Océanographie biologique/*Biological Oceanography*

Green photosynthetic bacteria associated with the deep chlorophyll maximum of the Sargasso Sea

Jacques NEVEUX, Daniel VAULOT, Claude COURTIES and Eri FUKAI

Abstract — Very small and very abundant red-fluorescing cells were detected with ship-board flow cytometry in the deep chlorophyll maximum of the Sargasso Sea. Their concentration decreased from a maximum of 10^5 cell.ml^{-1} at 100 m to fewer than 200 cell.ml^{-1} at 250 m. They had a size of less than 0.8 μm and contained photosynthetic pigments similar to eukaryotic chlorophylls *a* and *b*, with the exception of a slight shift in the fluorescence spectra. Although, key informations on these cells are still lacking, it is very likely that they are prokaryotes and play a fundamental role in the formation and maintenance of the deep chlorophyll maximum of oligotrophic oceans.

Bactéries photosynthétiques vertes associées au maximum profond de chlorophylle de la Mer des Sargasses

Résumé — *De nombreuses petites cellules (<0,8 μm) émettant une fluorescence rouge ont été mises en évidence, par cytométrie en flux, dans le maximum profond de chlorophylle de la mer des Sargasses. Elles présentent un maximum de concentration d'environ 10^5 cellules.ml^{-1} à 100 m, puis leur densité décroît avec la profondeur jusqu'à moins de 200 cellules.ml^{-1} à 250 m. Elles semblent bien adaptées à l'utilisation des faibles éclairements et contiennent des chlorophylles dont les spectres de fluorescence dans l'acétone à 90% sont grossièrement similaires à ceux que l'on rencontre habituellement chez les algues vertes eucaryotes (c'est-à-dire chlorophylles a et b), avec toutefois un glissement des maximums d'excitation de 5 à 7 nm vers les grandes longueurs d'onde. Ces cellules sont probablement des procaryotes qui jouent un rôle fondamental dans la formation et la maintenance du maximum profond de chlorophylle en mer oligotrophe.*

Version française abrégée — En mer oligotrophe, la distribution verticale de la chlorophylle *a* (chl *a*) présente un maximum profond (MPC) à un niveau photique compris entre 1 et 0,1 % de la lumière solaire arrivant en surface. Des travaux récents ont montré que la chlorophylle *b* (chl *b*), pigment accessoire présent chez divers groupes d'algues (Chlorophyceae, Prasinophyceae, Euglenophyceae), est généralement abondante dans le MPC ([1], [2], [3]). Ce pigment considéré longtemps comme négligeable dans le milieu océanique apparaît surtout associé à l'existence de fortes concentrations de très petites cellules récemment mises en évidence par la cytométrie en flux [4].

Au cours de la campagne Chlomax sur le *N.-O. Suroît* (14 septembre-13 octobre 1987), les recherches ont porté sur la structure et l'activité photosynthétique des communautés composant le MPC du Sud-Ouest de la mer des Sargasses (Atlantique Nord-Ouest). Les chlorophylles et les phéopigments ont été déterminés par spectrofluorométrie [5]. La microscopie en épifluorescence et la cytométrie en flux [6] ont permis de dénombrer et d'étudier les caractéristiques des différentes populations cellulaires. Une séparation chromatographique des chlorophylles a été réalisée sur colonne de cellulose [7].

Tous les profils verticaux montrent un MPC accusé (0,2 à 0,4 $\mu g.l^{-1}$ contre moins de 0,1 en surface) situé entre 100 et 130 m, au sommet d'une nitracline et à la base d'une thermocline prononcée (*fig.* A, B). Dans la partie supérieure de la zone euphotique, les rapports chl *b*/chl *a* (*b*/*a*) et chl *c*/chl *a* (*c*/*a*) sont pratiquement constants (*fig.* C), puis le rapport *b*/*a* augmente fortement pour atteindre un maximum 20 à 30 m en dessous du MPC. Le rapport *c*/*a* présente une variation inverse de celle du rapport *b*/*a*, mais avec une amplitude réduite.

