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DETECTION OF TOXIC PHYTOPLANKTON SPECIES BY IMMUNO-CHEMICAL PARTICLE ANALYSIS BASED ON FLOW CYTOMETRY

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KEYWORDS: dinoflagellates; flow cytometry; immunochemical labeling; toxic phytoplankton.

ABSTRACT

Particulate suspended matter in oceanic, coastal, and estuarine regions can be specifically marked immunochemically with a fluorescent probe using antisera recognizing antigens present on their surface. Of the particulate matter, phytoplankton is a major component. Toxic species that may form harmful blooms can be a direct threat to aquaculturing, tourism, sea-life and man. In order to detect such species in natural fixed phytoplankton populations, immunochemical tagging has been combined with flow cytometric evaluation. Microalgal cells can be labeled with a fluorescent probe (fluorescein isothiocyanate, FITC, is recommended). Labeled cells are counted using a flow cytometer. This method has proved to be applicable in a monitoring programme in the North Sea.

INTRODUCTION

Phytoplankton is a major component of particulate matter in oceanic, coastal and estuarine regions. Algal cells occur in a great variety of sizes, contributing most to suspended matter in the vegetative season while particles such as sand grains and dead organic matter (detritus) are present yearround. Distinction between living phytoplankton and dead material can be made by detection of substances typical for living particles, such as algal pigments, but also by tracing biomarkers, usually low-molecular weight organic compounds. In this contribution we will show that immunochemical methods can be a powerful tool when specific particles are to be detected. In principle, the antigens to be recognized can be a wide variety of molecules located on either living or dead matter.

Recently, much attention has been paid to the presence and abundance of potentially toxic phytoplankton species which may produce massive blooms often referred to as 'red tides' (SMAYDA and SHIMIZU, 1993). Since the last two decades the occurrence of blooms of toxic algal species has increased (ANDERSON, 1989; SMAYDA, 1990; HALLEGRAEFF, 1993). Nuisance-causing cells are normally not observed before the late-bloom stage of the various species, when biotoxins causing paralytic, amnesic, neurotic, and diarrhetic shellfish poisoning have already exerted their effect on sea-life and man. Also, ichthyotoxic species, which damage aquaculture, go unnoticed until the harm is done. Because there are numerous potentially toxic phytoplankton species, we now focus our interest on those (*i.e. Gyrodinium aureolum*, *Alexandrium tamarense*, *Pseudonitzschia* sp.) which are present in Dutch coastal waters and can be an imminent threat to human health and shellfish.

Monitoring of toxic phytoplankton species requires microscopy and detailed taxonomic knowledge. Other techniques, such as high performance liquid- or gas chromatography to detect biotoxins in dissolved natural substances and electron microscopic studies to analyse morphological details are time-consuming and expensive. In an attempt to look for toxic phytoplankton species at

rather dilute concentrations, which is the case at the early bloom stage, we have developed a method that combines selective immunochemical recognition of particles (in our case phytoplankton species) with automated enumeration by flow cytometry (VRIELING *et al.*, 1993a). How advantageous immunochemistry is has already been shown: resolution in particle recognition is high, *e.g.* within genera species can be identified (HIROISHI *et al.*, 1988; ANDERSON *et al.*, 1993; BATES *et al.*, 1993; VRIELING *et al.*, 1994). Furthermore, immunochemically 'tagged' phytoplankton species in mixed natural samples can be analyzed at high speed by a flow cytometer. We will illustrate the potential usefulness of this approach by overviewing results obtained during a project aimed at the detection of toxic algae in mixed phytoplankton populations.

