Initial results on the use of pH-stat titration (AIDA method) for the measurement of algal photosynthesis

V Mezzanotte[1]*, C Colombo[1], E Ficara[2] and A Rozzi[2]
[1] *Dept. DISAT, State University Milano-Bicocca, Milan, Italy*
[2] *Dept. DIIAR, Politecnico di Milano, Italy*

Abstract

The present work deals with the development of a method, named AIDA (algal inhibition detection analysis) for the measurement of algal photosynthesis by pH-stat titration. According to the pH probe signal, a titrator doses a saturated solution of CO_2 to maintain the pH at a set-point value, buffering variations induced by photosynthetic CO_2 consumption. The CO_2 titration curve, after an initial lag Phase, is well interpolated by a straight line, whose slope is proportional to the photosynthetic CO_2 uptake, until photosynthesis becomes limited probably due to nutrients limitation. Preliminary experimental results confirm that, under comparable test conditions, activity estimation was well repeatable. Moreover, the method could sense the photosynthetic response to nutritional changes (N:P ratio) and to the presence of toxicants. Therefore, AIDA shows potential applicability for both limnological studies and biomonitoring.

Keywords: photosynthesis, titration, pH-stat, activity, toxicity

Introduction

There is a growing interest for the assessment of algal photosynthesis for toxicological and for limnological investigations.

With respect to toxicity studies, toxicological analyses of industrial wastewater before discharge in water bodies are more and more frequently required. In Italy, the recent law on water protection (D.Lgs. 152/99) requires that the toxicity of a wastewater is tested by biomonitoring with organisms of various trophic levels, such as *Daphnia magna*, *Ceriodaphnia dubia*, *Selenastrum capricornutum*, bioluminescent bacteria, etc. According to the law, wastewater is acceptable for discharge into surface water when bioassays point out toxicity symptoms in no more than 50 % of the exposed organisms. For public sewers the limit is raised to 80%. Internationally, the most widely adopted algal inhibition test is the OECD (1981a) method, where algal growth is assessed by cell count normally made by a Coulter counter.

With reference to limnological studies, phytoplankton is particularly important since it is responsible for primary production in water and forms the first ring in the trophic chain of aquatic ecosystems. In these investigations, photosynthetic activity could be an important parameter which, however, is often disregarded due the non-availability of simple and reliable determination methods. In general, primary production is evaluated either by the oxygen method of dark and transparent bottles (Gaarder and Gran, 1927), or by the estimation of ^{14}C incorporation (Steemann-Nielsen, 1952). However, both methods involve practical problems due to the need for maintaining bottles submerged in the lake at a fixed depth, for a defined time interval, and results are not always reliable. The second method involves a more scientific approach, but it requires specific skills and equipment (Harris, 1986; Vollenweider, 1974). At present, in studies on eutrophication, primary production and algal blooms are evaluated on the basis of indirect parameters such as water transparency (Secchi disk) or chlorophyll concentration, which is a difficult analysis, the results of which are greatly affected by the extraction procedure and by the algal species.

This paper presents a new method to measure algal activity based on the estimation of CO_2 consumption by pH-stat titration. This is achieved by means of an instrument originally designed to evaluate microbiological activities, such as nitrification (Ramadori et al., 1980, Massone et al., 1998), on the basis of the pH variation they induce.

Materials and methods

AIDA: Principle of the method

The AIDA (algal inhibition detection analysis) method has been developed to measure activity and inhibition of photosynthetic micro-organisms. The principle of operation exploits the ability of these micro-organisms to increase the suspension pH by converting an acid substrate (CO_2) into a neutral product (biomass), according to the gross photosynthesis equation:

$$6\ CO_2 + 6\ H_2O \rightarrow C_6H_{12}O_6 + 6\ O_2 \qquad (1)$$

The method requires retaining constant pH by means of an automated titrator. As a matter of fact, in pH-stat conditions and in the absence of other pH-affecting reactions, the amount of CO_2 titrated (as CO_2- saturated solution) used to buffer the pH increase, equals the amount of CO_2 consumed by photosynthesis. Moreover, the rate of titrant addition is proportional to algal activity. Additionally, this method allows the measurement of algal activity at constant substrate (CO_2) concentration.