La cytométrie en flux a montré que les communautés de phytoplancton se caractérisent par trois types de cellules. Deux d'entre eux sont classiquement rencontrés dans les mers

Note présentée par Jean LAVOREL.

0249-6313/89/03080009 $2.00

oligotrophes et leur abondance est comparable à celles observées précédemment ([8], [9]): (1) 5.10^3 à 1.10^4 cell.ml^{-1} de petites Cyanobactéries du genre *Synechoccocus* (0,5-2 μm) mises en évidence par la fluorescence orangée de la phycoérythrine; (2) 5.10^2 à 1.10^3 cell.ml^{-1} de cellules eucaryotes de taille supérieure à 1 μm. Ces deux types dominent les communautés de phytoplancton dans la première centaine de mètres de la zone euphotique. Le troisième type est plus original et plus spécifiquement associé au MPC; les cellules n'émettent qu'une fluorescence rouge caractéristique de la chl *a* et leur taille est comprise entre 0,4 et 0,8 μm. Leurs caractéristiques d'excitation de fluorescence suggèrent qu'elles contiennent de la chl *b* comme pigment accessoire. Ce dernier type de cellule apparaît 20 à 30 m au-dessus du MPC, là où se produit l'augmentation marquée du rapport *b*/*a*. A cette profondeur, leur fluorescence et leur diffusion lumineuse sont aux limites de détection du cytomètre en flux utilisé et il n'est pas possible de dire si elles sont absentes de la couche supérieure (0-90 m) ou indétectables par l'instrument. Leur concentration au niveau du maximum varie entre 0,73 et $1,2.10^5$ cell.ml^{-1} et diminue exponentiellement en dessous du MPC (*fig.* D), alors que leur fluorescence augmente d'un facteur 10 (*fig.* D).

Des incubations *in situ* ont montré que ces cellules avaient un temps de génération net de 6 à 7 jours, ce qui est comparable à celui observé généralement pour l'ensemble de la communauté photosynthétique du MPC [12]. Leur temps de génération brut pourrait être encore beaucoup plus court, si le développement de cette population était contrôlé par une activité intense des brouteurs [13].

Ces cellules pourraient correspondre aux cellules procaryotes des types II et III observées en microscopie électronique, par Johnson et Sieburth [15]. Dans ce cas, leur composition pigmentaire les apparenterait plutôt à la nouvelle division des Prochlorophyta [16] qu'aux Cyanobactéries, comme ces auteurs le pensaient. Chisholm et coll. [4], qui ont également observé ces petites cellules dans diverses régions océaniques, montrent qu'elles ont une composition pigmentaire originale: la zéaxanthine et la chl *b* sont associées à l'α-carotène et à une chlorophylle particulière (divinyl-chl *a*) dont le maximum d'absorption dans le bleu est déplacé de 8 nm vers les grandes longueurs d'onde par rapport à la chl *a*. Un pigment analogue avait déjà été observé dans les eaux de surface de l'Atlantique nord [3], ce qui semble indiquer que la présence de ce type cellulaire n'est pas limité au MPC. Nous avons pu montrer que les caractéristiques de fluorescence de la chl *b* associée à ces petites cellules étaient elles aussi différentes de celles de la chl *b* des eucaryotes (déplacement vers les grandes longueurs d'onde des maximums d'excitation et d'émission, respectivement de 7 et 4 nm dans l'acétone à 90 %). Nous avons donc à faire à un nouveau type de procaryote photosynthétique appartenant soit à la division des prochlorophytes, soit à une autre division.

Le MPC se caractérise donc par l'existence d'une population très spécialisée, particulièrement bien adaptée aux faibles conditions lumineuses et nutritives. Intégrée sur l'ensemble de la colonne d'eau, elle représente environ 30-40 % de la biomasse chlorophyllienne et rend compte de 10 à 15 % de la production primaire totale. Contrairement aux autres Prochlorophyta qui sont associées et des habitats marginaux, ces cellules semblent largement répandues dans les océans et pourraient être les vrais précurseurs des chloroplastes des plantes.

