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REPORT OF THE ICES/IOC WORKSHOP ON INTERCOMPARISON ON *IN SITU* GROWTH RATE MEASUREMENTS (DINOFLAGELLATES)

Aveiro, Portugal, 25 - 29 July 1994

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ICES/IOC Workshop on INTERCOMPARISON ON *IN SITU* GROWTH RATE MEASUREMENTS (DINOFLAGELLATES)

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1. Opening the Workshop.

The workshop was opened by the chairman Dr. Maria Antonia Sampayo. The participants were welcomed by the director Dr. Manuel Sobral from the Aveiro Laboratory of the Portuguese Institute for Marine Research (IPIMAR), who hosted the workshop. Dr. Sobral also gave some information about IPIMAR and the work that is carried out at its regional center in Aveiro. It was pointed out by the chairman that the workshop was organized in co-operation with the Oceanographic Institute (IEO) from Vigo, Spain, the Aveiro University and the Hydrographic Institute (IH) from Lisbon.

1.1 Approval of the agenda and rapporteur.

The agenda was approved by the workshop. Dr. Odd Lindahl was appointed as rapporteur.

1.2 The purpose of the workshop.

The workshop was held according to Council Resolution 1993/2:48 in order to undertake an intercomparison study of *in situ* growth rates of dinoflagellates in support of the study of harmful algal blooms.

Good estimates of population dynamics parameters, such as growth rates, are essential to providing the means to quantify the detailed structure and processes which lead to a capability to model algal populations and bloom development. Ria de Aveiro has a model available, is physically uncomplicated which will facilitate intercomparison of techniques, and there is a certainty of finding relevant target species.

2. The Ria de Aveiro system.

Aveiro is situated 240 km north of Lisbon (N 40º 38.5', W 8º 44'). The Ria de Aveiro is a shallow lagoon with a wet area of 43-47 km^2. The Lagoon has a complex topography with three main channels radiating from the mouth, several branches, islands and mudflats (map 1). Organic pollution levels are high mainly from spring to autumn. Along its main channels and at some of the mudflats there is an important bivalve molluscs exploitation, mainly *Mytilus edulis*, *Cerastoderma edule* and *Venerupis pullastra*, which present almost yearly problems of PSP and DSP related with the presence respectiviely of *Gymnodinium catenatum* and *Dinophysis* spp.

The Ria de Aveiro system was presented by J. Dias, P. Silva, M.A. Esteves and M.A. Sampayo. The presentation began with the physical oceanography of the Ria and a numerical model on water currents, levels and tidal excursion was demonstrated. It was obvious from this presentation and the following discussion that the water in the Ria is usually well mixed.

The next presentation dealt with the nutrient status of the Ria, including the inputs from land run-off. Nutrient concentrations from 1992 and 1993 were presented, clearly demonstrating that the Ria is eutrophic. This was also obvious by the rich flora of diatoms which often is present.

The species composition of phytoplankton and the occurrence of toxic dinoflagellates were presented with particular emphasis on *Dinophysis* spp, and DSP in mussels in the Ria. From the phytoplankton monitoring programme it was shown that a rich variety of diatoms and dinoflagellates are generally found in the area and this was the case during the workshop. During this time *Dinophysis* cell numbers were low (<1000 cells·l^{-1}) in the Ria.

Finally, results from a cruise sampling outside the Ria de Aveiro four days prior to the Workshop were presented (T Moita, H. Cavaco and G. Vilarinho). Two sections were sampled on 21 July until Midshelf (Map 1). From temperature and salinity data it was obvious that the water column was stratified close to the coast, with higher salinities and lower temperatures observed innershelf (Fig. 1).

Cell numbers of *Dinophysis* were comparatively low also at sea reaching 800 cells·l^{-1} at the inner station (Fig. 2). Dinophysis was observed above 14 °C. High numbers of diatoms and dinoflagellates caracterized the phytoplankton community nearshore.

3. Logistics.

The aveiro laboratory was well equipped with the basic analytical equipment which was needed for the workshop. Two small research vessels was moored at fixed stations (map 2) for the sampling and the incubation studies: R/V MESTRE COSTEIRO (27 m) from Lisbon at the mouth of the Ria and R/V JOSE MARIA NAVAS (14 m) from Vigo, Spain in the commercial harbour. Two small boats were used for transfer between the laboratory and the vessels.

4. Presentation of techniques and measurements applied.

The participants presented the different methods and measurements which were applied for the intercomparison exercise.

4.1 Current meter measurements (P. Silva and J. Dias).

Instituto Hidrográfico collected current meter data in two stations at Ria de Aveiro (near the mouth - station 1 and inside the commercial Harbour - station 2) at three different levels in the water column (1 m above the bottom, middle water column depth and at 1 m below the surface).

4.2 Enclosed water column measurements (E. Dahl).

A main advantage of enclosed water column/mesocosm measurements is that the same waterbody with its organisms can be studied over time. In this experiment plastic bags mounted on aluminium frames with 1m diameter were used (Brockmann et al. 1977).

Five experiments were performed (Table 1). All the bags were filled and placed in the commercial harbour (map 2). The depth of all bags was approximately 2 m. When filled by pumping, water from 2 m depth in the bay was pumped into the bags using a Pumpex GA 200. On 25 July a natural water column was enclosed in

Bag 1-II by lowering the flattened plastic bag mounted on the frame to 2 m depth and then enclosing the upper 2 m water column by raising the bag to the surface. Zooplankton was removed by sieving the water through a 140 µm mesh. On 27 July nutrients were added to three bags. Parameters measured during the experiment were nutrients, chlorophyll and phaeopigments, particulate carbon and nitrogen and the phytoplankton composition with emphasis on selected species. Sampling was carried out with a tube to obtain integrated samples. When nutrients were added, the bag content was artificially mixed before sampling.

Table 1: An overview of the enclosed water column measurements

Bag number	Started	Filling technic	Zoopl. remov.	Nutr. added	Number of samplings	Ended
Bag 1	22th	Pump	Yes	No	4	25th
Bag 2	22th	Pump	Yes	27th	12	29th
Bag 3	24th	Pump	No	No	10	29th
Bag 4	24th	Pump	Yes	27th	10	29th
Bag 1-II	25th	Enclosure	No	27th	9	29th

Reference:

Brockmann, U.H., Eberlein, K., Hentzschel, G., Schöne, H.K., Siebers, K., Wandschneider, K. and Weber, A., 1977. Parallell plastic tank experiments with cultures of marine diatoms. - Helgol.Wiss.Meeresunters. 30:201-216.

4.3 Diffusion chamber method (M. Varela).

Primary production by phytoplankton is translated into population growth through increases in cell numbers by binary fission. General approaches have been taken to measure or, usually, estimate in situ growth rates of phytoplankton species or communities. One of these approaches consists in enclosing natural phytoplankton assemblages in containers that are incubated *in situ* or in simulated *in situ* conditions.

The method used here is based on that described by Furnas (1982) where the incubation chamber is made of clear acrylic plastic with polycarbonate filters or nitex mesh (10 µm) as the diffusion membranes.

Samples were taken at a fixed depth. A subsample was taken immediately and preserved with Lugol's solution for microscope counting to estimate the initial number of *Dinophysis* cells. Another subsample was poured into the chamber and incubated in situ for 48 h, after which the contents of chambers were poured into a plastic bottle and the content preserved with Lugol's solution. Microscope counting is made from this bottle to estimate the concentration of *Dinophysis* at 48 h. Daily growth rates (Furnas 1982) of *Dinophysis* will then be calculated from differences in concentration between T_{48} and T_0.

Reference:

Furnas, M.J., 1982. An evaluation of two diffusion culture techniques for estimating phytoplankton growth rates *in situ*. - Mar.Biol. 70: 63-72.

4.4 ^{14}C method *in situ* (O. Lindahl and L. Davidsson).

