

RED TIDES AND TOXIC ALGAL BLOOMS: WHO'S TO BLAME?

EDUARDO COSTAS

Abstract

Nigh on 5000 living phytoplankton species that make up the base of the marine food web, only 40 are known to be toxic. However, some dinoflagellates are able to produce such potent toxins that just a few dozen of cells per litre can be very harmful. While harmful algal blooms are natural phenomena that have occurred throughout recorded history, there is a conviction among many experts that the scale and complexity of this natural phenomenon are expanding. So, a major constraint to research and monitoring programmes for harmful algae stems from the need to identify the toxin-producing species within a mixed plankton sample. Invariably, microalgae maintain a fixed morphology whilst accumulating genetic variability within them. Hence, while considerable time, effort and skill are required to identify the alga responsible using microscopy, misidentification may appear if morphology is the sole criterion for the identification of toxin-producing species. A working alternative to morphological identification is the use of molecular probes that can bind to species-specific sites on thin target cells, to be visualized using spectrofluorimetry, flow-cytometry or epifluorescent microscopy. This communication will outline the recent progress in rooting out the dinoflagellates responsible for red tides and toxic algal blooms. The development of immunological, lectin-based and nucleic acid-based probes for harmful algal species goes hand in hand with this search. One such example of misplaced blame is the Mediterranean red-tide but non-toxic dinoflagellate *Gyrodinium impudicum*. It is so morphologically similar to the PSP toxin-producing *Gymnodinium catenatum* that neither species can be distinguished in formalin-fixed samples. Regular misidentification of these species has been long suspected. However both species can be rapidly and easily differentiated using the FITC-labelled WGA lectin, which specifically binds to *G. catenatum*. Thus the blame can be shifted back to the toxic species. Molecular probes can also clarify the taxonomy of toxigenic genera, for example, the genus *Alexandrium*. Further uses include facilitating rapid cell counts and separating toxic species, e.g. *A. minutum*, from mixed plankton assemblages from nature which can further aid the apparently unending search for the guilty algal species.

The Problem

“... all the waters that were in the river were turned to blood,
and the fish that were in the river died ...” (Exodus 7:20).

This biblical reference (about 1000 years BC) is believed to be the first written reference to a harmful algal bloom. Certain blooms of algae are called “red tides” when unicellular planktonic algae grow in such abundance (up to millions per litre) that they change the colour of the sea water to a reddish tone. In some cases algal cells become so dense that they cause anoxic conditions (at night or in dim light during the day, or by bacterial respiration during the decay of the bloom) resulting in the death of fish

and invertebrates. Red tides cause economic losses to aquaculture, fisheries and tourism. In other cases red tides are harmless or even beneficial to the surrounding fauna.

Obviously, the identification of the organism to blame for a red tide is very easy. However, some microalgal species produce such potent toxins that they can be deleterious even in low or moderate concentrations. Some microalgae are toxic to man at only a few hundred of cells per litre. Close to 2000 cases of human poisoning by phycotoxins from shellfish consumption are reported each year in the first world. Thousands of marine animals die every year by microalgal poisoning including vertebrates as big as manatees, porpoises and humpback whales (ANDERSON, 1994). So, rapid and unequivocal identification, counting and separation of toxic species has become one important focal point of toxic dinoflagellate research.

This however is not as simple as it may seem. Not all red tides are toxic, and not all toxic microalgae produce red tides. Of the thousands of phytoplankton species only a few dozen (about 6%) produce red tides, and only 40 (about 2%) are known to be toxic (SOURNIA, 1995). However, the majority of the toxic species, and about 50% of red tide species are dinoflagellates (SOURNIA, 1995).

While harmful algal blooms (or even the occurrence at low density) are natural phenomena occurring throughout recorded history, there is a conviction among many experts that the scale and complexity of this phenomenon are expanding. They have not failed to notice that toxic blooms, toxins and toxic species have all increased in number. Is this expansion real, or is it the result of an increase in scientific effort?. Many are convinced human activities such as the increased use of coastal waters in aquaculture, eutrophication, climatic change, or transport of resting cysts in ships' ballast water are causes of the expansion of harmful algae (HALLEGRAEF, 1993).

A major constraint to monitoring programs for harmful algae and marine biotoxins stems from the need to identify the alga responsible. The identification and enumeration of cells of potentially dangerous species within a mixed plankton assemblage is a serious problem. If the toxic alga is unknown, a lot of work is necessary to see whether toxin presence and algal occurrence are related. To prove toxicity one must use axenic cultures of the suspected alga.