A deep chlorophyll *a* (chl *a*) maximum (DCM) located near or below the 1 % surface solar irradiance is a general feature in oligotrophic oceans. Recently chlorophyll *b* (chl *b*), an accessory chlorophyll characteristics of green algae (Chlorophyceae, Prasinophyceae, Euglenophyceae), was found to be abundant in the DCM ([1], [2], [3]). This pigment,

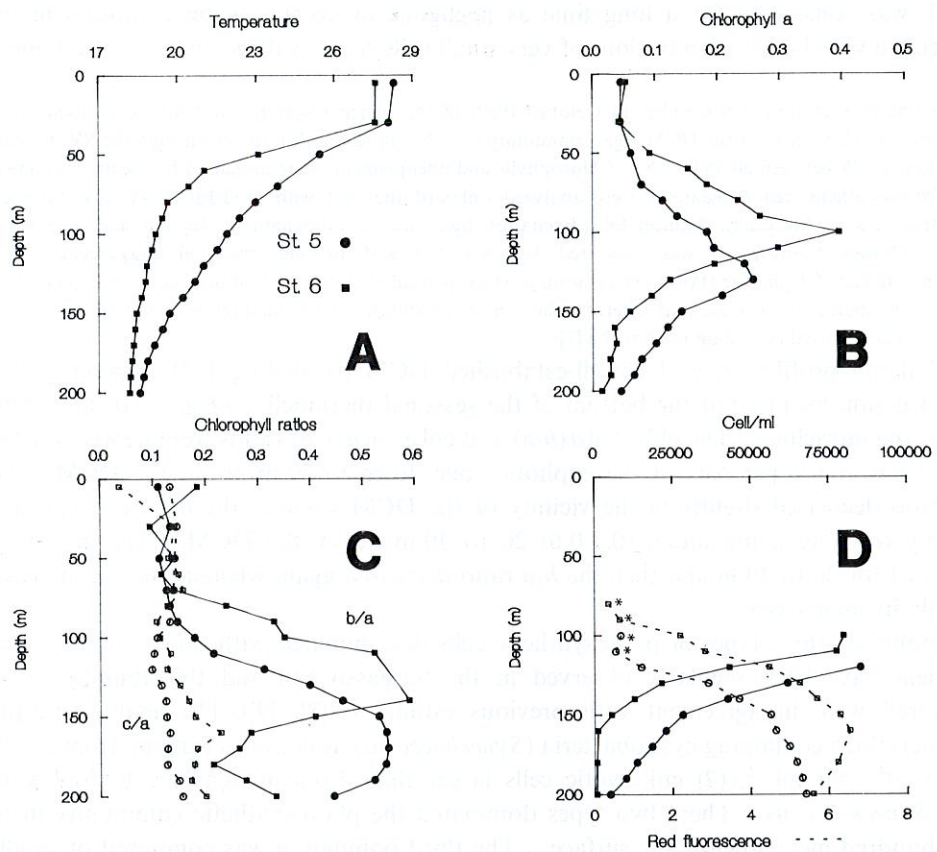
which was considered for a long time as negligible in oceanic waters, appears in fact associated with high concentrations of very small cells recently detected by flow cytometry [4].

The Chlomax cruise (14 September-13 October 1987) of the french research vessel *Suroît*, focused on the structure and physiology of the DCM algal community on a North to South transect through the SW Sargasso Sea, along 61°W between 20 and 30°N. Chlorophylls and pheopigments were measured by spectrofluorometry [5]. Photosynthetic cell populations were analyzed onboard the ship with an EPICS 541 flow cytometer (Coultronics s. a., Margency, France) [6]. Excitation light had a wavelength of 488 nm and a power of 1.5 W. Orange fluorescence was measured between 530 and 640 nm and red fluorescence above 640 nm. Beads of 1 μm size (Polysciences) were used as internal standards. Epifluorescence microscopy was used to enumerate autotrophic and heterotrophic cell populations. A chromatographic separation of chlorophylls was achieved according to Deroche [7].

