DEVELOPMENT AND EVALUATION OF RAPID AND SEMI-AUTOMATED DEVICES FOR THE DETECTION OF TOXIC ALGAE

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1. General Introduction

1.1 Harmful Algal Blooms

Oceans, the origin of life, harbour complex phytoplankton communities, which play an important role in marine biological ecosystems. Microalgae are the major producers of biomass and organic compounds in the oceans because of their photosynthetic activity and represent the base of the aquatic food chain. Filter feeding bivalve shellfish (oysters, mussels, scallops, clams, etc.), the larvae of crustaceans and finfish feed primarily on microalgae (Hallegraeff 2003). About 5000 species of marine microalgae are known to date (Sournia et al. 1991) and some 300 species can proliferate in such high numbers that they discolour the surface of the sea (Daranas et al. 2001; Hallegraeff 2003) as a so-called bloom (Figure 1).

Figure 1. Bloom of *Noctiluca scintillas* in October 2002, Leigh, New Zealand (photo: Miriam Godfrey)

This is regarded as a sudden increase in the microalgal population activated by suitable growth conditions so that concentrations of 10^4–10^5 cells per litre can be reached for a certain period of time (Masó and Garces 2006). A bloom can be dominated by a particular species or a group of species (Masó and Garces 2006). The initiation of a bloom requires an inoculum of cells, which can be from several sources and may involve different life stages, e.g., cysts (Steidinger and Garcés 2006), as well as favourable environmental conditions of temperature,

light, nutrients and water salinity (Zingone and Enevoldsen 2000; Daranas et al. 2001). Termination of a bloom is triggered by inappropriate environmental conditions, such as nutrient-deplete water, zooplankton predation or advection (Steidinger and Garcés 2006). Also viral termination of algal blooms of *Heterosigma akashiwo*, *Emiliana huxleyi* and *Phaeocystis globosa* have been observed (Bratbak et al. 1996; Brussaard et al. 2005).

Complex life cycles are described for numerous phytoplankton species and alternation between dormant, benthic stage and a motile, vegetative existence can take place. Dormant cysts or resting spores can be formed from many marine phytoplankton species during their life history and may play a an important role in bloom initiation (Zingone and Enevoldsen 2000). Most toxic or harmful species, dinoflagellates and diatoms, reproduce by asexual, binary division; however, sexuality can be induced under certain conditions. Morphological and physiologically distinct cell types (gametes, zygotes and cysts) are formed during the life cycle of most algae (Anderson et al. 2003). Blooms of dinoflagellates are annual events; the first increase of populations is usually documented in the spring. Sexual reproduction often occurs following the main period of vegetative growth and can last from a few hours several days. The resulting zygote is usually a resting stage or cyst. Cyst production is also assumed to be seasonal, because different dinoflagellate species become abundant at different times during the year. Some species attain their maximum abundance within the phytoplankton during the season spring and, therefore, form cysts in the late spring to early summer (e.g., *Protoperidinium oblongum*). Other species (e.g., *Alexandrium tamarense*, *Protoceratium reticulatum*) may exhibit two annual peaks in abundance and hence two peaks of encystment (Harland et al. 2004). Diatoms reproduce by asexual division until cell size reaches a minimum threshold level, usually below 30%–40% of the dimensions of the maximum cell size (Amato et al. 2005). This initiates sexual reproduction, which can be associated with increased photoperiod length (Steidinger and Garcés 2006). Life cycle investigations of diatoms have shown, that, within a population, sexual reproduction is a nearly synchronous event which occurs within a restricted size window, with a periodicity varying from 2 to 40 years (Mann 1988; Amato et al. 2005). Thick-walled resting cysts are occasionally formed from diatoms mainly towards the end of a bloom. They settle to the bottom or accumulate at pycnoclines (Anderson et al. 2003; Steidinger and Garcés 2006). Some dinoflagellate cysts can remain viable in the sediments for several years, ready to germinate when conditions permit (Zingone and Enevoldsen 2000; Daranas et al. 2001; Anderson et al. 2003).

In most cases, the proliferation of microalgae is a normal event and can be beneficial for aquaculture and fisheries operations. However if the bloom consists of harmful algae, it can also have a negative effect and cause severe economic losses to aquaculture, fisheries and tourism (Hallegraeff 2003). Three different types of HABs have been delineated by Hallegraeff (2003). The first type represents species that produce basically harmless discolorations of the water, but which can, under exceptional conditions, such as sheltered bays, form dense blooms that cause indiscriminate kills of fish and invertebrates through oxygen depletion (e.g., *Noctiluca scintillans*). Species that produce potent toxins form the second type are e.g., species of the genera *Alexandrium*, *Dinophysis* or *Pseudo-nitzschia*. Their toxins can find their way through the food chain to humans and cause a variety of gastrointestinal and neurological illnesses. The third type is presented by species that are non-toxic to humans but harmful to fish and invertebrates by damaging or clogging the gills or gill tissue (e.g., *Prymnesium parvum, Chrysochromulina polylepsis*) (Hallegraeff 2003). The impact of HABs is defined by the concentration of harmful species, even the most toxic species must occur with a minimum cell concentration to exert a harmful effect (Zingone and Enevoldsen 2000). About 200 noxious microalgal species and 97 toxic species (mainly dinoflagellates) are known to have the potential to form HABs (Zingone and Enevoldsen 2000; Moestrup 2004), a term introduced for the first time in 1974 at the 1st International conference of blooms of toxic dinoflagellates (Masó and Garces 2006). Harmful algae can be observed not only in a single class or in a few genera, but also can be found among six taxonomic groups (diatoms, dinoflagellates, haptophytes, raphidophytes, cyanophytes and pelagophytes) (Zingone and Enevoldsen 2000).

HABs are natural phenomena that have occurred throughout recorded history. However, in the past decades, the public health and economic impacts appear to have increased in frequency, intensity and geographic distribution (Daranas et al. 2001; Hallegraeff 2003). A worldwide increase of HABs cannot be verified because of missing time series data, but, numerous examples of HABs have been observed in areas where they were previously unknown (Zingone and Enevoldsen 2000), maybe through the transport in ballast water. Paralytic shellfish poisoning (PSP), triggered by blooms of *Alexandrium tamarense* and *A. catenella*, was only observed in the temperate waters of Europe, North America and Japan until 1970. By 2000, it was also well documented in the Southern Hemisphere (Hallegraeff 2003). The apparent increase of HABs can be explained, on one hand, by an increase of scientific awareness, reports in the press and electronic media and, on the other hand, by

increased aquaculture. This increase of fish and shellfish farming has been observed worldwide and consequently, the reports of harmful algae and human illnesses rise. Additionally some algal blooms appear to be stimulated by eutrophication activated by domestic, industrial and agricultural wastes. Also, climatological conditions can have an effect on the spatial distribution of a species. The dinoflagellate and PSP-producer *Pyrodinium bahamense* is presently known to be distributed in tropical seas, fossil cysts have been found in temperate regions of both hemispheres. Passive introduction of species from other areas by transport of cysts in ballast water as well as by currents and storms is considered as an explanation for extending the geographic range of a species. (Zingone and Enevoldsen 2000; Hallegraeff 2003)

1.1.1 Associated human illnesses

Harmful algae can affect human health in different ways. First, the ingestion of seafood contaminated with toxins produced by marine microalgae can cause a number of human illnesses like paralytic shellfish poisoning described below. Second, environmental exposures can occur when marine phytoplankton cells are disrupted by waves as they move onshore. Reports of skin irritation and respiratory distress have been associated with human exposure to water and aerosols containing toxins and cell fragments (Backer et al. 2003).

Paralytic shellfish poisoning (PSP) – One of the first recorded cases of paralytic shellfish poisoning was in 1793 when Captain Vancouver and his crew landed in the Pacific Northwest of the USA (Nishitani and Chew 1988). Early intoxications of humans have been recorded mostly in North America and Europe, but also in Malaysia, the Philippines, Indonesia, Venezuela, Guatemala, China and South Africa (Backer et al. 2003). The first isolated metabolite was saxitoxin (STX), whose origin was traced to plankton, and two major groups of toxins, saxitoxin and neosaxitoxin, have been identified. PSP symptoms are neurological and their onset is rapid. Neuronal and muscular sodium channels are blocked, which prevents propagation of the action potential in nerve axons and skeletal muscle fibres. A tingling or numbness around the lips is observed within 30 minutes, which gradually spreads to the face and neck. A prickly sensation in the fingertips, headache, fever, nausea, vomiting and diarrhoea usually follow. PSP is a life-threatening poisoning syndrome and the most severe cases result in respiratory arrest within 24 hours of consumption of the toxic shellfish. An antidote is non-existent and if supportive respiratory therapy can be carried out, survivors recover fully (Daranas et al. 2001). PSP toxins are produced by dinoflagellates of the genera

Alexandrium, *Gymnodinium* and *Pyrodinium*. 1,600 cases of intoxication were reported before 1970. Since that time an additional 900 cases have been diagnosed (Backer et al. 2003).

Diarrhetic shellfish poisoning (DSP) – The first report of DSP originated in 1976 from Japan, where it caused major problems in the scallop fisheries. The outbreaks in Japan were correlated with the appearance of the dinoflagellate *Dinophysis fortii.* Shortly after the outbreaks in Japan *Dinophysis* species and *Prorocentrum lima* were found to be responsible for DSP incidences in Europe (Hallegraeff 2003). The toxin responsible was named dinophysistoxin (DTX). The principal toxins responsible for incidents DSP are okadaic acid and its analogs, DTX1 and DTX2. Two other toxin groups, pectenotoxin and yessotoxin, are also placed in the "DSP" category, because of their co-occurrence with okadaic acid and DTX. Pectenotoxin (PTX) is named after the scallop genus *Patinopecten* from which it was first isolated and is the main toxin produced by *Dinophysis spp.*. Yessotoxins (YTX) are produced by the dinoflagellates *Protoceratium reticulatum* and *Lingulodinium polyedrum* (Quilliam 2003b). DSP toxins of the okadaic acid group produce gastrointestinal symptoms, including diarrhoea, nausea, vomiting and abdominal cramps. The symptoms can begin within 30 minutes after consumption of toxic shellfish and recovery takes place within three days without any medical treatment. Symptoms can easily be mistaken for those of bacterial gastric infections. However, some of the polyether toxins involved may promote stomach tumours and thus produce chronic problems in shellfish consumers (Daranas et al. 2001). In the 1970s and 1980s, altogether some 1,300 DSP cases were reported in Japan and more than 8,000 cases in Europe. By 2000, the global reports of diarrethic shellfish poisoning had extended to Japan, Europe, Chile, Thailand, Canada, Australia and New Zealand (Hallegraeff 2003).

Amnesic shellfish poisoning (ASP) – The first recognition of this phenomenon was in 1987 when three victims died and a hundred acute cases occurred after consumption of blue mussels from Prince Edward Island, Canada (Hallegraeff 2003). Domoic acid belongs to a group of amino acids called kainoids, a neuroexcitant, and interferes with the neurotransmission mechanisms in the brain. The diatom *Pseudo-nitzschia multiseries* was identified to be causative organism of the incident in Canada (Quilliam 2003a). Victims reported gastrointestinal symptoms, such as vomiting, abdominal cramps and diarrhoea, which usually occurred within 24 hours of the consumption of toxic shellfish. Additionally neurological symptoms can appear, usually within 48 hours. Dizziness, headache,

disorientation, short-term memory loss, respiratory difficulty and coma are also observed (Daranas et al. 2001; Backer et al. 2003). In 1991, brown pelicans and cormorants of California were victims of ASP from ingesting anchovies that had accumulated *Pseudo-nitzschia* species (Silver 2006). Domoic acid has been also isolated from *P. australis*, *P. delicatissima*, *P. multistriata*, *P. pseudodelicatissima*, *P. seriata*, *P. pungens* and *P. turgidula*. Reports of domoic acid are mainly restricted to North America and Canada, whereas only low concentrations have been found in Europe, Australia, Japan and New Zealand. (Hallegraeff 2003)

1.1.2 Aquaculture and harmful algal blooms

Because of the decrease in wild fishery catches, shellfish production and mariculture experience a worldwide expansion, especially in the Asia-Pacific region where seafood products are consumed in large amounts. In Europe, Spain, France, Italy, Denmark and the Netherlands are the main shellfish producers, with a total production of about one million tonnes in 1997. Mussel production is of great importance in these areas and the markets for fresh and frozen mussels are almost exclusively in Europe (Fernández et al. 2003). In 1998, worldwide production of mariculture fish was about 0.7 million tonnes (Rensel and Whyte 2003). Shellfish, such as bivalve molluscs, gastropods, crabs and lobsters, accumulate phycotoxins by direct filtration of the algal cells or by feeding on contaminated organisms. Regulation of accumulation of a particular toxin takes place by balancing toxin intake and loss from and to the environment as well as by the transformation to and from other toxins by microbial agents. Toxin accumulation rates as well as the rates of toxin loss by filter-feeding shellfish from toxic algae are toxin- and species-specific. (Fernández et al. 2003) Consequently, the duration of market closure depends on these rates. In 1984, the Swedish mussel industry was shutdown for almost a year because of DSP toxins (Hallegraeff 2003) that resided in the mussels depurated at slow rate (Svensson and Förlin 2004).