Some species are so toxic that they can produce serious problems occurring at such a low density that they are virtually invisible to cell counts in monitoring programs. Considerable time and effort are required to identify a particular species especially when its distinguishing characteristics are difficult to discern under the light microscope.

What, if anything, is a harmful algal species?

An old dictionary defines "dog" as "a well known animal species". Unfortunately, the species concept in microalgae is not as clear-cut as for domestic animals. The term "species" has been used and polemized more than almost any other in biology. The first taxonomists detected morphological variation and species boundaries were recognized from gaps in the form range. So, species were defined based on "ideal" morphotypes which were applied to actual individuals without doubt under the influence of a Platonic concept. At present, morphological criteria are the primary means to classify harmful

species. However, taxonomic problems within toxic groups of species, i.e. "tamarensis complex" of the *Alexandrium* genus in dinoflagellates or *Pseudonitzschia* in diatoms, led to the implementation of renewed morphological work. The in-depth examination of thousands of individuals has built up a knowledge of conservative versus variable morphological characters for unicellular algae, giving place to fine-scale morphology (BALECH, 1989; TAYLOR, 1993). Fine-scale morphology is such a potent tool that BALECH (1985, 1989) was able to recognise more than 30 morphospecies within the genus *Alexandrium*.

However, the validity of fine-scale morphology with respect to species - and strain-level classification is subject to debate (TAYLOR, 1993; SCHOLIN & ANDERSON, 1994; COSTAS & al., 1994). At a theoretical level the morphospecies concept has often been viewed with skepticism ever since Darwin. Indeed many evolutionary biologists share the opinion that the species concept is just pure fiction, lacking any objective reality, as the supposed species are nothing more than ever-changing, continually developing natural populations. Contrarily, most biologists recognize species to be real evolving biological units (MAYR, 1982). In addition at a practical level, the detail and skill required in the characterization of harmful algal species using fine-scale morphology is so great that is just too complex for routine monitoring programmes. So, some alternatives to morphospecies concept have been proposed for microalgae.

The Biological Species Concept (BSC) is commonly accepted in modern biology. A specie is said to be composed of a set of potentially interbreeding genotypes, where the ability to interbreed and produce viable offspring provides the tool for recognizing species (MAYR, 1982). Unfortunately, special problems come into existence when applying the BSC to unicellular algae, because sexual reproduction is an apparently infrequent event or great difficulties exist in its being studied. In short, the BSC cannot be applied to monitoring programs.

During recent years a set of molecular probes, such as enzyme electrophoresis, toxin composition profiles, cell surface antigens, surface glycan moieties and RFLP analysis and DNA gene sequences have been used to discriminate strains and to clarify the relationship between morphotype and species-level division, provoking debate on the species concept in unicellular algae (reviewed by CASTENHOND, 1992; MANHART & MCCOURT, 1992; WOODS & LEATHAN, 1992; TAYLOR, 1993, COSTAS & al. 1995). In some cases, molecular markers corroborate morphotaxonomic classification of microalgae but in many others they do not, and the relationship between morphotaxonomy and molecular-taxonomy remains obscure. The problems stemming from the relationship between morphospecies and genospecies are complex. Owing to the great importance that asexual reproduction can have in unicellular algae, natural populations of these organisms are able form clonal families rather than true Mendelian populations, with scarce little genetic flow between them (COSTAS, 1990). In this sense, these clones accumulate genetic differentiation with time. In those cases where differentiation chiefly affects the genes controlling morphology, differences will appear that could be interpreted as differences at the level of morphospecies. Contrarily, if genetic differentiation affected those genes unrelated with morphology, similar morphospecies could be very different genetically. From a morphologist's point of view, fine-scale morphology is superior to molecular probes since the morphotype is

considered to be the result of the expression of the whole genome while molecular probes only evaluate a small fraction of the genome. In contrast, molecular probes are regarded to be superior from a geneticists standpoint because the genome is measured directly without being affected by environmental effects which occurs for morphological characters. However, the amount of genome expressed by morphological characters of unicellular algae is currently unknown. It is possible that fine scale morphology underestimates the importance of the amount degree of fragmentary genome expression in algae, unlike current molecular comparisons. In this sense, it has been suggested that only a few genes control dinoflagellate morphology (COSTAS & al., 1995).