METHODS

For the detection of toxic phytoplankton species by immunochemistry, specific antisera can be prepared by using whole cells or purified components (certain proteins or toxins) of the species of interest as an antigen to immunize mammalian hosts. For immuno-flowcytometry and immunofluorescence microscopy, fixed, morphologically intact, phytoplankton mixtures should be analysed, so the antisera used are able to recognize antigens at the cell surface. Because of the immunochemical reaction between either antibody and antigen or antibodies, it is possible to 'tag' the species of interest with a fluorescent probe. In our experiments fluorescein isothiocyanate (FITC) is used as a probe. The emission spectrum of FITC (520-560 nm) differs from phytoplankton chlorophyll autofluorescence (>650 nm), so cross-talk, overlap of wavelengths of emitted light, of both signals to be analyzed by a flow cytometer can be avoided. For flow cytometric analysis of phytoplankton the Optical Plankton Analyzer (OPA) has been developed (DUBELAAR *et al.*, 1989; PEETERS *et al.*, 1989). In the course of the development of our method different configurations were used (Table 1): 1) preliminary work performed with *Prorocentrum micans* as model organism was done with the original configuration (VRIELING *et al.*, 1993b), 2) optimisation of the method was achieved by adapting the optical filtering and excitation source to increase FITC fluorescence-yield (VRIELING *et al.*, 1993a), and 3) measurements on particles of Lugol-fixed samples were accomplished by triggering on perpendicular light scattering (PLS). Data processing, using the DATADESK® program (DATA Description, New York, U.S.A.), of labeled mixtures can be performed on bivariate plots of green (FITC) versus red (chlorophyll) fluorescence in which labeled cells appear as clusters distinct from unlabeled cells.

Table 1. Configurations of the Optical Plankton Analyzer (OPA) used in different parts of the development of the immuno-flow cytometric identification of toxic phytoplankton species.

Configuration:	I	II	III
Excitation wavelength (nm)	442	488	488
Emission:			
Green (FITC) fluorescence (nm)	475-534	520-544	520-544
Red (chlorophyll) fluorescence (nm)	650-750	650-750	650-750
Triggering	red	red	PLS

RESULTS

Model organism

Preliminary studies were performed by using the non-toxic dinoflagellate *Prorocentrum micans* as a model organism using the original configuration of the flow cytometer and filter settings (Table 1). Cells of *P. micans* were immunochemically labeled with a polyclonal antibody raised against purified cell walls of this species (called PM1) which reacted genus-specifically with *Prorocentrum* species (VRIELING *et al.*, 1993b). Flow cytometric distinction of labeled cells, by comparing green FITC fluorescence with red chlorophyll fluorescence, was not good enough to identify *Prorocentrum* (Fig. 1A). A second polyclonal antibody raised against purified trichocystal cores (called PM2) and cross-reacting with trichocyst bearing dinoflagellates appeared to be effective in identification of naked dinoflagellates, as is shown for *Gymnodinium nagasakiense* (Fig. 1B).

In order to optimize the measurements, the immunochemical labeling has been improved by application of the biotin-streptavidin system resulting in an enhancement of fluorescence intensity due to the specific binding of FITC-conjugated streptavidin to antibodies conjugated with multiple biotin molecules (VRIELING *et al.*, 1993a).

At the same time, the performance of the flow cytometric analysis has been improved by optimizing the FITC fluorescence-yield (excitation wavelength) and better separation of FITC fluorescence and perpendicular light scattering (optical filtering), resulting in configuration II (Table 1). Both with confocal laser scanning microscopy (CLSM) and flow cytometry the enhancement of the fluorescent

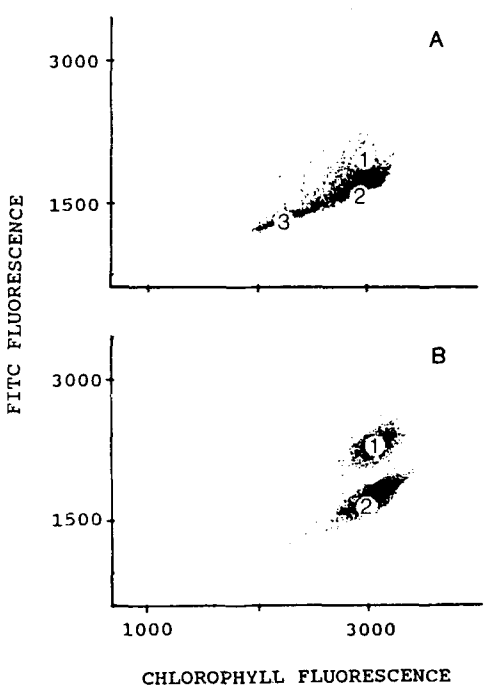

Fig 1. Flow cytrometric appearance of *Prorocentrum micans* (A) and *Gymnodinium nagasakiense* (B) cells in bivariates of FITC versus chlorophyll fluorescence after immunochemical labeling with either polyclonal antibody PM1 (A) or PM2 (B). In both bivariate plots, labeled and unlabeled cells are represented by clusters 1 and 2 respectively. Cluster 3 represents damaged cell debris present in the mixture.

