Harmful Algae
February 2009, Volume 8, Issue 3, Pages 538-545
http://dx.doi.org/10.1016/j.hal.2008.08.027

Monoclonal antibody against the surface of *Alexandrium minutum* used in a whole-cell ELISA

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Abstract:

Harmful algal blooms represent a major threat to marine production, and particularly to shellfish farming. *Alexandrium minutum*, which causes paralytic shellfish poisoning, is occurring with increasing frequency along European coasts. Current regulatory methods to analyze environmental samples are tedious and time consuming because they require taxonomists and involve animal experiments. New rapid detection methods, such as immunoassays, are needed to ensure a fast alert system and for field studies of algal ecodynamics. Rat monoclonal antibodies were raised and selected for their ability to specifically recognize a surface antigen for the *A. minutum* strain AM89BM from the Bay of Morlaix, France. A whole-cell ELISA was designed, leading to the selection of one AMI6 mAb that was selected for its performance in a large set of immunochemical formats. Moreover, AMI6 mAb displayed no detectable cross-reactivity with most algae found in similar biotopes, particularly those which might be mistaken during a conventional light microscope counting *Heterocapsa triquetra*, *Scrippsiella trochoidea*, *Karenia mikimotoi*, and two strains of *Alexandrium tamarense*, either toxic or not. Using colloidal gold conjugates on immunodecorated cells, we used electron microscopy to show that AMI6 mAb targets an exposed antigen at the surface of *A. minutum*. It was noted that this antibody could work with many preparations of *A. minutum* cells, i.e. fresh, frozen or dried cells. The detection limit in the whole-cell ELISA was found to be 10 cells per well. This assay displayed sensitivity and specificity when used for the analysis of natural seawater samples. A large set of immunochemical formats, using either AMI6 mAb or related antibodies from this series, could be further envisaged for designing environmental biosensors.

Keywords: Dinoflagellate; Enzyme-linked immunosorbent assay; Harmful algal bloom; Monoclonal antibody

Abbreviations: HAB, harmful algal bloom(s); ELISA, enzyme-linked immunosorbent assay; mAb(s), monoclonal antibody(ies); PSP, paralytic shellfish poison; SEM, scaning electron microscopy; PBS, phosphate buffer saline; BSA, bovin serum albumin; HRP, horseradish peroxidase

INTRODUCTION

Harmful algal blooms (HABs), also commonly known as "red tides", are natural phenomena, the frequency, intensity, and geographic range of which appear to have increased since the 1970s (Hallegraef 1993, 2003, VanDolah 2000). Furthermore, their economic impact is greater now than in the past, as a result of increasing consumption of seafood, growth of coastal populations and tourism. Different classes of toxins are produced by dinoflagellates; they accumulate in shellfish and are then responsible for severe human syndromes. HABs are also harmful to the marine ecosystem as a whole, because algal toxins can sicken and kill many forms of aquatic organisms (Landsberg 2002). Several hypotheses may explain these increasing occurrences of toxic blooms, such as the eutrophication of coastal waters which may support the growth of toxic endemic species, and the introduction, via human factors, of foreign competitive taxa (Hallegraeff 1993). Hence, there is an obvious need for suitable analytical methods with surveillance programs, both in areas which are dubious for shellfish farming and to understand algal dynamics in diverse coastal environments where dinoflagellates might proliferate.

The French network for phytoplankton monitoring, REPHY, was created by IFREMER (Institut Français de Recherche pour l'Exploitation de la Mer) in 1984 after several alerts on the Atlantic and Mediterranean coasts. Animal mortality or growth inhibition of marine fauna due to the presence of *Karenia mikimotoi*, which produces hemolytic cytotoxins and mucilage, were regularly detected during the period 1976–2000 (Erard-Le-Denn et al. 1990). The genus *Alexandrium* is among the most harmful since most species produce potent neurotoxins such as saxitoxins and gonyautoxins which are responsible for so-called "paralytic shellfish poisoning". *A. minutum*, was first detected on the French coast in 1985 and has recurrently formed toxic blooms along the North Brittany coast since 1988 (Le Doux et al. 1990). *A. catenella* and *A. tamarense* were previously considered to be rare in the Mediterranean Sea (Margalef and Estrada 1987). However *A.catenella* is now regularly detected along the French and Catalan coasts (Vila et al. 2001).

Monitoring of HAB species is tedious, often imprecise and still requires direct human expertise for species identification. The taxonomic classification of harmful dinoflagellates was discussed some twenty years ago by Taylor (Taylor 1987), and reviewed recently (Taylor et al. 2003). The methods used for identification are usually based on morphological studies using light microscopy. Unfortunately, algal morphology might change depending on environmental conditions and growth phases. We focused our study on two visually close species which may give rise to false identification by inexperienced taxonomists.

