SUMMARY.

During the last decades man has become increasingly aware of the danger of toxic microalgae that are present in the phytoplankton of coastal waters around the world. In Dutch coastal waters some toxic species have been known since the seventies, when outbreaks of diarrhetic shellfish poisoning caused by the dinoflagellate *Dinophysis* raised concern. Other species, such as the dinoflagellates *Gyrodinium aureolum* that is ichthyotoxic and *Alexandrium* spp. that produces toxins involved in paralytic shellfish poisoning, have been observed every year since 1989. In that year a phytoplankton monitoring programme was initiated by the government as part of a large-scale study of the effect of eutrophication. Results of laboratory experiments with several potentially toxic phytoplankton species that were grown under different nutrient conditions with respect to nitrogen and phosphate indicated that a high N/P-ratio favors growth of toxic dinoflagellates.

This is the reason why toxic events may be expected nowadays along the Dutch coast. However, most harmful algal blooms and outbreaks of shellfish poisoning still take us by surprise. The reason is that the environmental conditions that induce such blooms cannot be monitored continuously over the often vast regions where toxic algae may appear. In other words, the combination of ecophysiological, chemical and hydrographical conditions that initiate blooms is not known and cannot be demonstrated in the field easily, especially not since such a combination changes in time. Secondly, the species that are responsible for toxic outbreaks are often not observed during phytoplankton monitoring because they are sometimes hard to distinguish from morphologically closely related non-toxic species, or are only present at too dilute concentrations (the situation at the start of blooms). In order to detect toxic species in seawater samples even when only a few cells (less than 1000/L) are present, an immunological approach to species identification has been chosen. In this thesis the results of the application of immunofluorescence techniques are presented. The purpose of the project was to identify and enumerate important toxic phytoplankton species in Dutch coastal waters from the very beginning of blooms. The immunofluorescence method has been adjusted for flow cytometric analysis using the Optical Plankton Analyzer of the National Institute of Coastal and Marine Management (Chapters 2,3,4, and 5).

When the work was started at the end of 1990, immunofluorescence was only used for species identification of aquatic bacteria and some cyanobacteria. Immunofluorescence studies did focus on *physiological* aspects of phytoplankton, revealing the location and distribution of enzymes such as Rubisco and nitrate reductase, and of cytoskeleton and cell wall proteins. In chapter 1 the relevance of immunofluorescence in phytoplankton research is reviewed; it is made clear that this technique has become quite popular. Part of this chapter covers the principles of immunofluorescence, how antibodies can be obtained, and how the method can be applied. The methodological considerations that have to be kept in mind to achieve the "best" immunofluorescent labelling, free of interfering autofluorescent phenomena and also non-specific reactions are of great importance. In most of the studies that are reviewed, traditional epifluorescence microscopy has been used to evaluate fluorescence patterns; flow cytometry has been applied only since the beginning of the nineties to quantify fluorescence intensities or to identify immunochemically labelled species.

In order to complement traditional phytoplankton monitoring by microscopical observations, and to develop an early-warning system for presence of toxic algal species, an immunochemical method had to be developed to detect individual cells in multi-species populations in the field. Because such an approach was never explored properly before, it was decided to start with the non-toxic dinoflagellate *Prorocentrum micans* as a model organism.

The results of this introductory work is presented in chapter 2; it is concluded that a combination of immunofluorescent labelling of cells and flow cytometric detection is quite feasible. The polyclonal antisera derived against purified cell walls and trichynine (an excretion protein) of the trichocysts were genus and taxon specific, respectively. *P. micans* cells labelled with fluorescein isothiocyanate (FITC) could unfortunately not be distinguished unambiguously from unlabelled ones with the methods described in chapter 2. This poor separation was caused by the choice of the excitation wavelength of 532 nm (Helium/Cadmium laser) of the flow cytometer, which was suboptimal to excite FITC sufficiently. Also, the high chlorophyll autofluorescence of *P. micans* interfered. In contrast, FITC-labelled cells of the naked dinoflagellates *Gyrodinium aureolum* and *Gymnodinium nagasakiense* (both bearing trichocysts) could be separated clearly from unlabelled cells using the anti-trichynine antiserum.

