The following appendix accompanies the article

Whole-cell immunocytochemical detection of nitrogenase in cyanobacteria: improved protocol for highly fluorescent cells

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Aquatic Microbial Ecology 51:237–247 (2008)

Appendix 1. An immunocytochemical protocol for highly fluorescent cells of cyanobacteria was developed to detect nitrogenase proteins in a single cell

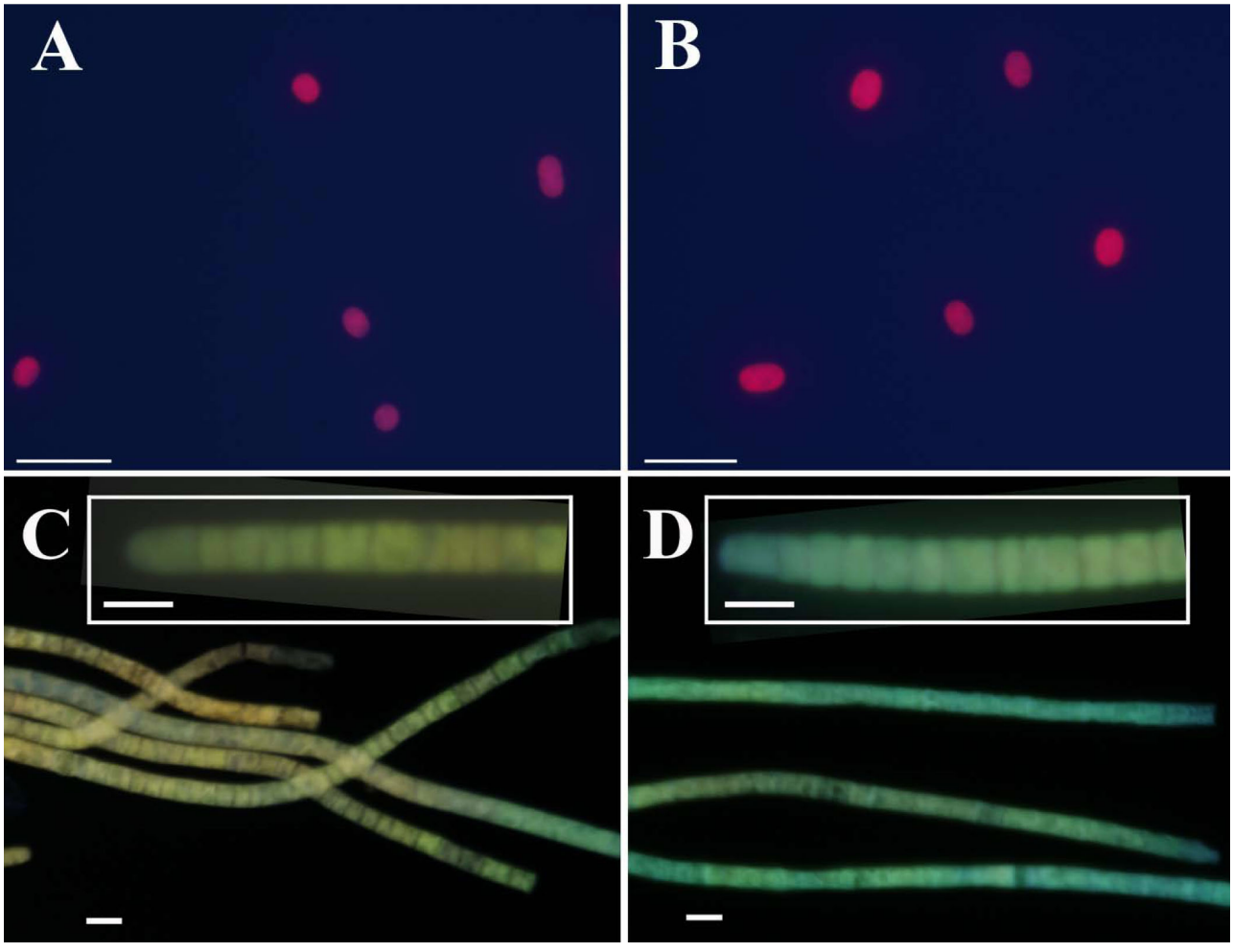

Fig. A1. Epifluorescence microscopy images of (A,B) *Gloeothece* sp. 68DGA and (C,D) *Trichodesmium* sp. NIBB1067 (A,C) before and (B,D) after incubation with an Alexa Fluor® 350-conjugated secondary antibody. Fixed cells were incubated with the Fe-protein antibody after permeabilization and blocking. Excitation light was 340 nm with a full width at a half maximum of 40 nm. Insets in (C) & (D): images with higher magnification. A single cell fluorescence spectrum of *Trichodesmium* sp. NIBB1067 under an epifluorescence microscope is available in Ohki (2008). Scale bars = 10 µm