The procedure is registered at the Patent Office of Politecnico di Milano (*Titrimetric Method to Assess Activity and Inhibition of*

* To whom all correspondence should be addressed.
☎+39-02-64482736; fax:+39-02-64482795;
e-mail: valeria.mezzanotte@unimib.it
Received 12 January 2004; accepted in revised form 13 May 2004.

Photosynthetic Organisms, Rozzi Alberto and Ficara Elena; Prot. nr.: 480/00, 9/22/2000).

Instrumentation

The instrumentation used in this experimentation (Figs. 1 and 2) comprised:

- An Erlenmeyer flask (V = 0.5 ÷ 2 ℓ) provided with openings for the titrant dosing line, the pH probe, the temperature probe and the gas outlet; the Erlenmeyer flask is placed into a bath fitted with a thermostat and mixed by a magnetic stirrer;
- A pH-stat titration unit (the commercially available MARTINA unit, SPES, Fabriano (AN), I) for the controlled addition of titrant; the titrant dosing line consists of a continuously working peristaltic pump and a micro-electrovalve, automatically activated according to the pH value, which directs the titrant flow rate either back to the titrant vessel or to the Erlenmeyer flask.
- A PC for data logging (pH, temperature values and volume of titrant added).

A light source provided a light intensity of 5 000 lux. The titrant solution was a CO_2 saturated solution, obtained by bubbling a 50% CO_2 to 50% N_2 gas from a gas bottle, in mineral water. CO_2 concentration, calculated from the CO_2 Henry constant at the working temperature (25°C), was 743.6 mg·ℓ$^{-1}$.

Photosynthetic organisms

Tests were performed on cells from pure algal cultures (*Scenedesmus quadricauda*), grown in a phytotrone, at constant light intensity (5 000 lux) and temperature (20°C), on a soil extract buffered by addition of solid $CaCO_3$ (Cosson, 1986).

Preparation of algal suspension

Measured aliquots of algal suspension were collected from the *Scenedesmus quadricauda* culture during exponential growth and diluted into 250 mℓ of either mineral water or filtered lake water in order to obtain 20 000 cells·mℓ$^{-1}$ algal density (D). Algal cells were counted by static citometry. Lake water was collected from a eutrophic lake (Lake Alserio, Northern Italy) in early spring (from the end of February until the end of April), while phytoplankton productivity was increasing after the winter season, but nutrients had not yet been significantly depleted. The lake water was then filtered on a 0.45µm membrane to remove phytoplankton. Waters were analyzed for nitrogen and phosphorus concentration according to Valderrama (1981).

For a first series of 7 tests, algal inoculum was diluted into 250 mℓ of the mineral water, while the filtered lake water was used for a second series of 3 tests. N and P concentrations were always analysed to assess their ratio and concentrations.

Activity and inhibition tests

The algal suspension was poured into the AIDA reactor, and pH was monitored until a stable reading was obtained. This value was entered as the set-point pH, which had to be maintained constant by the titration unit. Then, light was switched-on, triggering photosynthetic activity. As soon as the pH increased, titration started, allowing following the photosynthetic activity in the algal suspension.

A toxicity test was carried out with copper sulphate ($CuSO_4$).

