Vol. 9: 259-266, 1995 | AQUATIC MICROBIAL ECOLOGY Aquat microb Ecol | Published December 21

A simple method for the quantification of a class of labile marine pico- and nano-sized detritus: DAPI Yellow Particles (DYP)

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ABSTRACT: A simple and rapid method for microscopic quantification of an abundant class of labile pico- and nano-sized detrital particles (0.2 to 20 µm), using the fluorochrome stain DAPI, is described. Using an appropriate UV-filter set, examination of DAPI-stained samples revealed not only blue (DNA-containing) but also yellow particulate matter. We showed that DAPI yellow fluorescing particles resembled detritus made from plankton tow material or copepod faecal pellets and were almost exclusively organic, enzyme-degradable matter. Quantification of the total surface area of these particles in the Ligurian Sea (NW Mediterranean) revealed stocks averaging 80 mm^2 l^{-1} in surface layer waters. The possible origin and fate of these pico- and nano-sized DAPI Yellow Particles (DYP) are discussed.

KEY WORDS: Detrital particles · Detritus · Staining · DAPI Yellow Particles (DYP)

INTRODUCTION

Since at least as long ago as the early 1970s, it has been recognized that detritus in the water column ranges in size from colloids to macroscopic aggregates (Riley 1970) and that particle abundances are inversely related to particle size (e.g. Sheldon et al. 1972). In the past decade, however, attention has been focused on either macroscopic particles such as marine snow (e.g. Alldredge & Silver 1988), faecal pellets (Fowler & Knauer 1986), and phyto-aggregates (e.g. Lochte & Turley 1988) or submicronic particles (e.g. Koike et al. 1990, Sieracki & Viles 1992) and colloids (e.g. Wells & Goldberg 1994). Particles falling between the 2 extremes, i.e. microscopic particles (literally those visible using a light microscope), have received little attention in recent years, with the exception of a few studies on the production of faecal matter by protists (Gowing & Silver 1985, Nöthig & von Bodungen 1989, González 1992) and of 'Transparent Exopolymer Particles' (TEP) (Wiebe & Pomeroy 1972, Alldredge et al. 1993, Logan et al. 1994, Passow & Alldredge 1994).

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Studies of non-living organic particles in the pico- and nano-size range (largest dimension 1 to 20 µm) have been hampered by the lack of simple methods for enumeration and characterization. Earlier investigations, while underlining the probable importance of such particles and showing most of them to be organic, relied upon tedious, time-consuming, and expensive techniques such as histochemical staining (Gordon 1970a) or electron microscopy (Simpson 1982).

Here we present a rapid, simple method to quantify an abundant class of small, reactive and DAPI-yellow-fluorescing matter. The fluorescent dye DAPI (4'6'-diamidino-2-phenylindole), originally developed as an antibiotic for trypanosomes, is widely used today to enumerate bacteria (e.g. Turley 1993) which become visible due to a bright blue fluorescence of ultraviolet-excited DAPI conjugated with A-T rich nucleic acids (Lin & Alfi 1976, Lin et al. 1977). However, DAPI is not entirely specific for DNA but is known to bind to matter such as bacterial slime trails, flagellate mucocysts and polyphosphate granules (Coleman 1980). Recently, the usual DAPI protocol was described as yielding overestimates, by an order of magnitude, of concentrations of chromosome-containing bacterial cells, because of non-DNA-specific blue fluorescence

(Zweifel & Hagström 1995). In preparations for bacterial counts, besides bright blue fluorescing particles, yellow-fluorescing particles are often visible and have been assumed to represent detrital matter (Porter & Feig 1980). We describe the use of DAPI to enumerate small yellow-fluorescing particles and the results of experiments designed to determine their nature.

To examine the origin of the yellow fluorescence, we examined natural and artificially produced detritus with and without DAPI staining, and with and without fixation. We investigated the composition of DAPI Yellow Particles (DYP) by examining the effect of enzymatic degradation on DYP. We assessed the variability of DYP enumeration in subsamples and in replicate water samples. Finally we determined size-class distributions of DYP in the water column of a coastal oligotrophic system to estimate the total stock size of pico- and nano-sized DYP and the relative importance of different size classes of pico- and nano-sized DYP.

