BIODIVERSITY OF UNICELLULAR ALGAE: EXAMPLE OF PICO-AND ULTRAPLANKTONIC EUCARYOTES OF THE THAU LAGOON

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ABSTRACT. – Pico- and ultraplanktonic eucaryotes of the Thau Lagoon were investigated using flow cytometry and electron microscopy. Picoplanktonic species mostly belong to the Prasinophyceae (Chlorophyta). In such a size range, i.e. around 2 µm, they demonstrate a high diversity for a coastal community with seven different cell types. They appear dominant inside the Thau Lagoon, while procaryotic picoplankters (cyanobacteria) are more abundant outside the Lagoon. In the case of *Ostreococcus tauri*, the most abundant picoplankter, pigment analysis and molecular data were necessary to properly assign it to its taxonomic class. Ultraplanktonic forms include representatives of cryptophytes, rhodophytes, diatoms and chlorophytes. Very little is known about sexuality and life-cycles of these tiny algae that reproduce mainly as vegetative cells. A whole set of complementary techniques thus appears necessary to ensure a reliable identification and to assess the diversity of unicellular algae.

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RÉSUMÉ. – Les eucaryotes pico- et ultraplanctoniques de l'étang de Thau ont été étudiés à l'aide de la cytométrie en flux et de la microscopie électronique. Les espèces picoplanctoniques appartiennent pour la plupart aux Prasinophycées (Chlorophytes). Dans cette gamme de taille, c'est-à-dire aux environs de 2 µm, elles montrent une diversité élevée pour une communauté côtière, avec sept types cellulaires différents. Elles apparaissent dominantes à l'intérieur de l'étang, alors que les procaryotes picoplanctoniques (cyanobactéries) sont plus abondants à l'extérieur de l'étang. Des analyses pigmentaires et les données de la biologie moléculaire se sont avérées nécessaires pour placer correctement *Ostreococcus tauri* Courties et Chrétiennot-Dinet dans sa classe taxonomique. Les formes ultraplanctoniques sont représentées par des Cryptophytes, des Rhodophytes, des Diatomées et des Chlorophytes. On connaît peu de choses sur la reproduction et les cycles de vie de ces algues de très petite taille qui se multiplient principalement par voie végétative. Un ensemble de techniques complémentaires apparaissent aujourd'hui nécessaires pour obtenir une identification correcte de ces algues unicellulaires et permettre l'estimation de leur diversité.

INTRODUCTION

The algal community composed of minute eucaryotic phytoplankters – excluding the procaryotic cyanobacteria and the so called marine 'prochlorophytes' lacking a nucleus (Lewin 1976; Urbach *et al.* 1992) – has been poorly understood for a long time, and remains somewhat enigmatic mainly because of the lack of tools available for their detection and identification. Such cells are classified as pico- (< 2-3 µm) and ultra- (< 10 µm) planktonic (Thomsen 1986). Since Johnson & Sieburth (1982) revealed the presence of different very small eucaryotes on thin sections of samples from oceanic areas, picoeucaryotes have been recognized in many places, as reviewed by Stockner (1988). Limitations in the study of picoplankton are summarized in Sieburth & Johnson (1989), and recent ultrastructural studies of natural populations are rather scarce (Sieburth & Johnson 1989; Hargraves *et al.* 1989; Corpe & Jensen 1992). Cultures of these organisms are currently a necessity for a complete study including pigment