To overcome unsatisfactory separation between labelled and unlabelled cells of species that display high chlorophyll autofluorescence not only the flow cytometer had to be adapted: the labelling intensity had to be optimized too (Chapter 3). Instead of using the initial configuration of the Optical Plankton Analyzer (Chapter 2), the optimal excitation of FITC was achieved at 488 nm with an Argon-ion laser. In addition, optical filtering was adjusted to improve the measurements of green FITC fluorescence. The labelling intensity was enhanced by applying the so-called biotin/streptavidin system. It was shown that the intensity could be increased with enhancement factors of at least 1.5 and up to 6.9.; this was determined by flow cytometry and confocal laser scanning microscopy. Even labelled cells of *Prorocentrum* species were separated readily from unlabelled cells after enhanced labelling. Apparently, the enhancement method is effective in increasing the fluorescence intensities of weakly labelled phytoplankton cells to allow proper flow cytometric identification.

Results of the experiments presented in chapter 2 and 3 indicate that it is necessary to obtain species-specific antibodies. Therefore, it was decided to develop monoclonal antibodies (MAbs). As a start, the ichthyotoxic dinoflagellate *Gyrodinium aureolum* was chosen; it is an organism that can potentially create toxicity problems in Dutch coastal waters (Chapter 4). Sixteen MAbs were obtained which could be divided into three groups based on the fluorescence patterns observed at the cell surface. The two strongest of each group were examined further for their specificity in cross-reactivity experiments, binding capacity in relation to different methods of fixing cells, and applicability in flow cytometric identification. The results presented in chapter 4 indicate that all MAbs were species-specific to *G.* (cf.) *aureolum* and the two morphotypes *Gymnodinium mikimotoi* and *G.* (cf.) *nagasakiense*. Interestingly, the reactivity with both *Gymnodinium* species suggests a phylogenetic relationship of these species with *G. aureolum*. Of each group tested, MAbs of group II (showing very intense fluorescence of the cell surface and flagella) were overall the "best". The binding capacity remained stable, independent of the various fixation methods that were used; even cells fixed with Lugol's iodine were labelled. Group II MAbs seem to be useful in flow cytometric detection thanks to the excellent separation of labelled from unlabelled *G. aureolum* cells. An interesting fluorescence pattern was observed for MAbs of group III: a cloud of dots was concentrated at the cingulum and sulcus of cells (typical features of dinoflagellates) while others were distributed more randomly over the cell surface. MAbs of group I revealed a clear fluorescence of the whole cell surface (except the flagella), but with a lower intensity than was observed for MAbs of group II. Though MAbs of group I and III were not good enough to be used in flow cytometric identification of *G. aureolum*, they can be of interest in biochemical research aimed at the fate of the antigens recognized by these monoclonals.

Using one MAb described in chapter 4, an immuno-flow cytometric method for the identification and enumeration of the ichthyotoxic dinoflagellate *Gyrodinium aureolum* was developed and tested (Chapter 5). Two changes in methodology were introduced. First, a direct immunofluorescence (IF)-assay was followed instead of the indirect IF-assay described in chapters 2, 3, 4, 6 and 7. To reduce the number of processing steps, which saves time and causes less severe damage of cells, a direct IF-assay was developed for monoclonal GA_8. After purifying the antibodies from hybridoma supernatant they were conjugated with FITC. The final FITC/protein-ratio of 6.4 revealed an excellent binding capacity (although the titer dropped from 12500 to 3200) and a high fluorescence intensity. Also, the data acquisition of the flow cytometer was adjusted. Since chlorophyll autofluorescence can be used as a triggering parameter for the analysis of phototrophic phytoplankton (Chapters 2, 3 and 4), the effect of an increase in the threshold for this parameter was examined. Using a maximal threshold, the ratio of target cells over non-target cells increased from 0.02 (minimal threshold) to over 2.7. At the maximum threshold, data acquisition was only possible for cells with relatively high amounts of chlorophyll (normally the larger phytoplankton cells). Thus, identification of labelled *G. aureolum* cells in a data set of 10^4 events becomes more accurate.