MATERIALS AND METHODS

Characterisation of DYP. Preliminary observations on naturally occurring particulate matter and phytodetritus from a stationary phase culture of the diatom *Navicula* were made using the standard DAPI (4'6'-diamidino-2-phenylindole) protocol (Porter & Feig 1980) and formalin-fixed material. Following these observations, 3 different types of particulate matter were examined with and without DAPI staining, with and without fixation, and before and after enzymatic digestion. The particulate matter was (1) naturally occurring pico- and nano-detritus, (2) artificial detritus from desiccated, sonicated, plankton tow material, and (3) artificial detritus from ground, sonicated, copepod faecal pellets. The enzymatic digestion employed exposure to a cocktail, designed to break down most cellular structures (see Sambrook et al. 1989). Enzyme cocktail ingredients were added to small volumes of water containing particles (3 to 10 ml) to yield final sample concentrations of 2 mg ml^{-1} each of lysozyme and proteinase K, 25 mM Tris HCl, 0.5% SDS (sodium dodecyl sulphate) and 1 mM EDTA (pH = 8). For all 3 different types of particulate matter, the possible negative effects of the enzyme solution on DAPI was checked by adding fresh substrate and DAPI to an enzyme-degraded sample and preparing a slide.

Natural pico- and nano-detritus: Ten ml aliquots from a single surface water sample from Point 'B' in Villefranche Bay, France (43°41'10" N, 7°19'00" E) were distributed into 15 vials. Three vials received no treatment, 3 vials received formalin (final conc. 3%), 3 vials received DAPI alone (final conc. 0.25 µg ml^{-1}), 3 vials received both DAPI and formalin, and 3 vials received 1.27 ml of the enzyme cocktail. The vials with the enzyme cocktail were placed in an incubator at 37°C for processing the next day. The remaining vials were processed immediately following the protocol described below.

Ten ml was filtered onto 25 mm diameter 0.2 µm pore size black Nuclepore polycarbonate membranes using low vacuum (<0.2 bar). Filters were placed on a slide on top of a drop of low-fluorescence immersion oil, a drop of oil was added and was then covered with a cover slip. Slides were examined using a Zeiss Axiophot T/R with epifluorescence equipment, a 3Fl reflector, a DAPI filter set 487902: BP365, FT395, LP420, and a 100× Neofluar objective. A filter transect was scanned and yellow fluorescing particles in different size categories (0.2–2, 2–5, 5–10, 10–15, 15–20, 20–25, and ≥25 µm) noted. The vials containing the enzyme cocktail were processed after 24 h incubation. Samples were cooled for 10 min at −10°C, DAPI was added and the samples were then filtered and examined as above.

Detritus from desiccated plankton tow material: Organisms and particulate matter were collected in Villefranche Bay using a plankton net with 50 µm mesh size. The material was concentrated on a GF/F filter and stored in an oven at 60°C for 13 d. The resulting powder was sieved through a 280 µm sieve to eliminate large particles and 0.2 g of the sieved powder was added to 100 ml of 0.2 µm filtered and autoclaved seawater. This solution was sonicated for 10 min and then filtered through 30 µm mesh Nitex. The filtrate was used as a solution of detrital particles. Nine vials received 3 ml each of the detrital solution; of these 3 vials were not treated, 3 vials received DAPI, and 3 vials received enzyme cocktail reagents. Slides were prepared immediately from the first 6 vials, as described above, and the enzyme-added samples were processed the following day after 24 h of incubation at 37°C.

Detritus from faecal pellets: Three liters of plankton tow material was left undisturbed for 1 h to allow faecal pellet production and sedimentation. Supernatant water was removed and the sediment matter filtered through a 190 µm mesh size sieve to eliminate macroplankton, and then allowed to settle again. After 4 h, supernatant water was removed and the sedimented material drawn off and, after examination using a dissecting microscope, dispensed into a Teflon piston to be ground. The ground faeces and plankton was diluted with 100 ml of seawater (previously passed through a 0.2 µm filter and autoclaved). The solution was then sonicated for 10 min. Nine vials were prepared and processed as in the paragraph above.

