., extracellular polymeric substances, EPS) that was secreted by non-diazotrophic bacteria that grow under nutrient (N) stress (Marvasi et al. 2010; Wang et al. 2014). Concurrently, these diazotrophs may enhance their N_2 fixation rates per cell to compensate for the consumption of NH_4^+ by the non-diazotrophic bacteria. These type of synergistic interactions highlight the scientific potential of this newly developed approach, yet dedicated studies should be carried out to confirm the former observations.

Evaluating the abundance of unicellular diazotrophs in aquatic environments

Quantification of unicellular diazotrophs from marine and freshwater environments (i.e., Southeastern Mediterranean Sea, Jordan River, and the Sea of Galilee Lake) by immunolabeled flow cytometry resulted in a marked cluster (Fig. 4A). However, this cluster was slightly more scattered than in the monoculture controls. Complimentary imaging of subsamples by

CLSM indicated that only a small fraction of the cells collected from the Sea of Galilee Lake were tagged by nitrogenase immunolabeling (Fig. 4A, top circle). Additional CLSM images of immunolabeled bacteria from the Mediterranean Sea and the Jordan River are provided in the supporting information (Fig. S4).

The abundance of unicellular diazotrophs was $2 \pm 0.2 \times 10^7$ cells L^{-1} in the Mediterranean Sea, $1 \pm 0.7 \times 10^7$ cells L^{-1} in the Sea of Galilee Lake, and $6 \pm 0.9 \times 10^7$ cells L^{-1} in the Jordan River. The percent of unicellular diazotrophs from total bacterial abundance ranged between 0.1% in the Mediterranean Sea to 1.2 and 4.7% in the Sea of Galilee Lake and Jordan River, respectively (Fig. 4B). Corresponding N_2 fixation rates were between 0.2 and 1.2 nmole N L^{-1} (Fig. 4C), which are similar to previous reports (Halm et al. 2009; Marcarelli and Wurtsbaugh 2006, 2009; Rahav et al. 2022). Note that data on N_2 fixation rates in freshwater environments is highly limited (Marcarelli et al. 2022). Normalizing these rates to the number of diazotrophs detected by immunolabeling flow cytometry (DA) resulted in N_2 fixation rates per cell, ranging between 88 attomole N $cell^{-1}$ at the Mediterranean Sea to 0.3 attomole N $cell^{-1}$ at the Sea of Galilee Lake (Fig. 4C).

Limited concentrations of dissolved inorganic nitrogen were previously reported to enhance N_2 fixation rates per cell, thus providing new bioavailable nitrogen compounds (Zehr et al. 2003; Gruber and
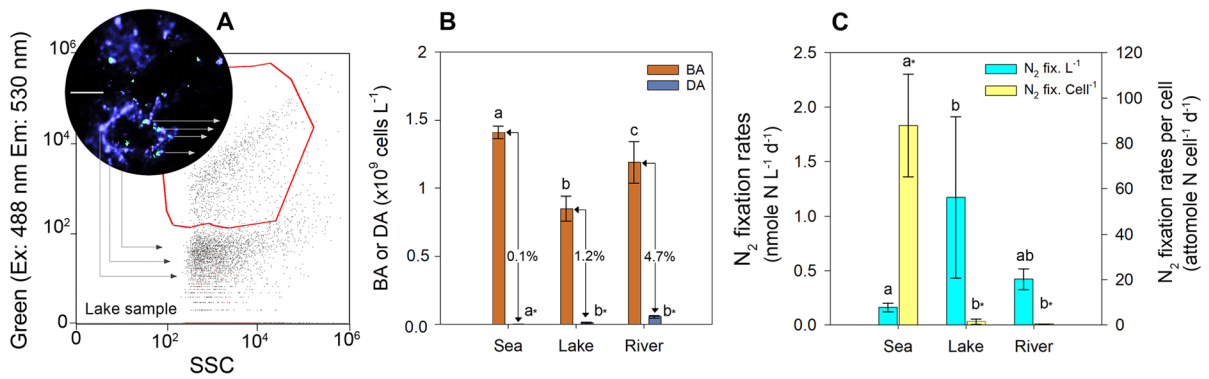
Fig. 4 Representative density plots of an immunolabeled subsample (Green as a function of SSC) that was collected from the Sea of Galilee Lake (**A**). A complimentary CLSM image was further captured from a lake subsample (A, top circle), whereas diazotrophs were identified by nitrogenase immunolabeling (Green), while total bacteria were detected by DAPI (Blue). The image scale bar is 10 µm. Bacteria and diazotroph counts (**BA** and **DA**, respectively) were determined from the Sea of Galilee Lake, the Jordan River, and the Mediterranean Sea (**B**). N_2 fixation rates were measured from the same natural environments (**C**). N_2 fixation rates per cell was calculated by normalizing the rates to the number of DA. The letters above the bars refer to ANOVA analysis with a confidence level of 95% ($\alpha = 0.05$) followed by a Tukey post hoc test. Letters without an asterisk correspond to the BA and total nitrogen fixation, while letters with asterisks coincide with DA and N_2 fixation rates per cell