Fish killing microalgae have caused high economic losses to aquaculture in the last decades. One example is the massive bloom of *Chrysochromulina polylepsis* that occurred in 1988 in the Skagerrak, the Kattegat, the Belt and the Sound between Denmark, Norway and Sweden and caused the deaths of 900 tonnes of fish, including cod, salmon and trout (Hallegraeff 2003). Similarly, losses from fish kills amounted to US$95.5 million in Korea and in North America to US$35 million (Rensel and Whyte 2003). Fish mortality is caused by a variety of physiological mechanisms. Gill clogging, irritation or mechanical damage to the gill tissue

leads to the production of mucus to relieve the abrasion or to clear the blocked filaments. That can be followed by blood hypoxia and respiratory dysfunction as the cause of fish death. Other reasons for fish death can be toxigenic reactions to ichthyotoxic reagents, blood hypoxia from environmental oxygen depletion or gas-bubble trauma from oxygen supersaturation (Rensel and Whyte 2003).

1.2 Monitoring of phytoplankton

Detection and enumeration of harmful algal species is important for the prevention of toxication of humans as well as from an ecological and economic point of view. On a global scale, approximately 2,000 cases of human poisoning through fish and shellfish consumption are reported each year with a mortality of 15 percent (Hallegraeff 2003). HAB monitoring programmes (e.g. GEOHAB) at the coastlines all around the world aim to prevent intoxication of humans and animals through the consumption of contaminated seafood. Additionally, the protection of humans from algal toxins delivered via sea spray or direct contact is aimed. The damage of living resources, such as shellfish and fish, as well as the economic losses to fisherman, aquaculturists and the tourist industry should be minimized (Andersen et al. 2003). Monitoring programmes include, in the majority of cases, the surveillance for potential toxic algal species (identification and quantification) and the monitoring of toxin content in shellfish. In addition, water temperature, salinity, nutrients, chlorophyll, water stratification, current circulation and other parameters are also observed for bloom prediction.

1.2.1 Methods

1.2.1.1 Mouse bioassay

The traditional method for the detection, analysis and control of toxicity in shellfish in European monitoring programmes is the mouse bioassay (MBA) (Yasumoto et al. 1978). It is currently the reference method under EU legislation (Aune et al. 2007). In this method, shellfish extracts are injected intra-peritoneal into three mice and the mice are monitored over a certain period of time. Should at least two of the mice die within the time frame, the shellfish are declared to be unsuitable for human consumption. The maximum permissible level of okadaic acid, dinophysistoxins and pectenotoxins in shellfish (the whole body or any part edible separately) is laid down to 160 µg of okadaic acid equivalents/kg, whereas for

yessotoxins this level is 1 mg of YTX equivalents/kg (Decision 2002/225/EC) (Mouratidou et al. 2006). For PSP toxins the mouse is only monitored for 60 minutes (Aoac 1999). If the mouse is still alive after this time the sample is regarded as negative. The detection limit for MBA is approximately 300 µg/kg of shellfish flesh and if the regulatory limit for PSP toxins of 800 µg/kg shellfish flesh is reached the harvesting area is closed (Holtrop et al. 2006). Toxin concentration or toxin type are not quantitatively or qualitatively measured and this assay is recognised as having poor reproducibility and variability (Flanagan et al. 2001). However, the use of animal assays induce ethical problems as even with non-toxic samples the injection of 1 ml of the acidic extract into the abdomen of a 20 g mouse causes considerable pain and suffering to the animal (Holtrop et al. 2006). This presents the urgent need to replace the mouse bioassay with a more suitable monitoring method.

1.2.1.2 Methods for the detection of toxins

High-performance liquid chromatography (HPLC) is a widely used technique for the analysis of shellfish toxins that provides excellent peak resolution and high sensitivity. A wide range of toxin structures can be separated with this instrument (Quilliam 2003b). The preferred analytical method is the use of HPLC in combination with UV absorbance detection, which has been used since 1987 in regulatory laboratories (Quilliam 2003a). Organic extract of shellfish tissue and plankton are complex and the toxins have to be extracted using organic solvents before analysis with the HPLC. A pre- or post-column alkaline oxidation treatment of a sample for the detection of PSP toxins is required for the fluoremetric detection (Franco and Fernández 1993; Luckas et al. 2003). A pre-column HPLC oxidation method proposed by Lawrence and Ménard, (1991) (Lawrence and Ménard 1991) and Lawrence et al. (1996) (Lawrence et al. 1996) can produce fast and sensitive results but does not separate all PSP toxins. The method of Oshima (1995) (Oshima 1995) can separate all PSP toxins, however, it is very time-consuming because of the need to perform three separate runs in order to determine all the toxins (Vale and De M. Sampayo 2001).

The analysis of marine toxins can also be carried out using capillary electrophoresis (CE); it provides fast and high-resolution separation (Quilliam 2003b). Separation by electrophoresis is based on differences in solute velocity in an electric field. Thibault et al. (1991) described the use of CE with UV detection for the separation and determination of PSP toxins. CE is a rapid and efficient method that needs only a small volume of sample (Thibault et al. 1991). However, a purified sample is required for an effective analysis (Zhao et al. 1997).

1.2.1.3 Counting techniques

Microscope-based methods can identify and quantify microalgae at the species or genus level. Compound microscopy is a simple and quick method to estimate cell numbers from a drop of seawater using counting cells, such as the Sedgewick-Rafter cell. For low cell numbers below 10^2-10^4 cells L^{-1} the cells have to be concentrated before counting. Another possibility for the quantification of low cell numbers uses an inverted microscope and Utermöhl sedimentation chambers to concentrate the algae in a sample (Utermöhl 1958). This method can last from a few hours to several days because of the time needed to settle cells in the sedimentation chamber, which depends on the sample volume, the fixative used and the linear dimension of the cells. Low cell numbers can also be counted using quantitative epifluorescence microscopy by concentrating the cells onto filters and staining. Several stains such as DAPI or Acridine Orange, can be used. (Andersen and Throndsen 2003). For the identification of unicelluar algae, using microscope-based methods, a broad taxonomic knowledge is required, because toxic and non-toxic strains can belong to the same species and thus are morphologically identical (e.g., *Alexandrium tamarense* species complex) (John et al. 2005).

1.2.1.4 Data buoys and remote sensing using satellites

Marine data buoys are used to monitor plankton as well as physical, chemical and meteorological variables *in situ* and in real-time. For example, the CytoBuoy (CytoBuoy, Bodegraven, Netherlands), can be used to conduct extended and/or high frequency time series of phytoplankton distribution and abundance on fixed locations. Several buoys from the Seawatch Buoy System are located along the Norwegian coast and forecasting of upstream blooms can be facilitated (Smayda 2003). Another new HAB buoy system identifies species using a high speed camera for in-flow acquisition (Culverhouse et al. 2006). Recently the environmental sampling processor (ESP) was introduced (Doucette et al. 2006). The ESP is an electromechanical/fluidic system that collects discrete water samples and concentrates microorganisms. An automated application of molecular probes is carried out that identifies microorganisms and their gene products (Doucette et al. 2006).

Also satellite images are used to achieve understanding of the regional influences of physical processes affecting local phytoplankton populations. Sea surface temperature images can aid the prediction of transport of noxious phytoplankton. Toxic phytoplankton cannot be

identified using remote sensing. However, detection of a monospecific bloom with high cell counts at the surface is possible by using species-specific chlorophyll *a* and ocean-colour imagery. For example, for the detection of *Karenia brevis* about 10^5 cells L^{-1} are necessary, which would result in early warning of fish kills but not shellfish toxicity (Franks and Keafer 2003).

1.2.1.5 Detection of harmful algae using molecular probes or antibodies

In the past decade, a variety of molecular methods have been adapted for the detection of harmful algae. The first review for the use of molecular probes as tools to aid the identification of harmful algal species was presented by Anderson (1995) (Anderson 1995). Today molecular probes are widely applied for the identification of micro-organisms. The usual targets for probes are the small and the large subunit ribosomal RNA genes, because of their high target number in the cell. More or less conserved regions in these genes make it possible to develop probes that are specific at different taxonomic levels (Groben et al. 2004).

Fluorescence *in situ* hybridization (FISH) uses a fluorescently labelled probe that is designed to recognize a specific sequence of a particular organism. The probe is hybridized inside the intact cells, the ribosomes and cells containing a fluorescently labelled probe can then be detected using epifluorescence microscopy (Hosoi-Tanabe and Sako 2005). FISH allows the rapid detection of different algal groups by epifluorescence microscopy and even the separation of closely related and morphologically similar species (Lim et al. 1993; Scholin et al. 1996; Scholin et al. 1997; Simon et al. 1997; Simon et al. 2000; Groben et al. 2004; Sako et al. 2004; Smit et al. 2004; Töbe et al. 2006). Sandwich hybridization assays (SHA) can also provide the possibility to identify and enumerate toxic algae rapidly. SHA relies on extracted nucleic acids from cell lysates. A capture probe bound to a solid surface immobilizes the target ribosomal RNA and forms a hybrid complex with a second signal probe. An antibody-enzyme complex binds to the signal moiety of the signal probe and reacts with a substrate forming a colorimetric product or an electrochemical current (Scholin et al. 1996; Tyrrell et al. 2002; Metfies et al. 2005). Just recently, the SHA was validated and accepted for international accreditation for commercial laboratory use in New Zealand in May 2004 (Ayers et al. 2005). DNA microarrays are used in many applications because of the possibility to analyze a large number of up to 250,000 different targets in parallel without a cultivation step (Lockhart et al. 1996; Graves 1999; Ye et al. 2001). This technology is also used to differentiate microalgae (Metfies and Medlin 2004; Metfies and Medlin 2005b; Ki and Han

2006; Godhe et al. 2007). A microarray consists of a glass-slide with special surface properties (Niemeyer and Blohm 1999) and is spotted with many copies of nucleic acids in a specific pattern, e.g., oligonucleotides, cDNAs or PCR-fragments (Graves 1999). The most common type of probes used in HAB research are antibodies (Scholin et al. 2003). Antibodies bind to different molecules, such as peptides, glycoproteins and toxins. Many of the developed antibodies for HAB species have been tested in laboratory but only a few in field studies. Primary as well as secondary antibodies are applied; however, many techniques for HAB species identification employ the indirect-labelling method using a fluorescent secondary antibody (Mendoza et al. 1995; Cordova and Muller 2002; Scholin et al. 2003; West et al. 2006). Detection of harmful species employing the polymerase chain reaction (PCR) is based on the binding of complementary strands of nucleic acids. Only a fragment of the genome is targeted, based on the use of oligonucleotide primers that define the size of the fragment as well as the taxonomic specificity of the reaction. PCR requires the extraction of nucleic acids from the sample, primers and an amplification protocol (Scholin et al. 2003). Direct quantitative PCR using fluorescent probes was recently used by Bowers et al. (2000) to detect *Pfiesteria* species. In this assay, the detection of amplified target DNA required the annealing of fluorescently labelled oligonucleotide probes. The 5'- to-3' exonuclease activity of the taq polymerase cleaves the probe and the quencher dye is released from the emitter dye, which in turn is then able to fluoresce (Bowers et al. 2000). The relative fluorescence is related the number of free fluorescent molecules in solution and the cycle of fluorescence detection is directly related to the number of target molecules in the initial reaction mixture. However, sensitivity and specificity of the assays has to be analyzed and the application for some field samples can be problematic, if sample composition inhibits DNA extraction and purification (Scholin et al. 2003).