Notwithstanding, everyone should recognise that microalgal species behave in the same fashion as day and night. Nobody has any difficulty in distinguishing between light or dark but, who can say precisely when day ends and night begins?

Toward a pragmatic species concept or "fuzzy species" in microalgae?

We have grown accustomed to a monotypic but still rather inflexible species concept. If an individual belongs to species A it cannot logically belong to species B. It is either one or the other but never both at the same time. However, in microalgae an abundance of asexual reproduction could easily lead to two clonal lines being maintained without genetic flow during many generations. In this case they will eventually become divergent as a result of the mutations they have accumulated. Also, interspecific reproduction can occur (COSTAS, 1986). In microalgae, the result of this would be species of a less rigid nature, in sense giving rise to what we could call "fuzzy species". An individual would not only belong in part to species A but also to species B at the same time.

In practice developing a set of probes that could assure sound species identification seems essential. Naturally these probes would have to be easy to apply for them to be of use in monitoring programmes. At present, the application of 3 molecular probes, cell surface antigens detected by blocking antibodies, cell surface glycan moieties detected by lectins and sequencing of genes-DNA probes, appear a powerful tool to clarify the species classification of the harmful algae.

Immunological techniques are increasingly being used to differentiate morphologically closely related toxic and non-toxic phytoplankton cells (SAKO & al. 1992; BATES & al., 1993; VRIELING & al. 1994; MENDOZA & al. 1995). Polyclonal (BATES & al., 1993) or monoclonal (SAKO & al. 1992; VRIELING & al. 1994) antisera recognize cell surface antigens at species-specific level. Recently, blocking and preadsorbed antibodies so potent that they are able to recognize among different clones of the same morphospecies have been developed (MENDOZA & al. 1995; COSTAS & LÓPEZ-RODAS, 1995).

Lectins have been also used in this sense (COSTAS & al. 1993; COSTAS & LOPEZ RODAS, 1994). Lectins recognize cell surface glycans and several of these glycans are species-specific and clone-specific (COSTAS & LÓPEZ-RODAS, 1994).

Nucleic acid-based probes show excellent promise with respect to specificity and ease of use. Segments of *Lsu* rRNA genes of several dinoflagellate species have been

successfully sequenced (LENAERS & al. 1989, 1991; MCNALLY & al. 1994). Recently a RFLP analysis of Ssu rRNA genes allowed SCHOLIN & ANDERSON (1994) to identify a group and strain-specific marker for *Alexandrium* species. Sequences of LsurRNA genes allowed COSTAS & al. (1995) and ZARDOYA & al. (1995) to clarify taxonomic and phylogenetic problems within the toxic genera *Alexandrium* and *Gymnodinium*.

From a practical point of view, these molecular probes have several advantages, because they allow for quick, precise and repeatable species identification.

***G. catenatum* vs *G. impudicum*, or similar morphology with genetic differentiation**

Gymnodinium catenatum Graham is one of the main dinoflagellates which causes PSP toxin production. In recent years, an athecate, chain-forming dinoflagellate, morphologically similar to *G. catenatum*, has bloomed in the Atlantic waters of Spain and the Mediterranean Sea. Using SEM to observe the fine-scale morphology of the dinoflagellate enabled FRAGA & al. (1995) to conclude that it was actually a different species to *G. catenatum*, and it was named *Gyrodinium impudicum*. In addition it was proved that *G. impudicum* is non-toxic. Although it is possible to distinguish species by fine-scale morphology observing live samples, when fixed it can be impossible. This fact presents serious problems in monitoring programmes because sometimes both species appear at the same time in the same waters, and several cases of misidentification are suspected (FRAGA & al. 1995).

Polyclonal antisera against each species were obtained in rabbits as described in MENDOZA & al. (1995). To obtain species-specific antibodies, antisera were blocked by preadsorption as described in COSTAS & al. (1995). The binding activity of the antibodies was detected using an immunofluorescence assay with a second FITC-conjugated antibody. Quality of stain was estimated by epifluorescence and quantity of antibody binding was measured by spectofluorometry. Cells from each clone were also treated with FITC-labelled lectins according to the protocol described by COSTAS & LÓPEZ-RODAS (1994). Quality of stain and quantity of binding was estimated just as in antibodies. In addition, D9 and D10 domains of Lsu rDNA gene were PCR amplified, cloning and sequencing as described in ZARDOYA & al. (1995) and COSTAS & al. (1995). Sequences were aligned with the program PILEUP of the GCG package.