(Lilly et al. 2002) showed that genetic studies could help taxonomists by improving phylogeny and providing a global genetic database. On this basis, very specific laboratory methods based on genetic information have been recently introduced, such as fluorescence *in situ* hybridization (FISH) (Sako et al. 2004, Anderson 2005) or real-time PCR (Dyhrman 2006, Galluzi 2005, Hosoi-Tanabe 2005). A method to detect *A. minutum* in a complex background using sandwich hybridization assay was reported, but required improved RNA isolation and method sensitivity (Diercks et al. 2008). Strategies for detecting HAB species using lectin have also been designed (Costas et al. 1993-1994).Fluorescently labelled lectins with different binding specificities have been used to differentiate among five different species including different serotypes, either toxic or not, of the same species.
Finally, antibodies still represent a great asset to detection assays and the literature reveals many attempts to generate specific antibodies against harmful algae. Polyclonal sera have been raised against *Aureococcus* (Anderson et al. 1989-1993), *Pseudonitzschia* (Bates et al. 1993). Such polyclonal antibodies may sometimes be adequate for detecting the species of interest, but they may also exhibit cross-reactivity as some antigens may be common to different HAB species (Mendoza et al. 1995). Monoclonal antibodies (mAbs) are more specific and reproducible, and can be reliably produced in large batches for large numbers of kits. MAbs were successfully obtained for *Chatonella* (Hiroishi et al. 1988, Nagasaki et al. 1991), *Gymnodinium* and *Gyrodinium* species (Vrieling et al 1994, Xin and al 2005), and *Scrippsiella trochoidea* cysts (Okasaki et al. 2001). Interestingly, mAbs could also be raised against *Alexandrium* dinoflagellates, from *tamarense* to *catenella* (Adachi et al. 1993a,b, Cordova 2002) or *fundyense* (Aiguilera et al. 1996, Sako 1993) species. Only one group reported blocking antibodies against *A. minutum* (Costas et al. 1996).
This report describes our strategy to obtain the first monoclonal antibodies specific to the surface of the French strain AM89BM (Bay of Morlaix) of *A. minutum* and their implementation in a reliable whole cell immuno-assay.

MATERIALS AND METHODS

Dinoflagellates strains and culture

All strains came from the IFREMER Centre de Brest collection: *Alexandrium minutum* AM89BM, *Heterocapsa triquetra* HT99PZ, *Scrippsiella trochoidea* ST97PZ and *Karenia mikimotoi* GATIN95. For *Alexandrium tamarense*, both a toxic and a non-toxic strain were studied: MOG835 and PLY497A, respectively. The culture inocula were maintained for transport at mid-exponential growth in f/2 Guillard and Ryther medium (Guillard, 1975). The cultures were grown axenically at 18 ± 1°C under cool-white fluorescence light, at a photon flux of 150 µE/m^2/s with 14:10 LD photoperiods. At the end of the exponential growth phase, cells were harvested by centrifugation (5000 g, 10 min) and could be kept frozen at -70°C for later use.

Natural samples

Natural samples containing phytoplankton and *A. minutum* were collected in the south of France by the IFREMER station at Sète, at the REPHY network coastal laboratory. Several water samples were taken at different spots during a bloom of *Alexandrium minutum* along the Mediterranean coast (January 2008). Each sample was observed under a light microscope by IFREMER to confirm whether or not *A.minutum* cells were present, kept at 2-8°C in the dark and then sent to the CEA laboratory at Marcoule. These natural samples containing 100 ml or 1Lwere then concentrated (10 to 500-fold) by centrifugation (4000 g 5 min) and resuspended in 1ml just before performing the whole cell ELISA (see below).

Generation of monoclonal antibodies

Three rats were immunized with live sonicated algae to induce the production of antibodies against cell surface antigens. Basically, one million *A. minutum* cells were suspended in 0.6 mL sterile PBS, then sonicated for 2min 30s to decrease toxicity. The cells were then centrifuged (4000 g 5min) and the pellet was rinsed in PBS. The resulting cell preparation, i.e. detoxified pellets, was then emulsified in 0.6 mL of complete Freund's adjuvant and 200 µL were injected subcutaneously (multiple points) into each Winstar rat. After 4 weeks, the animals were boosted using incomplete Freund Adjuvant at each injection. Every 2 weeks, after each injection, the sera were drawn from the immunized rats and sera titers were determined by whole cell ELISA (see below). Spleen cells from the rats with the highest antibody titter were harvested 4 days after the last boost (8 months). The cells were fused with 2.5 x 10^7 myeloma cells (P3X63Ag8.653) using 50% PEG (Poly Ethylen Glycol). Cell hybrids were resuspended with 200 µL/well of RPMI 1640 L-Glutamine, HT-Supplement (Gibco) selecting medium and 15% of fetal calf serum on 96 well tissue culture plates. The plates were incubated at 37°C in a moist atmosphere with 5% CO_2. Ten days after fusion, hybridoma supernatants were screened for secretion of specific antibodies, by ELISA as described below. All the supernatants were tested in a "double screening" during the ELISA test, i.e. first with *A.minutum* cells and a counter-selection vs *Heterocapsa Triquetra* cells to improve specific antibody selection. Only those giving an optical density 4 times higher than our negative control (non immunized rat) and no signal during the counter-selection process with *Heterocapsa triquetra* were kept. All positive cultures were expanded onto 24-well plates and hybrid samples were frozen in liquid nitrogen with a medium containing 10 % DMSO (Dimethyl Sulfoxyde) (Sigma) and 90% fetal calf serum. The different hybridoma supernatants (296) were screened against both species of algae.