All depth profiles showed a well-established DCM (0.2-0.4 $\mu g.l^{-1}$) between 100 to 130 m depth, localized at the bottom of the seasonal thermocline (*Fig.* A, B) and at the top of the nitracline. The chl *b*/chl *a* (*b*/*a*) and chl *c*/chl *a* (*c*/*a*) ratios were nearly constant (*Fig.* C) in the upper part of the euphotic zone down to 20 m above the DCM. The *c*/*a* ratio decreased slightly in the vicinity of the DCM whereas the *b*/*a* ratio increased sharply reaching a maximum (0.5-0.6) 20 to 30 m below the DCM. This maximum extended for 20 to 40 m and then the *b*/*a* ratio decreased again whereas the *c*/*a* increased slightly in some cases.

Among the three types of photosynthetic cells discriminated with flow cytometry, two of them have been regularly observed in the Sargasso Sea and the abundances we measured were in agreement with previous estimates ([8], [9]): (1) small (0.5-2 μm) phycoerythrin-containing cyanobacteria (*Synechoccocus*) at densities ranging from 5×10^3 to 1×10^4 cell.ml^{-1}; (2) eukaryotic cells larger than 1 μm at densities 10 fold lower than *Synechoccocus*. These two types dominated the photosynthetic community in the first hundred meters below the surface. The third population was composed of smaller cells containing chl *a* (fluorescence emission beyond 640 nm). They appeared 20 to 30 m above the DCM, at a depth where the *b*/*a* ratio started to increase. At this depth both their fluorescence and light scatter were very close to the detection limit of our instrument and we could not determine whether this population was absent from the upper layer, or cells too small and too faint to be recorded by flow cytometry. Their maximum concentration varied from 0.73 to 1.2×10^5 cell.ml^{-1} (*fig.* D). Below the DCM, cell concentrations decreased exponentially with depth. We established by size fractionation through Nuclepore membranes that these cells passed through a 0.8 μm membrane but were retained at 99.5% by a 0,2 μm membrane. The ratio of their right to forward angle light scatters was 5 times larger than for *Synechoccocus*, indicating that these cells have probably a higher refractive index. The ten-fold increase in red fluorescence (*i. e.* in chlorophyll content [10]) down the water column (*Fig.* D), was most probably a result of photoadaptation as observed previously for *Synechoccocus* in the mixed layer [6]. Assuming a median cell size of 0.6 μm, intracellular chl *a* concentrations could be estimated between 10 and 100 kg.m^{-3}, *i. e.* about 10 times larger than what is usually found in unicellular algae [11] suggesting that chlorophyll is very efficiently packed in these cells. Their high 488/457 excitation ratio and the fact that more than 80% of the chl *b* was recovered in the fraction below 1 μm suggested chl *b* as their major accessory chlorophyll.

Using column chromatography on cellulose [7], we analyzed chlorophylls from a sample taken at station 8 (63°20W, 26°17N; profile 840: 150 m). Pigments were characterized by their fluorescence excitation and emission spectra in 90% acetone. Besides chl *a*, we



Depth distributions at stations 5 (Circles: 26°48N, 62°32W, profile 501, 20 September 1987) and 6 (Squares: 29°37N, 61°42W, profile 606, 26 September 1987): (A) temperature (°C), (B) chl *a* ($\mu g.l^{-1}$), (C) ratios of accessory chl *b* (solid line) and *c* (dashed line) to chl *a*, (D) concentration (solid line), and average red fluorescence (chlorophyll content, dashed line) normalized to that of 1 μm beads, of small red-fluorescing cells measured by flow cytometry; symbols tagged with asteriks refer to samples for which the small cells could not be completely distinguished from noise, in which case their average red fluorescence was overestimated since cells with very low fluorescence were not taken into account.