1.3 Biosensors

Biochemical recognition with signal transduction for the detection of specific molecules is combined on electrochemical biosensors. The detection component, such as a probe sequence, an antibody, an enzyme or other biomolecules, catalyzes a reaction with or specifically binds to the target of interest. A transducer component transforms this detection event into a measurable signal. A specific detection of targets in a complex sample is possible. Biosensor types comprise optical, bioluminescent, thermal, mass and electrochemical recognition (Gau et al. 2005). Various sectors, such as clinical diagnostic, environmental monitoring, biothreat detection and forensics, apply single electrode sensors as well as arrays (Berganza et al. 2006;

Lermo et al. 2006; Taylor et al. 2006). Arrays of electrodes enable a simultaneous detection of multiple species with different molecular probes (Farabullini et al.; Dock et al. 2005). Biosensors can be used *in situ* and therefore circumvent the need to return samples into the laboratory. Rapid identification of aquatic microorganisms as well as physical and chemical measurements of the environment are important to understand coastal dynamics and processes that can impact marine ecosystems, such as the introduction and spreading of microbial pollutants and the initiation of HABs (Lagier et al. 2005). Metfies et al (2005) introduced a biosensor in combination with a hand held device for the detection and identification of the toxic dinoflagellate *Alexandrium ostenfeldii* (Metfies et al. 2005). The biosensor has the potential to serve as a quick and easy method for the identification of harmful algae.

1.4 Aim of thesis

My thesis was assigned to the development and evaluation of fast and reliable monitoring methods using molecular technologies. Harmful algal species are responsible for fish and shellfish kills and poisoning of consumers through ingesting of contaminated seafood. The detection and enumeration of harmful algal species is important from an ecological and economic point of view. The current monitoring methods are time consuming and require trained personnel and expensive equipment. Unicellular algae are taxonomically challenging and some of them have only few morphological markers for reliable identification. The aim of this thesis was to design and adapt molecular probes for the identification of toxic algae. Furthermore, the methods developed were adjusted and evaluated to serve as potential early warning systems for toxic algae.

1.5 Outline of thesis

1.5.1 Development and adaptation of molecular probes for sandwich hybridization

The species *Alexandrium minutum* belongs to the most potent PSP-toxin and other toxin producers (Taylor and Fukuyo 1998; Chen and Chou 2002; Nascimento et al. 2005). *A. minutum* can be observed world-wide and its geographic range as well as its bloom frequency are increasing (Lilly et al. 2005). Monitoring of toxic algae involves the accurate morphological identification and enumeration of species by using standard microscopy procedures. *A. minutum* is difficult to distinguish from other species of the same genus because it is characterized by minute details of its thecal plates (Taylor et al. 1995). The small

and the large subunit ribosomal RNA genes have more or less conserved regions that make it possible to design probes of varying target specificity (Groben et al. 2004). Molecular probes have been developed only for a small percentage of the toxic algal species. Sandwich hybridization methods using species-specific ribosomal RNA (rRNA) probes is a suitable tool for the rapid and reliable detection of harmful algae.

In **Publication I** a commercially available PCR ELISA Dig Detection Kit was adapted for the detection of the toxic dinoflagellate *Alexandrium minutum* using sandwich hybridization in a microtiter plate. For the detection of *A. minutum* a set of two 18S rRNA probes was developed using the ARB software package (Ludwig et al. 2004). The specificity of the probes was tested using the microtiter plate assay and also closely related species. An additional aim of this study was to investigate the potential of the modified assay for the detection of harmful algae without labour-intensive cell number determination. For the detection of *A. minutum* by means of standard calibration curve the total rRNA concentration per cell had to be determined. The assay and the standard curve were evaluated by using spiked water samples.

1.5.2 Design and evaluation of probe sets for toxic algae

Phytoplankton communities consist of assemblages of co-occurring species and the temporal and spatial variability in composition in the sea is substantial (Venrick 1999; Figueiras et al. 2006). The composition of the harmful algae species in different areas of Europe is complex and several algal genera include toxic species, such as *Alexandrium*, *Dinophysis*, *Gymnodinium* and *Pseudo-nitzschia* (Simon et al. 1997; John et al. 2003; Moita et al. 2003; Chepurnov et al. 2005). Molecular techniques for the detection of toxic algae require the use of probes targeting specific genes of the target species.

In **Publication II** probe sets for the species-specific identification of the toxic algal species *Gymnodinium catenatum*, *Protoceratium reticulatum, Lingulodinium polyedrum, Prymnesium parvum, Chrysochromulina polylepis*, *Pseudo-nitzschia multiseries, P. australis, P. seriata and P. pungens* were designed and adapted for the use in sandwich hybridization formats. An already existing probe set for the genus *Pseudo-nitzschia* was adapted. Target species as well as closely related species were utilized for the verification of specificity in the microtiter plate assay.

1.5.3 Improvement of detection protocol

Today biosensors are commonly used in clinical diagnostic, environmental monitoring, biothreat detection and forensics. The advantage of biosensors is the possibility to measure on-site and therefore, sample transport to the laboratory is unnecessary. Biosensors are used for the rapid identification of aquatic microorganisms. Metfies et al. (2005) introduced a biosensor for the identification of the toxic dinoflagellate *Alexandrium ostenfeldii* for the first time (Metfies et al. 2005).

In **Publication III** a description and illustrative visualization of the method introduced from Metfies et al (2005) is presented. The aim of this work was to bring up the method to a standard for ease of use through others. For this purpose it was necessary to adapt the method to sensor chips and a measuring device from another manufacturer. Furthermore, the equipment needed for a complete sample analysis was identified and modifications of protocols were presented.

1.5.4 Assessment of probe modification for signal enhancement

Identification of microbial species with probe-based methods requires sensitive and highly specific probes. The specificity of the probes depends on the number of sequences of the target gene available in databases. Probes designed from a low number of target species or for a group, which includes relatively unknown or unculturable species can detect also non-targeted species (cross-hybridization). Additionally, many non-targeted species exist whose sequences have not yet been determined. The frequently revise of probes is necessary because new sequences are added to databases on a daily basis. The introduction of locked nucleic acid (LNA) probe technology promises an enhancement of both specificity and sensitivity of molecular probes (Kongsbak 2002).

Publication IV involved the revision of probes for *Alexandrium ostenfeldii* and the comparison of specificity and sensitivity of conventional molecular probes and LNA modified probes. Two different solid phase hybridization methods, sandwich hybridization on biosensors and DNA-microarrays, were used for the detection of probe signals. The set of 18S-rRNA probes for *A. ostenfeldii* was applied to assess the impact of LNA-probes on the specificity of probes with the biosensor, thus, the sequence of the capture probe was

redesigned with locked nucleic acids. Three different species, *A. ostenfeldii*, *A. minutum* and *A. tamutum*, were tested with conventional probes and LNA modified probes. *A. minutum* previously showed low cross-hybridization signals (Metfies et al. 2005) and the 18S rRNA sequence of *A. tamutum* possessed only one mismatch to the capture probe. Five probes, that target the 18S-rRNA, were evaluated with the DNA-microarray. One of the probes targets the super kingdom of Eukarya and the other probes each of these four major phyla of algae: the Chlorophyta, Bolidophyta, Prymnesiophyta and Cryptophyta. For each probe, two different locked nucleic acid modifications were evaluated.

1.5.5 Development and evaluation of a biosensor

Monitoring programmes at the world-wide coastlines observe phytoplankton compositions and especially harmful algal species. The application of the mouse-bioassay is statutory for the monitoring of toxin contamination of shellfish, whereas toxin determination is performed by HPLC. The mouse-bioassay induces ethical problems because of the painful procedure for the animals; HPLC, in turn, is a very time-consuming and expensive method. Traditional methods, such as light microscopy, are time-consuming when numerous samples consisting of many species have to be routinely analyzed and require a broad taxonomic knowledge as well. Simultaneous detection of multiple species can be accomplished using e.g. DNA-microarrays with different molecular probes (Metfies and Medlin 2005b). The utilization of all described methods requires transportation of samples to specialised laboratories and high trained staff. The results are achieved within around five working days and therefore, preventive measures are not always possible. A fast identification of aquatic microorganisms is realized by the use of biosensors. The *in situ* investigation of coastal water for the presence of different toxic algae could provide a potential early warning tool for monitoring of bloom formation and thus, potential shellfish contamination.

In **Publication V,** the ability and adaptability of a biosensor for the rapid and reliable *in situ* detection of toxic algae was investigated. The aim of this study was the design and evaluation of a multiprobe chip and an automated device in order to facilitate the detection of several species simultaneously. For the design of the multiprobe chip, different materials for electrodes and the carrier material were tested to obtain accurate signal formation using sandwich hybridization and molecular probes. An adaptation of analysis and hybridization procedures was necessary for the use of the biosensor by layperson. Furthermore a portable device was designed, which performs the analysis in a semi-automated manner.

2. Publications

2.1 List of publications

This doctorial thesis is based on the following publications:

I. SONJA DIERCKS, LINDA K. MEDLIN AND KATJA METFIES
COLORIMETRIC DETECTION OF THE TOXIC DINOFLAGELLATE *ALEXANDRIUM MINUTUM* USING SANDWICH HYBRIDIZATION IN A MICROTITER PLATE ASSAY
Harmful Algae, to be submitted

II. SONJA DIERCKS, KATJA METFIES AND LINDA K. MEDLIN
MOLECULAR PROBES FOR THE DETECTION OF TOXIC ALGAE FOR USE IN SANDWICH HYBRIDIZATION FORMATS
Journal of Plankton Research, to be submitted

III. SONJA DIERCKS, KATJA METFIES AND LINDA K. MEDLIN
ELECTROCHEMICAL DETECTION OF TOXIC ALGAE WITH A BIOSENSOR
Manual and Guides: Microscopic and molecular methods for quantitative phytoplankton analysis, submitted

IV. SONJA DIERCKS AND CHRISTINE GESCHER, KATJA METFIES, LINDA K. MEDLIN
EVALUATION OF LOCKED NUCLEIC ACIDS FOR SIGNAL ENHANCEMENT OF OLIGONUCLEOTIDE PROBES FOR MICROALGAE IMMOBILIZED ON SOLID SURFACES
Limnology and Oceanography: Methods, submitted

V. SONJA DIERCKS, KATJA METFIES, STEFFI JÄCKEL AND LINDA K. MEDLIN
DEVELOPMENT AND OPTIMIZATION OF A SEMI AUTOMATED RRNA BIOSENSOR FOR THE DETECTION OF TOXIC ALGAE
Biosensors and Bioelectronics, to be submitted

Other publication prepared with contribution of the candiate from the period of time:

GODHE, A., AND OTHERS (2007)
INTERCALIBRATION OF CLASSICAL AND MOLECULAR TECHNIQUES FOR IDENTIFICATION OF *ALEXANDRIUM FUNDYENSE* (DINOPHYCEAE) AND ESTIMATION OF CELL DENSITIES
Harmful Algae, 6: 56-72.

2.2 Statement of my contribution to the publications

Publication I

The experiments were planned together with K. Metfies and L. K. Medlin. The experiments were carried out by myself and analyzed by myself. The manuscript was written by myself.

Publication II

The experiments were planned together with L. K. Medlin and K. Metfies and performed by myself. I have analyzed the data and wrote the manuscript.

Publication III

The experiments were planned together with L. K. Medlin and K. Metfies and performed by myself. I wrote the manuscript.

Publication IV

The experiments were planned together with K. Metfies, L. K. Medlin and C. Gescher and carried out from C. Gescher and myself. The manuscript was written equally with C. Gescher.

Publication V

The experiments were planned together with L. K. Medlin, K. Metfies. S. Jäckel was involved in the experiments for the development of the lysis buffer. All other experiments were performed and analyzed by myself. I wrote the manuscript.

2.3 Publication I:

COLORIMETRIC DETECTION OF THE TOXIC DINOFLAGELLATE *ALEXANDRIUM MINUTUM* USING SANDWICH HYBRIDIZATION IN A MICROTITER PLATE ASSAY

SONJA DIERCKS, LINDA K. MEDLIN AND KATJA METFIES
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Harmful Algae, to be submitted

Abstract

Rapid and reliable detection of harmful algae in coastal areas and shellfish farms is an important requirement of monitoring programs. Molecular technologies are rapidly improving the detection of phytoplankton and their toxins. Assays are based on the discrimination of genetic differences within different species. A commercially available PCR ELISA Dig Detection Kit was adapted for the detection of the toxic dinoflagellate *Alexandrium minutum* using sandwich hybridization in a microtiter plate. A set of two probes for the species-specific identification was developed for *A. minutum*. The specificity of the probes was successfully demonstrated with the microtiter plate assay. A standard calibration curve for different RNA concentrations and thus cell counts was determined for the assay. Total rRNA was isolated from three different strains of *A. minutum* and the mean concentration of RNA per cell of was determined to be 0.028 ng. The assay and the standard curve were evaluated by using spiked water samples. The results demonstrate that the molecular assay was able to detect *A. minutum* cells at different cell counts in the presence of a complex background. The experiments with spiked natural samples present a proof of principle of this assay. These tests also provided the necessary specificity tests prior to the probes being adapted to an automated biosensor using a sandwich hybridization format (Metfies et al. 2005).