Galloway 2008). Therefore, compared to the mesotrophic Jordan River (Rom et al. 2014) and the Sea of Galilee Lake (Hadas et al. 2015), measuring the highest N_2 fixation rates per cell in the oligotrophic, nitrogen poor, Mediterranean Sea were expected (Rahav et al. 2013; Kress et al. 2014). Nevertheless, it should be pointed that recent studies have reported that N_2 fixation rates per cell could be somewhat higher after the addition of dissolved inorganic nitrogen (Martínez-Pérez et al. 2018; Mills et al. 2020). These studies, as well as the above, indicate that cellular mechanisms controlling N_2 fixation rates are not clear and likely change according to the environmental conditions and the different metabolic (i.e., heterotrophic/autotrophic) pathways used by the diazotrophic community (Mills et al. 2020).

Conclusion

Coupling immunolabeling and flow cytometry can be used as a high throughput approach to quantify the number of unicellular diazotrophs that were likely fixing N_2 since the nitrogenase enzyme was synthesized. This approach can be applied to count unicellular diazotrophs in controlled lab-scale experiments and in various aquatic environments. Counting the total and immunolabeled cells of a diazotrophic

monoculture indicated that even under anaerobic and N-limiting conditions, only a fraction (15–20%) synthesize the nitrogenase enzyme. That discrepancy was likely due to the experimental conditions (e.g., carbon liability and nutrient concentrations) and yet highlights the importance of counting diazotrophs (even from monocultures under controlled conditions) to determine fundamental aspects such as the N_2 fixation rates per cell. Moreover, direct quantification of unicellular diazotrophs from natural environments can provide novel insights related to the aquatic nitrogen cycle. For example, the field results indicate that N_2 fixation rates per cell were highest in the oligotrophic Mediterranean Sea compared to the Jordan River and the Sea of Galilee Lake, pointing on their potential significance for total biological N production in N-limited environments.

It should be highlighted that this approach could be further developed: (i) differentiate and specifically count unicellular heterotrophic vs. photoautotrophic diazotrophs. Enhancing the specificity could provide new quantified estimation over the contribution of the different diazotrophic groups to total N_2 fixation; (ii) compare the number of diazotrophs using this immunolabeled flow cytometry approach to molecular, *nifH*-based approaches that are currently applied at various aquatic environments (Zehr and Capone 2020). Nevertheless, we suggest that adopting this

approach could provide information on specific N_2 fixation capacity of freshwater and marine diazotrophs. Moreover, quantifying diazotrophs will likely provide new insights on the contribution of these microorganisms to the aquatic nitrogen cycle.

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Data availability   Any enquiries related to data availability should be directed to the corresponding authors.

Declarations

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