Estimating *in situ* concentrations of DYP. ***Standard protocol:*** The standard protocol adopted for natural

samples consisted first of formalin fixation (3% final conc.), then DAPI was added to a 10 ml aliquot (final conc. 0.25 µg ml^{-1}), and the sample immediately drawn down onto a 25 mm black Nuclepore polycarbonate membrane (0.2 µm pore size) using low vacuum (<0.2 bar). As described above, the filter was placed on a slide, examined using a DAPI filter, and particles enumerated in different size classes (0.2–2, 2–5, 5–10, 10–15, 15–20, 20–25, and ≥25 µm). Fields were examined until a minimum of 100 particles in each size class <15 µm (0.2–2, 2–5, 5–10, 10–15 µm) were enumerated. To calculate the surface area of particles they were considered as circles with diameters as following for each of the above size classes, respectively: 1.25, 3.5, 7.5, 12.5, 17.5, 22.5 µm; for the particles ≥25 µm, diameters were measured for each particle.

Sampling error: To examine sampling error, 5 replicate Niskin bottle casts were taken from 20 m depth at Point 'B'. One 10 ml aliquot was taken each time except for the last bottle from which 3 subsamples were taken. DYP particles were counted in different size classes and standard errors, as percentage of mean concentration, calculated for the average concentrations found.

Spatial variability: Samples for DYP were taken at 4 stations on May 12, 13, and 17, 1993, along a transect running perpendicular to the coast in the Ligurian Sea (Fig. 1). At each station 3 depths were sampled, corresponding to the thermocline, chlorophyll maximum, and deep water. Sample aliquots (10 ml) were formalin-fixed and processed as given above.

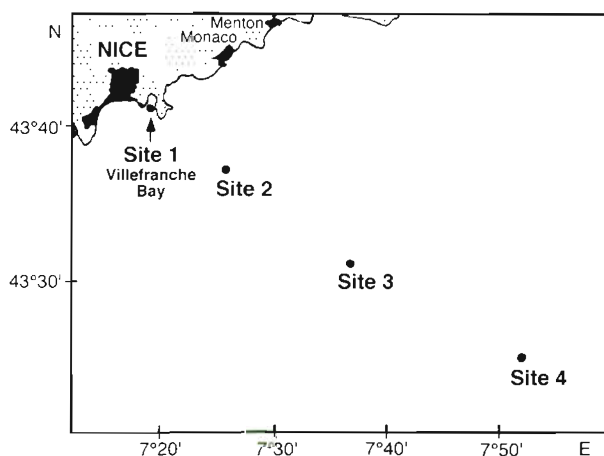

Fig. 1. Sampling sites in the Ligurian Sea (NW Mediterranean). Site 1: mouth of Villefranche Bay (43°41'10" N, 07°19'00" E); Site 2: 5.5 nautical miles offshore (43°37'45" N, 07°26'05" E); Site 3: 15.5 nautical miles offshore (43°31'50" N, 07°37'80" E); Site 4: 28 nautical miles offshore (43°24'70" N, 07°52'30" E)

RESULTS

Characterisation of DYP

Preparations of naturally occurring particulate matter (Fig. 2A) and detritus from a diatom culture (Fig. 2B) both showed yellow-fluorescing particles. Regardless of sample origin, in samples prepared without the addition of DAPI, there were no yellow particles, only light chlorophyll autofluorescence (red), cyanobacteria (orange) and some light blue particles such as crustacean exoskeleton parts. The addition of DAPI to samples containing natural particles, or desiccated plankton tow material (Fig. 2D) or ground faecal pellets (Fig. 2E), yielded preparations showing abundant and very similar DYP particles. The addition of formalin had no effect on particle fluorescence or on the number of DYP (chi-squared test, data not shown).