Introduction

Over the last decades the occurrence of harmful algal blooms (HABs) has increased. Coastal systems around the world have had fish kills, outbreaks of shellfish poisonings, deaths of marine mammals and loss of quality of coastal waters for recreational use. Phytoplankton blooms are defined as a sudden increase in the microalgal population initiated by suitable conditions for growth, and reach cell concentrations up to 10^4-10^5 L^{-1} (Maso and Garces 2006). Two types of causative organisms are considered harmful: the toxin producers and the high-biomass producers. Around 4000 marine planktonic microalgae are described so far. Of these, around 97 are toxic species (mainly dinoflagellates) and about 200 can be noxious (Zingone and Enevoldsen 2000; Moestrup 2004). These harmful/noxious species belong to six algal groups: diatoms, dinoflagellates, haptophytes, raphidophytes, cyanophytes, and pelagophytes, which differ greatly in terms of morphological, physiological and ecological characteristics (Maso and Garces 2006). Among the dinoflagellates, 23 species are known to produce potent toxins, such as saxitoxins. Saxitoxins are responsible for the life-threatening paralytic shellfish poisoning (PSP), which can be caused by the consumption of molluscs that have filtered toxic dinoflagellates of the genus *Alexandrium* (Daranas et al. 2001; Chou et al. 2004) as their food source. The identification of the genus *Alexandrium* by means of morphological characteristics, such as general form, cell size and shape of the apical pore is difficult and labour-intensive. The morphological characteristics cannot be used alone for *Alexandrium s*pecies identification because of their similarity to other microalgae, and, in addition, intermediate morphological forms (Cembella and Taylor 1985; Hosoi-Tanabe and Sako 2005; John et al. 2005). Consequently, an improved monitoring, rapid detection and enumeration of toxic algae is crucial. Within the genus *Alexandrium*, the species *Alexandrium minutum*, which has been observed world-wide (Lilly et al. 2005), belongs to the most potent algal group of PSP-toxin and other toxin producers (Taylor and Fukuyo 1998; Chen and Chou 2002; Nascimento et al. 2005). The geographic range and bloom frequency of *A. minutum* is increasing (Lilly et al. 2005). Monitoring methods based on light microscopy are time-consuming and costly if a large number of samples need to be processed. For the identification of some species, highly-trained staff and expensive equipment are needed. Molecular techniques, such as whole cell fluorescent *in situ* hybridization or FISH (Anderson et al. 2005; Hosoi-Tanabe and Sako 2005; Kim and Sako 2005), sandwich hybridization assays or SHA (Tyrrell et al. 2002; Matweyou et al. 2004; Metfies et al. 2005), PCR-based assays (Penna 1999; Guillou et al. 2002) and monoclonal antibody probes (Anderson et al.

1999) can identify phytoplankton species. The principle of the sandwich hybridization was introduced by (Zammatteo et al. 1995; Rautio et al. 2003) and represents a DNA probe-based method for rapid identification of micro-algae that uses two species specific oligonucleotide probes targeting ribosomal RNA (rRNA) (Ayers et al. 2005), one to capture the target molecule and the other to carry the detectable signal (Figure 1). Oligonucleotide DNA probes are designed to bind to complementary sequences of the small or the large subunit ribosomal RNA algal genes and have a length of 18-25 base pairs. The possibility to design probes of varying target specificity is possible because of more or less conserved regions of the rRNA molecule (Groben et al. 2004). It is necessary that the specificity of probes is extensively tested, so that false positives are not encountered. The probes must be tested so, that close neighbours (clade tests) and probe neighbours (probe tests, target sequence close, but phylogenetically unrelated) do not bind to the probe. Such extensive tests require a rapid and easy to use format so that the many variations in hybridization conditions and test organisms can be verified as non-reactive. For FISH probes, the dot blot hybridization with chemiluminescent detection provides this vehicle for probe specificity testing prior to FISH applications (Groben and Medlin 2005).

In this study a fast and simple method for the detection of *Alexandrium minutum* is presented, whose principle is based on a sandwich hybridization with the capture oligonucleotide probe bound to the well of a microtiter plate. The commercially available PCR ELISA Dig Detection Kit from Roche Diagnostics (Mannheim, Germany) was adapted to the sandwich hybridization assay as a rapid, cost-effective, easy-to-use method that requires minimal handling. The assay presented here comprises a biotinylated target specific capture probe that binds to the streptavidin-coated well of a microtiter plate. Target nucleic acid hybridizes to the capture probe and a second digoxigenin-labelled signal probe hybridises to this complex. The detection and visualisation take place via an anti-digoxigenin peroxidase conjugate and colorimetric substrate. This method provides an easy to use method to test for probe specificity and has potential to be used for routine monitoring of field samples.

Material and Methods

Cultures and growth conditions - The algal strains used in this study were cultured under sterile conditions in seawater-based K-medium (Keller et al. 1987), IMR-medium (Eppley et al. 1967), F2-medium (Guillard and Ryther 1962; Guillard 1975) and Prov (Provasoli et al. 1957; Guillard and Ryther 1962; Guillard 1975) at temperatures listed in Table 1. All cultures were exposed to a photon irradiance rate of 150 µEinstein –200 µEinstein provided by white lamps at a light:dark cycle of 14:10 h.

Cell counts of algae cells - Aliquots were taken from the different algae cultures prior to harvesting and counted using the Multisizer 3 Coulter Counter (Beckman Coulter GmbH Diagnostics, Krefeld, Germany).

Isolation of RNA - Total RNA was isolated from all algal cultures with the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and the isolation protocol from Qiagen was modified for quality enhancement. Having applied the cell lysate to the QIAshredder spin column, the centrifugation time was increased from 2 to 15 minutes to improve separation of supernatant from cell debris. The first washing step with buffer RW1 was repeated twice and modified by adding an incubation time of one minute on the RNeasy spin column. Furthermore, the first wash step with buffer RPE was repeated. A Nanodrop Spectrophotometer (Peqlab, Erlangen, Germany) was used to measure the RNA concentration.

Fragmentation of RNA - Prior to hybridization, the total rRNA was fragmented in fragmentation buffer (40mM Tris, pH 8.0/100mM KOAc/30mM MgOAc) for 5 minutes at 94 °C and then chilled on ice.

PCR ELISA (DIG Detection) kit contents and preparation of working solutions - The PCR ELISA Dig Detection Kit from Roche Diagnostics (Mannheim, Germany) contains several reagents; however, only the hybridization buffer, conjugate dilution buffer, substrate buffer, Anti-digoxigenin-POD conjugate (anti-DIG-POD), washing tablets and ABTS tablets were used in these experiments for the identification of *A. minutum*. The microtiter plates are provided as plate modules (8 wells each), pre-coated with streptavidin and post-coated with blocking reagent. The kits are stored at 4 °C. Prior to the experiments, the washing solution

was prepared by dissolving one washing tablet in two litre deionized water. The Anti-DIG-POD is lyophilised and was dissolved in 250 µL of double distilled water.

Hybridization - The biotinylated capture probe, the digoxigenin-labeled signal probe and the positive control were diluted to a concentration of 10 µM prior to hybridization. For the sandwich hybridization, 4 µL of each probe and different concentrations of rRNA were added to the hybridization buffer resulting in a final volume of 250 µL. A negative control was prepared containing only both probes and hybridization buffer, whereas the positive control included additionally the test DNA (target sequence of the probes). Hybridization solution containing the RNA, the negative and positive controls were added into the wells of the microtiter plate and incubated on a shaker for 1 hour at 46 °C.

Incubation with antibody - The Anti-DIG-POD working solution (1 volume Anti-DIG-POD and 99 volumes conjugate buffer) was prepared at least one hour prior to the incubation step and stored in dark to equilibrate to room temperature before use. Subsequent to the hybridization, the wells of the microtiter plate were washed with washing solution three times. 200 µL of antibody solution were added to each well and incubated for 30 minutes at 37 °C with agitation in the dark. The antibody is directed against the digoxigenin label on the signal probe.

Incubation of substrate solution - Substrate solution was prepared by adding one tablet of ABTS to 5 mL of substrate buffer and stored protected from light. The substrate solution was allowed to equilibrate to room temperature before use. After the incubation with the Anti-DIG-POD, the wells were washed again three times with washing solution, 200 µL of substrate solution were filled in the wells and incubated in the dark on a shaker at 37 °C for 30 minutes. The hybrids are detected using an anti-digoxigenin antibody conjugated to horseradish peroxidase that reacts with substrate to produce a green colorimetric product.

Reading of microtiter wells - Each well of the microtiter plate was read out at 405 nm using a quartz cuvette with a Varian Cary 4000 UV-Vis Spectrometer (Varian Inc., Darmstadt, Germany).

Preparation of spiked water samples - A water sample was taken from the estuary of the Weser River (German Bight) with a natural phytoplankton population as a matrix. The water

sample was pre-filtered over a 180 µm nylon filter (45 mm diameter, Millipore, USA) to remove larger particles, such as zooplankton. Sedimentation was allowed over night and subsequently the water sample was filtered through a 10 µM polycarbonate filter (45 mm diameter, Millipore, Billerica, USA). 500 mL of the supernatant was filtered over a 5 µm polycarbonate filter (45 mm diameter, Millipore, USA) to collect the remaining matrix and spiked with three different cells counts of *Alexandrium minutum* and other algae cells with different cells counts (Table 2). The samples were prepared in triplicate. RNA was isolated from the samples as described above and analyzed with the microtiter plate assay.

Results

Design of oligonucleotide probes - From the probe design option within the ARB software package (Ludwig et al. 2004) two probes were designed for the sandwich-hybridization that bind to the 18S rRNA of *Alexandrium minutum* (Table 3) from a database consisting of more than 3000 published and unpublished algal 18S rRNA sequences. Two probes were chosen next to each other in the target sequence in case the target nucleic acid was degraded and the sites were no longer accessible from the same length strand of rRNA. *In silico*, probe AMINC is specific for *A. minutum* and has at least one mismatch against *A. insuetum* and two mismatches against all other non-target organisms listed in the ARB database. Probe AMINCNEXT recognizes not only *A. minutum*, but also *A. ostenfeldii*, *A. tamutum* and *A. insuetum*. Furthermore, it only has one mismatch against *A. affine*, but two mismatches against all other species. A BLAST search (Altschul et al. 1990) was conducted to test the overall specificity of the probes against all available sequences. Positive control and probes were synthesized from Thermo Electron Corporation (Ulm, Germany). Thus, from these *in-silico* tests, AMINC was defined as capture probe and AMINCNEXT as signal probe.

Specificity of probes - The specificity of the *Alexandrium minutum* probes was tested using the sandwich-hybridization-assay in a microtiter plate well. Total RNA was isolated from different strains of the target species *Alexandrium minutum* and more distantly related species of the genus *Alexandrium* and *Gonyaulax spinifera*. The obtained signals were normalised to a target concentration of 350 ng RNA and compared to one another. Signals were observed for all *A. minutum* strains, whereas no signals were determined for the non-targeted species (Table 4).

Total RNA concentration per cell - In a range of 10,000 to 500,000 *Alexandrium minutum* cells, total RNA was isolated in triplicate from different cell counts of three strains (AL3T, AMP4, AL5T) to determine the RNA concentration per cell (Figure 2) at optimum growth conditions, because this corresponds most closely to bloom development in the field (Ayers et al. 2005). The curves of the different strains show variations in the RNA concentration for the different cell numbers. However, all three curves of the different strains show a straight proportional development. For each strain, a mean RNA concentration per cell was calculated from the RNA concentration of the different cell counts. Strains AMP 4, AL3T and AL5T contained 0.017 ng, 0.027 ng and 0.036 ng RNA per cell, respectively. The mean concentration of total RNA per cell for the *Alexandrium minutum* strains was determined to be 0.028 ng.

Standard Curves of photometer readings to cell counts - The microtiter plate assay using a sandwich-hybridization and specific probes for *Alexandrium minutum* detected hybridization signals for different RNA concentrations and thus these values could be converted to cell numbers of *A. minutum*. The photometer readings (Figure 3) for isolated total RNA of three *A. minutum* strains showed a linear increase in signals from a mean absorbance of 0.0297 for 10,000 cells to 1.7757 for 500,000 cells. Strain AL5T produced higher probe signals than strains AL3T and AMP4; however, average values of the tested strains were observed to be in the same range as the signals for strain AL3T.