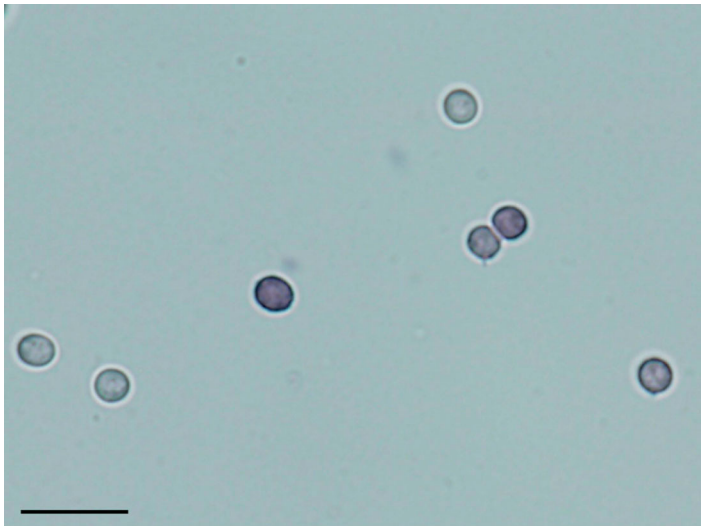

Fig. A2. Immunocytochemical detection of nitrogenase (Fe-protein) visualized with an alkaline phosphatase-conjugated secondary antibody and the chromogenic substrates 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate. *Gloeothece* sp. 68DGA grown without combined nitrogen was used. Scale bar = 10 µm