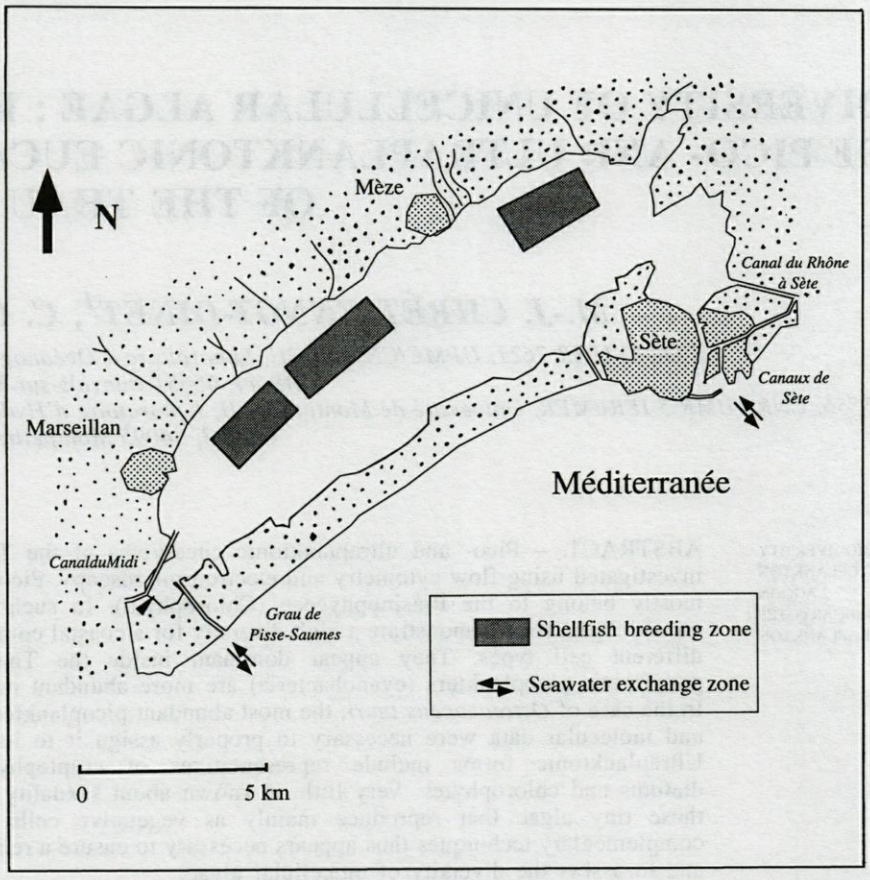

Fig. 1. – Schematic representation of the Thau Lagoon showing the exchange zones with Mediterranean waters and shellfish location.

analysis, ultrastructure and eventually molecular data for new taxa (Andersen *et al.* 1993, Shimada *et al.* 1995a). Selective methods, such as the serial dilution culture (Throndsen, 1993) or size fractionation and cultures in adapted media (Keller *et al.* 1987) led to isolation of picoeucaryotes, mostly coccoid cells. Several marine species have been recently described and new genera introduced, such as *Bathycoccus* (Eikrem et Throndsen 1990), *Resultor* (Moestrup, 1991), *Pycnococcus* (Guillard *et al.* 1991), *Prasinococcus* (Miyashita *et al.* 1993), *Ostreococcus* (Chrétiennot-Dinet *et al.* 1995) and *Prasinoderma* (Hasegawa *et al.* 1996) for the Chlorophyta; *Aureococcus* (Sieburth *et al.* 1988) and *Pelagomonas* for the new class Pelagophyceae (Andersen *et al.* 1993) within the Chrysophyta. Although fluorescence microscopy is still in use for the study of natural picoplankton (Kuylenstierna & Karlson 1994), the adaptation of flow cytometry to the marine environment (Frankel *et al.* 1990) allows a better detection and enumeration of the smallest photosynthetic cells based on their fluorescence properties and cell size and shape. This was the case for procaryotes, particularly *Prochlorococcus marinus* discovered in the Atlantic Ocean (Chisholm *et al.* 1992) but also found in the Pacific Ocean (Shimada *et al.* 1993, 1995b) and in the Mediterranean waters (Vaulot *et al.* 1990), but also for picoeucaryotes (Li *et al.* 1992; Li 1995; Blanchot & Rodier 1996; Partensky *et al.* 1996). Nevertheless a precise identification of these populations is usually lacking and has only been possible in a few occasions (Chrétiennot-Dinet *et al.* 1995). Increasing attention has been paid to pigments of natural populations and HPLC techniques greatly improved our knowledge on pigment signatures, particularly for coccoid strains (Hooks *et al.* 1988; Fawley 1992). An attempt to better characterize oceanic picoeukaryotes was recently carried out on a wide range of strains obtained in culture (Simon *et al.* 1994).