Method application to spiked samples - For method evaluation, a natural water sample was taken and spiked with different numbers of cells to simulate real samples as closely as possible. The photometer readings from the microtiter plate assay and *Alexandrium minutum* probes were compared using a lab culture of *A. minutum* and field samples spiked with *A. minutum*. Signals for 10,000 cells of *A. minutum* for both samples were slightly above the background but still measurable (Figure 4). The spiked sample with 50,000 cells of *A. minutum* gave a signal of 0.055, which was fourfold lower than the signal of 0.199 for a lab culture at a similar cell concentration. Also the signal for the spiked sample with 100,000 cells was threefold lower than that for the lab culture.

Discussion

In this study, a new method for the detection of the toxic dinoflagellate *Alexandrium minutum* is presented. The PCR ELISA Dig Detection Kit (Roche Diagnostics, Mannheim, Germany) in a microtiter plate was successfully adapted to a sandwich hybridization format using two differently labelled probes. The capture probe is biotin-labelled and the signal probe is digoxigenin-labelled. The probes used in the sandwich hybridization presented here are targeted against the 18S-rRNA of *A. minutum.* Sandwich hybridizations and rRNA targeted probes are used in different applications for the detection of microalgae (Scholin et al. 1996; Tyrrell et al. 2002; Ayers et al. 2005; O'halloran et al. 2006).

Probes were designed using the software ARB software package (Ludwig et al. 2004). The specificity of the probes for *A. minutum* was shown using sandwich hybridization in a microtiter plate well. The signals for all *A. minutum* strains were always significantly above the signals for the non-target species as predicted by the *in-silico* tests. Moreover, *Alexandrium* species with a single mismatch in the target sequence were not detected with the sandwich hybridization even without the use of a competitor to block these non-target species and prevent the RNA from hybridising with the capture probe. More distantly related species were not tested with the assay assuming that the species with the fewest number of mismatches would present the highest possibility of unspecific binding. Distantly related species have even more mismatches to the probe sequences and probe binding would be unlikely. The probes were designed to be in close proximity to one another in the target 18S-rRNA sequence to avoid a loss of signal if the target RNA molecule was degraded.

To develop a standard calibration curve of the microtiter plate assay for *A. minutum,* the total rRNA concentration per cell was determined at optimum growth conditions for three different strains as this was expected to correspond most closely to bloom development in the field (Ayers et al. 2005). A mean concentration of 0.028 ng rRNA per cell was found. The different strains were not synchronised, consequently a part of the culture could have been in the lag or stationary phase. This calculated rRNA concentration per cell of *A. minutum* also corresponded to that obtained for *A. fundyense* (data not shown) and *A. ostenfeldii* (Metfies et al. 2005). Additionally, similar findings were achieved for different growth conditions for *A. minutum* (personal communication L. Carter, Westminster University, London, UK). A standard calibration curve for different rRNA concentrations and consequently different cell

counts of *A. minutum* strains with the microtiter plate method was calculated. The signal increases with higher RNA concentrations and thus with higher cell numbers. The measured signal for 10,000 *A. minutum* cells is just above the background and can also be regarded as a negative signal. A signal that is clearly distinguishable from the background was observed for 12,500 cells of *A. minutum*. A low signal with an absorbance of 0.07 presents either 12,500 cells of *A. minutum* or a very high amount at least 500,000 cells of *A. ostenfeldii* or *A. fundyense* with high concentrations of RNA, thus the signal can not be misinterpreted. RNA isolation limits the detection method because of high user variability in the ability to isolate rRNA from the same number of algal cells and thus resulting in lower RNA concentrations per cell. Signal intensities of these RNA concentrations would not reflect the correct cell numbers. 10,000 cells of *A. minutum* present the smallest possible number of cells for RNA isolation in this study, however, RNA isolation is not limited by cell numbers but rather by limitations of the extraction kit. But these cell numbers result in the lowest measurable concentration; otherwise the standard error is too high. Thus, the detection limit of the microtiter plate assay for 12,500 *A. minutum* cells with an average yield of 0.028 ng RNA per cell the sampling volume would imply that 50 litres with 250 cells per litre would have to be concentrated before a reliable detection value is measured. More work is needed to reduce the detection limit.

The microtiter plate assay using a sandwich hybridization was evaluated with the analysis of spiked samples. Phytoplankton communities often consist of several different species and the temporal and spatial variability in composition in the sea is substantial. The experiment with spiked samples revealed that for 50,000 and 100,000 cells of *A. minutum,* the signal was lower than the signals for the same number of cells of a lab culture. One reason for the lower signals of the natural sample can be the composition of the sample. Large amounts of natural sediment were observed at the sampling location and this sediment seems to disturb the RNA isolation. The concentration of total rRNA may be improved by changing the RNA isolation protocol. Therefore, future experiments should also include the development of an independent system without RNA isolation as described by Tyrrell et al. (2002) and Ayers et al. (2005). As a result of the lower signals in the microtiter plate assay for natural samples, the correlation of signal to cell numbers is limited, only an estimation of cell numbers can be done. Hence, samples with high sediment loads are inappropriate for analysis with the microtiter plate assay. However, the method presented here using a sandwich hybridization in a microtiter plate is reliable, and in comparison to other molecular methods, inexpensive, fast

and easy to handle. It provides a rapid assay for testing of probe specificity, much in the same way that dot blots provide the vehicle for testing probe specificity for FISH probes.

Conclusion

A microtiter plate assay was adapted for the detection of the toxic dinoflagellate *Alexandrium minutum* using a sandwich hybridization. The assay has the potential to be a fast and reliable method for the detection of toxic algae by eliminating the need to count algae manually. The assay takes only two and a half hours to examine up to 30 different samples. The experiments with spiked natural samples present a proof of principle of this method. Clearly additional work is required to improve RNA isolation from natural samples and to optimize the sensitivity of the method for *A. minutum* probes. For the routine testing of probe specificity, it can provide a rapid assay for assessing probe specificity at both the clade and target sequence level.

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Table 1. Culture conditions and geographical origin of algae strains used in this study

Species	Strain	Culture medium	Temperature	Origin
Alexandrium minutum	AL1V	K	15 °C	Ria de Vigo, Spain, 1987, S. Fraga
Alexandrium minutum	AMADO6	K	15 °C	Australia, South Australia, Hallegraeff
Alexandrium minutum	AMITA	K	15 °C	Adriatic, Mediteranean Sea
Alexandrium minutum	AMP4	K	15 °C	Mediterranean Sea, Spain, Santiago Fraga
Alexandrium minutum	AL1T	K	15 °C	Gulf of Trieste, Italy, A. Beran
Alexandrium minutum	AL3T	K	15 °C	Gulf of Trieste, Italy, A. Beran
Alexandrium minutum	AL5T	K	15 °C	Gulf of Trieste, Italy, A. Beran
Alexandrium minutum	AL8T	K	15 °C	Gulf of Trieste, Italy, A. Beran
Alexandrium minutum	AL9T	K	15 °C	Gulf of Trieste, Italy, A. Beran
Alexandrium minutum	Nantes	K	15 °C	Atlantic Ocean, France
Alexandrium minutum	AL 7 V	K	15 °C	Atlantic Ocean, Spain
Alexandrium minutum	PALMIRA1	K	15 °C	Mediterranean Sea, Spain
Alexandrium minutum	AL 4V	K	15 °C	Ria de Vigo, Spain, 2000, S.Fraga
Alexandrium minutum	AL 2V	K	15 °C	Ria de Vigo, Spain, Bravo
Alexandrium insuetum	CCMP 2082	Prov	20 °C	Uchiumi Bay, Kagawa, Japan, 1985 S. Yoshimatsu
Alexandrium sp.	CS 001	K	15 °C	Scotland, M. Grieve
Alexandrium tamutum	SZNB029	K	15 °C	Gulf of Naples, Italy, M. Montresor
Alexandrium fundyense	CA 28	f2	15 °C	Woods Hole, Oceanographic Institution, D.M. Anderson
Alexandrium tamarense	SZNB 01	IMR	15 °C	Gulf of Naples, Italy 1999, M. Montresor
Alexandrium tamarense	SZNB 019	IMR	15 °C	Gulf of Naples, Italy 1999, M. Montresor
Alexandrium ostenfeldii	AOSH 1	K	15 °C	Ship Harbour, Nova Scotia, Canada, A. Cembella
Alexandrium ostenfeldii	CCMP 1773	K	15 °C	Limfjordan, Denmark, Hansen
Alexandrium catenella	BAH ME 255	IMR	15 °C	Spain, M. Delgado
Alexandrium taylorii	AY 2T	K	15 °C	Lagoon of Marano, Italy, A. Beran
Gonyaulax spinifera	CCMP409	f2	15 °C	Gulf of Maine, North America, 1986R. Lande
Protoceratium reticulatum		f2-Si	15 °C	Helgoland,North Sea, Germany, M. Hoppenrath
Lingulodinium polyedrum		IMR	15 °C	Norway, T. Castberg
Prymnesium parvum	K-0081	K	15 °C	Flade Sø, Denmark
Rhodomonas sp.	CCMP 768	K	22 °C	North Island, New Zealand, South Pacific, F. Chang

Table 2. Algal species and cell counts used for spiked samples

Species	Strain	Cell counts
Alexandrium minutum	AL3T	10000, 50000, 100000
Alexandrium ostenfeldii	CCMP 1773	50000
Alexandrium fundyense	CA 28	25000
Alexandrium tamutum	SZNB029	50000
Protoceratium reticulatum		10000
Lingulodinium polyedrum		10000
Prymnesium parvum	K-0081	590000
Rhodomonas sp.	CCMP 768	100000

Table 3. Sequences of probes for *Alexandrium minutum*

Probe	Probe sequence
A MIN C	GAA GTC AGG TTT GGA TGC
AMIN C NEXT	TAA TGA CCA CAA CCC TTC C
positive control (target sequence)	GCA TCC AAA CCT GAC TTC GGA AGG GTT GTG GTC ATT A

Table 4. Specificity of probes for *Alexandrium minutum*

Species	Strain	Signal	Average value OD 350 µg/µL
Alexandrium minutum	AL1V	+	2.3476
Alexandrium minutum	AMADO6	+	2.8662
Alexandrium minutum	AMITA	+	4.9956
Alexandrium minutum	AMP4	+	4.6426
Alexandrium minutum	AL1T	+	5.1715
Alexandrium minutum	AL3T	+	3.2775
Alexandrium minutum	AL5T	+	2.2989
Alexandrium minutum	AL8T	+	3.4521
Alexandrium minutum	AL9T	+	1.9837
Alexandrium minutum	Nantes	+	2.1611
Alexandrium minutum	AL 7 V	+	2.8885
Alexandrium minutum	PALMIRA1	+	1.8488
Alexandrium minutum	AL 4V	+	1.5897
Alexandrium minutum	AL 2V	+	4.6268
Alexandrium insuetum	CCMP 2082	-	0.0304
Alexandrium sp.	CS 001	-	0.0075
Alexandrium tamutum	SZNB029	-	0.0971
Alexandrium fundyense	CA 28	-	0.0000
Alexandrium tamarense	SZNB 01	-	0.0000
Alexandrium tamarense	SZNB 019	-	0.0351
Alexandrium ostenfeldii	AOSH 1	-	0.1215
Alexandrium ostenfeldii	CCMP 1773	-	0.0201
Alexandrium catenella	BAH ME 255	-	0.1701
Alexandrium taylorii	AY 2T	-	0.0161
Gonyaulax spinifera	CCMP409	-	0.0188