Variation de divers paramètres en fonction de la profondeur aux stations 5 (*Cercles:* 26°48*N*, 62°32*W*, *profil* 501, 20 *septembre* 1987) *et* 6 (*Carrés:* 29°37*N*, 61°42*W*, *profil* 606, 26 *septembre* 1987): (*A*) *température* (°*C*), (*B*) *chl a* ($\mu g.l^{-1}$), (*C*) *rapports pigmentaires chl b/chl a* (*ligne continue*) *et chl c/chl a* (*ligne brisée*), (*D*) *concentration* (*ligne continue*) *et fluorescence moyenne dans le rouge* (*ligne brisée*) *de ces petites cellules* $<0,8$ μm *mesurées au cytomètre de flux; la fluorescence est normalisée par rapport à celle de billes de* 1 μm.

also found a chl *a*-like pigment with a fluorescence excitation maximum shifted about 5 nm toward higher wavelengths (437 nm instead of 432 nm) and a fluorescence emission spectrum nearly identical to that of chl *a*. Chl *b* also showed spectral shifts of its fluorescence excitation (466 nm instead of 459 nm) and emission (659 instead of 655 nm) maxima, suggesting that this pigment was not really chl *b* but rather a chl *b*-like pigment.

In situ incubations of bottle-confined samples demonstrated potential changes in the concentration of these cells over 24 hrs. ranging from -7 to 40% with nearly identical average values at 120 m (11%, $n=4$) and 140 m (12%, $n=5$). This corresponds to an average net population doubling time of 6-7 days, which is consistent with previous estimates of global phytoplankton growth rates in the DCM [12]. However actual cell

generation time might be shorter if grazers are actively controlling population development [13].

What are these cells? The three main features we observed were: (1) their small size (0.2-0.8 μm), (2) their chl *b*-like pigmentation and (3) their high concentration (10^5 cell. ml^{-1} in the DCM). They are probably not chl *b* containing eukaryotes since recent analysis have established that the carotenoids usually associated with Chlorophyceae and Prasinophyceae are not observed in abundance in the DCM ([3], [4]). Using transmission electron microscopy, Johnson and Sieburth [15] observed at the same location that the deep photosynthetic community was dominated by two types of prokaryotes (Types II: diameter = 0.6-0.9 μm, and III: diameter = 0.4-0.5 μm), which mainly occurred in oceanic samples and primarily at 100 m depth. It is likely that these cells and those we described here are identical. Their pigment content characteristics make it highly probable that they are not Cyanobacteria as Johnson and Sieburth [15] initially thought, but are rather related to the recently founded division of Prochlorophyta [16], which is characterized by chl *b* ([17], [18]) as the main accessory pigment. HPLC analyses of carotenoids in the DCM have indicated the presence of zeaxanthin [3] which characterizes some groups of Cyanobacteria like *Synechoccocus* but also prochlorophytes.

Similar conclusions have been recently reached by Chisholm *et al.* [14] who observed the same type cells in various marine areas of the N. Atlantic and Pacific. They showed that these cells exhibited an unusual pigment pattern: zeaxanthin and chl *b* were associated with a divinyl-chl *a*-like pigment and α-carotene. The divinyl-chl *a*-like pigment appears similar to that observed by Gieskes and Kraay [3] in surface waters of the North Atlantic, suggesting that the small red-fluorescing cells are not restricted to the DCM. In addition, we also found that the chl *b* of these small cells is probably different from the eukaryotic one. In summary, these cells contain at least three main pigments (α-carotene, divinyl-chl *a*-like and chl *b*-like pigments) which differ from those of known Prochlorophyta, suggesting that we are in presence of a novel cell type belonging probably to a new division of the photosynthetic prokaryotes.

The discovery of these very small cells has important implications from both ecological and evolutionary standpoints. First these findings invalidate previous theories of DCM formation, such as the sedimentation hypothesis, and previous assessments of the nature of the picoplanktonic community in the DCM ([8], [9]). The cells we found form a very specialized population which is particularly well adapted to low light conditions through their pigment composition. They represent up to 30-40% of the integrated water column chlorophyll and from 10 to 15% of the integrated primary production; they certainly account for a higher proportion of the new nitrate-based production. Second, these small cells might be mistaken for heterotrophic bacteria when DNA staining techniques are used to routinely estimate concentrations of the latter, since their red fluorescence is too weak to be detected by epifluorescence microscopy. In fact, during the Chlomax cruise, estimates of DAPI-stained bacteria in the DCM were of the same order of magnitude ($1\text{-}1.40 \times 10^5$ cell. ml^{-1}) than the small prochlorophytes. Lastly, the two types of Prochlorophyta previously found are restricted to fairly marginal habitats ([16], [17], [18]), while this new type, if it is confirmed that it is a Prochlorophyta, appears to be widespread in the oceans [14]. Localized in a very stable environment, it could be the true precursor of plant chloroplasts. However full investigation on the nature, physiology, taxomony and evolutionary role of these cells must wait until attempts to cultivate them are successful.