In all sample types, slides prepared from enzyme-treated samples showed a reduction of about 90% of the DYP. An example of DYP concentrations before and after enzyme treatment is shown in Fig. 2E, F. Quantitative reductions in DYP concentrations in natural samples are given in Table 1. In general, the DYP remaining after enzymatic treatment were large (>20 µm). Interestingly, although DYP were clearly affected by the enzymes, chlorophyll autofluorescence was still evident inside diatoms. The reactivity of DAPI in the enzyme-treated samples was evidenced by the appearance of DYP when fresh detritus was added to enzyme-treated samples and the presence of DYP in non-incubated enzyme-treated samples (i.e. processed immediately following the addition of enzyme cocktail ingredients).

Estimating *in situ* concentrations of DYP

Estimates of variability between replicate water samples and subsamples for particles of different sizes are given in Table 2. Standard error, as a percentage of

Table 1. Concentration (number per ml) of DAPI Yellow Particles (DYP) (untreated) and Enzyme Resistant DYP (ERDYP) (i.e. after enzymatic degradation), in 8 samples before and after enzymatic degradation

Depth (m)	DYP	ERDYP	% ERDYP
0	7502	481	6.411
0	7148	309	4.322
0	7215	322	4.462
0	8865	29	0.327
10	8247	47	0.569
30	17112	61	0.356
50	8865	31	0.349
75	43914	27	0.061