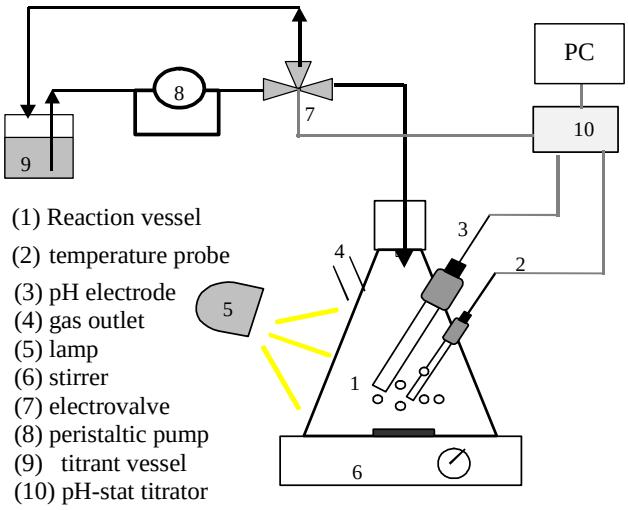

Figure 1
Instrumentation used to perform AIDA tests

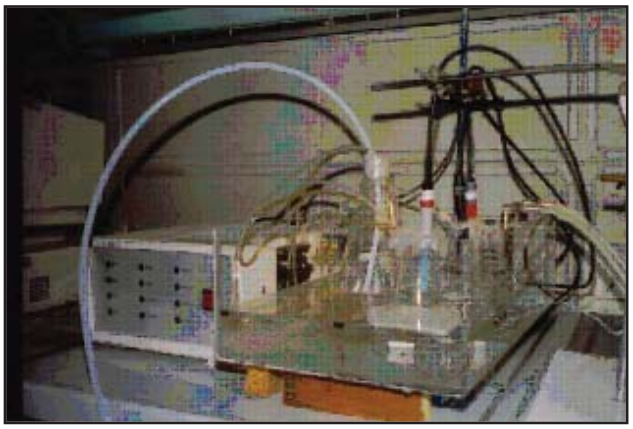

Figure 2
Picture of AIDA test apparatus

First, algal activity was allowed to reach a constant rate, then the toxicant was spiked and the consequent decrease in titration rate was monitored. The toxicant solution was spiked twice in order to obtain 1 and 2 mg·ℓ$^{-1}$ in the algal cell suspension.

Results and discussion

Activity tests

A typical titration curve obtained from an AIDA activity test is plotted in Fig. 3. The first part of the curve (Phase (1) in Fig. 3) represents the lag Phase; in the second part (Phase (2)), the titration curve is nearly a straight line, indicating a constant CO_2 consumption. During the last part of the test (Phase (3)), the curve reaches a horizontal asymptote probably because photosynthesis stops due to the depletion of nutrients during the test.

Carbon dioxide assimilation rate was calculated on the basis of the slope of the titration curve ($Q_{titrant}$ in Fig. 2) assessed during Phase (2). Photosynthetic activity, referred to the test volume, was calculated as follows:

$$PA_V = Q_{titrant} \cdot [CO_2]_{titrant} / V_{sample,} \quad (2)$$

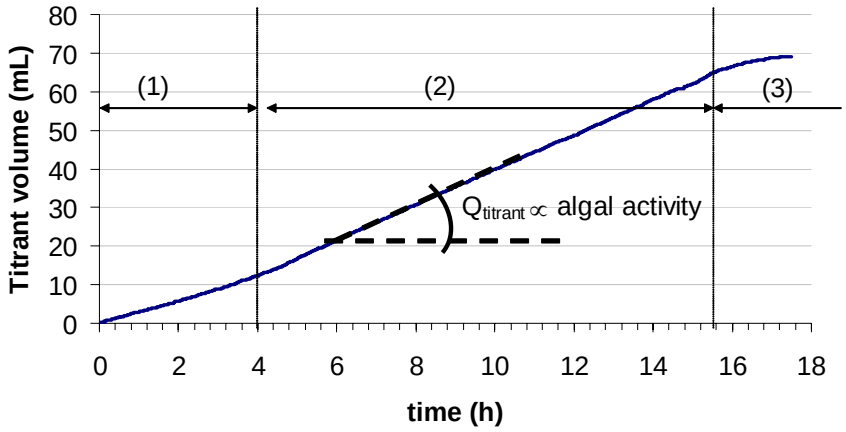

Figure 3
Typical trend of a titration curve during AIDA tests: (1) lag phase; (2) unlimited growth phase; (3) limited growth phase

TABLE 1
Results and test conditions of activity tests on *Scenedesmus quadricauda*. Inoculum diluted in mineral (M) and lake (L) water.