Positive hybridomas were re-cloned using the limiting dilution technique. And thanks to all these, the criteria were met by 13 positive clones. MAbs were produced *in vivo* by intraperitoneal injection of hybridoma cells into nude mice. The mAbs were then purified from ascites fluids using ammonium sulfate precipitation at 50 % saturation. After centrifugation for 30 min at 3500 g, at 2-8°C, the pellets were solubilized in PBS. Desalting was achieved by 3 consecutive dialysis cycles in PBS, using MWCO:6-8000 Spectrum® dialysis membranes. A final centrifugation was performed (3500 g, 2-8°C) for 20 min. The supernatant was injected into a protein G HiTrap1® column (Pharmacia). Protein concentration was determined by a Bradford assay (Bio-Rad).

Whole cell ELISA

The selection of mAbs that recognize only external surface antigens was performed by indirect enzyme immunoassay. Conic well microplates (Greiner) were saturated with PBS containing 1 % BSA at 2-8°C overnight to avoid non-specific coating. Briefly, 2x10^5 *A. minutum* cells were added to each well (100 µL). Antibody was added as a 100 µL solution, diluted in PBS, 1% BSA (1/100 to 1/5000) depending on the experiment. The immune reaction was left to proceed for 2 hours at room temperature on an orbital shaker at 900 rpm. After incubation, 3 washes were performed in PBS 0.1% tween. Between each wash, the cells were settled by microplate centrifugation (4000 g, 2min) and resuspended in 200 µL of wash buffer. Then, the second antibody, an HRP-labeled goat anti-rat antibody (Sigma), was diluted 1/50000 in PBS 1% BSA and incubated for 1h30 at room temperature (RT) with shaking at 900 rpm. The cells were again subjected to 3 washes in 200 µL of PBS 0.1% tween and the immune complex was revealed by adding 100 µL TMB reagent (Tetra Methyl Benzidin, Sigma). The colorimetric reaction was stopped 30 min later by adding 25 µL 1 M H_2SO_4 per well. The optical density was recorded at 450 nm.

Dot immunoassay
Pellets of algal cells were resuspended in PBS with approximately $5X10^6$ cells/µL. Two µL of each solution were applied onto a 15 µm nitrocellulose sheet (Advanced Microdevices Pvt) and allowed to dry at room temperature. The membrane was blocked with PBS 3% BSA for 2h at RT and incubated with specific monoclonal antibody (around 1 µg/mL) in PBS 1% BSA for 2h at RT with shaking. After washing four times in PBS 0.1% tween (10 min each time) the rat mAb was revealed with a colloidal gold labelled-antibody diluted to 1/100 in PBS containing 1% BSA. Goat anti-IgG conjugated with 30 nm gold micro-beads were purchased from British Biocell International. Staining developed after 2h at RT.

Scanning electron microscopy
Many supports were assayed for the SEM analysis. Interestingly, the nitrocellulose membranes used in the dot assay gave the best results. A colloidal gold marker system was used for SEM because colloidal gold solutions have a high secondary electron (SE) and backscattered electron (BSE) coefficient, are electron dense, and have a distinctive X-ray signal. The scanning electron analysis was performed at IFREMER Brest, using a Quanta 200 instrument (FEI Company, Hillsboro, Oregon, USA).

RESULTS

1. Monoclonal antibodies specific to *A. minutum*

Our objective was to raise suitable monoclonal antibodies in a whole cell assay. The protein structure of the *A. minutum* theca is still unknown so we could not refer to any specific protein immunogen. The immunization strategy was then based on intradermic multipoint injection of cell extracts, after inactivation by sonication to avoid toxicity to rats. Immunization of mice was also attempted but failed, possibility due to acute toxicity of the algal emulsion.
The serum from each immunized animal was tested monthly by ELISA over an 8 month period. Only one rat in three displayed a stable and positive immune response with a significant titer around $8x10^{-3}$ (**Figure 1)**. The selection of hybridoma clones secreting antibodies to *A. minutum* surface antigens was achieved by double screening on whole algae. We focused our primary screening on the ability of mAbs to discriminate between *A. minutum* and *Heterocapsa triquetra,* two species with very comparable features under light microscopy but different toxicity patterns**.**
The different hybridoma supernatants (296) were screened against both species of algae and at last we obtained 13 positive clones. This report deals with AMI6, the best mAb within a subset of 5 clones which displayed a high production yield in ascites.