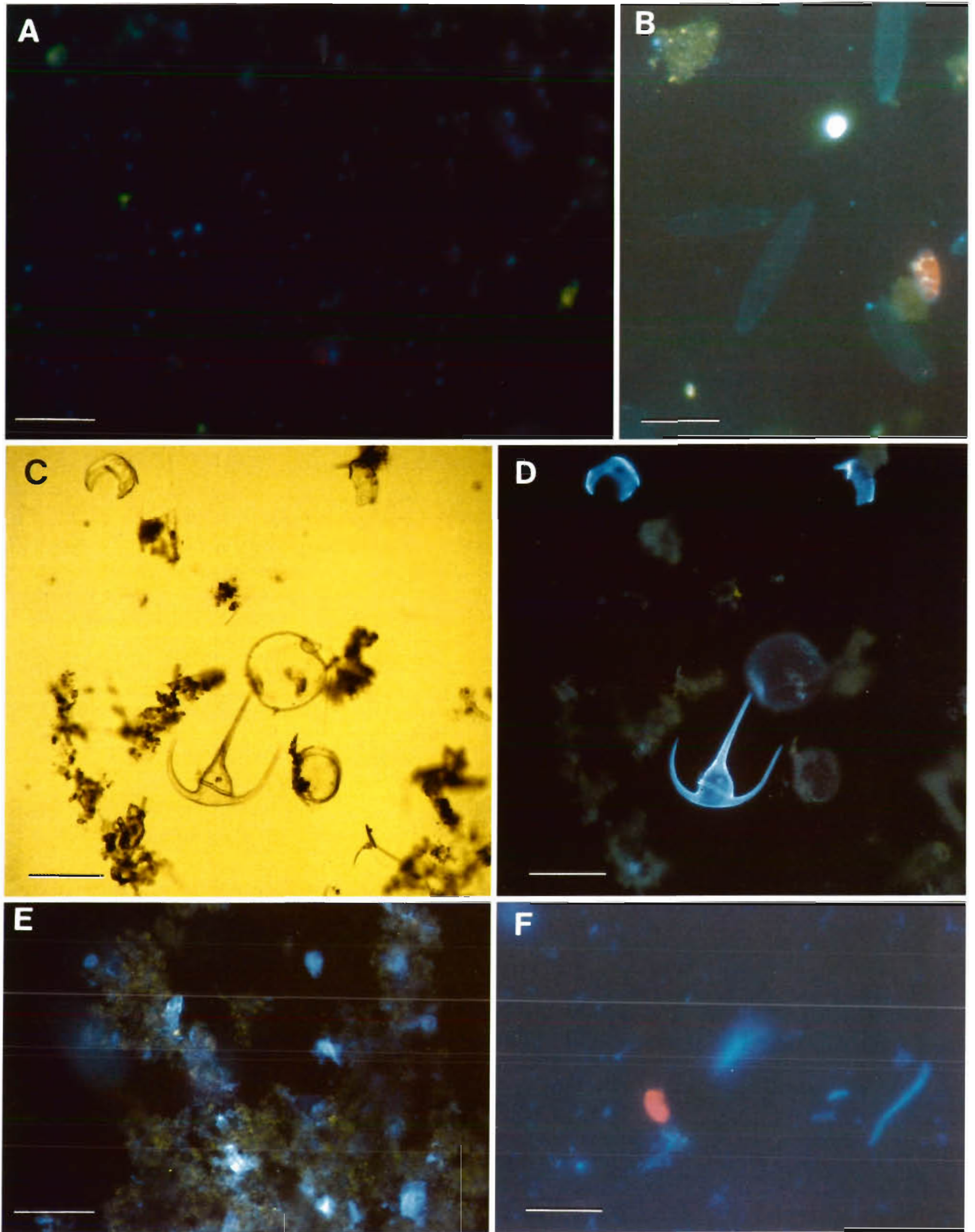
A
B
C
D
E
F

Fig. 2. Photomicrographs. (A) Natural water sample, formalin-fixed and DAPI-stained; note the 4 yellow-fluorescing particles 1–3 µm in size. (B) DAPI-stained detritus from a culture of the diatom *Navicula*. (C) Desiccated plankton tow material, DAPI-stained as seen with transmitted light and (D) the same material with UV excitation; note that the yellow-fluorescing matter in D corresponds with the amorphous matter in C. (E) Ground copepod faecal pellets, DAPI-stained and UV excited. (F) Ground copepod faecal pellets, DAPI-stained after digestion with the enzyme cocktail; note the red-fluorescing particle, presumably chlorophyll-containing, which resisted enzymatic degradation. Scale bars: (A) 5 µm; (B) 10 µm; (C) 100 µm; (D) 100 µm; (E) 100 µm; (F) 10 µm

the mean, ranged from 3 to 66%. Variability in concentration estimates was higher for larger particles relative to small particles and higher for the means of replicate bottle casts compared to subsamples from the same bottle. The higher variabilities associated with DYP >10 µm appeared to correspond with their lower concentrations. It should be noted that for the relatively abundant DYP particles ≤10 µm in size, standard errors represented <20% of estimated mean concentrations.

Preliminary sampling along the offshore transect showed that different size classes of detrital particles exhibited remarkably variable vertical and horizontal distributions (Fig. 3). In general, DYP ≤15 µm were much more abundant than the other size classes considered. In our samples, 15–20 µm detrital particles were almost absent. Table 3 illustrates the concentration of detrital particles per liter. Larger particles appeared sporadically; 20–25 µm detritus particles were observed only once at Site 3 at 20 m and particles ≥25 µm were observed twice at Sites 1 and 2, at 20 m and 600 m, respectively. The standard protocol presented here with 10 ml of sample volume is designed for pico- and nano-DYP in the Mediterranean Sea. Enumerations of DYP ≥20 µm would require a volume larger than 10 ml.

The most abundant size class found almost at all sites and all depths was 0.2–2 µm. All sizes of particles showed highest values at the nearshore Site 1, with a gradual decrease towards Site 4 in the neritic zone (Table 3, Fig. 3). There appeared to be a discontinuity in size-class distributions with virtually no particles 15–20 µm in diameter observed. However, beyond these generalities, few consistent trends were apparent in the distributions of detritus. Vertical distributions were particularly complex.

For example, concentrations of the 0.2–2 µm size class particles decreased in abundance with depth at Site 1, increased with depth at Site 2, and were relatively constant with depth at Sites 3 and 4 (Table 3, Fig. 3A). The quantities of 2–5 µm particles were highest at 40 m at the nearshore Site 1, peaked at 600 m at Site 2, declined with depth at Site 3 and increased with depth at Site 4 (Fig. 3B). Particles 10–15 µm in size were absent at 40 and 75 m at Site 1, from 15 m at Site 2, from 600 m at Site 3 and from 50 m at Site 4 (Fig. 3D). The total surface area of ≤15 µm detritus decreased with increasing depth at Sites 1 and 3. In contrast, at Site 2, their abundance increased between 15 and 600 m.