In the Thau Lagoon, situated on the Mediterranean French coast and used for oyster production (Fig. 1), no picoplankton was mentionned until flow cytometric analyses revealed the great abundance of a picoeukaryotic population (Courties *et al.* 1994; Vaquer *et al.* 1996). We report here on a study focused on the diversity of small eucaryotes from this coastal marine lagoon.

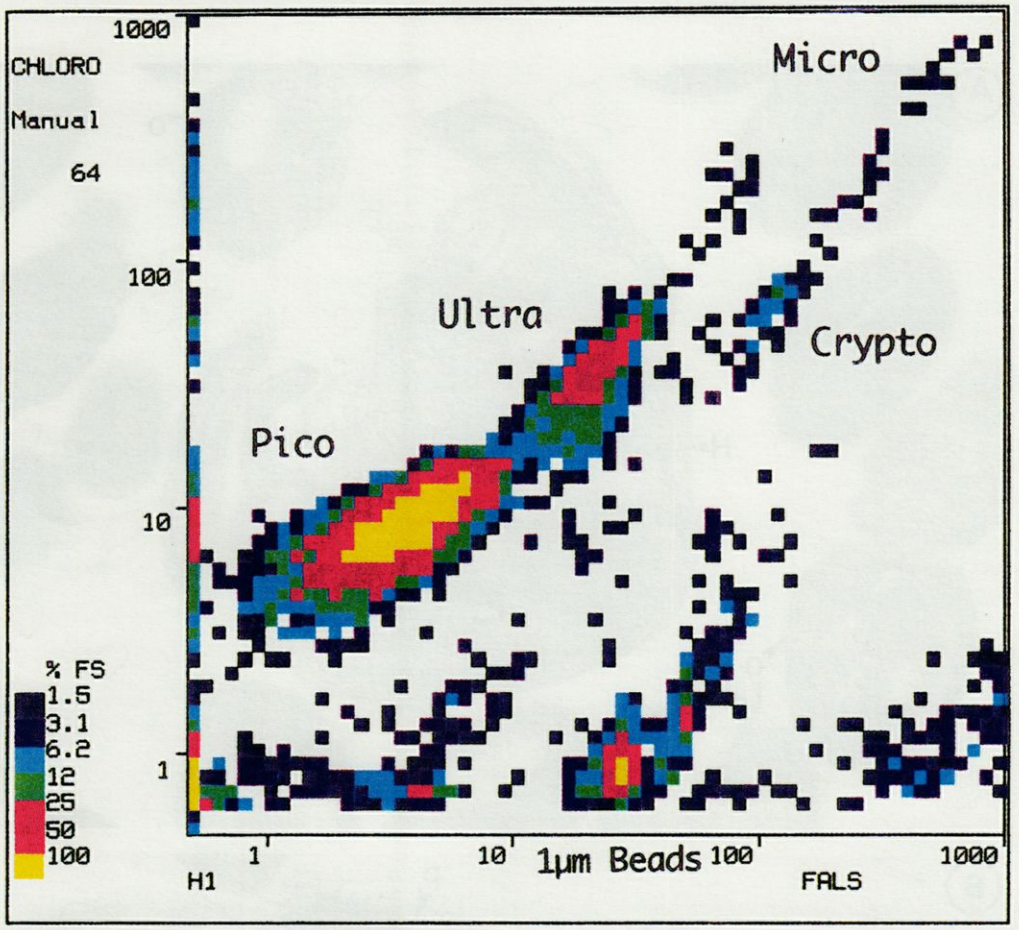

Fig. 2. – Flow cytometric analysis of a natural phytoplankton sample from the Thau Lagoon. Cells were discriminated according to light scatter (FALS), abscissa and red fluorescence (CHLORO), ordinate. Internal standard is given by 1 µm fluorescent beads (1 µm Beads). Most of the cells are picoplanktonic (Pico) with a typical signature, almost identical to that of *Ostreococcus tauri*. Ultraplankton (Ultra) is also present and Cryptophyceae (Crypto) are clearly delineated. Microplankton (Micro) is less abundant and without specific information.