This work was supported by the Centre national de la Recherche scientifique (I.N.S.U., GRECO n° 34, U.A. n° 117) and the University Pierre-et-Marie-Curie. We thank C. Giguet from Coultronics S.A. for technical assistance. We are grateful to the officers, crew members and scientists of the research vessel *Suroît* (IFREMER) for their cooperation during the Chlomax cruise.

Note remise le 11 août 1988, acceptée après révision le 30 novembre 1988.

REFERENCES

[1] S. W. JEFFREY, *Mar. Biol.*, 37, 1976, pp. 33-37.

[2] J. NEVEUX and G. DE BILLY, *Deep Sea Res.*, 33, 1986, pp. 1-14.

[3] W. W. C. GIESKES and G. W. KRAAY, *Mar. Biol.*, 91, 1986, pp. 567-576.

[4] S. W. CHISHOLM, R. J. OLSON, E. R. ZETTLER, J. WATERBURY, R. GOERICKE and N. WELSCHMEYER, *Nature*, 334, 1988, pp. 340-343.

[5] J. NEVEUX and M. PANOUSE, *Archiv. Hydrobiol.*, 109, 1987, pp. 567-581.

[6] R. J. OLSON, D. VAULOT and S. W. CHISHOLM, *Deep Sea Res.*, 32, 1985, pp. 1273-1280.

[7] M. E. DEROCHE, *Chim. anal.*, 53, 1971, pp. 704-709.

[8] H. E. GLOVER, A. E. SMITH and L. SHAPIRO, *J. Plank Res.*, 7, 1986, pp. 519-535.

[9] L. S. MURPHY and E. M. HAUGEN, *Limnol. Oceanogr.*, 30, 1985, pp. 47-58.

[10] P. H. BURKILL, *Microbes at sea*, M. A. SLEIGH Ed., Ellis Horwood Ltd, Chicester, England, 1987, pp. 139-166.

[11] A. MOREL and A. BRICAUD, *Can. Bull. Aquat. Sci.*, 214, 1988, pp. 521-559.

[12] R. W. EPPLEY, E. SWIFT, D. G. REDALJE, M. R. LANDRY and L. W. HAAS, *Mar. Ecol. Prog. Ser.*, 24, 1988, pp. 289-301.

[13] L. CAMPBELL and E. J. CARPENTER, *Mar. Ecol. Prog. Ser.*, 33, 1986, pp. 121-129.

[14] R. GOERICKE and N. A. WELSCHMEYER, *E.O.S.*, 68, 1987, pp. 1706.

[15] P. W. JOHNSON and J. MCN SIEBURTH, *Limnol. Oceanogr.*, 24, 1979, pp. 928-935.

[16] R. A. LEWIN, *Nature*, 261, 1976, pp. 697-698.

[17] R. A. LEWIN and N. W. WITHERS, *Nature*, 256, 1975, pp. 735-737.

[18] T. BURGER-WIERSMA, M. VEENHUIS, H. J. KORTHALS, C. C. M. VAN DE WIEL and L. R. MUR, *Nature*, 320, 1986, pp. 262-264.

J. N.: *Laboratoire Arago, Université Pierre-et-Marie-Curie*, 66650 *Banyuls-sur-Mer;*

D. V. et C. C.: *C.N.R.S., LP* 4601, *Station biologique*, 29211 *Roscoff;*

E. F.: *C.E.R.O.V., Station zoologique*, 06230 *Villefranche-sur-Mer.*