DISCUSSION

Early quantifications of 'microscopic' detrital (as in non-living, organic) particles relied on examining untreated material with standard transmitted light microscopy and yielded abundance estimates for easily visible, relatively large particles >30 µm (e.g. Kane 1967 and references therein). Later studies (reviewed in Riley 1970, Wiebe & Pomeroy 1972, Simpson 1982) employed techniques such as histochemical staining or the use of a standard scanning electron microscope or one coupled with Energy Dispersive X-Ray (EDXR). These techniques allowed examination of particles in the pico- and nano-size range and provided higher abundance estimates as well as compositional data. However, they are not widely employed today due to their very time-consuming nature. For example, Gordon (1970a), whose efforts were described as heroic (Wiebe & Pomeroy 1972), used histochemical stains to enumerate and characterize organic particles (5 to 50 µm in largest dimension) as either largely protein or carbohydrate in the North Atlantic; he found 10 to 300 particles ml^{-1}, with small particles more abundant than large ones, and revealed that most appeared to be

Table 2. Standard errors, given as percentage of the mean, for replicate water samples and subsamples from 1 sample

	Size class (µm)						
	0.2–2	2–5	5–10	10–15	15–20	20–25	≥25
5 samples from 5 replicate Niskin bottles	11	10	18	41	–	–	66
3 subsamples from 1 Niskin bottle	3	6	7	9	50	–	–