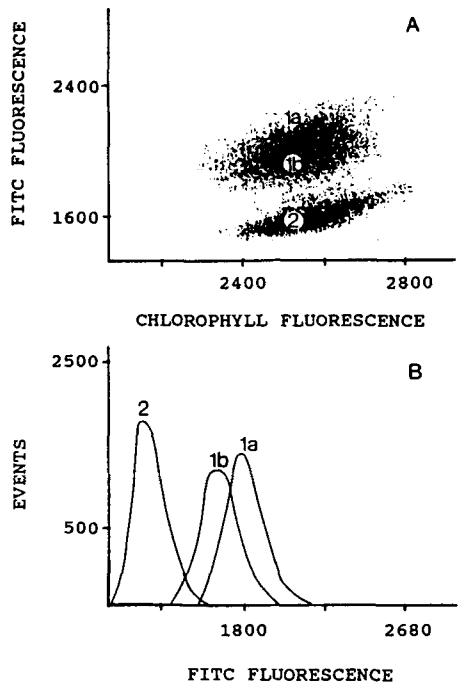

Fig 2. Flow cytometric appearance of labeled and unlabeled cells of *Gymnodinium nagasakiense* in A) the bivariate plot of FITC versus chlorophyll fluorescence and B) the histogram of registered events versus FITC fluorescence. Clusters 1a and 1b represent enhanced labeled or normally labeled cells, respectively. Cluster 2 represents unlabeled cells.

signal was obvious (VRIELING *et al.*, 1993a) and distinction of clusters of labeled and unlabeled cells of *Gyrodinium aureolum* in the bivariates of FITC versus chlorophyll fluorescence improved (1a in Fig. 2A and B). Enhancement of labeling intensity is more obvious when the number of cells (events) per cluster has been compared with FITC fluorescence (Fig. 2B), to overcome the slight overlap of clusters 1a and 1b (Fig. 2A). Although the fluorescent signal increased, enhancement of FITC fluorescence of *P. micans* was not perfect yet for automated data processing. Probably, steric hindrance caused by cross-linking of antibodies and streptavidin molecules at local binding sites at the top site of the pores of the cell wall masks the available antigens at the inside of the pores of the cell wall (VRIELING *et al.*, 1993a).

Monoclonal antibodies.

In order to achieve a very selective identification, monoclonal antisera recognizing just one part of an antigen have been prepared. For the ichthyotoxic dinoflagellate species *Gyrodinium aureolum*, 16 monoclonal antibodies were prepared, which could be subdivided into three groups according to their different immunochemical reaction observed by epifluorescence microscopy (Fig. 3). Antibodies of group I (Fig. 3A) and II (Fig. 3B) both revealed labeling of the cell surface, although labeling of group II antibodies was of higher intensity besides fluorescence of flagella. Group III antibodies showed a granular-like pattern distributed over the cell surface but especially at the edges of the sulcus and cingulum (Fig. 3C). The antibodies reacted only with their target, *G.* (cf.) *aureolum* and two morphologically closely related ichthyotoxic *Gymnodinium* species which were supposed to be synonymous (VRIELING *et al.*, 1994). Antibodies of each group and a pooled antiserum (combined antibodies of group I, II, and III) were applied to investigate the separation of labeled cells from unlabeled ones by flow cytometry using configuration II (Table 1). Only