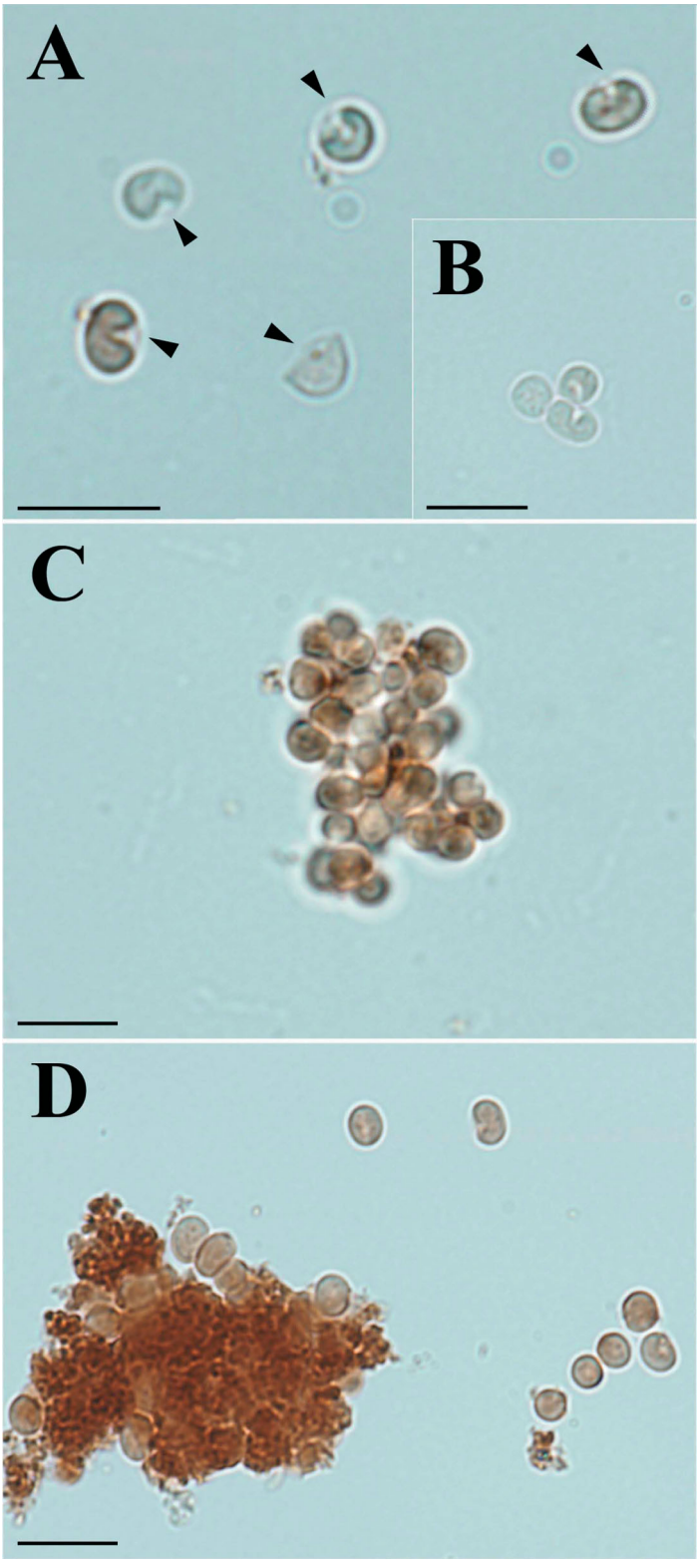

Fig. A3. Immunocytochemical detection of nitrogenase (Fe-protein) in *Gloeothece* sp. 68DGA grown without combined nitrogen after various treatments. (A,B) The cells were preserved in methanol (at –30°C) without paraformaldehyde fixation. Collapsed cells are indicated by arrowheads in (A). (C) Permeabilization with 1% SDS in phosphate-buffered saline (PBS) containing NaOH (10^{-1} M). (D) Permeabilization with 1% Triton X-100 in PBS. Cells were incubated with the Fe-protein antibody after blocking, and immunoreactivity was visualized with a horseradish peroxidase-conjugated secondary antibody and 3-3′-diaminobenzidine tetrachloride. Scale bars = 10 µm