The test for specificity and cross reactivity of pre-adsorbed antisera is shown in Table 1. It was possible to obtain species-specific pre-adsorbed antisera binding for *G. catenatum* and *G. impudicum*. The binding assay of fluorescent lectins (Table 2) makes it easy to differentiate between the toxic dinoflagellate *G. catenatum* and the morphologically similar species *G. impudicum* which is non toxic. In practice, the use of WGA lectin (about 10 minutes of work) could prevent all the cases of misidentification between the toxic *G. catenatum* and the non-toxic *G. impudicum*.

The alignment of nucleotide sequences corresponding to a fragment of the LsuRNA gene comprising of two divergent domains (D9 and D10) and the corresponding flanking conserved region is shown in Fig. 2. The two variable domain sequences represent about 120 nt, while the conserved flanking region comprises of approximately

200 nt. The nucleotide sequence of *G. mpudicum* is clearly different from that determined for *G. catenatum*. Both species differ in 22 nucleotide substitutions, and 1 nt insertion in position 58 in *G. impudicum* and a 2 nt deletion in position 190.

Although, *G. catenatum* and *G. impudicum* are so similar morphologically that they are frequently misidentified and it is not possible differentiate between them in Lugol's fixed samples, the extent of their genetic differentiation is so great that it seems very likely both species diverged more than fifty million years ago.

Clones/ Species	Antisera			
	<i>G. catenatum</i> preadsorbed on <i>G. impudicum</i>	<i>G. impudicum</i> preadsorbed on <i>G. catenatum</i>	<i>A. minutum</i> preadsorbed on <i>A. lusitanicum</i>	<i>A. lusitanicum</i> preadsorbed on <i>A. minutum</i>
<i>G. catenatum</i>	3.67±0.18 +++	0.57±0.07 -		
<i>G. impudicum</i>	0.47±0.09 -	3.15±0.17 +++		
<i>A. minutum</i>			2.08±0.11 ++	0.62±0.07 -
<i>A. lusitanicum</i>			0.50±0.10 -	2.16±0.10 ++

Table 1. Specificity and cross reactivity of pre-adsorbed blocked antibodies. Values of fluorescence as mean ± SD. Quality of staining: (+++) bright stain, (++) moderate bright, (+) low intensity stain, (-) non-detectable reaction.

	Lectins		
	WGA	DBA	Con A
<i>G. catenatum</i>	3.34±0.18 +++	2.47±0.07 ++	1.48±0.16 +
<i>G. impudicum</i>	0.33±0.06 -	0.32±0.07 -	0.43±0.04 -
<i>A. minutum</i>	0.71±0.05 -	1.61±0.12 +	2.65±0.25 ++
<i>A. lusitanicum</i>	0.81±0.04 -	3.41±0.28 +++	2.37±0.18 ++

Table 2. Binding patterns of FITC-labeled lectins. Quantitative values of fluorescence are expressed as mean±SD. Fluorescence unit=fluorescence of 10 pg mL⁻¹ of FITC. Index of optical quality of staining: (+++) bright stain, (++) moderate bright, (+) low intensity, (-) non detectable reation.

***A. minutum* vs. *A. lusitanicum*, or different morphology yet genetically identical.**

A. minutum is a widely distributed PSP toxin-producing dinoflagellate. At least on two occasions a new dinoflagellate called *A. lusitanicum* by BALECH (1989) using fine-scale morphology to distinguish them apart, has occurred in Portuguese waters (Sampayo, pers. comm.). *A. lusitanicum* is also PSP toxin producing but exhibits slight differences in toxin pattern with regard to *A. minutum* (FRANCO & al. 1995).

Cultures of both species were treated with antibodies and lectins, and sequenced just as *G. catenatum*-*G. impudicum*. The results are summarized in Tables 1 and 2 and Fig. 1. In this case it was not possible to obtain species-specific pre-adsorbed antibody binding for *A. minutum* and *A. lusitanicum*. It was also very difficult to differentiate between *A. minutum* and *A. lusitanicum* using lectins. In addition, the PCR-amplified fragments from *A. minutum* and *A. lusitanicum* share the same nucleotide sequence.