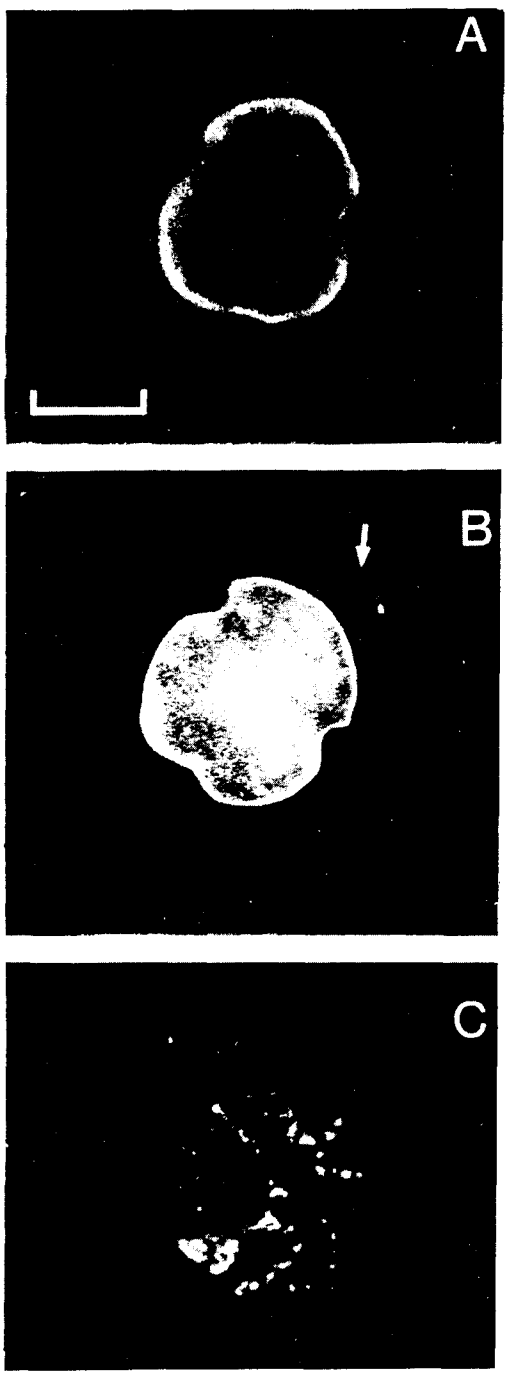

Fig 3. Immunofluorescence micrographs of the ichthyotoxic dinoflagellate *Gyrodinium aureolum* labeled with monoclonal antibodies. A) labeling of the cell wall (group I), B) very intense labeling of the cell wall including the flagella (arrow) with antibodies of group II, and C) granular-like labeling of particles on the cell surface, but especially in the cingulum and sulcus (group III). Bar: 20 µm.

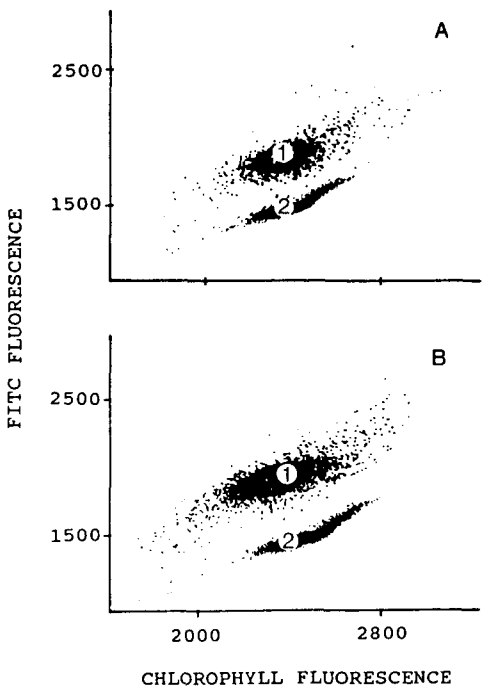

Figure 4. Flow cytometric appearance of labeled and unlabeled cells of *Gyrodinium aureolum* in bivariates of FITC versus chlorophyll fluorescence after labeling with either monoclonal antibodies of A) group II or B) a pooled antiserum (combined antibodies of groups I, II, and III).

antibodies of group II (Fig. 4A) and the pooled antiserum (Fig. 4B) were sufficient for flow cytometric identification. Because of the excellent separation of labeled and unlabeled cells, these antisera were used in the following experiment.