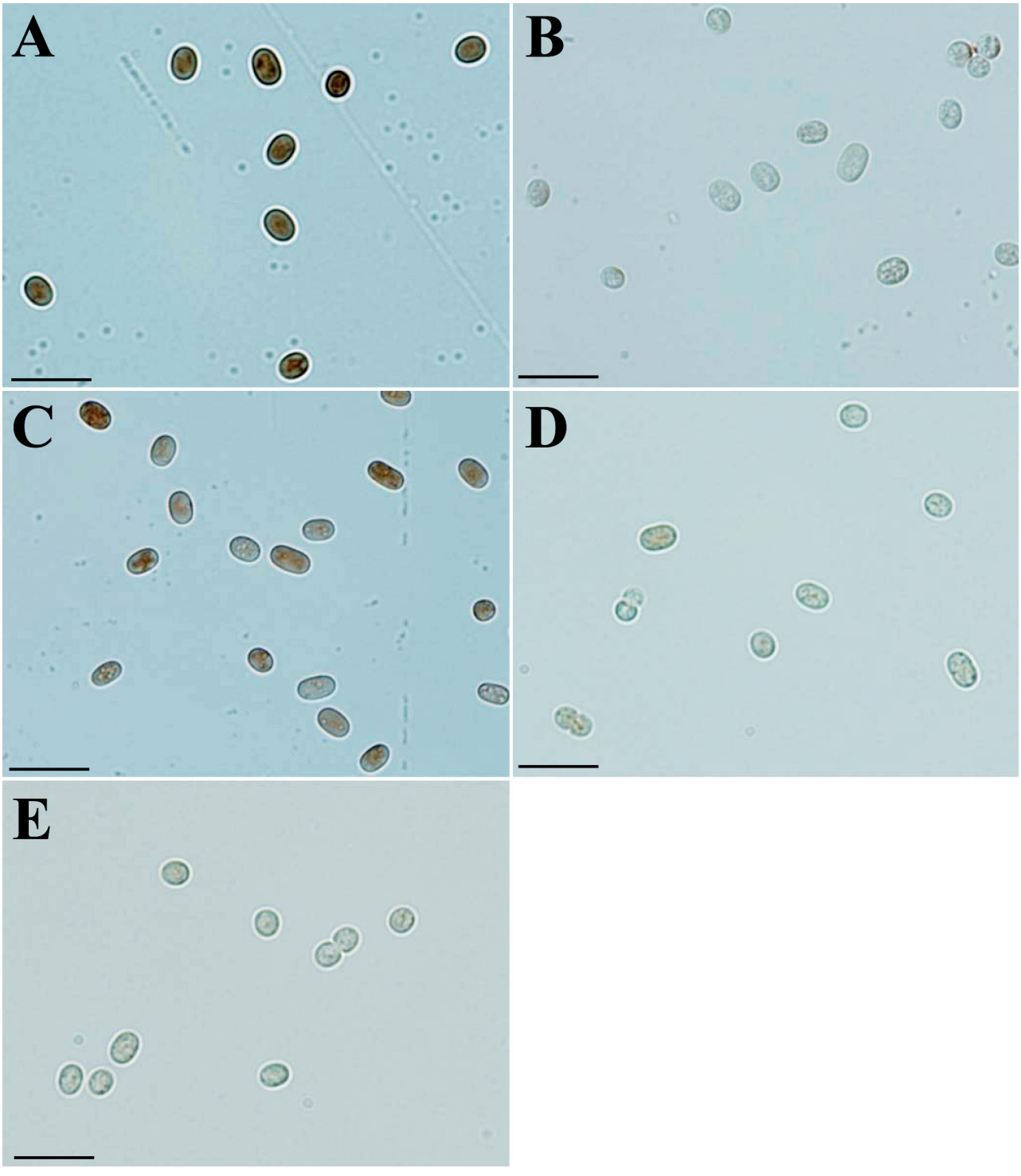

Fig. A4. Immunocytochemical detection of nitrogenase proteins in *Gloeothece* sp. 68DGA grown (B,D) with or (A,C,E) without-combined nitrogen. Fixed cells were incubated with (A,B) the Fe-protein antibody or (C,D) the MoFe-protein (α-subunit) antibody after permeabilization (in 10% dimethylsulfoxide for 4 h) and blocking (in 10% normal rabbit serum for 4 h). Immunoreactivity was visualized with a horseradish peroxidase (HRP)-conjugated secondary antibody and 3-3′-diaminobenzidine tetrachloride. (E) Same as in (A), but incubation with the HRP-conjugated secondary antibody was omitted. Scale bars = 10 µm