Test	PA_V ($mgCO_2 \cdot \ell^{-1} \cdot h^{-1}$)	PA_{cell} ($ngCO_2/cell \cdot h^{-1}$)	pH	Temp (ºC)	N:P	N ($mg \cdot \ell^{-1}$)	P ($mg \cdot \ell^{-1}$)
1-M	4.99	0.25	8.02	24.4	14.5	1.69	0.12
2-M	2.83	0.14	8.24	24.7	15.9	1.59	0.10
3-M	3.5	0.18	8.25	23.9	21.1	1.43	0.07
4-M	2.99	0.15	8.11	23.8	24.5	1.37	0.06
5-M	2.74	0.14	8.33	24.6	23.2	1.39	0.06
6-M	5.49	0.28	7.93	26.4	7.0	0.70	0.10
7-M	5.26	0.26	8.13	25.7	7.0	0.70	0.10
mean	***3.97***	***0.2***	***8.1***	***26.2***	***16.2***	***1.27***	***0.09***
st .dev.	***1.23***	***0.06***	***0.14***	***0.9***	***7.24***	***0.40***	***0.02***
1-L	6.22	0.31	7.93	24.7	6.6	1.75	0.27
2-L	5.27	0.26	8.08	25.1	6.6	1.73	0.26
3-L	11.54	0.58	7.77	26.0	7.1	1.53	0.20
mean	***7.68***	***0.38***	***7.93***	***25.3***	***6.8***	***1.67***	***0.24***
St. dev.	***3.38***	***0.017***	***0.16***	***0.6***	***0.30***	***0.12***	***0.04***

where:

PA_V = photosynthetic activity (mg $CO_2 \cdot \ell^{-1} \cdot h^{-1}$) per unit volume
V_{sample} = sample volume (ℓ)
$Q_{titrant}$ = titrant flow rate ($m\ell \cdot h^{-1}$)
$[CO_2]_{titrant}$ = CO_2 concentration in the titrant solution (mg $CO_2 \cdot m\ell^{-1}$)

In order to obtain more specific information, PA can be referred either to the number of algal cells (PA_{cell}) or to biomass unit (PA_B), as follows:

$$PA_{cell} = PA_V / D \quad (3)$$

where:

PA_{cell} = specific photosynthetic activity per algal cell (mg $CO_2 \cdot cell^{-1} \cdot h^{-1}$)
D = algal cell density in the test suspension ($cells \cdot \ell^{-1}$)

or:

$$PA_B = PA_V / B \quad (4)$$

where:

PA_B = specific photosynthetic activity per unit of biomass (h^{-1})
B = biomass concentration ($mg \cdot \ell^{-1}$).

Biomass concentration can be obtained either gravimetrically or, in the case of pure cultures, by calculation, from the number of cells, being known the average cell biovolume for a given species and assuming that cell density for all phytoplankton species is 1 $kg \cdot \ell^{-1}$.

Table 1 reports experimental conditions and activities measured in 10 tests carried out by suspending the algal inocula in mineral and lake waters. In both cases pH and temperature varied within a relatively narrow range (pH range = 7.77 to 8.33 and T range = 23.8 to 26.4). In mineral waters nutrient concentrations and N: P ratio (considering inorganic nitrogen and soluble phosphorus) were considerably different from test to test (N range = 0.70 to 1.69; P range = 0.06 to 0.12; N: P range = 7.0 to 24.5), while in lake waters variations were less important (N range = 1.53 to 1.75; P range = 0.20 to 0.27; N: P range = 6.6 to 7.1). As a consequence, lower mean values were obtained for PA_v and PA_{cell} in mineral water than in lake waters: mineral water had, in some cases, N:P ratio far from the optimal range (6 to 15), while lake water was richer in P and, probably, in micronutrients, positively affecting