Application in biomonitoring

In 1993, studies were performed on Lugol-fixed mixed natural samples, which were taken in 1991 at three North Sea stations 100, 135, and 175 km northwest of the island Terschelling between July and October (Dutch monitoring programme of Rijkswaterstaat). Routine observation of these samples by light microscopy revealed *Gyrodinium aureolum* to be present at these stations at cell densities more than 10^5 cells l^{-1} forming a 'small' bloom (KOEMAN *et al.*, 1992). Because the samples were fixed with Lugol's iodine, chlorophyll autofluorescence had disappeared. Therefore, the flow cytometric triggering of phytoplanktonic particles performed on the basis of chlorophyll fluorescence (see above) had to be changed. Instead of triggering on red

chlorophyll fluorescence which remains stable after paraformaldehyde fixation (HALL, 1991), perpendicular light scattering (side scatter) was used (configuration III in Table 1) and all particles present in the mixtures were evaluated for green fluorescence. With the aid of a control sample (labeled and unlabeled *G. aureolum* cells) a selected area in which only labeled cells of the samples appeared in the bivariate plot could be fixed at this position during data analysis. Fig. 5 indicates the position of labeled cells in samples taken from the surface (Fig. 5A) and the thermocline (Fig. 5B) at station 135 on 14 August 1991.

The seasonal increase and decrease of the cell densities of *G. aureolum* could be observed at the 135 and 175 Km stations (not shown), although cell densities were much lower compared to those observed by microscopical counting by KOEMAN *et al.*, (1992). The most probable cause of this difference is the amount of cell loss following the performance of the immunochemical labeling and this cell loss has yet to be quantified. The separation of labeled and unlabeled particles seems to be less good when triggered on side scatter, because green fluorescence of particles induced by the laser interferes with the measurements. Furthermore, registered events of green fluorescing particles may appear close to the events of the target organism, whereas the signal to noise ratio decreased as judged from the enormous amounts of dots indicating small particles (arrows Fig. 5A and B). When triggered on chlorophyll autofluorescence such an interference will not be observed. With triggering on side scatter, the cell density of *Gyrodinium aureolum* can be determined. However, because all particles are evaluated for side scattering (including bacteria, inorganic matter, etc.), the percentage of this species within the natural phytoplankton population cannot be estimated, since fluorescence of chlorophyll disappeared due to fixation with Lugol's iodine.

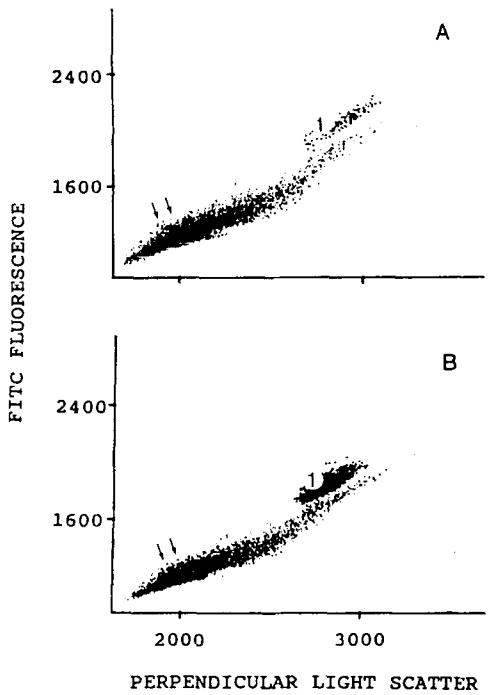

Fig. 5. Flow cytometric appearance of immunochemically labeled *Gyrodinium aureolum* cells in bivariates of FITC fluorescence versus perpendicular light scattering. The latter was used as triggering parameter. Samples from North See station 135 taken on 14 August 1991 from the surface (A) and from the thermocline (B). Note: the position of the events of cluster 1 coincides with the position of events of labeled control cells of *G. aureolum*.

CONCLUSIONS

Overlooking the results obtained until now, we conclude that flow cytometric identification of potentially toxic phytoplankton species is feasible, although some restrictions have to be kept in mind. Pretreatment of samples using paraformaldehyde fixation is recommended. Firstly, signals of interfering suspended matter can be avoided by triggering on chlorophyll fluorescence instead of forward or side scattering. Due to this fixation not only chlorophyll fluorescence remains stable, but also the abundance of the target species within the natural phytoplankton population can be determined. Nevertheless, the results obtained after applying antibodies on Lugol-fixed samples in biomonitoring are promising, but in order to quantify cell counting more work has to be done to prevent the cell loss that we observed. Because of the high resolution of immunochemical identification, target particles can be observed even at dilute concentrations. In the case of algal particulate matter, prediction of massive toxic phytoplankton blooms and the issue of early warning for toxic species presence becomes feasible when cells can be detected before the bloom starts. We imply that immuno-flowcytometry presented here as a tool to identify toxic phytoplankton species can be used in particle analysis in general, because both biotic and abiotic particulate matter carries molecules that can be used as antigens.