Although, *A. minutum* and *A. lusitanicum* show sufficient morphological differentiation to be classified as different morphospecies, it is not possible to differentiate between them using molecular probes. The extent of their genetic differentiation is so small and very little genetic change is implied in their morphological differentiation that both morphospecies are the same genospecies.

Morphospecies vs genospecies debat. The extinction of the morphospecies concept?

The destiny of all species on Earth is to become extinct. In science the destiny of every idea is to be replaced (even the good ones). Perhaps, we are now presiding at the birth of a new paradigm, the birth of the genospecies, one which could possibly replace the concept of morphospecies. The proof of the pudding is in its tasting.

The extent of morphological differentiation between the dinoflagellates *G. catenatum* and *G. impudicum*, compared to that between *A. minutum* and *A. lusitanicum*, is, in all appearances, of the same order of magnitude. *G. impudicum* and *A. lusitanicum* were only recently recognised as new species using fine-scale morphology. In fact, *G. impudicum* was recognised apart from *G. catenatum* using scanning electron microscope by FRAGA & al. (1995), and *A. lusitanicum* was recognised as a new species distinct to *A. minutum* by BALECH (1989) using differences plate morphology. The morphological differences exhibited between these species are incredibly difficult to perceive to a trained eye. However, the extent of genetic differentiation between the dinoflagellates *G. catenatum* and *G. impudicum* is significantly very large, that is, two well defined genospecies are easily recognisable while the extent of genetic differentiation existing between *A. minutum* and *A. lusitanicum* is virtually non-existent, showing that they are the same genospecies.

The sequence data analysis reveals that the nucleotide sequence of *G. catenatum* is clearly different from that determined for *G. impudicum*. There is a high degree of genetic differentiation in both species which is not reflected in their morphology. In this respect, their similar morphology may indicate an adaptation to the same ecological strategy for swimming and active vertical migration to reach the thermocline. In the

1
AGCCAAATGCCCTCGTCACTAATCAGTGACGCGCATGAATGGAATTAACGAGA-TGCCCACTGTCCCTACTACTATCTAGCGA
- - - - - T - - - - - C - - - - - T - C -
AACACAGCCACGGGAACGGGCTTGGGATAATCAGCGGGAAAGAACCCCTGTGAGCTTGACTCTAGTCCGACTTTGTGAA
- C - G - - A -
ATGACTTAGGAGGTGGAGCATAAGTGGGAGCTTTGGCGATCTTGAAATACCACTTAAATGCCATTTTATTATTCGGTT
- - - - - T -
331
GAACTGAAGCTTGCGTCACAGTGAGTGTGGCTTTAAGTTGTCTTCTGATGGTTGATTTGTGCGGAAAGACATAGTCAGG
- - - - - A - - - - - T - - - - - A - - - - - A - - - - - G C - C - - - - - C C - - - - - G - - - - -

G. catenatum
G. impudicum

1
AGCCAAATGCCCTCGTCACTAATCAGTGACGCGCATGAATGGAATTAACGAGA-TGCCCACTGTCCCTACTACTATCTAGCGAA
-
ACCACAGGCAAGGGAACGGGCTTGGGATAATCAGCGGGAAAGAACCCCTGTGAGCTTGACTCTAGTCTGACATTTGTGAAA
-
TGACTTAGGAGGTGATAGGATAAGTGGGAGCTTTGGCGATCTTGAAATACCACTACTCTTAAATGTCATTTACTTATCTGTGA
-
330
AGCTGGAACCTTCTGAACCTGTGAGTTTGGATTTAAGGCCGTTCTTCTGAATGGTTGATCTGTGGAAAGACATAATCAGG
- -

A. minutum
A. lusitanicum

Fig. 2. Sequence alignment of D9 and D10 domains of Lsu rDNA from *G. catenatum*, *G. impudicum*, *A. minutum* and *A. lusitanicum*

opposite case, the PCR-amplified fragments of *A. minutum* and *A. lusitanicum* share the same nucleotide sequence providing sufficient evidence to suggest that these two described species may actually be one unique species. In this respect, RFLP analysis supports the evidence that *A. minutum*/*A. lusitanicum* are only morphological variants of the same species (SCHOLIN & ANDERSON, 1994). Moreover, given that both species are toxic with PSP toxin profiles of a close nature, and that between any two clones of *A. minutum* it is virtually impossible to detect viable differences in their PSP toxin profiles (FRANCO & al. 1995), there is no practical reason for continuing to view these species as separate and distinct.