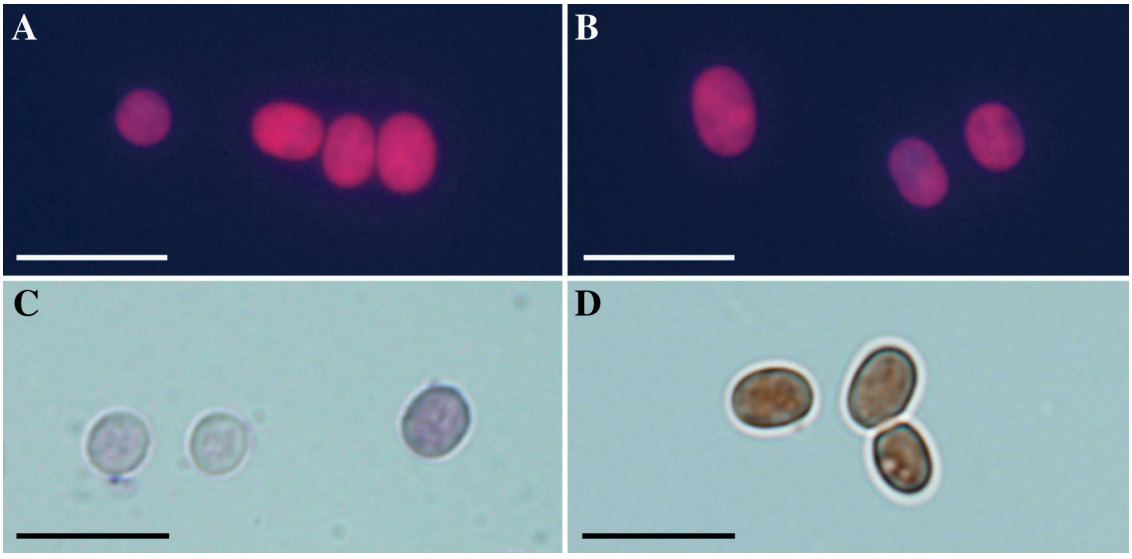

Fig. A5. Immunostaining with Alexa Fluor 350®, 4-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) and 3-3′-diaminobenzidine tetrachloride (DAB)/H_2O_2 in *Gloeothece* sp. 11DGA grown without combined nitrogen. (A) Epifluorescence microscopy image of control cells without secondary antibody. After cells were treated with nitrogenase (Fe-protein) antibody, immunoreactivity was visualized with (B) Alexa Fluor 350®-conjugated secondary antibody, (C) an alkaline phosphatase-conjugated secondary antibody and NBT/BCIP or (D) a horseradish peroxidase-conjugated secondary antibody and DAB/H_2O_2. Scale bars = 10 µm