2. Performances of AMI6 mAb in whole cell assay

Comparison between fresh and frozen cells
We evaluated the whole cell ELISA on different types of samples, namely a frozen cell sample which was similar to the one used for immunization, and a fresh culture of *A. minutum*. The results showed that AMI6 mAb detection was comparable in both sample preparations and almost identical at higher antibody dilutions (**Figure 2A**). Consequently, we chose to perform subsequent cell assays using frozen cells to facilitate the transport of biological materials between the laboratories involved in this study, and to somehow standardize assay conditions.

Antibody specificity
Purified monoclonal antibody AMI6 mAb was tested for cross-reactivity in the whole cell ELISA. The assay was designed to be compatible with the various cell densities usually found in environmental samples. Cross-reactivity experiments were determined at 2,000-fold the alert level in France. Experiments were then performed at 20,000 frozen cells per well, i.e. $2 X 10^8$ cells per liter. We first assayed two *Alexandrium tamarense* strains collected in Plymouth (UK), with very close morphologies but described either as a toxic, PSP producing strain (MOG835), or as a non-toxic (PLY497A) strain. The *Heterocapsa triquetra* (HT99PZ) strain was included in the assay. We also tested two other species: *Scrippsiella trochoidea* (ST97PZ) that can bloom without toxicity, and *Karenia mikimotoi* (GATIN95). As shown in (**Figure 2B),** AMI6 mAb recognized only *A. minutum*, and all the other species gave a signal around 0.5 OD, i.e. close to the background signal. This experiment shows that AMI6 mAb specifically recognizes *A. minutum* and gives no cross-reaction with any of the other algae tested. Moreover, this absence of cross-reaction is also confirmed by western blot analysis of total algal cell lysates (data not shown).

Detection limit of the whole cell assay

The reference assay for antibody screening used 20,000 *A. minutum* cells per well. To evaluate the detection limit of AMI6 mAb in the whole cell ELISA, we performed serial dilutions of *A. minutum* cells down to around 5 cells per well. Antibody dilutions ranged from 1/100 to 1/1000.As shown in (**Figure 2 C)**, the background level in this assay (first performed without antibody) was around 0.2 OD. Even at 1/1000 dilution, and for 5 cells per well, a signal 3 times higher (0.8 OD) than the baseline could be observed.

The detection limit for the whole cell assay was also approached using 10 replicates of the experiment at 10 cells per well, two antibody dilutions (1/100 and 1/1000) and at the OD value of the control (without specific antibody). The detection limit values (blank + 3 Standard Deviation) were found to be 0.21 cells at 1/100 mAb dilution, and 2.08 at 1/1000. The intra-assay reproducibility, at 10 cells per well and at 1/100 of diluted AMI6 mAb, displayed a coefficient of variation of 6.89% (Data not shown). However, a compromise had to be found between sensitivity and selectivity and we won't reasonably quantify samples at less than 150 cells per well.

3. Assay of natural seawater samples

To evaluate the performance of AMI6 mAb towards environmental samples, we used the whole cell assay with seawater samples. The alert level in France was established by REPHY within the range 10^4-10^6 cells/L *for Alexandrium minutum.* As previously mentioned, a compromise had to be found between sensitivity and selectivity. AMI6 dilution at 1/5000 was found to avoid high background due to the matrix effect. The range for the quantitative assay was set at 500 to 10^4 frozen cells (*A. minutum* AM 89MB) per well. The standard plot was based on the means from 3 independent experiments, and displayed very good linear correlation: $y = 0.0002 X + 0.3883$; $R^2 = 0.9872$ (**Figure 3A**). The value at 0.3883 for the average of background signals was a good match for previous data **(see Figure 2B)**. We tested different samples harvested by the REPHY network during the January 2008 bloom of *A. minutum* along the Mediterranean coast. Two samples, Baccares and Sète, proved negative, both by optical microscopy counting (lack of any *A. minutum* cell) and whole cell ELISA (**Table 1**). Due to the choice of antibody dilution 1/5000 (described previously) and because of the volume of the sea-water sample (often around 1L) all the natural samples were concentrated by centrifugation before being tested by whole cell ELISA.

Interestingly, none of the many phytoplankton cells contained in these media gave rise to any false positives with AMI6 mAb. Even with a concentration of the negative sample, the optical density read was always under 0.3883. Positive samples obtained by microscopy counting also proved positive in ELISA. These positive samples gave an optical density of 0.5 to 3 within the linear range of the standard curve. The value was then corrected by applying the concentration factor to obtain the initial number of cells per liter in the seawater sample. Moreover, although different, all the measurements were globally within the same order of magnitude. The ELISA values were higher in the 10^3 range, either due to recurrent over-estimation effect or increased sensitivity at low cell density.