From a phylogenetic standpoint practically nobody doubts the existence of species. Recently, a set of molecular probes have been used to discriminate strains and to clarify the relationship between morphotype and species-level division, in turn provoking debate on the species concept in unicellular algae. This debate has been the subject of several review papers (CASTENHOLZ, 1992; MANHART & MCCOURT, 1992; WOOD & LEATHAM, 1992; TAYLOR, 1993) since in some cases, molecular markers corroborate the morphotaxonomic classification of algae, but in many others they do not. Consequently, the relationship between morpho-taxonomy and molecular-taxonomy remains obscure. In this respect, it's necessary to bear in mind the frequent lack of relationship between the classical morphospecies and modern genospecies, because in general, no obvious correlation appears to exist between the extent of morphological differentiation and the extent of genetic distance. The lack of correlation between the degree of morphological variation and the extent of genetic variation should not be discarded when it seems ever more apparent that marine phytoplankton species have remained extremely conservative in form while accumulating genetic variability within them (TAYLOR, 1993).

Morphology is the result of a genetic base affected by environmental, stochastic and other interacting factors. Yet only recently have we had the tools to measure the direct nature of the genetic base, something which has evaded biologists for decades. In this sense, no-one should doubt the value of the genetic base over morphology at the hour of species classification. Using genetic markers there is no risk of classifying an environmentally-mediated variation of the same genotype as two different species.

In the past, geneticists analysed only morphological characters. But nowadays genetics is based on molecular study of genome. So, it is possible in a near future that microalgae will be classified in species based on molecular probes. At present, bacteria, parasitic protists and several harmful protists are classified based on molecular probes. In algae the use of purely molecular taxonomy still seems remote. At present only 6 papers have worked with dinoflagellate gene sequences. Yet conversely a continued classical morphological classification will still require several hundred people working decades at the best to keep it up to date. Perhaps it is only a matter of time. In this interim, the usefulness of molecular probes can be of great help. Ten minutes hard-work with the lectin WGA can prevent all the misidentifications between *G. catenatum* and *G. impudicum*. Whatismore, molecular probes can be useful to count and separate toxic cells.

Counting *Alexandrium minutum* cells using molecular probes

A major constraint of monitoring programmes for harmful algae stems from the need to identify, enumerate and separate cells of dangerous species within a mixed plankton assemblage. Generally, morphological criteria are sufficient to classify unicellular algae to species, and to identify toxin-producing algae. However, considerable time and effort are required to identify and count a particular species using light microscopy. Although some morphospecies have proven to be consistently linked to toxicity, other morphospecies are known to exist in both toxic and non-toxic strains (TAYLOR, 1993). These facts present serious problems in monitoring programmes. An alternative is the use of molecular and cellular probes that can bind to sites on the target species. Using immunological procedures (immunofluorescence + flow cytometry and immunobeads) the quick counting and separation of *Alexandrium minutum* from seawater samples becomes very easy.

Natural seawater samples containing *A. minutum* cells were collected in the Rias of Arosa and Vigo (NW Spain shores) and fixed in 4% buffered formaldehyde. The samples were treated with an anti *A. minutum* species-specific blocked antibody and a secondary FITC-labelled antibody, according to the protocol described by MENDOZA & al. (1995) and examined by a EPIC 541 flow cytometer (Coultronics Inc. Florida). Furthermore, another aliquot was treated with an anti *A. minutum* species-specific blocked antibody and a secondary magnetic bead-conjugated antibody. The magnetic bead-target cell complex was isolated using immunomagnetic separation.

Table 3 summarizes the cell densities of *A. minutum* from different sampling locations and periods estimated by immunofluorescence and flow-cytometry, which showed excellent agreement with the direct counts. Counts at 1/10 and 1/100 dilutions of seawater samples allowed the repeatability of flow cytometer counts to be estimated with respect to direct counts for low cell density. Flow cytometer counts (by

	seawater	1/100 dilution
<i>Arosa (1993)</i>		
flow citometry	43±8,8	0,4±0,1
direct count	43±5,1	0,2±0,2
<i>Vigo (1993)</i>		
flow citometry	52,6±5,3	0,6±0,4
direct count	58,0±6,2	0,4±1,8
<i>Vigo (1994)</i>		
flow citometry	599±31	6,1±0,1
direct count	794±11	7,0±2,8

Table 3. Cell density (m±sd) of *A. minutum* estimated by immunofluorescent procedures using flow cytometry and by direct counting in an inverted microscope.

immunological) procedures appeared to have a higher repeatability than direct counts for diluted low-density samples (maximum error for flow cytometer about 10% versus 28% for direct counts).