One of the purposes with the workshop was to compare the "old" ^{14}C-method with newly developed methods. The ^{14}C-method is known to give relatively good estimates of the gross production of the whole phytoplankton community in the

experimental bottle (Williams, 1993). Thus, in this workshop the community growth rates were going to be compared with growth rates of single species measured by both ^{14}C-uptake and by other methods, obviously a difficult task. However, according to the local experience the summer phytoplankton flora in the Ria de Aveiro is often dominated by a few species and a comparison between community and single species growth rates could be possible.

The ^{14}C measurements were performed in the traditional way by taking water from different depths with a water-bottle and incubated in a single glass bottle (125 ml) at each depth for 2 to 4 hours (BMB, 1976). 10 μCi of ^{14}C was added to each bottle. Immediately after the incubation three parallell subsamples of 10 ml were taken out of each bottle into a scintillation bottle and acidified and bubbled with air for 15 minutes. The carbon uptake of the whole phytoplankton community was thus measured.

14C-measurements *in situ* are time consuming and may introduce errors due to that water from different depth are brought to the deck of the ship and then back again. Especially cells which are dark adapted may become distributed by this handling. To reduce this problem Dandonneau (1993) developed an automated sampling and incubation device which closes while being lowered. This closing principle is suitable for homogenous and clear waters. However, in coastal stratified waters with low visibility and a high abundance of thin subsurface chlorophyll and production maxima an *in situ* incubator should contain a water representative for a certain depth or a thin layer. An *in situ* incubator which hopefully will meet these needs has been constructed (Lindahl and Haamer, unpubl.) and is still under development. This incubator is like a small water-bottle made of acrylic plastic and kept horizontal. The closing is triggered by a small hydraulic plunger after approximately 5 minutes. ^{14}C is added from a syringe after the incubator has closed. After incubation the *in situ* incubator and its sample is treated like an ordinary ^{14}C-bottle. Some parallel measurements were made with this *in situ* incubator.

References:

Baltic Marine Biologists, 1976. Recommendations on methods for marine biological studies in the Baltic Sea. - BMB Publ. no. 1, 98 pp.

Dandonneau, Y., 1993. Measurements of *in situ* profiles of primary production using an automated sampling and incubation device. - ICES Marine Science Symposia, Vol. 197:172-180.

Williams, P.J.leB., 1993. Chemical and tracer methods of measuring plankton production. - ICES Marine Science Symposia, Vol. 197: 20-36.

4.5 Single cell ^{14}C uptake method (M. Varela, B. Reguera and I. Bravo).

The basic method is that of Rivkin and Seliger (1981). The purpose of the experiment is simply to conduct a typical ^{14}C productivity incubation, but in chambers of sufficient size that *Dinophysis* is not disturbed. Polycarbonate bottles of about 1 l volume are used. Water samples are gently poured into these bottles and alkalinity measured. Initial cell counts are taken and the ^{14}C is added at a rate of 1 μ Ci per ml. These are incubated *in situ* or simulated *in situ*.

Immediately after ^{14}C was added and mixed, an aliqout was taken to measure activity added to sample. After 24h incubation the samples were poured through a large sieve (130 μm) into a beaker. The material collected was then poured through a second 20 μm sieve, followed by at least 2 liters of filtered seawater. The sieve content was washed into a small tube, which was placed in a beaker on ice in a cooler and kept dark.

1 ml sub-samples were taken from this suspension and placed on slides in order to isolate the cells. Cells were washed thoroughly in drops of filtered sea water before placing them into scintillation vials, keeping track of the exact number of cells isolated. Around 50 cells should be isolated into each vial to give good statistics. It is also necessary to have control vials, in which you draw 50 samples of the background water (i.e. no cells) approximately equal in volume to the amount drawn with each cell that is isolated. This is also placed in a scintillation vial for counting.

In order to estimate a growth rate , it is necessary to estimate the amount of carbon in *Dinophysis* cells. Therefore, we need to measure a number of them so that calculations of cell volume and cell carbon can be made.

References:

Rivkin R.B.and Seliger H.H. 1981: Liquid scintillation counting for ^{14}C uptake of single algal cells isolated from natural populations. - Limnol.Oceanogr., 26: 780-784.

Granéli E., Anderson D.M., Maestrini S.Y. and Paasche E. 1992: Light and dark carbon fixation by the marine dinoflagellate genera *Dinophysis* and *Ceratium*. - ICES Marine Science Symposia, vol. 197: 274.

4.6 Species-specific division rates via morphological differences in cells undergoing mitosis (I. Bravo, E. Garcés and B. Reguera).

Our objective was to estimate *in situ* division rates of *Dinophysis* spp by applying the model of McDuff and Chisholm (1982). The application of this model is based in the observation and quantification of morphological differences observed in cells undergoing mitosis. The observations to be quantified were:

i) Frequency of double nucleated cells.

ii) Frequency of paired cells.

iii) Frequency of just divided cells.

Double nucleated cells will be recorded by epifluorescence of cells stained with a DNA-specific dye, DAPI (4'6-diamindino-2 phenylindole, Sigma Chemical) at a final concentration of 1-2 $\mu g \cdot ml^{-1}$.

Paired cells of *Dinophysis* spp can be easily observed before the end of cytokinesis when sampling at the appropriate hours of the day. In the case of *Dinophysis acuminata* division in natural populations seems to be very synchronized, and is observed during a narrow window of time, between 5 am and 7am (GMT) , both in Atlantic and Mediterranean waters of the Iberian peninsula (unpubl. data).

Just divided cells of *Dinophysis* spp show complementary sulcal lists, each daughter cell missing either the left or the right sulcal list. These marked morphological differences will allow a good application or even a refinement of McDuff and Chisholm's model.

Samples are taken every hour or every other hour, except between 2.^{00} am and 8.^{00} am (GMT) when the frequency is increased (every half an hour). Some parameters and processes that will be under study and need further refinement in the course of the present (this Workshop) and future monitorings of *Dinophysis* cell cycle are:

i) Determination of the division time (T_D) .

ii) Constancy of T_D under varying environmental conditions and different seasons.

iii) Time lag for the full development of the sulcal lists in the daughter cells.

iv) Possible existence of bimodal cycles when hypothetical gamet production

takes place at different hours of the day (MacKenzie, 1992) or different stages of the population growth (Reguera et al, 1990).

References:

McDuff, R.E. and Chisholm, S.W., 1982. The calculation of *in situ* growth rates of phytoplankton populations of cells undergoing mitosis: a clarification. - Limnol.Oceanogr. 27: 783-788.

MacKenzie, L., 1992. Does *Dinophysis* (Dinophyceae) have a sexual life? - J.Phycol. 28: 399-406.

Reguera, B., Bravo, I. and Fraga, S., 1990. Distribution of *Dinophysis acuta* at the time of a DSP outbreak in the Rias of Vigo and Pontevedra. ICES C.M. 1990/L:14.

4.7 RNA and DNA Measurements as Indicators of Growth Rate (D.M. Anderson and D. Kulis).

RNA and DNA measurements can be used in several different ways to obtain estimates of growth rates in phytoplankton. For example, the ratio of RNA:DNA is used extensively in studies of fish, fish larvae, and other larger marine organisms as an indicator of physiological condition. The concept has been explored for marine bacteria (Delong et al., 1989) and phytoplankton (Dortch et al., 1983). For some of these organisms, it is clear that the ratio varies systematically with growth rate (e.g. Dortch et al., 1983; Delong et al., 1989). Nevertheless, considerable work remains, especially with microorganisms, to determine whether the environmental variables that limit growth affect the ratio in different ways (Dortch et al., 1985; Berdalet et al., 1992, 1994).

With respect to toxic or harmful dinoflagellates, relatively little is known about the utility of the RNA:DNA ratio as an indicator of physiological condition or growth rate. One of the objectives of this subproject within the workshop was to investigate how this ratio might vary in a *Dinophysis* population.

Another potentially useful measurement would be of DNA alone, as shown by Chang and Carpenter in a series of papers (Chang and Carpenter 1988, 1991, 1994; Carpenter and Chang 1988). DNA-specific stains are used to quantify the amount of DNA in individual cells through time which can then be used to estimate growth rate using the mitotic index approach (McDuff and Chisholm 1982; Weiler and Chisholm 1976).