4. Localization of the antigen recognized by AMI 6

Dot blot analysis

To validate the fact that AMI6 mAb recognizes an epitope located on the antibody specificity in a dot blot assay using whole cells. This format has already been described for fast detection of *Gymnodinium* sp. using a polyclonal antibody (Xin et al. 2005). Algae were spotted onto nitrocellulose membrane and immunodecorated with AMI6 mAb. The immune complex was revealed with a goat anti-rat IgG antibody coupled with colloidal gold beads, average diameter 30 nm, resulting in direct coloration of the membrane. No significant signal or background was observed with *Heterocapsa triquetra* spots (**Figure 4A**), while an obvious pink signal appeared at the level of *A. minutum* spots (**Figure 4B**). We also verified the specificity of AMI6 in dot blot analysis by adding an anti-rat coupled HRP antibody revealed by the OPD substrate instead of the previous one with colloidal beads. (Data not shown) This observation confirmed the specificity of AMI6 mAb for the surface of *A. minutum* and also validated the accessibility of the epitope in the membrane immobilized algae. This property was exploited for the electronic microscopy analysis described below.

Scanning Electron Microscopy analysis (SEM)

The structural integrity of *A. minutum* was checked for cells immobilized on the nitrocellulose membrane. The morphology proved identical to that frequently reported (**Figure 5A**). The different thecal plates signing *A. minutum* were correctly maintained despite a harsh drying procedure. SEM's ability to detect the 30 nm colloidal gold beads used for antibody labelling was correct (**Figure 5B**). Scanning electron micrographs of immunodecorated *A. minutum* cells, with AMI6 mAb and goat anti-rat IgG coupled to gold colloidal beads (30nm), clearly displayed the location and accessibility of AMI6's mAb antigen at the surface of the thecal plates (**Figures 5C** and **5D**).

DISCUSSION

Current regulatory methods for analyzing environmental samples are tedious and time-consuming because they require experienced taxonomists and involve animal experiments. New rapid detection methods, such as immunoassays, are needed, both to ensure a fast alert system and to study algal ecodynamics in the field. Only one group, twelve years ago, reported that they obtained suitable polyclonal antibodies against *A. minutum* using flow cytometry (Costas et al. 1996). Our strategy was to produce specific monoclonal antibodies directed against the surface of toxic algae and use them in a reliable ELISA compatible with use in coastal laboratories.
Rats and mice were envisaged as possible animals for raising antibodies. We could not raise a sustainable immune response in mice, probably because very minute amounts of live *A. minutum* cells still contain enough toxins to sicken animals. For the record, the Mouse Bio Assay, a reference procedure for evaluating PSP content in suspicious shellfish samples (AOAC 1990), indicates that toxin doses in the µg/kg range are lethal. Although our immunogens were thought to be non-toxic, we have to conclude that a significant dose was reached in the animal's body, via an unknown mechanism. Previous reports have already suggested that immunization would be better using partially inactivated algae (Sako 1993, Cordova et al. 2002). This was successful in our case too. The key point in our study was the thorough selection process using live *A. minutum* cells and the counter-selection of mAbs against *Heterocapsa triquetra.* In this way, we eliminated more than 52% of the initial clones that were also positive in *Heterocapsa triquetra*. This original and selective screening method was efficient because no mAbs cross-reacted with frozen strains of *A. tamarense* (Asiatic algae), *K. mikimotoi* or *S. trochoidea.*

A colorimetric whole cell ELISA was developed. We deliberately made the choice of a colorimetric format instead of using fluorescent technology like flow cytometry (Costas et al. 1996). Many harmful algae, such as *Alexandrium,* display high fluorescence in a broad spectrum that interferes with detection and often leads to high background signals (data not shown). We also favored a simple design, i.e. a direct sandwich assay for whole cells, over competitive ELISA. The polyclonal antibody-based colorimetric c ELISA developed for *Gymnodinium* sp required overnight incubation (Xin et al. 2005). Our direct assay was faster (4h), straightforward, and suitable for the technical level of most coastal laboratories in the surveillance network. Taking into account the criterion of a 3-fold background signal, the evaluation of the detection limit using our method with cultured cells is at ~10 cells per 100 µL sample, i.e. 10^5 cells/L and matches the alert level at 10^4-10^6 cells/L. However, for the natural sample, because of the antibody dilution that must be 1/5000 to avoid the seawater background we know that a concentration with only centrifugation of the natural sample (100 fold or 500 fold) is necessary to reach the 10^4 or lower sensitivity level. It is worth recalling that the IFREMER reference method involves several hours of phytoplankton sedimentation for the 10 mL sample. In the whole cell ELISA, centrifugation might be envisaged to replace the smoother sedimentation procedure. In any case, the convenience of a whole cell ELISA over optical microscopy enumeration might lead to it being used as a complementary tool in HAB detection.

Performing the assay relies mainly on the fact that mAb-AMI6, displays specific surface recognition on *A. minutum* cells, at the level of a theca antigen. Scanning electron microscopy experiments confirmed this property and also suggested that the antigen was of low abundance. Few colloidal gold spots were detected. Investigations are requested to characterize the nature and structure of this protein. Genomic and proteomic data are desperately lacking for *Alexandrium* sp., and for phytoplankton in general. So, the isolation and characterization of the AMI6 mAb epitope might be a substantial task.