Table 4 summarizes percentages of successful separation of *A. minutum* cells from natural samples (defined as the ratio: 100 x number of *A. minutum* cells by immunobeads / total of *A. minutum* cells in the sample estimated by direct count) as well as the percentage of mistakenly separated cells (defined as the ratio: 100 x number of non-*A. minutum* cells separated by immunobeads / total of cells separated by immunobeads). Apparently, immunobeads were able to successfully separate a high percentage (between 92% and 61%) of *A. minutum* cells from natural samples with a relatively low incidence of mistaken separation (between 9% and 1%).

The accuracy of cell density estimation of *A. minutum* using immunofluorescent procedures suggests that this procedure could be an important tool for counting potentially toxic cells from natural samples. Although direct counts by light microscopy are more precise at high cell densities, immunological counts appeared more precise at low cell densities. In practice, this could be of interest to detect early bloom stages, as suggested by VRIELING & al. (1994). With this in mind, the percentages of successful separation of *A. minutum* cells using immunobeads means it would also be suitable to use immunomagnetic separation.

	seawater	1/100 dilution
<i>Arosa</i> (1993)		
successful recuperation	83%±4	91%±7
mistaken recuperation.....	9%±2	2%±1
<i>Vigo</i> (1993)		
successful recuperation	61%±13	75%±5
mistaken recuperation.....	8%±2	4%±2
<i>Vigo</i> (1994)		
successful recuperation	92%±7	95%±5
mistaken recuperation.....	3%±0	1%±1

Table 4. Percentages (m±sd) of successful separation and mistaken recuperation of a *A. minutum* cells from natural samples.

References

- ANDERSON, D. M. (1994). Red Tides. *Sc. Amer.* **271**: 52-59.
- BALLECH, E. (1985). The genus *Alexandrium* or *Gonyaulax* of the *tamarensis* group. In D. M. Anderson, A. W. WHITE & D. G. BADEN (eds.) *Toxic Dinoflagellates*: 33-38. Elsevier Science Publishing, New York.
- (1989). Redescription of *Alexandrium minutum* Halim (Dinophyceae), type species of the genus *Alexandrium*. *Phycologia* **28**: 737-745, 206-211.