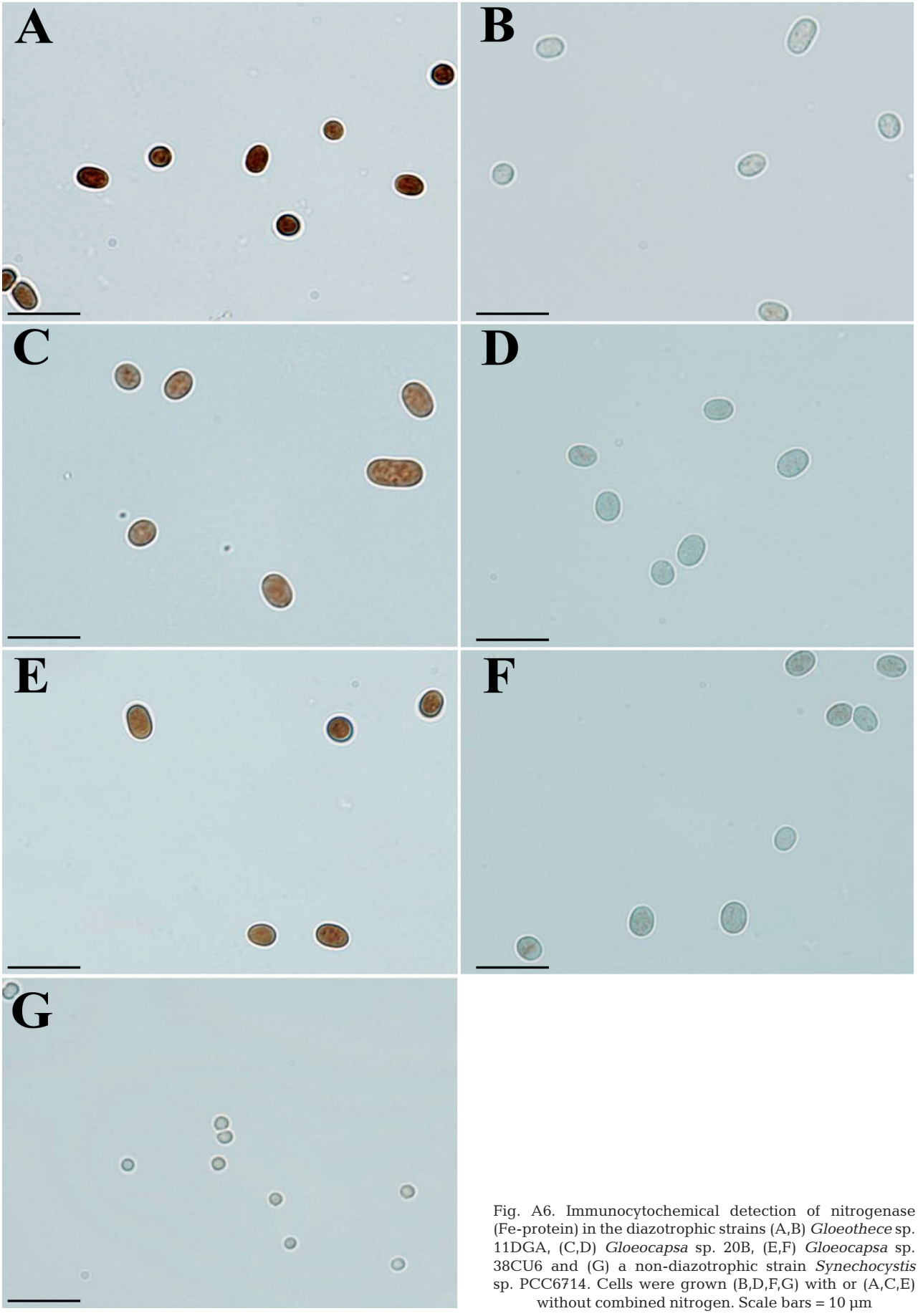

Fig. A6. Immunocytochemical detection of nitrogenase (Fe-protein) in the diazotrophic strains (A,B) *Gloeothece* sp. 11DGA, (C,D) *Gloeocapsa* sp. 20B, (E,F) *Gloeocapsa* sp. 38CU6 and (G) a non-diazotrophic strain *Synechocystis* sp. PCC6714. Cells were grown (B,D,F,G) with or (A,C,E) without combined nitrogen. Scale bars = 10 µm