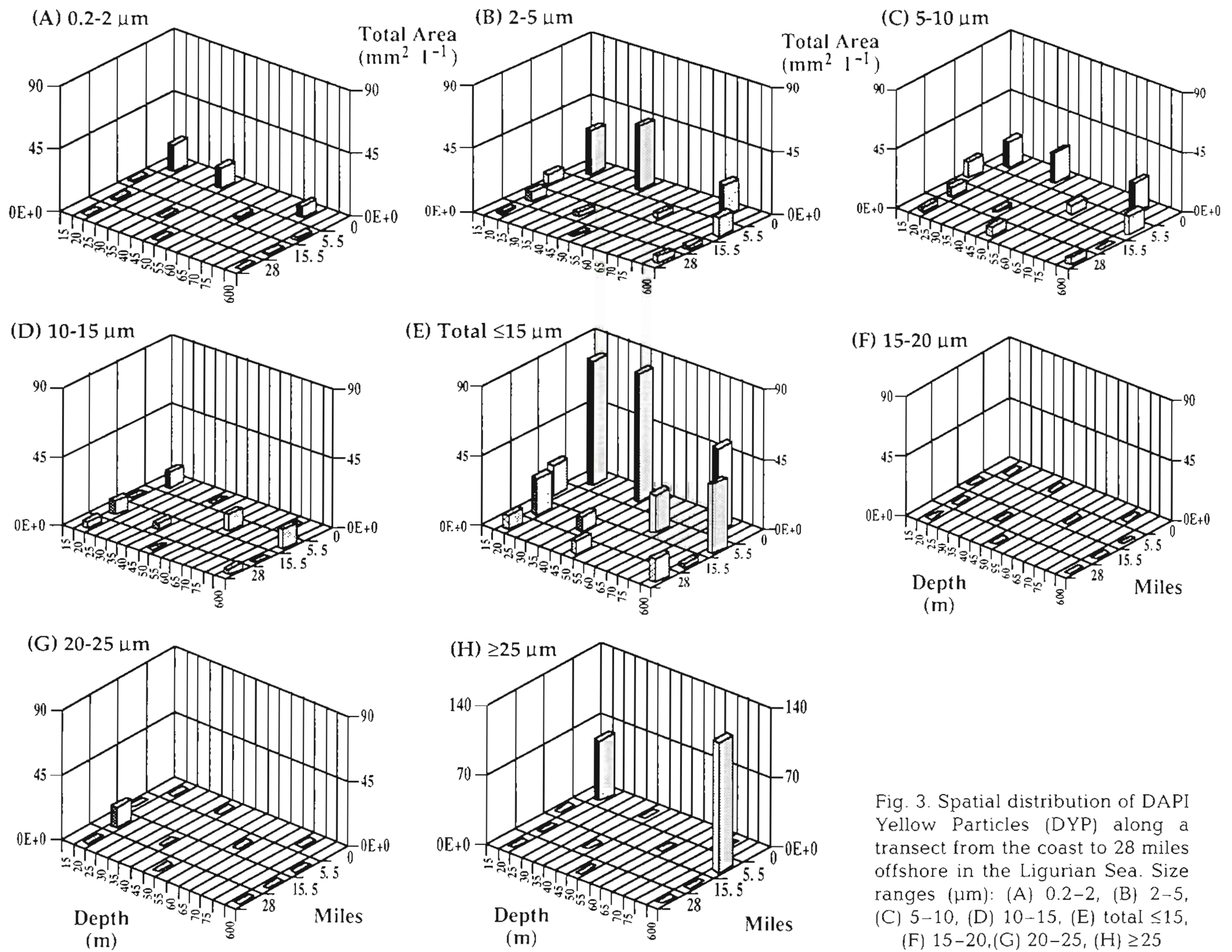

Fig. 3. Spatial distribution of DAPI Yellow Particles (DYP) along a transect from the coast to 28 miles offshore in the Ligurian Sea. Size ranges (µm): (A) 0.2–2, (B) 2–5, (C) 5–10, (D) 10–15, (E) total ≤15, (F) 15–20, (G) 20–25, (H) ≥25

Table 3. Concentration of DAPI Yellow Particles (DYP) $\times 10^5$ l^{-1} at 4 sites and different depths

Site	Depth (m)	Size class (µm)							
		0.2–2	2–5	5–10	10–15	<15	15–20	20–25	≥25
1	20	77.5	16.2	2.18	0.43	96.3	0	0	0.43
1	40	57.8	23.2	2.62	0	83.6	0	0	0
1	75	31.5	11.4	2.62	0	45.5	0	0	0
2	15	3.7	3.7	1.39	0	8.79	0	0	0
2	60	6.95	1.39	0.92	0.46	9.72	0	0	0
2	600	8.71	7.22	1.66	0.55	18.1	0	0	0.55
3	20	3.15	3.33	0.74	0.37	7.59	0	0.18	0
3	40	1.48	1.48	0.18	0.18	3.32	0	0	0
3	600	2.4	1.48	0	0	3.88	0	0	0
4	20	0.55	0.37	0.37	0.18	1.47	0	0	0
4	50	0.74	0.74	0.74	0	2.22	0	0	0
4	600	0.55	2.78	0.55	0.18	4.06	0	0	0

amorphous, predominately carbohydrate-containing, aggregates. Gordon (1970b) also showed that the particles were hydrolyzable using an enzyme cocktail of alpha-amylase, trypsin and chymotrypsin.

The method presented here of using DAPI is relatively simple and inexpensive. Our data indicate that DYP resemble particles of detritus from desiccated plankton or faecal pellets, or phyto-detritus, are enzyme-degradable and very abundant. Similar to trends found in previous studies using other methods (e.g. Riley 1970, Simpson 1982), small particles are more abundant than large particles. In surface waters of the NW Mediterranean, for example, particles <5 µm in size were often present in concentrations of 10^3 ml^{-1} while larger particles 5 to 20 µm were found in numbers of 55 to 262 ml^{-1} (Table 3). Interestingly, the latter concentrations are very similar to the 50 to 125 ml^{-1} concentrations of aggregates, from 5 to 20 µm in size, found in different areas of the North Atlantic from the Sargasso Sea to the Nova Scotia Shelf (Gordon 1970a). While these results suggest that pico- and nano-DYP may correspond to small amorphous aggregates of earlier studies (e.g. Riley 1970, Wiebe & Pomeroy 1972), it should be noted that we did not examine all organic non-living material and, consequently, DYP cannot be considered as accounting for all non-living organic particles.