Given the potential utility of RNA:DNA ratios and DNA measurements by themselves, an approach was pursued during this workshop to obtain both types of data. In order to obtain simultaneous measurements of RNA and DNA in the same cell, double-labeling with DNA-specific stains (propidium iodide, DAPI, or Hoechst) will be used in conjunction with fluorescently-labeled ribosomal RNA probes. The latter are short segments of synthetic DNA designed to bind to the rRNA of target organisms. Since rRNA represents the vast majority of total RNA (Kemp et al., 1993), this provides a useful estimate of the RNA content in a cell and avoids the problem of attempting to find a RNA-specific general stain that does not bind to DNA and does not vary stoichometerically due to conformation of the rRNA (Danzynkiewicz et al., 1987). In an ideal case, the rRNA probe could also be species-specific, and thus serves two purposes; identifying the target species and quantifying its rRNA at the same time. With respect to *Dinophysis*, no rRNA probes yet exist, so a "universal" probe (Giovannoni et al., 1988) that binds to rRNA of all organisms will be used instead. The bright orange phycoerytherin fluorescence of *Dinophysis* in combination with size information from 90° or forward light scatter measurements will be used to distinguish this organism from the rest of the mixed population.

Since it is not clear whether simultaneous RNA and DNA measurements will be possible on most standard flow cytometers, a fall-back position was pursued to measure DNA content alone and to use the distributions of cells going through mitosis through time to calculate growth rate.

Procedures

Every two hours for 36 hours a 20 M plankton net was lowered to within 2 meters of the bottom of the water column and raised vertically twice in sucession to provide a nonquantative, integrated plankton sample. The sample was then screened through a 130 µm nitex sieve and the effluent was rinsed through a 20 M sieve to concentrate dinoflagellate species. Cells were preserved in 2.5% formaldehyde, and stored at 4 °C in the dark until analysis.

To quantify the RNA/DNA ratio by flow cytometry a subsample was removed and rinsed again through a 35 µm sieve to further purify the dinoflagellate cell component. The washed cell slurry was resuspended in a 15 ml centrifuge tube and was centrifuged at 7500 x g for 5 minutes. The supernatant was aspirated and 0.5 ml hybridization buffer containing 5X SET (750 mM NaCl, 100 mM tris-HCl, 5 mM EDTA, pH 7.8), 0.1mg/l polyadenylic acid, 0.1% Tergitol NP-40, 10% formamide was added to the cell pellet. The sample was prehybridized at 37 °C for 30 minutes. 50 l of a FITC conjugated universal or negative shipworm bacterium control (Distel et al., 1991) rRNA probe (final conc. 5 ng/l) were added and the sample was incubated for an additional 2 hours at 37 °C. The sample was then centrifuged as described above and the cell pellet was washed in 0.2X SET buffer for 10 minutes at 37 °C. Following the wash the sample was again centrifuged, the supernatant aspirated, and the hybridized pellet was resuspended in 5X SET containing a DNA specific stain such as PI, DAPI, or Hoechst. These samples will be analyzed on a flow cytometer or microscope photometer to quantify the rRNA and DNA fluorescence of *Dinophysis* sp.

References:

Delong, E. F., G. S. Wickham, and N. R. Pace. 1989. Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. *Science* 243:1360-1363.

Distel, D. L., E. F. Delong, and J. B. Waterbury. 1991. Phylogenetic characterization and in situ localization of the bacterial symbiont of shipworms (Teredinidae: bivalvia) by using 16S rRNA sequence analysis and oligonucleotide probe hybridization. *Appl.Environ. Microbiol.* 57:2376-2382.

Giovannoni, S. J., E. F. Delong, G. J. Olsen, and N. R. Pace. 1988. Phylogenetic group-specific oligodeoxynucleotide probes for identification of single microbial cells. *J. Bacteriol.* 170:720-726.

Darzynkiewicz, Z., J. Kapuscinski, F. Tranganos, and H. A. Crissman. 1987. Application of pyronin Y (G) in cytochemistry of nucleic acids. *Cytometry* 8:138-145.

Berdalet, E., M. Latasa, and M. Estarada. 1992. Variations in biochemical parameters of *Heterocapsa* sp. and *Olisthodiscus letus* grown on a 12:12 h light:dark cycle. I. Cell cycle and nucleic acid composition. *Hydrobiologia.* 238:139-147.

Berdalet, E., M. Latasa, and M. Estarada. 1994. Effects of nitrogen and phosphorus starvation on nucleic acid and protein content of *Heterocapsa* sp. *J. Plankton Res.* 16:303-316.

Dortch, Q., T. L. Roberts, J. J. R . Clayton, and S. I. Ahmed. 1983. RNA/DNA ratios and DNA concentrations as indicators of growth rate and biomass in planktonic marine organisms. *Mar. Ecol. Prog. Ser.*, 13:61-71.

Dortch, Q., J. J. R . Clayton, S. S. Thoresen, J. S. Cleveland, S. L. Bressler and S. I. Ahmed. 1985. Nitrogen storage and use of biochemical indices to assess nitrogen defiency and growth rate in natural populations. *J. Mar. Res.*, 43: 437-446.

Kemp, P. F., S. Lee and J. LaRoche. 1993. Evaluating bacterial activity from cell- specific ribosomal RNA content measured with oligonucleotide probes. *In* XXX [Ed.] *Handbook of Methods in Aquatic Microbial Ecology*. Lewis Publishers, XXX, pp. 415-422.

Carpenter E. J., and J. Chang. 1988. Species-specific phytoplankton growth rates via diel DNA synthesis cycles. I. Concept of the method. *Mar. Ecol. Prog. Ser.* 43:105-111.

Chang J., and E. J. Carpenter. 1988. Species-specific phytoplankton growth rates via diel DNA synthesis cycles. II. DNA quantification and model verification in the dinoflagellate *Hetreocapsa triquetra*. *Mar. Ecol. Prog. Ser.* 44:287-296.

Chang J., and E. J. Carpenter. 1991. Species-specific phytoplankton growth rates via diel DNA synthesis cycles. V. Application to natural populations in Long Island Sound. *Mar. Ecol. Prog. Ser.* 78:115-122.

Chang J., and E. J. Carpenter. 1994. Active growth of the dinoflagellate *Ceratium teres* in the Caribean and Sargasso Seas estimated by cell cycle analysis. *J. Phycol.* 30:375-381.

McDuff, R. E., and S. W. Chisholm. 1982. The calculation of in situ growth rate of phytoplankton populations from fractions of cells undergoing mitosis: a clarification. *Limnol. Oceanogr.* 27:783-788.

Weiler, C. S., and S. W. Chisholm. 1976. Phased cell division in natural populations of marine dinoflagellates from shipboard cultures. *J. Exp. Mar. Bio. Ecol.* 25:239-247.

4.8 DNA/PCNA cell cycle method (E. Carpenter and S. Lin).

We measured growth rates of phytoplankton using a cell cycle technique. Basically, we obtained the growth rate by sampling the phytoplankton at 2 hr intervals over a 24 h period, then determining the percentage of cells which are dividing. From this information and a determination of the length (duration) of the division phase (or some other "terminal event"), growth rate was calculated. A terminal event is defined as being a marker occurring at the end of cell division cycle. It can be a microscopic observation of the number of paired cells, a measure of cells with 2x DNA or the presence of a chemical which might only be present at one stage of the cell cycle or some other type of observation. We used two methods for determining the percent which will divide, DNA and PCNA.

For the DNA technique, we collected phytoplankton and preserved them in methanol. The methanol serves to remove photosynthetic pigments which might fluoresce and also preserves the cells. Next we add the DNA- specific fluorochrome DAPI. DAPI fluorescence is proportional to DNA content, and we measure DNA in single cells using a TV-computer-based microscope system. After the DNA content of about 300 cells of a selected species is saved on the computer, we can plot a histogram of the DNA profile of the population. By examining profiles at 2 hr intervals through the day we can see how the population progresses through the cell division cycle. Equations are then used to deconvolute the histograms and extract each of the cell cycle phases: G, S, G_2+M. The G, S, G_2+M phases are used as the "terminal event" and we calculate growth rate by comparing those which are dividing (with a "terminal event") with those that are not.