A calibration experiment showed that the Optical Plankton Analyzer was accurate to a level of at least 91.8 % (introducing an underestimation of 8.2 %) when labelled *G. aureolum* cells were identified after backgating (identifying cells which fit in a selected window) on green (FBG) and orange (FBO) fluorescence values. Other backgating methods were less accurate. A series of artificially mixed phytoplankton populations spiked with decreasing concentrations of *G. aureolum* were analyzed to determine the recovery and sensitivity of the immuno-flow cytometric method. When cell numbers of *G. aureolum* in the mixed phytoplankton populations were over 1000 cells/L, the mean accuracy in the recovery of these cells was 76.7 %; the coefficient of variation of 0.20 suggests quite some variation in recovery, but not worse than variations that are normal when counting is done by microscopy. In these experiments an unexpected low recovery of the total cell count was observed. At higher concentrations of *G. aureolum* the total cell loss was about 58 %, whereas at low concentrations of the target cells this value increased to 90 %. Examination of samples revealed that mainly cells smaller than *G. aureolum* were lost. Apparently, cells of *G. aureolum* are not too sensitive to sample manipulations, whereas others (such as *Chattonella*, *Alexandrium*) may be damaged severely. Consequently, *G. aureolum* cells were identified readily. Altogether it can be concluded that the immuno-flow cytometric method can now be applied in field studies or monitoring programmes.

While the method for immuno-flow cytometric identification of the toxic *G. aureolum* was developed, a number of other toxic species was observed in Dutch coastal waters (Chapter 6 and 7). In chapter 6 the distribution of three Raphidophytes (*Chattonella marina*, *C. antiqua*, and *Fibrocapsa japonica*) is described. The three species were never really abundant. Only once *C. marina* was observed at cell numbers over 2.10^5/L. Monoclonal antibodies assisted in distinguishing both *Chattonella* species. This was necessary because cells of the longer elliptical species *C. antiqua* easily transform to the obovoid *C. marina* type when samples are manipulated. Based on these immunofluorescence studies it was concluded that *C. marina* was present more often than *C. antiqua*. The other type, *F. japonica*, could only be identified in live samples and was recognized by its size and the presence of numerous mucocysts. The distribution of Raphidophytes suggests that they favor the warmer coastal region, namely the Wadden Sea and the estuarine area south of the river Rhine. It is not known how these Raphidophytes survive colder periods, or if they transform into cysts, as is observed in Japanese strains. More work should be done to investigate the presence of

these species (and their survival stages) and to determine if isolates are able to produce neurotoxins. Immunochemistry is an appropriate best tool to distinguish the *Chattonella* species.

The final chapter (Chapter 7) deals with the isolation and identification of some diatoms of the *Pseudo-nitzschia* species complex present in the Wadden Sea. Observations by electron microscopy show that four species can be identified taxonomically by their frustule architecture. Not only *P. fraudulenta*, *P. delicatissima* and *P. pungens* forma *pungens* were found in field samples, but also the domoic acid-producing *P. pungens* f. *multiseries*. This latter species was isolated and cultured to confirm the production of domoic acid, a toxin involved in amnesic shellfish poisoning (ASP). The Dutch strain of the f. *multiseries* type was shown to be able to produce the toxin to levels of 19.0 pg/cell during late stationary growth, comparable to strains isolated in the United States and Canada in regions that are known for ASP outbreaks since 1987. For both *P. pungens* types the reactivity to species-specific molecular probes (polyclonal antibodies and oligonucleotides derived to North American strains) was examined. The probes reacted in a way that suggests a strong phylogenetic relationship between the Dutch and the North American strains. Recent rDNA sequencing results performed in Monterey Bay (California, USA) confirm this relationship; further work has to be done to complete the data set in order to draw firm conclusions.

Immunofluorescence appears to be a powerful tool in species identification; specific antibodies even allow distinction of morphologically very closely related species (Chapter 6 & 7). The probe development against the ichthyotoxic dinoflagellate *Gyrodinium aureolum* was easily extended to other toxic species (*Alexandrium tamarense* and *Pseudo-nitzschia pungens* forma *multiseries*). Following worldwide progress with identification of toxic phytoplankton species, more species-specific molecular probes are being prepared. These probes will allow identification of toxic species. Thus in time, ecophysiological, chemical and hydrographical data explaining mass occurrence of these species can be interpreted. Moreover, only when it is possible to detect very low numbers of toxic cells is it feasible to issue warnings for upcoming outbreaks of blooms that can threaten aquacultures or the health of man.