AMI6 MAb was raised against the Brittany strain *A. minutum* AM89BM, harvested in the Bay of Morlaix. We demonstrated that it also detected the strain naturally occurring in the Mediterranean, which, despite its morphotype, is known to differ from the Atlantic strain, i.e. presence of a ventral pore and no smooth thecal plates (Hansen et al. 2003). The whole cell ELISA was used for analysis of Mediterranean seawater samples. It proved to be qualitatively equivalent to the manual counting method for counting *A. minutum* but tended to overestimate the number of cells by a factor close to 10. The epitope might be more abundant and/or more accessible in the Mediterranean variant. One precaution would be to build a specific standard curve for the assay to correct this strain effect.
Finally, the potential of AMI6 mAb to operate in a large variety of immunometric formats and conditions for use, including detection of live cells, makes it highly suitable for designing antibody-based environmental biosensors.

ACKNOWLEDGMENTS

The authors wish to thank Philippe Crassous at IFREMER Brest for skillful technical assistance in SEM experiments and Eric Abadie at the IFREMER coastal station, Sète, for providing us with natural seawater samples.
This work is part of the HAB-SEACHIP project, supported by a grant from the ANR PRECODD program.

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FIGURE CAPTIONS

Figure 1. Titration of anti-*A. minutum* rat sera.
Serum samples 25 µL of immunized rat (8 months old ♦) and a non-immunized serum (■), as a control, were diluted serially from 1/100 to 1/100000 and tested in whole cell ELISA. Bound antibodies onto the surface of captured *A. minutum* cells were revealed by HRP labelled-anti-rat and TMB as a colorimetric substrate.The absorbance was read at 450 nm. The background with the non-immune serum was similar to control buffer (▲).

Figure 2A. Comparison between fresh or freezed cells.
A. *minutum* coming from fresh culture (gray bars) and (white bars) frozen cells (at - 70°C) of *A. minutum* were checked in whole cell ELISA.
The control contains all reagents without AMI6 mAb. The values represent the mean of 6 experiments +/- SD.

Figure 2B. Cross reactivity profile of AMI6 mAb.
Cells of various algae were tested in the whole cell-ELISA test to check the immunospecificity of AMI6 mAb.
Alexandrium minutum strain (-■-), *Heterocapsa triquetra* strain (-●-), *Alexandrium tamarense* toxic strain (-▲-) or non-toxic strain (-✦-), *Scrippsiella trochoidea* strain (-✕-) and *Karenia mikimotoi* strain (-▬-) were tested. The antibody was diluted from 1/1000 to 1/20000 and the cells sample (100 µL) contain 20,000 cells of each algae species. The immunoreaction between AMI6 mAb vs antigen was revealed by an anti-rat labelled HRP and measured spectrophotometrically at 450 nm. All data are presented as mean +/- Standard Deviation (SD) (n=4).

Figure 2C. Detection limit assay.
The detection limit of assay was approached by decreasing the number of *A. minutum* cells, i.e. from 20,000 to 5 cells per well in the 100 µL sample used for the whole cell ELISA.
Different dilutions of AMI6 antibody from 1/100, 1/200, 1/400, 1/600 and 1/1000 were checked for all cells quantity tested. The absorbance (450nm) was plotted against the number of cell per well.

Figure 3. Standard curve in whole–cells ELISA assay.
The absorbance was plotted against the number of cells. All data are presented as mean +/- Standard Deviation (n=3). A linear regression was used to approach a standard curve.
The dilution of AMI6 mAb is always constant at 1/5000. The *A. minutum* cells were diluted with PBS BSA 1% from 10,000 to 500 cells.

Figure 4. Dot blot immunoassay.
Cells of *Heterocapsa triquetra* (A) or *A. minutum* (B) were spotted on nitrocellulose membrane. After blocking reaction, and incubation with the specific antibody (AMI6 mAb), the sheet was incubated with an anti-rat labeled by colloidal beads.

Figure 5. Direct visualization of gold-labelled mAb-AMI6 on *A. minutum* surface.
(A) *A. minutum* cells directly spotted on membrane ; antibody free sample.
(B) Colloidal gold-labeled anti-rat IgG antibodies. Isolated molecules are visible, showing the monodispersity of the beads and confirming they are visible at that magnification.
(C) Immunodecorated *A. minutum* cell. AMI6 mAb on the surface was revealed by the colloidal gold conjugate.
(D) 5-fold magnification of the electromicrography, in the area of the AMI6's label.

TABLE

Table 1. Comparison of whole cells-ELISA assay and cell counting for seawater samples analysis.
The ELISA standard curve (Fig. 3) was used to give an estimation with a Coefficient variation of the measurements (n=3) of *A. minutum* cells under 100 µl. We applied the concentration factor and approximate in cells by liter for all seawater samples. The IFREMER results were given by counting under optical microscopy. For the absence of values in optical counting (samples Baccares and Sète) we presented the results of no significant Optical density signal in ELISA (< 0.3883) by a cut-off < 1.5×10^3 cells/L.