Since the above method is time consuming and involves a lot of expensive equipment, we have developed an antibody method to substitute as the "terminal event". The presence of the cell cycle protein PCNA (proliferating cell nuclear antigen), a cyclin compund is used as the event. All that is required is to add fluorescent labeled antibodies to PCNA to a sample and then to visually examine the sample using a standard epifluorescence microscope. This way, the investigator can visually examine the species composition of the whole phytoplankton population and obtain growth rates for all species. Sample collection and formulas for determining growth rates are identical to that used for the DNA method.

4.9 Monoclonal antibodies, species specific diel DNA measurements and bioassay (L. Peperzak).

1. Collection of Dinophysis spp, to be used for the production of monoclonal antibodies (Vrieling et al, 1994).

2. 48 hours of sampling for flowcytometric species and DNA measurements. Samples will be labelled with a species specific label and a DNA dye. The species label will trigger the f.c.m. that will then measure the amount of DNA present. Growth rates can then be calculated with the Carpenter-cell cycle method (Chang and Carpenter, 1988). (Species labels: *Prorocentrum micans, Alexandrium tamarense, Pseudonitzschia pungens f. multiseries*).

3. Samples for bioassay experiment were incubated in bottles that were moored *in situ*. The following additions were made: 1.) none, 2.) growth factors, 3.) pH lowering, 4.) chelator, 5.) PEP-Si growth medium with extra vitamins, 6.) All (6 bottles in duplicate). Effects were measured as *in vivo* chlorophyll fluorescense and cell (*P. micans, Dinophysis spp*) concentration.

References:

Chang, J. and Carpenter, E.J., 1988. Species-Specific phytoplankton growth rates via diel DNA synthesis cycles. II. DNA quantification and model verification in the dinoflagellate *Heterocapsa triquetra*. - Mar.Ecol.Prog.Ser. 44:287-296.

Vrieling, E.G., Peperzak, L., Gieskes, W.W.C. and Veenhuis, M., 1994. Monoclonal antisera: an immunochemical tool for the specific detection of the ichtyotoxic dinoflagellate *Gyrodinium aureolum* and morphologically related *Gymnodimium* species. - Mar.Ecol.Prog.Ser. 103: 165-174.

5. Preliminary results and some comments.

5.1 Current meter data.

Station 1 (near mouth of Ria de Aveiro):

1. The values of the currents observed were highly related to ocean tidal wave, as expected.

2. The velocity of the current near the mouth was approximately constant in he vertical, although the values were bigger near the surface. The maximum values occurred in ebb situations. The ebb mean time was longer than the flood mean time, 5h 40 and 6h 30 respectively.

3. The maximum velocities were observed at intermediate tide (±2 h after the high and low tide), which showed the tidal wave in the Ria, at least near this location, was a mixture between a progressive and stationary wave.

Station 2 (Commercial Harbour):

1. In flood situations the velocity currents had a significant value while in the ebb situations the velocity was almost zero. This showed that the harbour could be considered as a reservoir that filled fast end emptied slowly during the tidal cycle.

2. The currents were not constant in the vertical; they were more intense near the surface and decreased with depth.

5.2 Enclosed water column measurements.

The results from counting *Dinophysis* spp. on filters in microscope with epifluorescence attachment are shown in tables 2-6. From each sampling two or three subsamples of 50 ml were concentrated by filtration and counted. This method should theoretically detect concentrations of *Dinophysis* spp. down to 10 cells/l. Only *D. acuminata* was present in numbers high enough to get reliable data of their concentration. In all bags this species increased in numbers during the first 24 h.

From data in Tables 2-6 during the first 24 h of the experimental period, the following growth rates for *D. acuminata* was calculated

Bag no.	Div./day
1	0.09
2	0.11
3.	0.23
4	0.40
1-II	0.5-1.1

according to the formula (Eppley and Strickland, 1968):

$$k = 3.32 \cdot (\log n_t - \log n_{t0}) \cdot (t-t_0)^{-1}$$

where k = growth rate as divisions per day (24 h)
t_0 and t = point of time for two different measurements of cell concentration, unit days
n_{t0} and n_t = the corresponding concentration of cells
log = $\log_{10}$

After about 24 h, however, the concentration of *D. acuminata* decreased in all bags. Even if the concentration of the other species of *Dinophysis* were too low to get reliable data on growth one may, from Table 2-6, get the general impression that the heterotrophic species, *D. rotundata*, showed somewhat better survival in the bags than during the experiment. The addition of nutrients, 11 a.m. on 27 July, to bag 2, 4 and 1-II did not stimulate growth of *Dinophysis* spp. during the next 48 h, while phytoplankton biomass measured as chlorophyll increased significantly during the same period.

Accompanying species in all the bags were dominated by diatoms, mainly *Leptocylindrus danicus*, *Thalassionema nitzschioides* and *Pseudonitzschia* sp. Their content of chlorophyll per cell became less and less untill 27 July when nutrients were added to the bags. After the addition of the nutrients the chloroplasts recovered and the diatom population very soon showed a much more healthy condition. This, together with the immediate increase of chlorophyll biomass after addition of nutrients indicate nutrient limitation during the first days of the experiment.

As harmful dinoflagellates occurred in rather low numbers during the experiment, more abundant dinoflagellates as *Ceratium fusus*, *Helgolandinium subglosum* and *Prorocentrum micans* were also counted. Such data together with data on *Dinophysis* counted by other technics and data on nutrients and particulate carbon and nitrogen will be presented in a later report.

By the end of the experiment the sediment in each bag was qualitatively checked for algae, and the preliminary results revealed a rather strong sedimentation in the bags during the experiment, especially of diatoms.

Reference:

Eppley, R.W. and Strickland, J.D.H., 1968. Kinetics of marine phytoplankton growth. In: Droop, M. and Ferguson Wood, E.J. (eds.) Advances of Microbiology of the Sea 1:23-62.

5.3 Diffusion chamber method.

Cell counts of the inverted bottle sample used for in situ incubations for the single cell ^{14}C uptake, and to fill the diffusion chambers showed a very low concentration of *Dinophysis* spp (100-300 cells/l), but much more abundant

populations of *Prorocentrum micans* and *Helgolandinium* sp. Therefore, attention will be focused in these two species besides the attention on *Dinophysis* spp.

The low concentration of *Dinophysis* spp will not affect the method based on mitotic indices, because this is based on frequencies (not on concentrations) and because the net haul (20 µm) sampling will assure the supply of enough cells.

Preliminary counts of the dinoflagellate populations at time zero (t_0) and after 48h of incubations (t_{48}) incubated at 0 and 5 m depth, showed that all phytoplankton populations had a drastic decrease in numbers. The content of the diffusion chamber had a very high proportion of detritus that prevented any growth and caused damage to the surviving cells that did not look very healthy. This was due to the high content of detritus in Ria de Aveiro combined with the use of 20 µm mesh size in the extremes of the chamber.

5.4 ^{14}C method in situ.

Three measurements on 26 July and one on 27 July were carried out at the station situated in the mouth channel of the Ria. The very strong tidal currents involved that only the samples incubated close to the surface (0.5 m) were accurate.

Day	Time	Chlorophyll *a*	Prim. prod. 0.5 m	Chl./Pp.
		$\mu g \cdot l^{-1}$	$mgC \cdot l^{-1} \cdot h^{-1}$	
26	08.15 am	11.6	128	10.8
	11.10 am	9.6	55	5.5
	14.30 pm	15.1	131	8.5
27	08.15 am	no data	107	no data

Both the chlorophyll *a* concentration and the primary production were high, i.e. in a range typical for an eutrophied area. However, the chlorophyll to primary production ratios (assimilation number) were comparatively low, indicating that the phytoplankton community at this station was not growing at a high rate. At present there is no other explanation than patchiness to the large variation in chlorophyll and productivity between the different measurements.