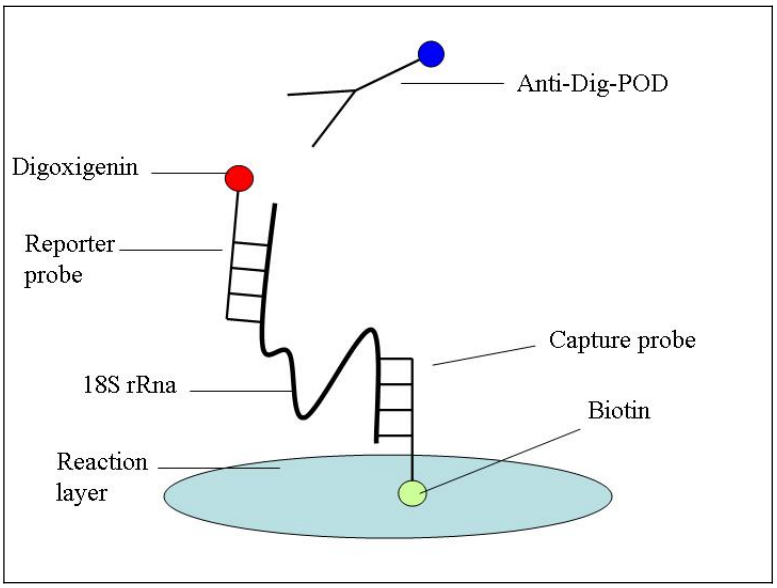

Figure 1. Sandwich hybridization

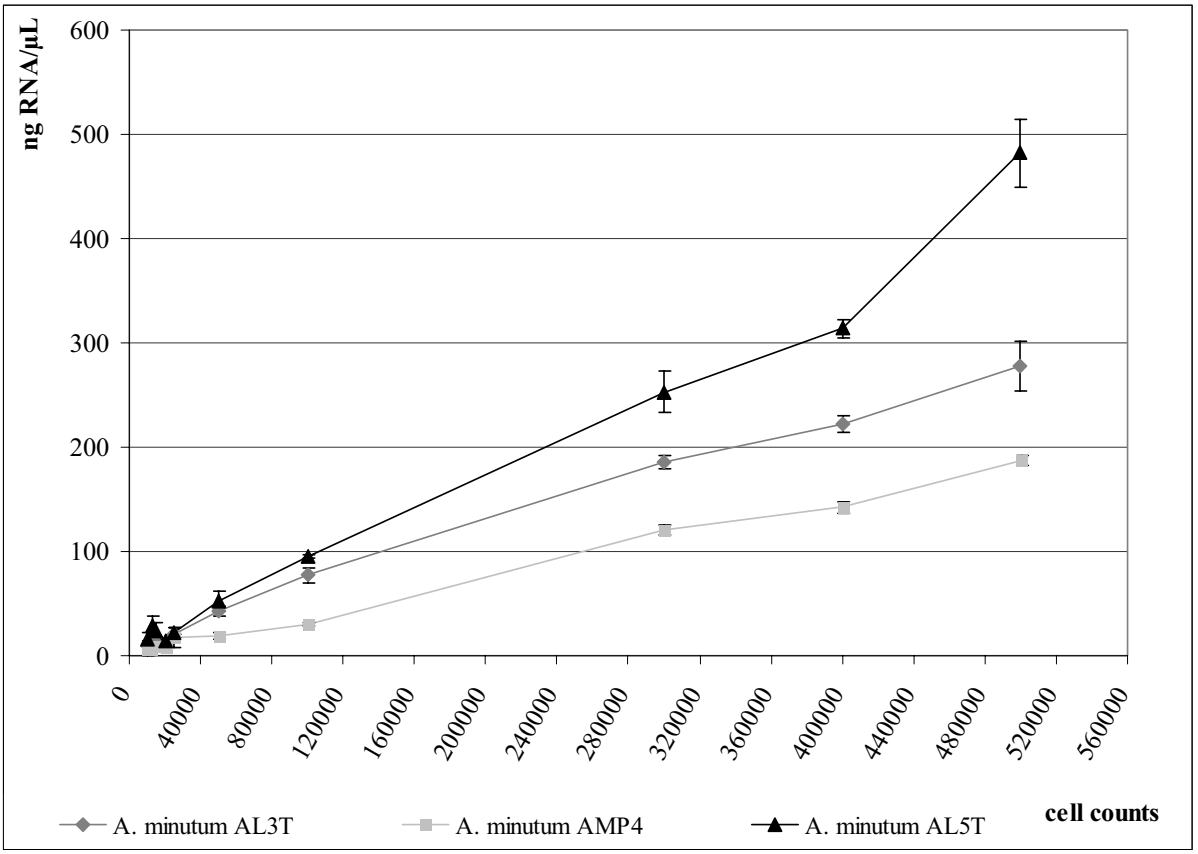

Figure 2. Total RNA concentration for three *Alexandrium minutum* strains in ng/µL at different cell counts

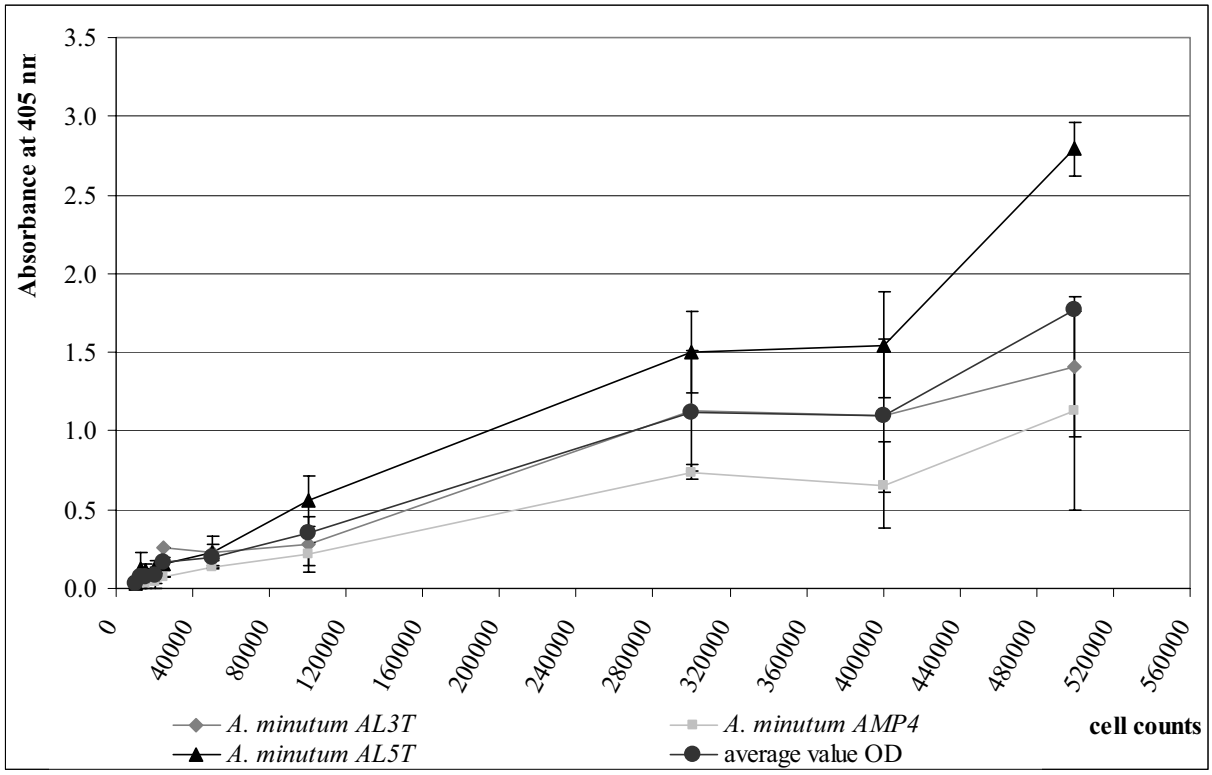

Figure 3. Photometer readings for cell numbers of three different *Alexandrium minutum* strains

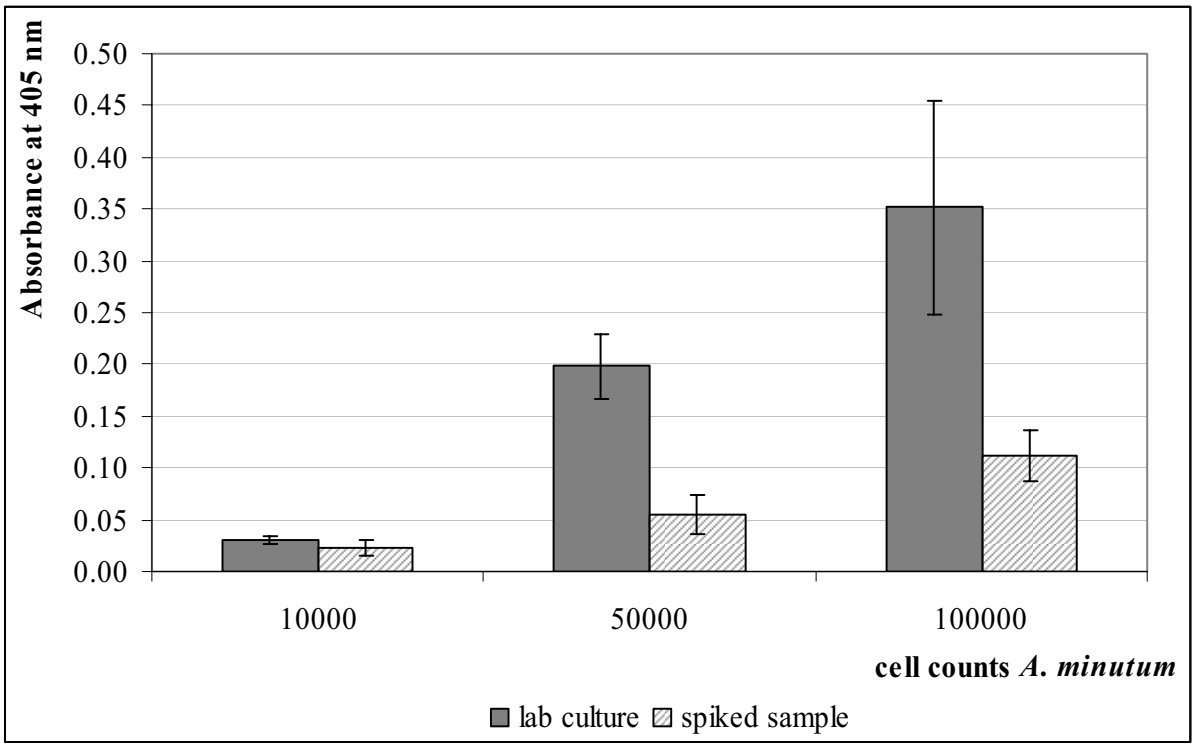

Figure 4. Comparison of photometer readings at 405 nm for a lab culture of *A. minutum* and spiked samples with *A. minutum*

2.4 Publication II

MOLECULAR PROBES FOR THE DETECTION OF TOXIC ALGAE FOR USE IN SANDWICH HYBRIDIZATION FORMATS

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Abstract

Molecular probes can be used for early and rapid detection of toxic algae species. The sandwich hybridization requires two probes for each species, a capture probe and a nearly adjacent signal probe. Probe sets for the species-specific identification of the toxic algal species *Gymnodinium catenatum*, *Protoceratium reticulatum, Lingulodinium polyedrum, Prymnesium parvum, Chrysochromulina polylepis*, *Pseudo-nitzschia multiseries, P. australis, P. seriata and P. pungens* were designed. A genus probe set for *Pseudo-nitzschia* species was adapted and all probe sets were tested for specificity. The target molecules for the probe sets are the large and the small subunit ribosomal RNAs. The specificity of the different probes sets was tested using a sandwich hybridization in a microtiter plate assay with ribosomal RNA isolated from laboratory strains of the target species and closely related species. The assay showed the eight probe sets to be highly specific. Detection of one other species, in addition to the target species, was observed for two of the probe sets. These ten probe sets are valuable tools for identifying and monitoring different toxic algae. The microtiter plate assay is a cheap and effective means of testing probe specificity.

Introduction

Harmful algae can produce powerful toxins that cause fish kills and shellfish poisoning. Early and rapid detection of toxic algal species in coastal areas and aquaculture is the most effective way to mitigate their negative effects on human populations. A variety of detection techniques using molecular probes, such as fluorescence *in situ* hybridization (FISH) (Scholin et al. 1996; Simon et al. 2000; Smit et al. 2004; Kim and Sako 2005), DNA microarrays (Metfies and Medlin 2005a; Metfies and Medlin 2005b) and sandwich hybridization assays (SHA) (Scholin et al. 1996; Ayers et al. 2005; Metfies et al. 2005) can be applied for this purpose. Usually, targets for the molecular probes are the small and the large subunit ribosomal RNA (rRNA) genes because they can be found in high numbers in the cell and contain more or less conserved regions (Groben et al. 2004). The relative conservation of the 18S and 28S gene can complicate the search for suitable probes at the species level (Gagnon et al. 1996; Ki and Han 2006). Specific probes for several algal taxa have been developed recently (Scholin et al. 1999; Tyrrell et al. 2002; John et al. 2003; Kim and Sako 2005; Metfies et al. 2005; Töbe et al. 2006), however, still only a small percentage of all toxic algal species is covered. For sandwich hybridization formats (Zammatteo et al. 1995; Rautio et al. 2003) two probes are needed, and at least one of the probes has to be specific for the target. One of the probes, the capture probe can be immobilized on solid surfaces as in combination with DNA biosensors (Metfies et al. 2005) or in the well of a microtiter plate (see Publication I) and bind to target RNA or DNA. A second probe, the detection probe, carries the signal moiety and binds near the binding site of the capture probe. Here, we present the results of the application of 10 probe sets for the detection of the different toxic algal species *Gymnodinium catenatum*, *Protoceratium reticulatum, Lingulodinium polyedrum, Prymnesium parvum, Chrysochromulina polylepis*, *Pseudo-nitzschia multiseries, P. australis, P. seriata and P. pungens* and for all species of the genus *Pseudo-nitzschia.* The probes were tested for specificity applying the sandwich hybridization in a microtiter plate well described in Publication I with laboratory strains.