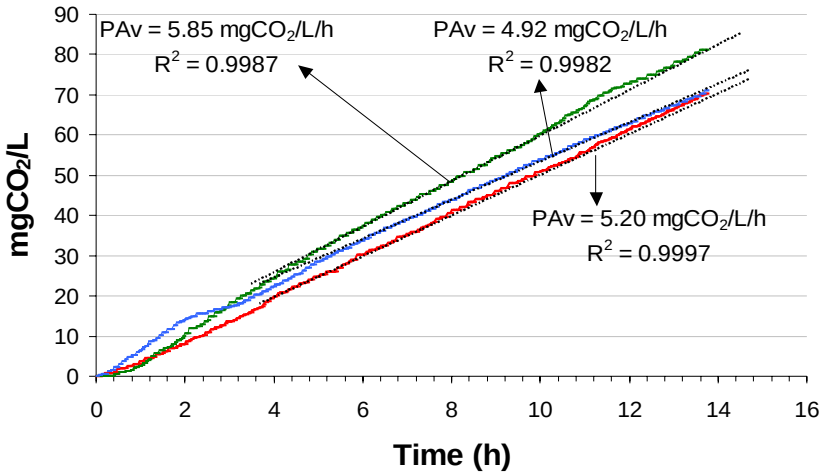

Figure 4
Comparison between photosynthetic activity measured in three tests, in similar conditions: N: P = 6,7 (w/w), light intensity = 5 000 lux, $pH_{set\text{-}point}$ = 7.8 ,8.2, algal density = 20 000 cells ml $^{-1}$

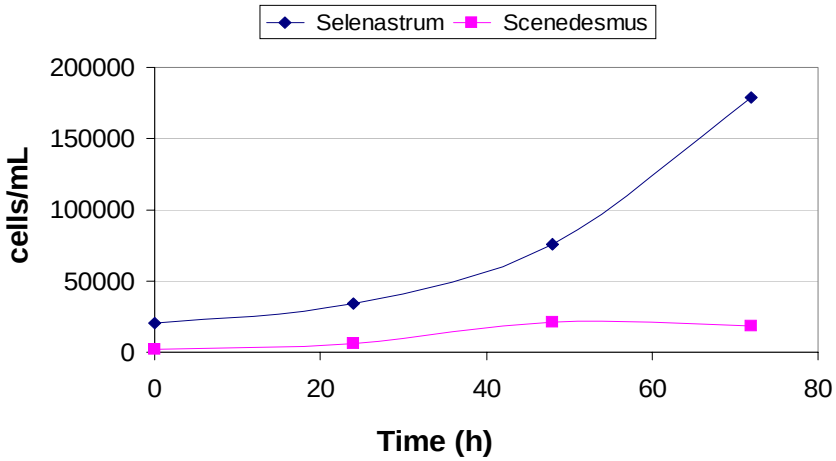

Figure 5
Growth curves for Selenastrum capricornutum *and* Scenedesmus quadricauda

algal activity.

In order to assess the repeatability of photosynthetic activity estimation, tests were carried out in comparable conditions (N: P ratio, light intensity, pH, algal density and temperature). In Fig. 4, three repetitions are plotted. As it can be observed, the activity (i.e. the slope of the titration curve measured after the lag Phase) is similar for the three tests. The coefficient of variation (CV = 9.8%) indicates a satisfactory repeatability of the test results.

In the literature only limited information is available on photosynthetic CO_2 assimilation by algae. Mingazzini et al. (1997) quantified algal oxygen production by *Selenastrum capricornutum* as 1.2 to 1.6·10 $^{-3}$ nmol·cell^{-1}·min^{-1}. Considering that, during photosynthesis, the production of 1 mole of oxygen derives from the assimilation of 1 mole of CO_2 (Eq.(1)) and that 1 nmol of CO_2 corresponds to 44 ng, PA_{cell} could be calculated as 2.6 to 3.5 ng CO_2·cell^{-1}·h^{-1}. AIDA estimates that *Scenedesmus quadricauda* were in the range 0.1 to 0.6 ngCO_2·cell^{-1}·h^{-1}. This difference is reasonable taking into account the slower growth rate of *Scenedesmus quadricauda* that is clearly shown in Fig. 5, reporting two series of experimental data obtained in comparable conditions.

The influence of environmental factors such as N:P ratio and working pH was investigated.