In order to avoid the strong currents an incubation was carried at the raft with the bags in the afternoon on the 27th. One bottle was incubated at each 0.5 m down to 4 m depth (figure 2). Light inhibition at the surface involved that a maximum productivity of 340 $\mu gC \cdot l^{-1} \cdot h^{-1}$ was found at 0.5 m depth. This was a very high value. Still at 2 m depth the productivity was around 200 $\mu gC \cdot l^{-1} \cdot h^{-1}$ and at 4 m (just above bottom) a productivity of 22 $\mu gC \cdot l^{-1} \cdot h^{-1}$ was measured. (As a comparison it could be mentioned that a high spring bloom value may reach 75 $\mu gC \cdot l^{-1} \cdot h^{-1}$ and high summer values are around 25 $\mu gC \cdot l^{-1} \cdot h^{-1}$ in Scandinavian coastal waters). When integrated over depth the productivity was 699 $mgC \cdot m^{-2} \cdot h^{-1}$ and the daily production was estimated by the light factor method (BMB, 1976) to 7700 $mgC \cdot m^{-2} \cdot d^{-1}$, which indicated that the primary production was very high on this occasion.
Unfortunately, no chlorophyll samples were taken during this day. The Secchi-depth was 1.5 m at all primary productivity measurements.

5.5 Monochlonal antibodies, species specific diel DNA measurements and bioassay.

Because *Dinophysis* spp abundance was low, there was no opportunity to collect enough cells for monochlonal antibody production. Therefore, *Dinophysis* specific growth rates can probably not be measured with the NICMM flowcytometer in the near future.

The 48 h sampling programme was reduced to 36 h. Two vertical net hauls were taken with a 20 µm plankton net at two stations. Sample processing will begin in 1995. Division rate measurements will be focussed on *Prorocentrum micans*.

Bioassay samples were incubated for 49 h at 1 m depth. *In vivo* fluoresence measurements suggested that GF, Chel and pH were not significantly different from NONE ($p \geq 0.05$) and that PEP-Si and ALL were not significantly different from NONE ($p > 0.05$). However, they were all different from NONE, GF, Chel and pH as a group ($p < 0.05$). PEP-Si and ALL were not significantly different from each other.

The preliminary cell counts showed as a general trend that *Helgolandium subglosum* and *Leptocylindrus danicus* increased during the incubation, while *Ceratium fusus* declined. The effect of the different treatments seems negligible or even negative. A complete report, including references, will become available later this year at the National Institute for Coastal and Marine Management (Holland).

6. Action list.

The participants of the workshop agreed on the following action list:

6.1 To prepare results so that a comparison and evaluation of methods and techniques used during the workshop can be made. This work should be done within a year and finally be presented as an ICES Co-operative Research Report.

6.2 To prepare a poster regarding the workshop for the7th International Conference on Toxic Marine Phytoplankton in Sendai, Japan, 1995. Dr M.A. Sampayo and Dr O. Lindahl agreed to co-ordinate this work.

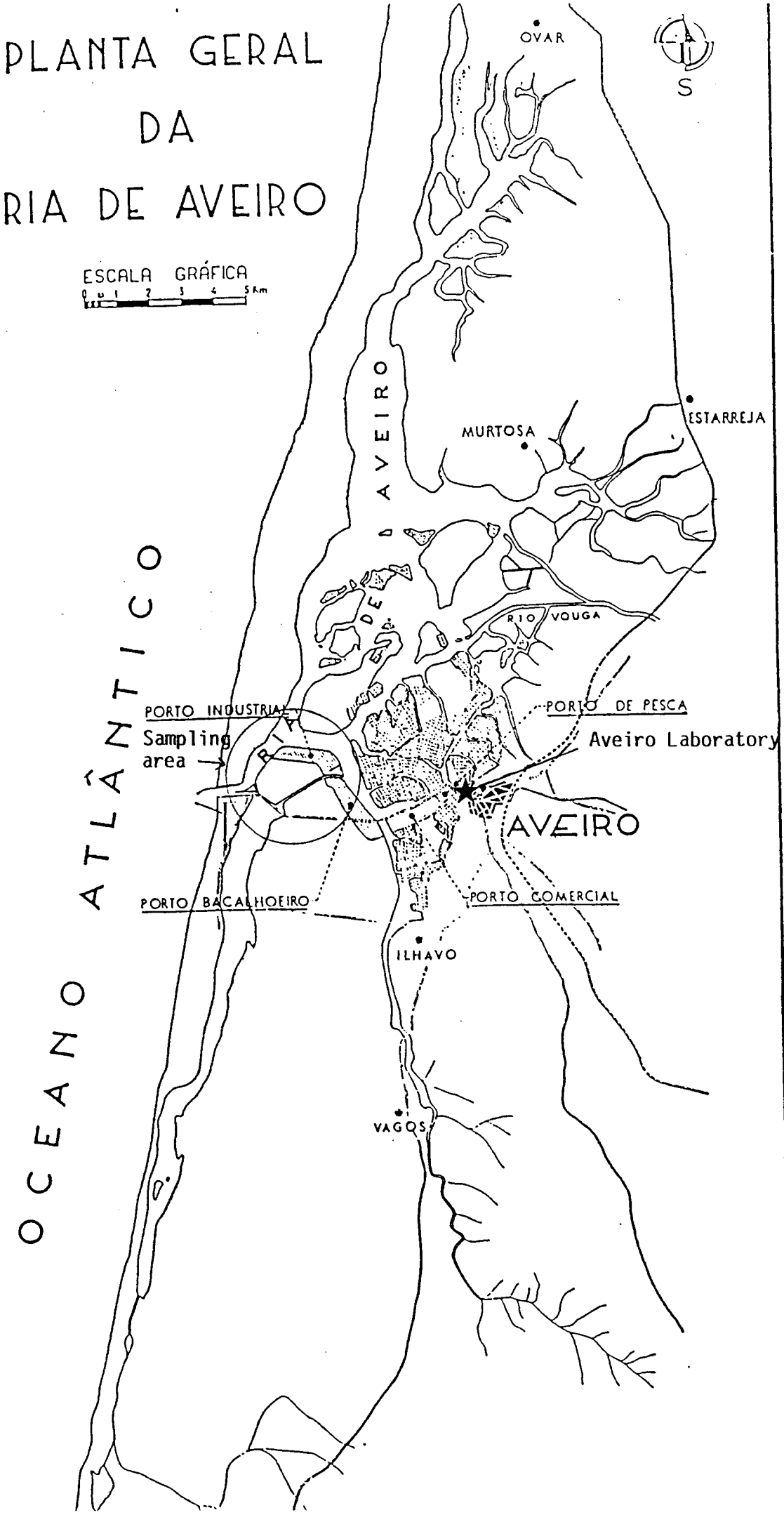
7. Recommendations.

7.1 The Workshop strongly recommends that a final report of the obtained results and a comparison and evaluation of the different methods which were used, are made. It is suggested that this report shall be in the ICES Co-operative Research Report series.

7.2 In order to effectively fulfil recommendation 7.1 the Workshop suggests that the participants of the workshop reconvene for two full days, just before the meeting of the WG on "The dynamics of algal blooms" in Helsinki, Finland in May 1995.