MATERIAL AND METHODS

Flow cytometry

Phytoplanktonic populations were screened by flow cytometry on living material. For detailed protocols, see Troussellier *et al.* (1993) and Chrétiennot-Dinet *et al.* (1995). Cytograms were established for the determination of taxonomic groups, according to their pigment composition (chl *a* versus phycobilins for example) and size.

Cultures

Cell cultures were initiated on a F/2 medium modified for nutrient concentration and removal of silicate (Chrétiennot-Dinet *et al.* 1995). Size fractionation on Nuclepore membranes (porosity : 3 µm, 1 µm and 0.8 µm) was carried out on a crude sample prior to inoculation in culture medium.

Electron microscopy

Mono- or plurispecific cultures were obtained after one or two weeks and checked by light microscopy and/or flow cytometry. Selected cultures were pre-fixed in glutaraldehyde (final concentration 1 %), then centrifuged at 2 650 g and the cell pellets were embedded in agar before treatment for electron microscopy (Chrétiennot-Dinet *et al.* 1995). Thin sections were later examined with a Hitachi H 600 electron microscope for identification.

RESULTS

Identification of a chl *a*-containing population, whose size was below the 1 µm beads as determined by flow cytometry (Fig. 2), was initially attributed to a very small eukaryote (Courties *et al.* 1994), later described as *Ostreococcus tauri*