Figure 1

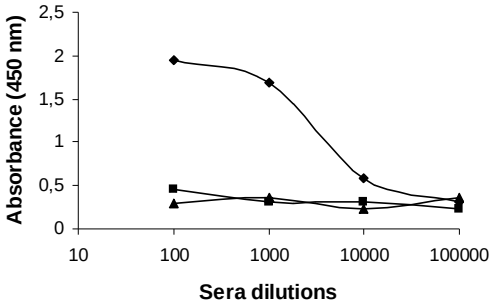
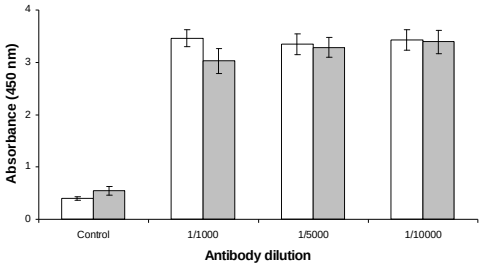


Fig. 1

Figure 2



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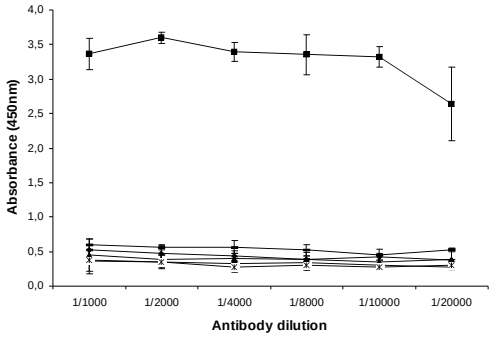

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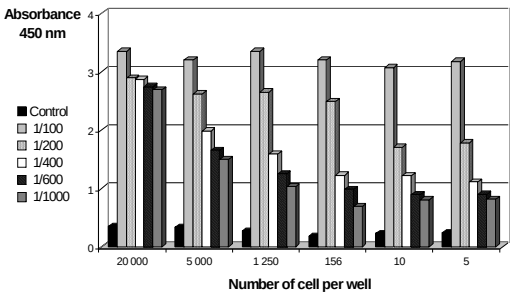

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Fig. 2

Figure 3

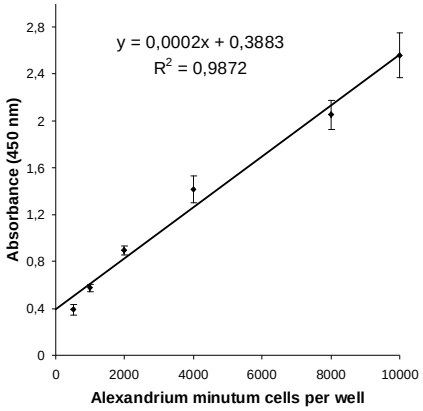

Fig. 3

Figure 4

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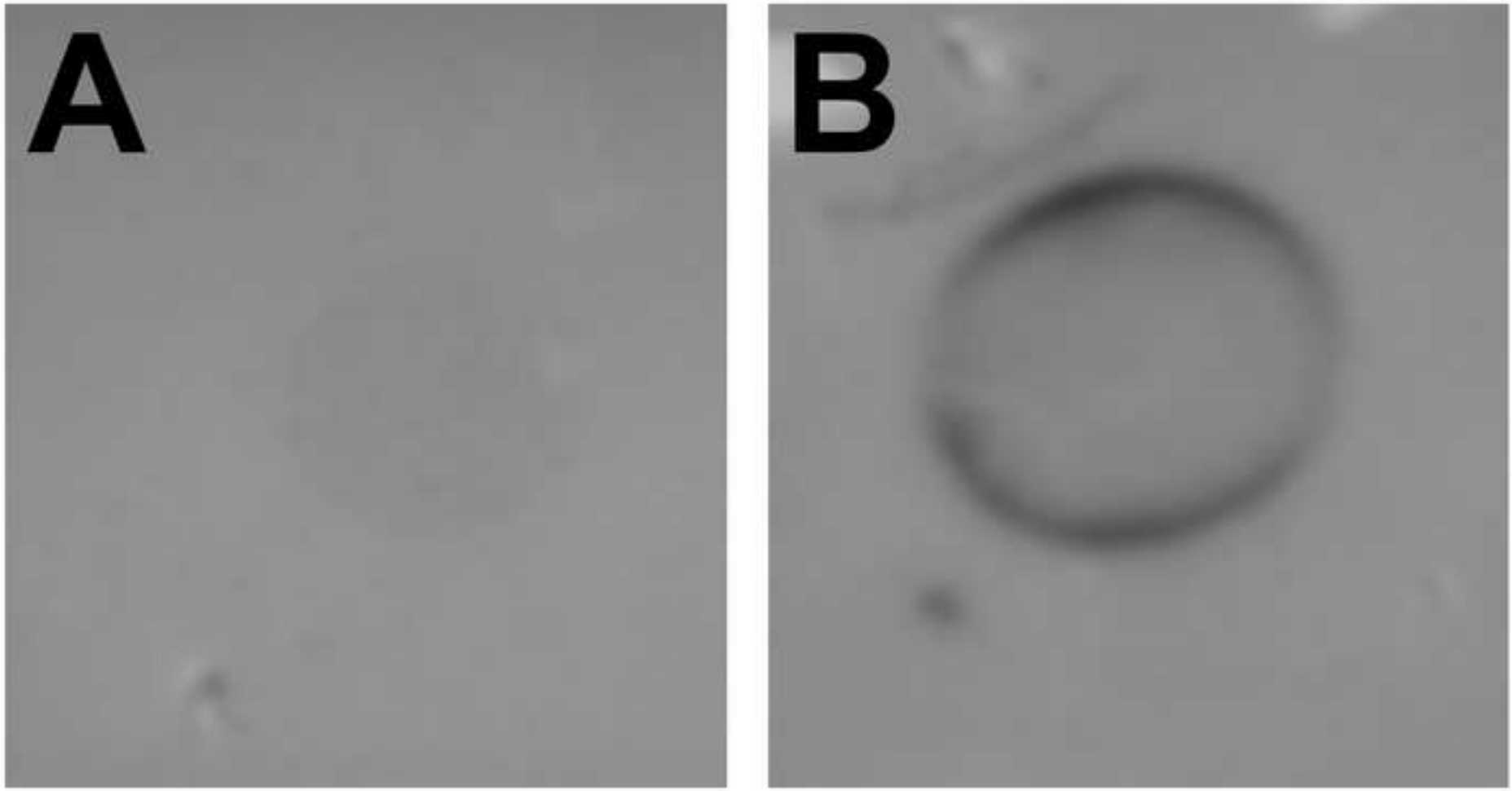