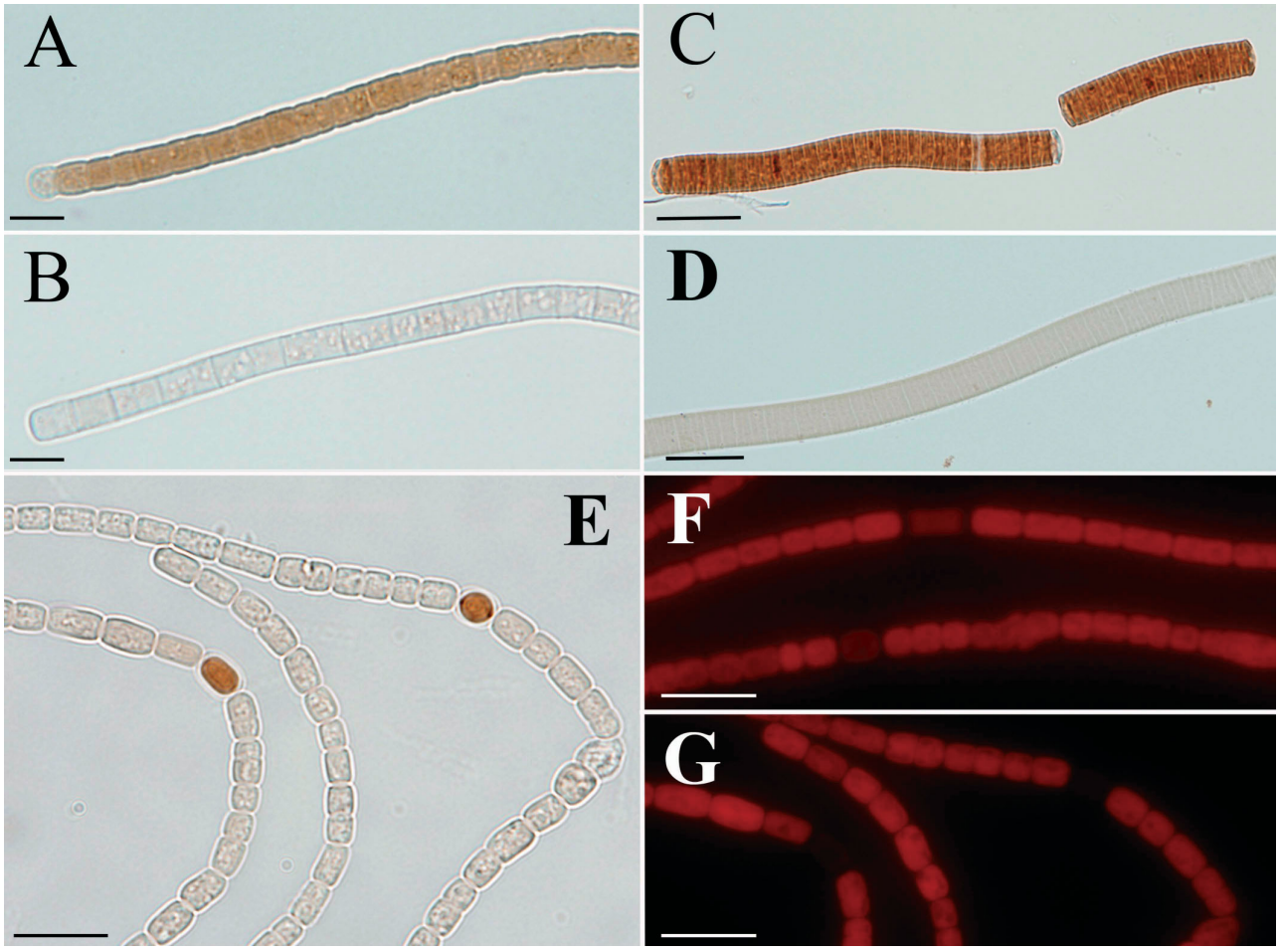

Fig. A7. Immunocytochemical detection of nitrogenase (Fe-protein) in non-heterocystous strains (A,B) *Trichodesmium* sp. NIBB1067 and (C,D) *Lyngbya* sp. 10B and (E) heterocystous strain *Anabaena cylindrica* IAM-M1, and epifluorescence microscopy images of *A. cylindrica* (F) before and (G) after permeabilization. Cells were grown (B,D) with or (A,C,E–G) without combined nitrogen. For *A. cylindrica*, 1% SDS in phosphate-buffered saline containing 10^{-1} M NaOH was used for permeabilization of cells. Scale bars = 10 µm

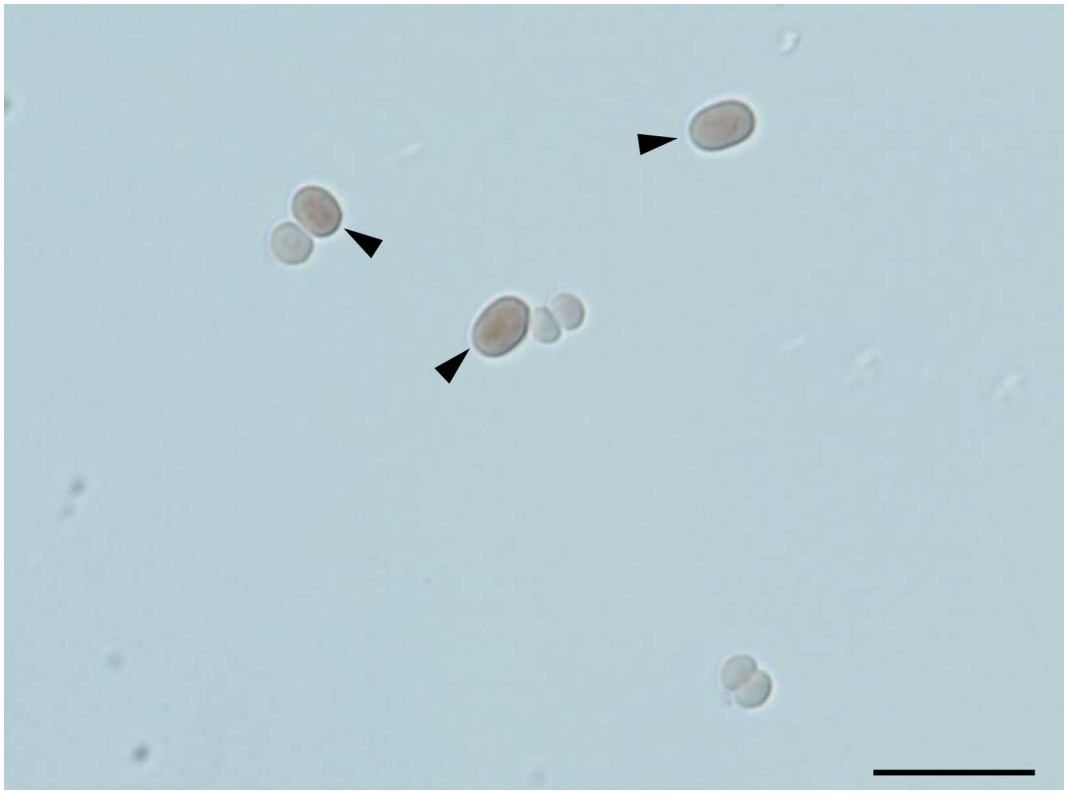

Fig. A8. Immunocytochemical detection of nitrogenase (Fe-protein) in a mixed cell suspension of the diazotrophic strain *Gloeothece* sp. 68DGA (arrowheads) and the non-diazotrophic strain *Synechocystis* sp. PCC6714. Scale bar = 10 µm