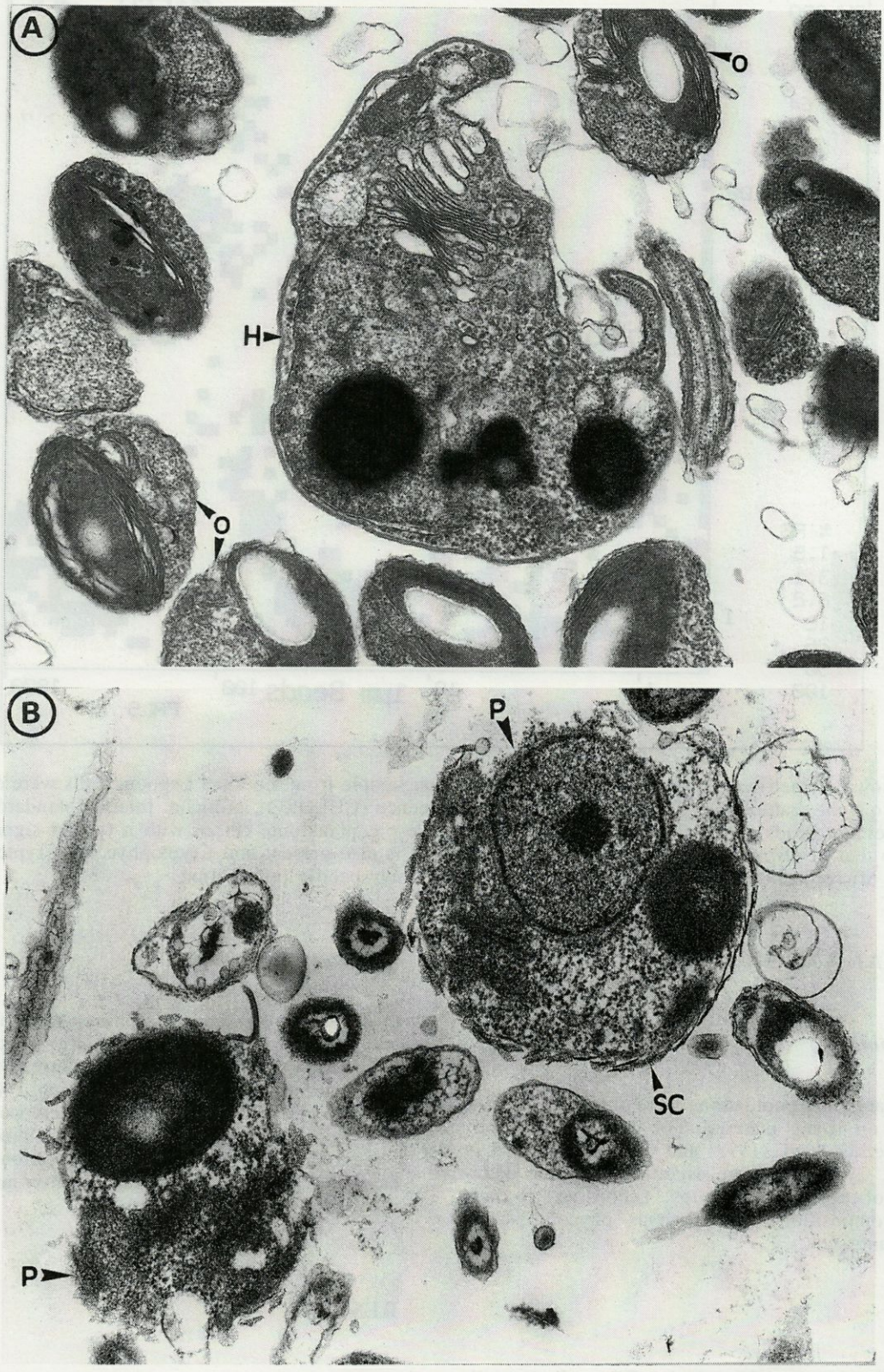

Fig. 3. – A, Ultrathin section of a mixed culture containing the picoplanktonic *Ostreococcus tauri* (O) and an ultraplanktonic *Hemiselmis* species (H), transversally sectioned. Magnification : × 31 200. B, Ultrathin section of two prasinophytes (P) belonging to the Mamiellales because of their scaly covering (SC). Magnification : × 31 200.

Courties et Chrétiennot-Dinet (Chrétiennot-Dinet *et al.* 1995) (Fig. 3a). A full description of this organism was only possible after production of a culture allowing examination of its ultrastructure and pigment composition. A preliminary gene sequence analysis was necessary to place it within the Prasinophyceae. Thin sections revealed the presence of starch that clearly placed it in the Chlorophyta but the absence of scales was misleading. Pigments indicated affinities with Prasinophyceae, although prasinoxanthin, usually present in Prasinophyceae (Foss *et al.* 1986), was absent. Gene sequence analysis placed it close to *Mantoniella* (Courties *et al.* in prep.), a typical Prasinophyceae belonging to the family Mamiellaceae (Moestrup 1984). Further work carried out on other cultures led to a more careful examination of the pico- and ultraplanktonic populations (Fig. 3). Seven different cell types of picoeucaryotes were identified on thin sections. Some of them were scaly and belonged unambiguously in the Prasinophyceae (Fig. 3b). Among them, one type was identified as *Bathycoccus prasinos*, described from Naples waters (Eikrem & Throndsen 1990). Others were close to *Resultor mikron* (Moestrup 1991) and to *Micromonas pusilla* (Butcher) Manton et Parke (1960) or were reminiscent of *Pycnococcus* Guillard (Guillard *et al.* 1991) if not unidentified species. They will be described in detail in another publication (Chrétiennot-Dinet *et al.* in prep). Ultraplanktonic cells belonged to different taxonomic groups : diatoms were represented by *Skeletonema* cf. *costatum* (Grev.) Cleve (Medlin *et al.* 1991), *Thalassiosira conferta* Hasle and *Thalassiosira stellaris* Hasle et Guillard; two Prasinophyceae were found in sections and identified from their scale pattern : *Pyramimonas cirolanae* Pennick and *Pyramimonas* cf. *grossii* Parke. We have also to mention a very small Cryptophyceae (Fig. 3a) belonging to the genus *Hemiselmis* Parke (Parke 1949) and the unicellular Rhodophyceae *Porphyridium purpureum* (Bory) Drew et Ross (Ott 1987).