As in natural conditions, during AIDA tests, N:P ratio was found to be the most significant factor affecting the photosynthetic activity. Actually, tests were all carried out with nutrients concentrations above their limiting thresholds (N = 100 mg·ℓ$^{-1}$, P = 10 mg·ℓ$^{-1}$ according to Mur and Schreurs, 1988).Within the investigated range (from 6 to 25), the highest activity was obtained for an N:P ratio between 6.5 and 15, in agreement with the general assumptions on nutrient limitation (OECD, 1981b), as it is shown in Fig. 6.

The second factor considered for its influence on PA_{cell} was pH. As a matter of fact, the availability of CO_2 as such depends on pH. That is why CO_2 photosynthetic uptake decreased with the increase in the set-point pH, as indicated in Fig. 6. For pH>8, the equilibrium shifts towards HCO_3^- (97% of the total inorganic carbon), which is not directly available for algal photosynthesis.

Toxicity tests

As previously mentioned, a possible application of the AIDA method is the measurement of photosynthetic activity for toxicity assessment. Preliminary long-term tests (10 to 20 h) were carried out which gave encouraging results. However, for practical reasons, it was decided to study toxicity in shorter-term experiments. Figure 7 reports a titration curve derived from a 6 h test with two subsequent additions of $CuSO_4$, a well-known algal toxicant. At the end of the lag phase, the value of the curve slope (PA_v = 5.18 mg CO_2·ℓ$^{-1}$·h^{-1}) was very similar to those reported in Fig. 4. After the first $CuSO_4$ addition, the algal activity decreased slightly (PA_v = 5.04 mg CO_2·ℓ$^{-1}$·h^{-1}), while it was appreciably lower after the second addition (PA_v = 1.65 mg CO_2·ℓ$^{-1}$·h^{-1}). This result indicates the possible application of this procedure to estimate toxicity by comparing the slope of the titration curve obtained in standard conditions to the one obtained in the presence of the tested toxicant.

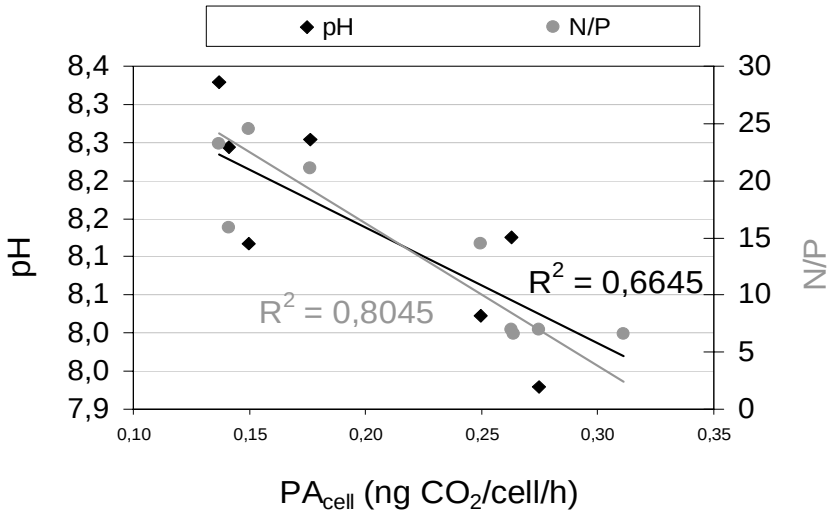

Figure 6
Effect of N: P and pH on algal activity (PA_{cell})