Figure 5

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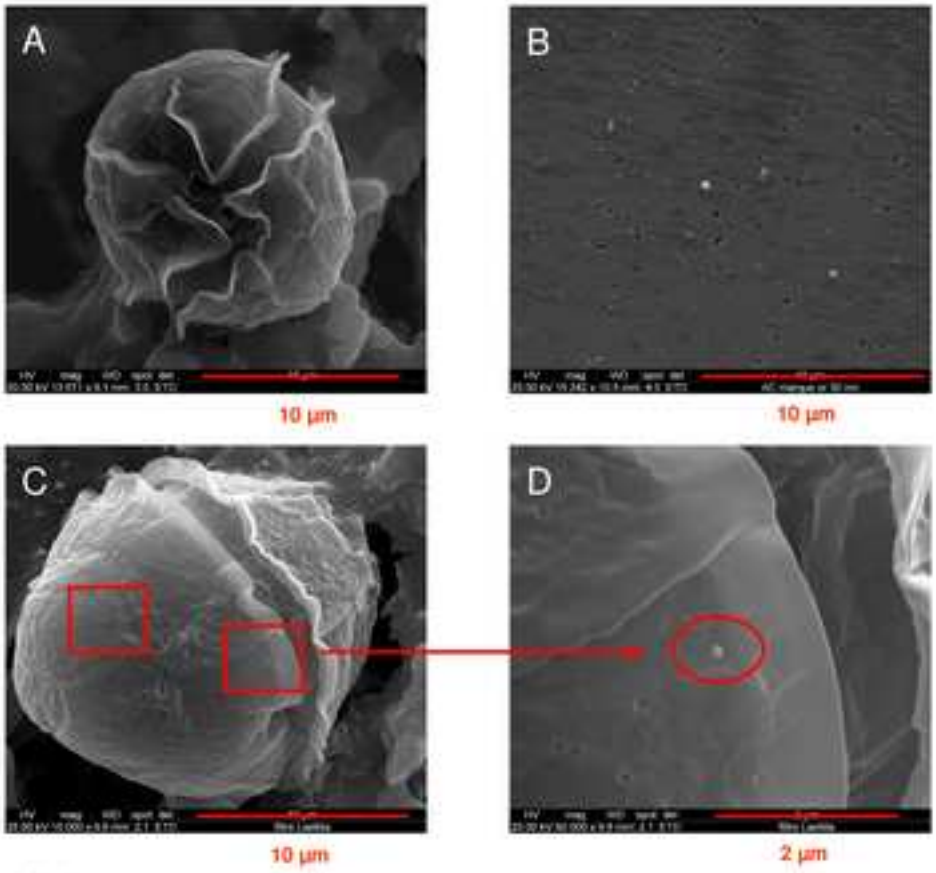

Fig. 5

Table

TABLE 1

Comparison of ELISA assay and cell counting for seawater samples analysis

SEAWATER SAMPLE	Optical counting (*A. minutum* cells/L)	ELISA counting (*A. minutum* cells/L)
Salses 1	$3,6 \times 10^3$	$43.1 \pm 8.6 \times 10^3$
Baccares	-	$< 1.5 \times 10^3$
Sète	-	$< 1.5 \times 10^3$
Salses 2	$1,2 \times 10^3$	$45.9 \pm 9.2 \times 10^3$
Salses 3	$1,817 \times 10^6$	$2,20 \pm 0.4 \times 10^6$