Materials and Methods

Culture conditions - All algal strains were cultured under sterile conditions in seawater-based media K (Keller et al. 1987), IMR (Eppley et al. 1967), Drebes (Stosch and Drebes 1964), Prov. (Provasoli et al. 1957; Guillard and Ryther 1962; Guillard 1975), L1 (Guillard and

Ryther 1962; Guillard 1975; Guillard and Hargraves 1993), DY IV (Andersen et al. 1997), f2-Si (Guillard and Ryther 1962; Guillard 1975) and GP%50 (Loeblich and Smith 1968) at different temperatures and approximately 100 µEinstein with a light: dark cycle of 14:10 hours (Table 1).

RNA-extraction - Isolation of total rRNA from *Pseudo-nitzschia* strains was carried out using the protocol for the Tri Reagent kit (Sigma, Taufkirchen, Germany). Glass beads (212-300 µm, Sigma, Germany) were also added to the isolation solution to break open the cells with a bead Mini-Beadbeater (Biospec products, Biospec products Inc, Bartlesville, USA) for 20 seconds. Subsequent to the cell lysis steps, the Clean up protocol from Qiagen (Hilden, Germany) was used for RNA purification. Total RNA from all other algal cultures was isolated according to a modified protocol from the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Modifications of this protocol were done to enhance the quality and quantity of the extracted rRNA by improved removal of polysaccharides and proteins. For quality enhancement, the centrifugation step of two minutes for separation of supernatant and cell debris was extended to 15 minutes. Buffer RW1 was applied two times to the RNeasy column, incubated for one minute and then centrifuged. The first wash step with buffer RPE was repeated. RNA concentration was measured with a Nanodrop Spectrophotometer (Peqlab, Erlangen, Germany).

Probes and probe synthesis - Specific FISH probes for several species have been previously developed (Table 2) and were used in this study. A second probe in close proximity to the first probe was developed for these probes for the sandwich hybridization. The previously developed probes for the Genus *Pseudo-nitzschia* were both used in combination; PNFRAGA was adapted as a capture probe and PNEXDELIB as a detection probe. All probes and positive controls (Test DNA) were synthesized from Thermo Electron Corporation (Ulm, Germany).

Sandwich Hybridization using a microtiter plate assay (MTPA) - The probe sets for the detection of the different algae were tested for specificity using a sandwich hybridization in a microtiter plate assay as described in Publication I. In this assay the capture probe is biotinylated and the signal probe is digoxigenin-labelled. Prior to the experiments, the different buffer solutions from the PCR ELISA Dig Detection Kit from Roche Diagnostics (Mannheim, Germany) were prepared for use. Total rRNA from the different algae was

fragmented in a fragmentation buffer (40mM Tris, pH 8.0/100mM KOAc/30mM MgOAc) for 5 minutes at 94°C and then chilled on ice prior to hybridization. Biotinylated probes and digoxigenin labeled probes at a concentration of 10 µM and different concentrations of rRNA were added to the hybridization buffer. A negative control was prepared containing only probes and hybridization buffer, whereas the positive control contained also Test DNA (synthesized target sequence of both probes). The different hybridization solutions were added into the wells of the microtiter plate and incubated on a shaker for 1 hour at 46°C. Subsequently to the hybridization, the wells of the microtiter plate were washed with washing solution. Antibody solution was applied into each well and incubated 30 minutes at 37°C with agitation in the dark. After incubation with the antibody solution, the wells were re-washed with washing solution and substrate solution was filled into the wells and incubated in the dark on a shaker at 37 °C for 30 minutes. The anti-digoxigenin antibody conjugated to horseradish peroxidase reacts with substrate to produce a green colorimetric product. The wells of the microtiter plate were read out at 405 nm using a quartz cuvette with a Varian Cary 4000 UV-Vis Spectrometer (Varian Inc., Darmstadt, Germany).

Results

Probe design - Probes were developed for *Gymnodinium catenatum,* four *Pseudo-nitzschia* species, *Chrysochromulina polylepis*, *Prymnesium parvum/patelliferum*, *Lingulodinium polyedrum* and *Protoceratium reticulatum* (Table 3) using the probe design option in ARB software package (Ludwig et al. 2004). Additionally a BLAST search (Altschul et al. 1990) was conducted to test the overall specificity of the probes against all publically available sequences. It was possible to design two specific probes that are located in sufficient proximity for the sandwich-hybridization approach for *G. catenatum*, *L. polyedrum* and *P. reticulatum.* However, it was not possible to design two specific probes for *Pseudo-nitzschia australis*, *Pseudo-nitzschia pungens*, *Chrysochromulina polylepis* and *Prymnesium parvum/patelliferum.* Therefore an unspecific signal probe for these target species was chosen that bind in close proximity to the previously developed specific capture probe. Thus, the specificity of the reaction was determined by the capture probe. The close proximity of the capture probe and the detection probe minimizes possible degradation effects of the target nucleic acid. Positive controls are the synthesized target sequences of each respective probe set.

Specificity of probes - The specificity of 10 probe sets for toxic algae was tested using a sandwich hybridization assay in a microtiter plate as described in Publication I with closely related species (Tables 4 and 5). Total rRNA was isolated from the target species and more distantly related species for each probe set. The signals obtained were normalised to a target concentration of 350 ng RNA and compared. A probe set for the toxic algal species *Chrysochromulina polylepsis* was tested with two strains of *C. polylepsis* and three closely related species (Table 4). It showed specific signals only for the target species. The target species *Gymnodinium catenatum* and the non-target species *G. impudicum* showed both positive signals for the GCAT probe set, whereas other non-target species showed no signal at all (Table 4). However, *G. impudicum* also showed a signal, but the signal for *G. catenatum* was threefold higher than the one of *G. impudicum*. *Lingulodinium polyedrum* gave a signal for the LPOLY probe set and all non-target species did not (Table 4). The PRETI probe set showed specific signals for *Protoceratium reticulatum* (Table 4). Signals for all *Prymnesium* species were achieved with the capture probe PRYM 694 and the detection probe PRYM 694 NEXT. The Genus *Pseudo-nitzschia* probe set was tested for specificity with all available *Pseudo-nitzschia* species (Table 5), except *P. multiseries* strain Oroe13 and *P. seriata* strain CCMP 1309, which were not available. *P. pseudodelicatissima* strains AL-93 and SAL-5 were not detected with the *Pseudo-nitzschia* genus level probes. The species probe sets PSN AUS, PSN MULTI, PSN PUNG and PSN SERI were tested with their respective *Pseudo-nitzschia* target species and with representative strains of the other *Pseudo-nitzschia* species. Signals of all probe sets were only observed for the target species (Table 5).

Discussion

Probes sets for 10 toxic algal species were developed and tested for specificity using a microtiter plate assay and a sandwich hybridization. Single probes for some species had already been developed and tested for specificity with dot blot and FISH. Thus, only a second probe was needed for these species to complete the sandwich hybridization. The combination of both probes needed to be tested for specificity. Our capture probe for *Chrysochromulina polylepsis* was developed for FISH and tested for specificity by Simon et al. (1997). The detection probe for *C. polylepsis* is unspecific; however, in combination with the specific capture probe only *C. polylepsis* is detected. Although this probe set was only tested with few species, its specificity should be confirmed with further tests e.g., spiked samples. High detection signals were observed with the probe set GCAT and the target *Gymnodinium*

catenatum. A threefold lower signal was determined for the non toxic *G. impudicum*, a worldwide occurring species, that can form massive red tide blooms (Fraga et al. 1995). A high signal for *G. catenatum* presents only 10,000 cells, whereas for the same signal intensity at least 250,000 cells of *G. impudicum* are needed, thus a misinterpretation of signal is unlikely. The probe set for *Prymnesium parvum* detected all tested *Prymnesium* species including *P. nemamethecum*, which is a non-toxic species occurring in marine waters (Pienaar and Birkhead 1994). The majority of *Prymnesium parvum* blooms have been recorded in brackish waters (Edvardsen and Paasche 1998) and there have not been any reports of blooms caused by *P. nemamethecum* (West et al. 2006). In water samples from brackish water, the detection of *P. nemamethecum* cannot be ruled out but seems unlikely. Some false-positive results are almost impossible to avoid with a monostrigent hybridization approach, because the stability of mismatched probe-target hybrids cannot easily be predicted *in silico* (Loy et al. 2005b).

The specificity tests using species of the genus *Pseudo-nitzschia* and probes for the different species turned out to be difficult because of the difficulty in maintaining cultures long term under laboratory conditions. Consequently only a few representative strains of each species could be examined. The *Pseudo-nitzschia* genus probes were tested with all available strains and were observed to detect only one of the three *P. pseudodelicatissima* strains. However, the 18S gene of the three strains was sequenced and sequences of all *Pseudo-nitzschia* strains were compared to the probe sequences. The sequences of strains AL-93 and SAL-5 revealed two mismatches to the capture probe sequence, whereas no mismatch was found in strain AL-19. The sequence of AL-19 was identical to that of other *P. delicatissima*, thus, this strain was determined to be *P. delicatissima* rather than *P. pseudodelicatissima*. Hence, our *Pseudo-nitzschia* genus probes are not able to detect *P. pseudodelicatissima* and the absence of a signal can be used as a determinate marker for *P. pseudodelicatissima,* which can be difficult to separate from *P. delicatissima* at the light microscopic level. The search for suitable probes can be difficult for some recently evolved species because of the relative conservation of the 18S gene (Gagnon et al. 1996; Ki and Han 2006), therefore a new probe set should be developed for the detection of *P. pseudodelicatissima*. Signals of all other probe sets were observed only for the target species. Even when probes are designed from a large database, a frequent revision of probe sequences is necessary because new sequences are added almost daily to databases.

Conclusion

Ten probe sets for different toxic algal species were designed and eight probe sets proved to be highly specific in our sandwich hybridization assay. Two probe sets, GCAT and PRYM 694, detect another species in addition to its target species. All designed probe sets can be applied for the monitoring of toxic algae using solid surface, such as biosensors and the microtiter plate assay. The microtiter plate assay is a fast and efficient way to test probes for use in sandwich hybridization much in the same way that dot blots are used to screen for specificity for FISH probes.

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Table 1. Culture conditions and geographical origin of strains