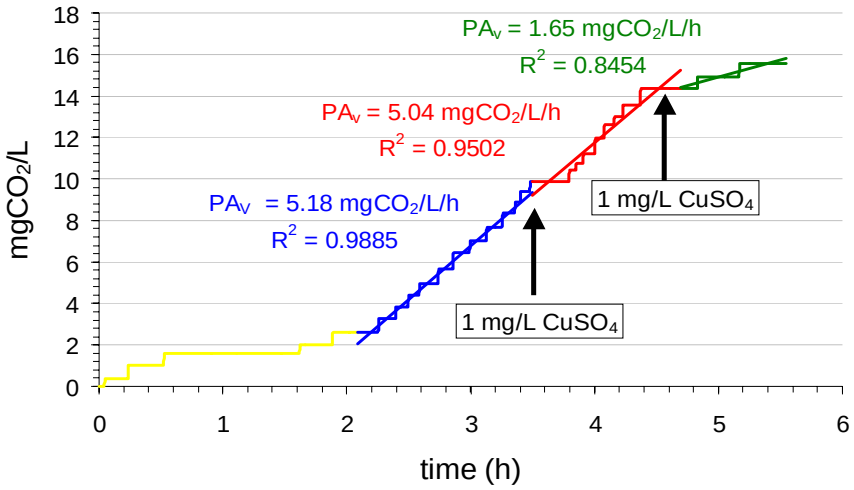

Figure 7
Titration curve obtained in a 6 h test with two $CuSO_4$ additions. Test conditions: N: P = 7 (w/w), light intensity = 5 000 lux, pH = 8.0, algal density = 20 000 cells $m\ell^{-1}$

Conclusions and perspectives

An original method (AIDA) is presented to estimate algal activity and inhibition. The algal CO_2 uptake rate is quantified by using a pH-stat titrator, which compensates CO_2 consumption by controlled additions of a CO_2 saturated solution maintaining constant both pH and CO_2 concentration in the algal suspension.

Experimental results from a limited number of preliminary tests suggest that AIDA may be used for assessing algal photosynthesis in natural environments (primary production in lakes) and for toxicity testing. The work is still in progress with the aim of standardizing the test procedure and to find out possible interfering factors. With reference to toxicity assessment, the work will include the definition of the duration of the test and the comparison with standard methods. For this purpose, AIDA will also be used with pure cultures of other species, such as *Selenastrum capricornutum*, which is indicated as a reference species for algal toxicity assays by the Italian law.

References

COSSON J (1986) Croissance des sporophytes résultants d'hybridations interspécifiques et intergénériques chez les laminaires. *Cryptogamie-Algologie* **8** 61-72.

DECRETO LEGISLATIVO n.152, 11/5/1999 *Disposizioni sulla tutela delle acque dall'inquinamento e recepimento della Direttiva 91/271/CEE concernente il trattamento delle acque reflue urbane e della Direttiva 91/676/CEE relativa alla protezione delle acque dall'inquinamento provocato dai nitrati provenienti da fonti agricole.*

GAARDER T AND GRAN HH (1927) Investigation of the production of plankton in the Oslo Fjord. *Rapp. et Proc.-Verb., Cons. Int. Explor. Mer.* **42** 1-48.

HARRIS GP (1986) Photosynthesis, productivity and growth: the physiological ecology of phytoplankton. *Ergebn.Limnol.* **10** 1-163.

MASSONE A, GERNAEY K, ROZZI A and VERSTRAETE W (1998) Measurement of ammonium concentration and nitrification rate by a new titrimetric biosensor. *WEF Res. J.* **70** 343-350.

MINGAZZINI M, SÀENZ ME, ALBERGONI FG and MARRÉ MT (1997) Algal photosynthesis measurements in toxicity testing. *Fresenius Environ. Bull.* **6** 308-313.

MUR, LR and SCHREURS H (1988) Response of algal biomass to reduction in P concentration. *Proc. Int. Symp. Lake Restoration by Reduction of Phosphorus Loading.* Noorwijkerhout (NL), April 17-19, 1988.

RAMADORI R, ROZZI A and TANDOI W (1980) An automated system for monitoring the kinetics of biological oxidation of ammonia. Technical Note. *Water Res.* **14** 1555-57.

OECD (1981a) *Algae Growth Inhibition Test. Guidelines for testing of chemical. Effects on Biotic Systems, Test 201.*

OECD (1981b) *Eutrophication of Lakes.* Geneva, 1981.

STEEMANN-NIELSEN E (1952) The use of radioactive carbon (^{14}C) for measuring organic production in the sea. *J. Conseil* **18** 117-140.
VALDERRAMA J (1981) The simultaneous analysis of total nitrogen and total phosphorus in natural waters. *Mar. Chem.* **10** 109-122.
VOLLENWEIDER RA (ed.) (1974) *A Manual On Methods for Measuring Primary Production in Aquatic Environments* (2nd edn.). Blackwell Sci., Oxford.