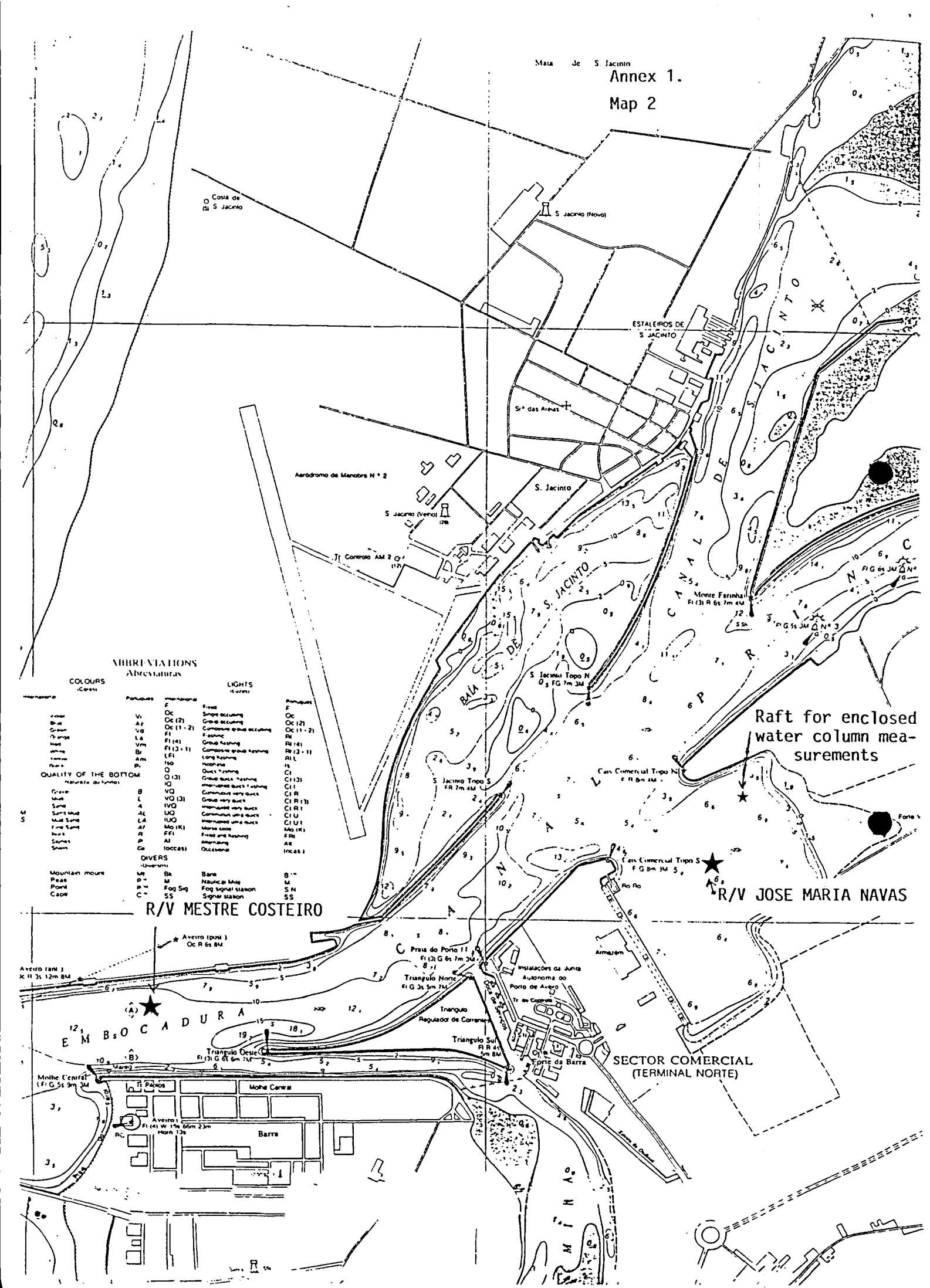
7.3 The Workshop finally recommends that more workshops on phytoplankton growth rates are carried out, where intercalibration of existing methods are tested and evaluated against new ones.

Annex 1.
Map 1


PLANTA GERAL
DA
RIA DE AVEIRO
ESCALA GRÁFICA
0 1 2 3 4 5 Km
N
S
OVAR
ESTARREJA
MURTOSA
RIA DE AVEIRO
OCEANO ATLÂNTICO
RIO VOUGA
PORTO INDUSTRIAL
Sampling area
PORTO DE PESCA
Aveiro Laboratory
AVEIRO
PORTO BACALHOEIRO
PORTO COMERCIAL
ILHAVO
VAGOS

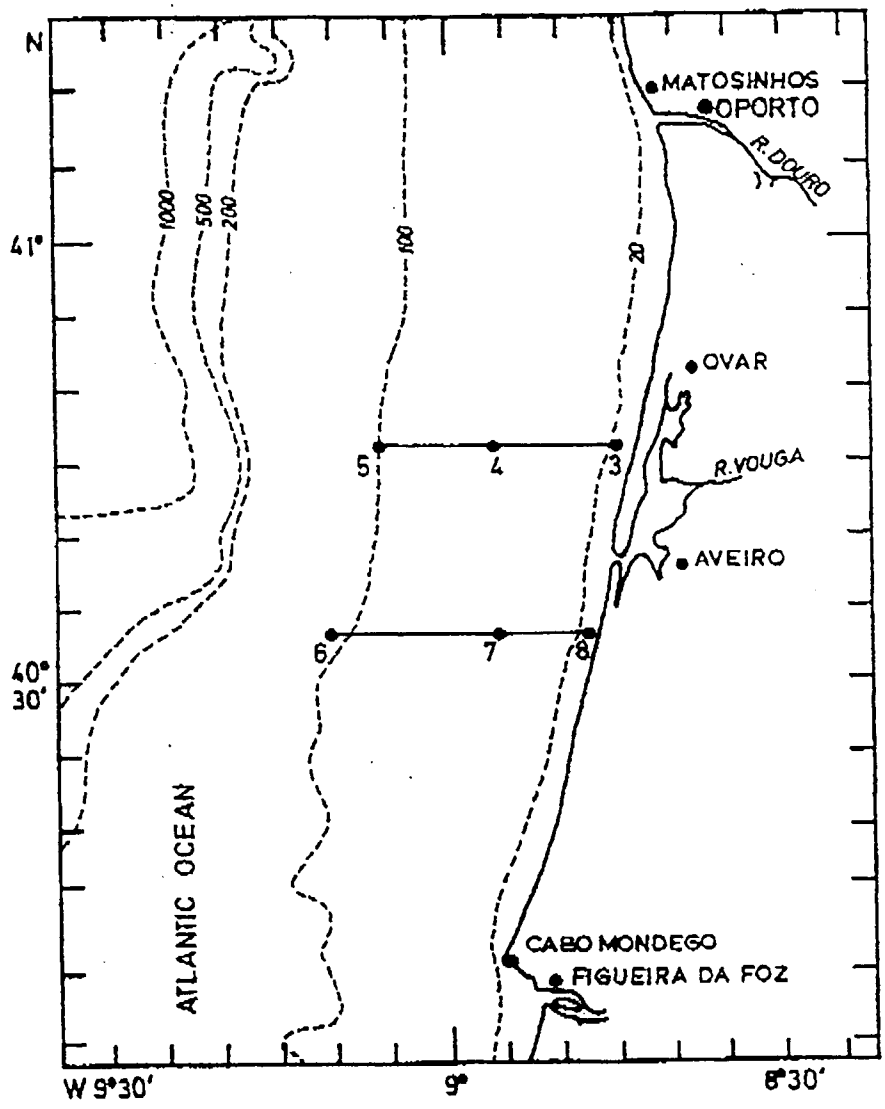
Annex 1.