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REFERENCES

ANDERSON, D.M., 1989. Toxic algal blooms and red tides: A global perspective. In: T. Okaichi, D.M. Anderson and T. Nemoto, Eds., Red Tides: Biology, environmental sciences, and toxicology. Elsevier, New York, p. 11-16.

ANDERSON, D.M., B.A. KEAFER, D.M. KULIS, R.M. WATERS and R. NUZZI, 1993. An immunofluorescent survey of the brown tide chrysophyte *Aureococcus anophagefferens* along the northeast coast of the United States. J. Plankton Res., 15: 563-580.

BATES, S.S., C. LÉGER, B.A. KEAFER, and D.M. ANDERSON, 1993. Discrimination between domoic-acid producing and non-toxic forms of the diatom *Pseudonitzschia pungens* using immunofluorescence. Mar. Ecol. Prog. Ser., 100: 185-195.

DUBELAAR, G.B.J., A.C. GROENEWEGEN, W. STOKDIJK, G.J. VAN DEN ENGH and J.W.M. VISSER, 1989. The optical plankton analyser (OPA): a flow cytometer for plankton analysis. II: Specifications. Cytometry, 10: 529-539.

HALL, J.A., 1991. Long-term preservation of picoplankton for counting by fluorescence microscopy. British Phycol. J., 26: 169-174.

HALLEGRAEFF, G.M., 1993. A review of harmful algal blooms and their apparent global increase. Phycologia, 32: 79-99.

HIROISHI, S., A. UCHIDA, K. NAGASAKI and Y. ISHIDA, 1988. A new method for identification of inter- and intra-species of the red tide algae *Chattonella antiqua* and *Chattonella marina* (Raphidophyceae) by means of monoclonal antibodies. J. Phycol., 24: 442-444.

KOEMAN, R.P.T., T.W.M. RADEMAKER and A. BUMA, 1992. Biomonitoring of phytoplankton in Dutch salt and brackish waters. TRIPOS rapp., by order of Ministry of Public Works and Transport, Tidal Waters Division (In Dutch).

PEETERS, J.C.H., G.B.J. DUBELAAR, J. RINGELBERG and J.W.M. VISSER, 1989. The optical plankton analyser (OPA): a flow cytometer for plankton analysis. I: Design considerations. Cytometry 10: 522-528.

SMAYDA, T.J. 1990. Novel and nuisance phytoplankton blooms in the sea: Evidence for a global epidemic. In: E. Granéli, B. Sundström, L. Edler and D.M. Anderson, Eds., Toxic marine phytoplankton. Elsevier, New York, p. 29-39.

SMAYDA, T.J. and Y. SHIMIZU, 1993. Toxic phytoplankton blooms in the sea. Elsevier, Amsterdam.

VRIELING, E.G., A. DRAAIJER, W.J.M. van ZEIJL, L. PEPERZAK, W.W.C. GIESKES and M. VEENHUIS, 1993a. The effect of labeling intensity, estimated by real time confocal laser scanning microscopy, on flow cytometric appearance and identification of immunochemically labeled marine dinoflagellates. J. Phycol., 29: 180-188.

VRIELING, E.G., W.W.C. GIESKES, J.W. HOFSTRAAT, F. COLIJN, L. PEPERZAK and M. VEENHUIS 1993b. Immunochemical identification of toxic marine algae: First results using *Prorocentrum micans* as a model organism. In: T.J. Smayda and Y. Shimizu, Eds., Toxic phytoplankton blooms in the sea, Elsevier, Amsterdam, p. 925-931.

VRIELING, E.G., L. PEPERZAK, W.W.C. GIESKES and M. VEENHUIS, 1994. Detection of the ichthyotoxic dinoflagellate *Gyrodinium* (cf.) *aureolum* and morphologically related *Gymnodinium* species using monoclonal antibodies: a specific immunological tool. Mar. Ecol. Progr. Ser., 103: 165-174.

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