These populations are present all year long and are replaced outside the lagoon by cyanobacteria (Vaquer *et al.* 1996). Their relative importance is related to the season, but from chl.*a* concentrations, they represent about 1/3 of the chlorophyll biomass (Chrétiennot-Dinet *et al.* 1995, Vaquer *et al.* 1996).

DISCUSSION

Biodiversity of algae in general is difficult to evaluate (Norton *et al.* 1996) but biodiveristy of picoeucaryotes in particular is far from being fully catalogued. Results presented here indicate a high number of small cell types for a coastal area, in the lowest size range. Most studies on picoplankton come from oceanic waters and assert the dominance of procaryotic cells (Johnson & Sieburth 1979; Iturriaga *et al.* 1986) with an apparently low diversity because of the dominance of *Prochlorococcus* and *Synechococcus* in the water column. Very little is known about the number of species of picoeucaryotes in coastal areas. Described species were obtained as clonal cultures, sometimes after toxic bloom events, as for *Aureococcus* (Sieburth & Johnson 1989) or after an oil spill with *Pelagococcus* (Throndsen & Kristiansen 1982). In coastal and estuarine environments, ultraplankton dominance is often considered as indicative of a stressed environment (Shapiro & Guillard 1986). A characteristic feature of the Thau Lagoon is the dominance of picoeucaryotes over the procaryotes, mainly represented by cyanobacteria. Outside the lagoon, cyanobacteria (*Synechococcus*) are abundant and dominate the picoplanktonic fraction. On the contrary, the abundance and diversity of picoeukaryotes is noteworthy inside the lagoon. Why do we have so little information on coastal picoeucaryotes ? Because their detection is difficult, specially in coastal waters where procaryote numbers usually dominate at all depths (Shapiro & Guillard 1986). Autofluorescence was used for a long time to enumerate photosynthetic cells in light microscopy but special techniques were developed for smaller cells (Booth 1987). Picoeucaryotes are easily overlooked because of their faint fluorescence and *Ostreococcus*, for example, cannot be detected by light microscopy. Epifluorescence added to our knowledge of micro-organisms (Davis & Sieburth 1982; Haas 1982), however flow cytometry proved to be invaluable for detection and quantification of picoplankton, particularly for picoeucaryotes as mentioned previously. Immunological methods are now applied to marine species, for the detection of ultraplankton (Campbell *et al.* 1994) and antibodies probes, or nucleic acids probes are in use for toxic species (Anderson 1995). Oligonucleotide probes for marine eucaryotes were recently attempted (Lim *et al.* 1993), and taxon-specific probes tested (Simon *et al.* 1995; Lange *et al.* 1996). But information is also scarce because cultures are necessary for a complete study including ultrastructure, pigment signature and eventually gene sequence analysis. All these techniques require special equipment and expertise in the field, and comprehensive studies are therefore more difficult to achieve. Finally, these tiny cells reproduce only by vegetative division and have very few morphological features that can be used to assess their phylogenetic position. The absence of sexuality raises the concept of species, discussed in an ecological context by Wood & Leatham (1992). According to Manhart & McCourt (1992), morphological, biological or phylogenetic species can be defined. As for bac-

teria, light microscopy does not allow an adequate estimation of biodiversity and our results clearly show that a whole set of techniques is required for a proper identification. Estimation of ecological importance and biodiversity of picoeucaryotes is just in its infancy, in coastal as in open waters, and will only be achieved if adequate tools for investigation are provided and if a team of specialists, including trained taxonomists, is working in the field.

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