Map 2

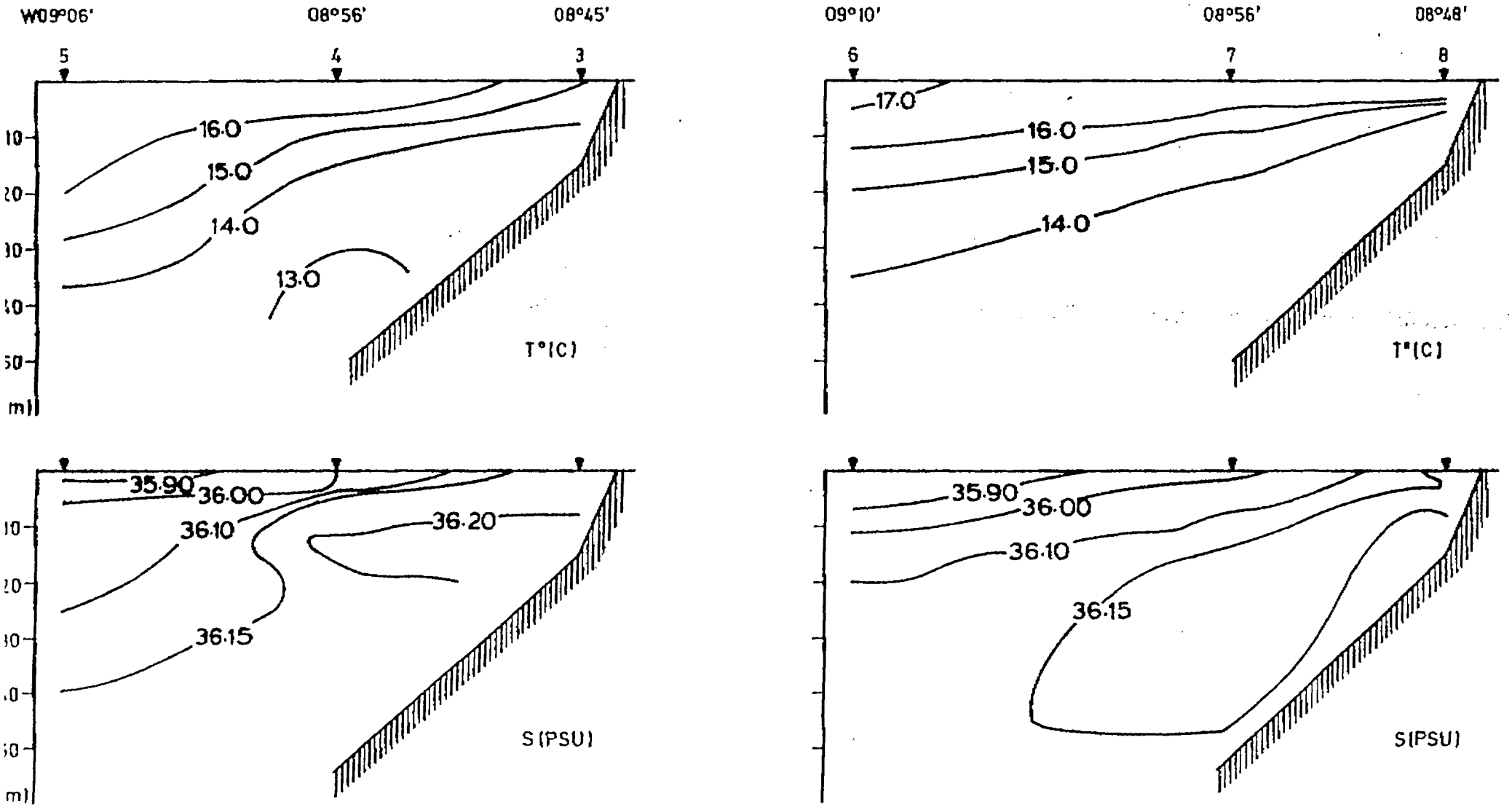

Mata de S. Jacinto
Casa de S. Jacinto
S. Jacinto (Nova)
ESTALEIROS DE S. JACINTO
CANAL DE S. JACINTO
Sr.ª das Areias
Aeródromo de Manobra N.º 2
S. Jacinto
S. Jacinto (Velha)
Torre de Controlo AM 2
BAIA DE S. JACINTO
S. Jacinto Topo N
Monte Farinha
Fl (3) R 6s 7m 4M
S. Jacinto Topo S
Cais Comercial Topo N
Cais Comercial Topo S
Raft for enclosed water column measurements
R/V JOSE MARIA NAVAS
R/V MESTRE COSTEIRO
CANAL DE AVEIRO
Aveiro (ext.)
Oc R 6s 8M
Aveiro (int.)
Praia do Porto
Triangulo Norte
Triangulo Regulador de Corrente
Triangulo Sul
Triangulo Oeste
EMBOCADURA
Molhe Central
Barra
Aveiro
Fl (4) W 15s 65m 23m
Instalações da Junta Autónoma do Porto de Aveiro
Armazém
Torre da Barra
SECTOR COMERCIAL (TERMINAL NORTE)
ABBREVIATIONS
Abreviaturas
COLOURS
LIGHTS
QUALITY OF THE BOTTOM
DIVERS

Annex 1.

Map 3

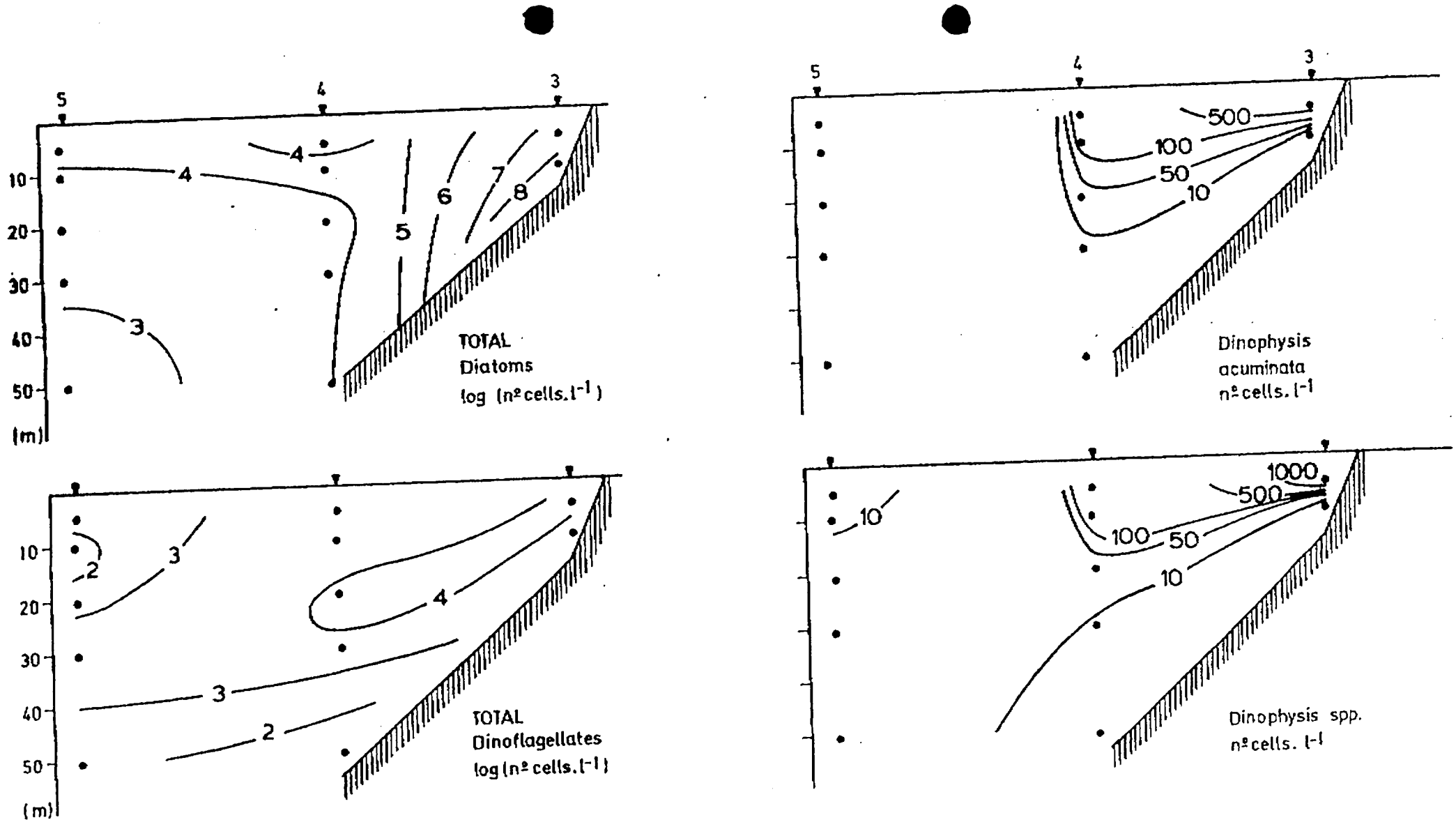


- Sampling stations on 21st July.