- BATES, S. S., C. LÉGER, B. KEAFER & D. M. ANDERSON (1993). Discrimination between domoic-acid producing and nontoxic forms of the diatom *Pseudonitzschia pungens* using immunofluorescence. *Mar. Ecol. Prog. Ser.* **100**: 185-195.
- CASTENHOLZ, R. W. (1992). Species usage, concept, and evolution in the cyanobacteria (blue-green algae). *J. Phycology* **28**.
- COSTAS, E. (1986). Aparición de híbridos interespecíficos de *Prorocentrum micans* y *Prorocentrum triestinum* en cultivos experimentales. *Boletín Inst. Esp. Oceanog.* **3**: 61-66.
- 1990. Genetic variability in growth rates of marine dinoflagellates. *Genetica* **83**: 99-102.
- , E. GONZÁLEZ CHÁVARRI, A. AGUILERA, S. GONZÁLEZ-GIL & V. LÓPEZ-RODAS (1993). The use of lectins to recognize and differentiate unicellular algae. *Bot. Marina* **36**: 1-4.
- & V. LOPEZ-RODAS (1994). Identification of marine dinoflagellates using fluorescent lectins. *J. Phycol.* **30**: 987-990.
- , V. LÓPEZ-RODAS (1996). Enumeration and separation of the toxic dinoflagellate *Alexandrium minutum* from natural samples using immunological procedures with blocking antibodies. *J. Exper. Mar. Biol. Ecol.* **198**: 81-87.
- , R. ZARDOYA, J. BAUTISTA, A. GARRIDO, C. ROJO & V. LÓPEZ-RODAS, 1995. Morphospecies vs. genospecies in toxic marine dinoflagellates: an analysis of *Gymnodinium catenatum*/*Gyrodinium impudicum* and *Alexandrium minutum*/*A. lusitanicum* using antibodies, lectins, and gene sequences. *J. Phycol.* (in press).
- FRAGA, S., I. BRAVO, M. DELGADO, J. M. FRANCO & M. ZAPATA (1995). *Gyrodinium impudicum* sp. nov. (Dinophyceae) a non toxic, catenate, red tide dinoflagellate. *Phycol.* (in press).
- FRANCO, J. M., S. FRAGA, M. ZAPATA, I. BRAVO, F. P. VILA & I. RAMILO (1995). Comparison between different strains of genus *Alexandrium* of sp. the *minutum* group. In Lassus, P. (ed.) *Toxic Marine Phytoplankton*: 53-58. Lavoisier, Paris.
- HALLEGRAEF, G. M. (1993). A review of harmful algal blooms and their apparent global increase. *Phycol.* **32** (2): 79-99.
- LENAERS, G., L. MAROTEAUX, B. MICHOT & M. HERZOG (1989). Dinoflagellates in evolution. A molecular phylogenetic analysis of large subunit ribosomal RNA. *J. Molecular Evol.*, **29**: 40-51.
- , C. Scholin, Y. Bhaud, D. Saint-Hilaire & M. Herzog (1991). A molecular phylogeny of dinoflagellate protists (Pyrrophyta) inferred from the sequence of 24 S/rRNA divergent domains D1 and D8. *J. Mol. Evol.* **32**: 53-63.
- MANHART, J. R. & R. M. MCCOURT (1992). Molecular data and species concepts in the algae. *J. Phycol.*, **28** (in press).
- MAYR, E. (1982), The growth of biological Thought: diversity, Evolution and inheritance. *Harvard University Press*, Cambridge, Massachusetts.
- MCNALLY, K., N. S. GODVIN, P. E. THOME & R. K. TRENCH (1994). Small-subunit ribosomal DNA sequence analyses and reconstruction of the inferred phylogeny among symbiotic dinoflagellates (Pirrophyta). *J. Phycol.* **30**: 316-329.
- MENDOZA, H., V. LÓPEZ-RODAS, S. GONZÁLEZ-GIL, A. AGUILERA & E. COSTAS (1995). The use of polyclonal antisera and blocking of antibodies in the identification of marine dinoflagellates: species-specific and clone-specific antisera against *Gymnodinium* and *Alexandrium*. *J. Exper. Marine Biol. and Ecol.* (in press).
- SAKO, Y., C. KIM & Y. ISHIDA (1992). Mendelian inheritance of paralytic shellfish poisoning toxin in the marine dinoflagellates *Alexandrium catenella*. *Biosc. Biotechn. Biochem.*, **5**: 692-694.
- SCHOLIN C. A. & D. M. ANDERSON (1994). Identification of group and strain-specific genetic markers for globally distributed *Alexandrium* (Dinophyceae). I. RFPL analysis of SSU rRNA genes. *J. Phycol.* **30**: 744-754.

- SOURNIA, A. (1995). Red-tide and toxic marine phitoplankton of the world ocean: an inquiry into biodiversity. In L. PATRICK (ed.), Harmful Marine Algal Blooms. 103-112.
- TAYLOR, F. (1993). The species problem and its impact on harmful phytoplankton studies. In T. J. SMAYDA & Y. SHIMIZU, *Toxic Phytoplankton Blooms in the sea*, Elsevier Sci. Publ., New York, 81-86.
- VRIELING, E., L. PEPPERZAK, W. W. C. GIESKES & M. VEENHUIS (1994). Detection of the ichthyotoxic dinoflagellate *Gyrodinium* (cf.) *aureolum* and morphologically related Gyrodinium species using monoclonal antibodies: a specific immunological tool. *Marine Ecol. Progress Ser.* **103**: 165-174.
- WOODS, A. M., T. LEATHAM, 1992. The species concept in phytoplankton ecology. *J. Phycol.* **28**: 723-729.
- ZARDOYA, R., E. COSTAS, V. LÓPEZ-RODAS, A. GARRIDO-PERTIERRA & J. M. BAUTISTA (1995=). Revidied dinoflagellate phylogeny inferred from molecular analysis of large subunit ribosomal RNA gene sequences. *J. Molecular Evol.* **41**: 637-645.

Address of the author:

Prof. E. Costas Costas, GeneticaFacultad de Veterinaria, U.C.M. 28040 Madrid, Spain.