However, DYP, at least in the NW Mediterranean, represent a considerable stock of organic matter. In nearshore surface waters, average total area of DYP, dominated by particles 0.2 to 15 µm in size (Fig. 3), was 80 mm^2 l^{-1}. This figure is about 1 order of magnitude lower than those of TEP, ranging in size from 3 to several hundred µm, based on recent reports from other systems; however it should be noted that TEP concentrations can vary by 4 orders of magnitude (10^0 to 10^4 TEP ml^{-1} and 0.2 to 2000 mm^2 l^{-1} respectively) (Passow & Alldredge 1994). Micro-sized DYP also appear important relative to total micro-detrital particles, those with a smallest dimension of 30 to 55 µm, based on data from Kane (1967) from the same system. Average micro-aggregate abundance in coastal surface waters in the NW Mediterranean was estimated as 12.5 ml^{-1} (Kane 1967) compared to 43 ml^{-1} for DYP ≥25 µm in nearshore surface waters (Table 3).

The data on water column distributions of DYP (Fig. 3) reveal complex distributional patterns. The irregularities in abundances with depth or distance from shore are unlikely to be the result of sampling error, given the reasonable reproducibility of DYP estimates (Table 2). The patterns suggest then that DYP are a dynamic part of the water column stock of particulate matter and not a conservative component with a single origin. Nagata & Kirchman (1992) hypothesized that flagellates release their own digestive enzymes and incompletely digested membranes and probably other cellular components from bacterial prey. Together with studies of faecal production by protozoa (Stoecker 1984, Nöthig & von Bodungen 1989, Buck et al. 1990, Elbrächter 1991, González 1992, Buck & Newton 1995), the idea is reinforced that one of the possible sources of DYP could be particles released from protozoa. The reason for the absence of larger DYP in deep waters may also be the fact that they are scavenged by even larger aggregates. While DYP resemble particles from faecal matter or micro- and mesoplankton, we have no direct evidence on the origin of pico- and nano-sized DYP *in situ* from large particles. However, consideration of the size-class distributions of DYP and low abundances of associated fauna supports the hypothesis that pico- and nano-sized DYP represent a distinct stage in the processing of detrital matter in the water column.

Biddanda & Pomeroy (1988) proposed a scheme in which phytoplankton-derived detritus, and by extension other detritus, passes through distinct phases of aggregation, disaggregation, and re-aggregation. In the scheme, fresh particles are colonized by bacteria, followed by particle aggregation and then the appearance of protozoans. The combined activities of bacteria and protists lead to particle disaggregation, that is the production of small particles. The small particles may either combine with fresh particulate matter to renew the cycle or form aggregates which sink out of the water column.

In our samples, remarkably few bacteria were evident on pico- and nano-sized DYP (Fig. 1A). In contrast, micro-sized (>20 µm) DYP, although rare in our samples which were not designed to quantify them, often appeared to support considerable bacterial populations (unpubl. obs.). It is noteworthy that few particles 15 to 20 µm were found. The discontinuity in size-class distribution and differences in bacterial colonization indicate that pico- and small nano-DYP probably differ from micro-sized DYP in more than just size. The observations suggest that, within the Biddanda & Pomeroy (1988) scheme, pico- and nano-sized DYP could be small, used, detrital particles from the disaggregation of detritus. Their eventual fate then would be re-aggregation, perhaps with a maximum size of 15 µm and sedimentation out of the water column.

Acknowledgements. We thank L. Legendre, F. Thingstad, P. Verity and B. Avril as well as 3 anonymous reviewers for their constructive comments, V. Boivin for her help in enzymatic experiments, and D. Betti, G. Gorsky and L. Stemmann for their aid in sampling at sea. The work presented here is part of the doctoral dissertation of B.M. Financial support was provided by the CNRS/INSU (URA 716) and E.E.C. MAST II-MTP (Mediterranean Targeted Program, contract MAST II-CT93-0063, Medipelagos).

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Responsible Subject Editor: F. Azam, La Jolla, California, USA

Manuscript first received: July 31, 1995
Revised version accepted: October 19, 1995