Annex 1.
Fig. 1

Fig. 1 - Vertical distribution of temperature and salinity at two sections.



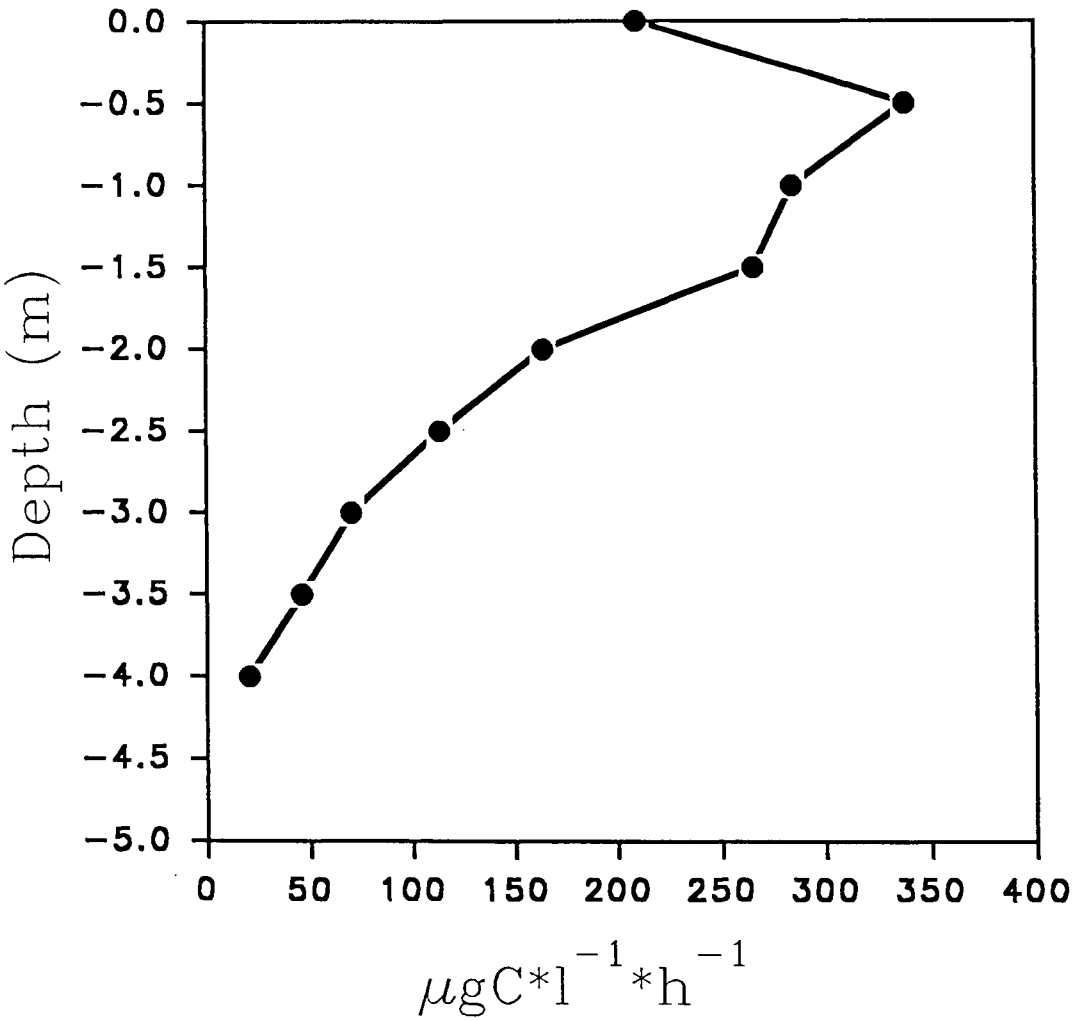
Annex 1.
Fig. 2

Fig. 2 - Vertical distribution of phytoplankton at one section.

Primary productivity

"Einars raft"*, Ria de Aveiro

94.07.27



* the raft where the enclosed water column measurements were carried out.

Table 2. Occurrence of Dinophysis spp. (cells/l) and chlorophyll (µg/l) in bag 1

Date	Time	Hours	D. acuminata	D. acuta	D. caudata	D. rotundata	D. tripos	Chloroph.	Phaeopig.
22 July	1800	0	1070	30	0	20	20	6.39	2.57
23 July	1200	18	1120	60	0	20	60	7.10	2.67
24 July	1000	40	1060	160	0	60	30	5.56	1.91
25 July	1300	63	800	30	10	30	0	3.98	1.34

Table 3. Occurrence of Dinophysis spp. (cells/l) and chlorophyll (µg/l) in bag 2

Date	Time	Hours	D. acuminata	D. acuta	D. caudata	D. rotundata	D. tripos	Chloroph.	Phaeopig.
22 July	1800	0	1020	40	0	30	20	6.70	2.32
23 July	1200	18	1080	50	20	30	0	7.26	2.60
24 July	1000	40	1060	100	10	50	0	5.34	1.74
25 July	1300	63	760	40	10	40	0	2.25	0.68
26 July	700	81	640	70	10	130	10	3.80	1.97
26 July	1300	87	400	40	0	70	0		
26 July	1900	93	280	10	0	50	0		
26 JUly	2400	98	210	0	0	40	0		
27 July	700	105	300	10	0	60	10	2.64	1.16
27 July	1400	112	280	10	0	90	0	2.62	0.69
28 July	1200	134	200	20	0	30	0	14.51	3.38
29 July	1400	160	90	10	0	90	0	14.16	3.06

Table 4. Occurrence of Dinophysis spp. (cells/l) and chlorophyll (µg/l) in bag 3

Date	Time	Hours	D. acuminata	D. acuta	D. caudata	D. rotundata	D. tripos	Chloroph.	Phaeopig.
24 July	1000	0	700	40	0	100	10	5.73	2.22
25 July	1300	27	840	20	0	60	0	7.31	2.08
26 July	700	45	610	60	0	160	20	4.58	1.93
26 July	1300	51	650	30	0	80	20		
26 July	1900	57	570	40	0	110	0		
26 July	2400	62	430	40	0	150	0		
27 July	700	69	520	100	0	140	0	4.09	3.48?
27 July	1400	76	540	40	0	150	20	5.64	1.58
28 July	1200	98	180	0	0	110	10	4.80	2.24
29 July	1400	124	40	0	0	150	0	2.60	0.83

Table 5. Occurrence of Dinophysis spp. (cells/l) and chlorophyll (µg/l) in bag 4

Date	Time	Hours	D. acuminata	D. acuta	D. caudata	D. rotundata	D. tripos	Chloroph.	Phaeopig.
24 July	1000	0	820	80	0	50	0	7.16	2.26
25 July	1300	27	1120	20	0	70	30	11.03	2.90
26 July	700	45	670	120	0	50	10	5.68	2.54
26 July	1300	51	860	30	20	50	10		
26 July	1900	57	400	0	0	50	10		
26 July	2400	62	320	40	0	70	0		
27 July	700	69	370	50	0	20	30	6.65	2.46
27 July	1400	76	180	10	0	110	0	6.08	0.34
28 July	1200	98	240	30	0	40	0	18.21	4.07
29 July	1400	124	130	30	0	70	0	18.60	3.36

Table 6. Occurrence of Dinophysis spp. (cells/l) and chlorophyll (µg/l) in bag 1-II

Date	Time	Hours	D. acuminata	D. acuta	D. caudata	D. rotundata	D. tripos	Chloroph.	Phaeopig.
25 July	1800	0	670	160	0	30	10		
26 July	700	13	690	90	0	20	40	15.32	3.64
26 July	1300	18	1180	90	10	10	0		
26 July	1900	24	950	20	0	30	0		
26 July	2400	29	-	-	-	-	-		
27 July	700	36	640	100	0	70	30	12.49	3.80
27 July	1400	43	870	80	0	0	0	5.29?	3.37
28 July	1200	65	870	70	0	40	0	22.33	6.00
29 July	1400	91	270	50	0	30	10	19.80	3.36

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