Species	Strain	Culture-medium	Tempera-ture	Origin
Pseudo-nitzschia australis	PS 195 V	K	15 °C	Ría de Vigo, Baiona, Spain, 05/05/2005, S. Fraga
Pseudo-nitzschia australis	PS 193 V	K	15 °C	Ría de Vigo, Baiona, Spain, 05/05/2005, S. Fraga
Pseudo-nitzschia australis	PS 191 V	K	15 °C	Ría de Vigo, (E14B), Spain, 04/05/2005, S. Fraga
Pseudo-nitzschia calliantha	CL 187	K	15 °C	S. Bates
Pseudo-nitzschia calliantha	CL 190	K	15 °C	S. Bates
Pseudo-nitzschia multiseries	CL 174	K	15 °C	Cardigan River, USA, S. Bates
Pseudo-nitzschia multiseries	CL 195	K	15 °C	Deadman's Harbour, Bay of Fundy, USA, S. Bates
Pseudo-nitzschia delicatissima	AL-23	Drebes	15 °C	Naples, Italy, 2004, A. Amato
Pseudo-nitzschia delicatissima	AL-63	Drebes	15 °C	Naples, Italy, 2004, A. Amato
Pseudo-nitzschia delicatissima	Al-86	Drebes	15 °C	Naples, Italy, 2004, A. Amato
Pseudo-nitzschia fraudulenta	AL-104	Drebes	15 °C	Naples, Italy, 2005, A. Amato
Pseudo-nitzschia delicatissima	AL-18	Drebes	15 °C	Naples, Italy, 2004, A. Amato
Pseudo-nitzschia delicatissima	AL-47	Drebes	15 °C	Naples, Italy, 2004, A. Amato
Pseudo-nitzschia pseudodelicatissima	AL-93	Drebes	15 °C	Naples, Italy, 2004, A. Amato
Pseudo-nitzschia pseudodelicatissima	SAL-5	Drebes	15 °C	Naples, Italy, 2004, A. Amato
Pseudo-nitzschia pseudodelicatissima	Al-19	Drebes	15 °C	Naples, Italy, 2004, A. Amato
Pseudo-nitzschia pungens	238	K	15 °C	S. Kühn, K. Evans
Pseudo-nitzschia pungens	Oroe 5	K	15 °C	Bot. Inst. Uni Kopenhagen, Denmark, N. Lundholm,
Pseudo-nitzschia pungens	Thisted 37 19/8-97	K	15 °C	Bot. Inst. Uni Kopenhagen, Denmark, N. Lundholm,
Pseudo-nitzschia pungens		K	15 °C	Sylt, Germany
Pseudo-nitzschia pungens	708	K	15 °C	Bristol, UK, K. Evans
Pseudo-nitzschia spec		K	0 °C	Resolute Passage, Barrow Strait, Northwest Territories, Canada, R. Smith
Pseudo-nitzschia seriata	CCMP 1309	K	0 °C	Resolute Passage, Barrow Strait, Northwest Territories, Canada, R. Smith
Chrysochromulina ericina	CCMP 281	K	15 °C	North Pacific
Chrysochromulina kappa	CCMP 288	K	20 °C	Bigelow Laboratory dock, West Boothbay Harbor, Maine USA, M. Keller
Chrysochromulina polylepis	B11	IMR	15 °C	Norway, B. Edvardsen
Chrysochromulina polylepis	B1511	IMR	15 °C	Norway, B. Edvardsen
Phaeocystis globosa		K+soil	15 °C	A. Dauelsberg
Gymnodinium nagasakiense (K. mikimotoi)	PLY 561	IMR+soil	15 °C	not known
Gymnodinium fuscum	CCMP 1677	DY IV	15 °C	Pond, LaTrobe University, Melbourne, Victoria, Australia, D. Hill
Gymnodinium simplex	CCMP 418	K	15 °C	Plymouth, England, United Kingdom
Gymnodinium catenatum	GC 12V	Dreb/IMR	20 °C	Ría de Vigo, Baiona, Spain, S. Fraga
Gymnodinium impudicium	CCMP 2214	K	20 °C	Valencia, North Atlantic, Spain, I. Bravo
Karenia papilionaceae	CAWD 91	GP%50	15 °C	Hawkes Bay, New Zealand, A. Haywood
Lingulodinium polyedrum	Norway	IMR	15 °C	Norway, T. Castberg
Ceratocorys horrida	CCMP 157	L1	22-26 °C	Banda, Banda Sea, South Pacific, South East Asia, B. Sweeney
Ceratium longipes	CCMP 1770	K	15 °C	Bigelow Laboratory dock, West Boothbay Harbor, Maine, USA, S. L. Morton

Thecadinium inclinatum	CCMP 1890	K	15 °C	Boundary Bay, British Columbia, Canada, E. Simons
Gonyaulax spinifera	CCMP 409	f2-Si, L1	15 °C	Gulf of Maine, North America, 1986, R. Lande
Protoceratium reticulatum	Sylt	f2-Si	15 °C	Helgoland,North Sea, Germany, M. Hoppenrath
Alexandrium minutum	AMP4	K	15 °C	Mediterranean Sea, Spain, Santiago Fraga
Alexandrium minutum	AL3T	K	15 °C	Gulf of Trieste, Italy, A. Beran
Emiliana huxleyi	CCMP 1516	f2	20 °C	South Pacific, L. Polans
Prymnesium parvum	K-0081	K	15 °C	Flade So, Denmark
Prymnesium parvum f. parvum	RL10parv93	IMR	15 °C	Bergen, Norway, A. Larsen
Prymnesium parvum f. patelliferum	K-0252	IMR	15 °C	Wilson Promontory, Norman Bay, Victoria Australia, SCCAP
Prymnesium nemamethecum		K	15 °C	South Africa St James False Bay, South Africa
Prymnesium patelliferum	K-0374	K	15 °C	Norway
Prymnesium patelliferum	K-0082	K	15 °C	Brackish; Thornham, Hunstanton, Norfolk, England, T. Christensen
Fragilariopsis cylindrus		K	0 °C	A. Krell

Table 2. Sequences of capture and detection probes

Probe name	Used as the	Probe sequence	Target	Citation
PNEXDELIB	Detection probe	GCG CAA TCA CTC AAA GAG	Genus *Pseudo-nitzschia* 18S	Eller & Medlin, unpublished
PNFRAGA	Capture probe	ATT CCA CCC AAA CAT GGC	Genus *Pseudo-nitzschia* 18S	Eller, Töbe & Medlin, unpublished
PSNAUS A-8	Capture probe	AAC GTC GTT CCG CCA AT	*Pseudo-nitzschia australis* 18S	Eller & Medlin, unpublished
PSNPUNG A-12	Capture probe	GGG CAC CCT CAG TAC GAC	*Pseudo-nitzschia pungens* 18S	Eller, Töbe & Medlin, unpublished
CPOLY01	Capture probe	GAC TAT AGT TTC CCA TAA GGT	*Chrysochromulina polylepis* 18S	(Simon et al. 1997)
PRYM694	Capture probe	CAG CCG ACG CCG AGC GCG	*Prymensium parvum* 28S	(Töbe et al. 2006)

Table 3. Sequences of the probes and positive control 5’ to 3’

Probe name	Probe sequence 5'-3'	Target	Positive control
Capture probe: GCAT FNEXT	TTT TTA AAA GAT TAC CCA ATC	*Gymnodonium catenatum* 18S	TAC GAC CTT GTC CGA CAG GAT TGG GTA ATC TTT TAA AAA
Signal probe: GCAT F	CTG TCG GAC AAG GTC GTA	*Gymnodonium catenatum* 18S	
Capture probe PSNAUS			ATT GGC GGA ACG ACG TTA CGT CTC CGT CAG CAC CTT G
Signal probe: PSNAUS ANEXT	CAA GGT GCT GAC GGA GAC GT	*Pseudo-nitzschia australis* 18S	
Capture probe: Psnmult A-17	GCA TGC GAT CCG CAA TTT	*Pseudo-nitzschia multiseries* 18S	AAA TTG CGG ATC GCA TGC CCT TTT GGC GGC GAT GGA
Signal probe: Psnmult A+14	TCC ATC GCC GCC AAA AGG	*Pseudo-nitzschia multiseries* 18S	
Capture Probe PSNPUNG			GTC GTA CTG AGG GTG CCC TTG CGC TGT ACT GGT CTG
Signal probe: PSNPUNG ANEXT	CAG ACC AGT ACA GCG CAA	*Pseudo-nitzschia pungens* 18S	
Capture probe: PSN SERI E	GAC AGG TTC TCG TGG TCA GAT TC	*Pseudo-nitzschia seriata* 18S	GAA TCT GAC CAC GAG AAC CTG TC TTG TGG TTG GTT TCC TTT ATT
Signal probe: PSN SERI E NEXT	AAT AAA GGA AAC CAA CCA CAA	*Pseudo-nitzschia seriata* 18S	
Capture Probe C poly			ACC TTA TGG GAA ACT ATA GTC CGG AAG TCC TTT TTG ACT CC
Signal probe: CPOLY01 NEXT	GGA GTC AAA AAG GAC TTC CG	*Chrysochromulina polylepsis* 18S	
Capture Probe Prym694			CGC GCT CGG CGT CGG CTG GAG CCT GGA TGG ATG GCG
Signal probe: PRYM694NEXT	CGC CAT CCA ACC AGG CTC	*Prymnesium parvum/patelliferum* 28S	
Capture probe: LPOLY J	GGC CAT CTA AAG CAG AAG	*Lingulodinium polyedrum* 18S	CTT CTG CTT TAG ATG GCC ATC TGG CTT GTC TTG GGC
Signal probe: LPOLY C	GCC CAA GAC AAG CCA GAT	*Lingulodinium polyedrum* 18S	
Capture probe: PRETI K NEXT	TGT AAC TAA TAA AAA CAG CCCT	*Protoceratium reticulatum* 18S	TTC TTG GCC CGA CTT TCG CGGA AGG GCT GTT TTT ATT AGT TACA
Signal probe: PRETI K	TCC GCG AAA GTC GGG CCA AGAA	*Protoceratium reticulatum* 18S	

Table 4. Specificity of probes for *Chrysochromulina polylepsis*, *Gymnodinium catenatum*, *Lingulodinium polyedrum*, *Protoceratium reticulatum* and *Prymnesium parvum*

CPOLY			**GCAT**		
Species	**Strain**	**Signal**	**Species**	**Strain**	**Signal**
Chrysochromulina ericina	CCMP 281	-	*Gymnodinium nagasakiense*	PLY 561	-
Chrysochromulina kappa	CCMP 288	-	*Gymnodinium fuscum*	CCMP 1677	-
Chrysochromulina polylepis	B15	+	*Gymnodinium simplex*	CCMP 418	-
Chrysochromulina polylepis	B1511	+	*Gymnodinium catenatum*	GC 12V	+
Phaeocystis globosa		-	*Gymnodinium impudicium*	CCMP 2214	+
			Karenia papilionaceae	CAWD 91	-
LPOLY			**PRETI**		
Species	**Strain**	**Signal**	**Species**	**Strain**	**Signal**
Lingulodinium polyedrum	Norway	+	*Lingulodinium polyedrum*	Norway	-
Ceratocorys horrida	CCMP 157	-	*Ceratocorys horrida*	CCMP 157	-
Ceratium longipes	CCMP 1770	-	*Ceratium longipes*	CCMP 1770	-
Thecadinium inclinatum	CCMP 1890	-	*Thecadinium inclinatum*	CCMP 1890	-
Gonyaulax spinifera	CCMP 409	-	*Gonyaulax spinifera*	CCMP 409	-
Protoceratium reticulatum	Sylt	-	*Protoceratium reticulatum*	Sylt	+
Alexandrium minutum	AMP4	-	*Alexandrium minutum*	AL3T	-
PRYM 694					
Species	**Strain**	**Signal**			
Chrysochromulina polylepis	B1511	-			
Phaeocystis globosa		-			
Emiliana huxleyi		-			
Prymnesium parvum	K-0081	+			
Prymnesium parvum f. parvum	RL10parv93	+			
Prymnesium parvum f. patelliferum	K-0252	+			
Prymnesium nemamethecum		+			
Prymnesium patelliferum	K374	+			
Prymnesium patelliferum	K-0082	+			

Table 5. Specificity of probes for the Genus *Pseudo-nitzschia*, *P. australis*, *P. multiseries*, *P. pungens* and *P. seriata*

Genus Pseudo-nitzschia			**PSN MULTI**		
Species	**Strain**	**Signal**	**Species**	**Strain**	**Signal**
P. australis	PS 195 V	+	*P. australis*	PS 195 V	-
P. australis	PS 193 V	+	*P. calliantha*	CL 187	-
P. australis	PS 191 V	+	*P. calliantha*	CL 190	-
P. calliantha	CL 187	+	*P. multiseries*	CL 174	+
P. calliantha	CL 190	+	*P. multiseries*	Oroe 13	+
P. multiseries	CL 174	+	*P. delicatissima*	AL-23	-
P. delicatissima	AL-23	+	*P. delicatissima*	Al-86	-
P. delicatissima	AL-63	+	*P. pseudodelicatissima*	AL-93	-
P. delicatissima	Al-86	+	*P. pseudodelicatissima*	SAL-5	-
P. fraudulenta	AL-104	+	*P. pseudodelicatissima*	Al-19	-
P. delicatissima	AL-18	+	*P. pungens*	238	-
P. delicatissima	AL-47	+	*P. pungens*	Oroe 5	-
P. pseudodelicatissima	AL-93	-	*F. cylindrus*		-
P. pseudodelicatissima	SAL-5	-			
*P. pseudodelicatissima**	Al-19	+			
P. pungens	238	+			
P. pungens	Oroe 5	+			
P. pungens	Thisted 37	+			
P. pungens		+			
P. pungens	708	+			
F. cylindrus		-			
PSN AUS			**PSN PUNG**		
Species	**Strain**	**Signal**	**Species**	**Strain**	**Signal**
P. australis	PS 195 V	+	*P. calliantha*	CL 187	-
P. australis	PS 193 V	+	*P. calliantha*	CL 190	-
P. australis	PS 191 V	+	*P. multiseries*	CL 174	-
P. calliantha	CL 187	-	*P. delicatissima*	AL-23	-
P. calliantha	CL 190	-	*P. delicatissima*	Al-86	-
P. multiseries	CL 174	-	*P. fraudulenta*	AL-104	-
P. delicatissima	AL-63	-	*P. pseudodelicatissima*	AL-93	-
P. delicatissima	AL-18	-	*P. pseudodelicatissima*	Al-19	-
P. pseudodelicatissima	AL-93	-	*P. pungens*	238	+
P. pseudodelicatissima	Al-19	-	*P. pungens*	Oroe 5	+
P. pungens	238	-	*P. pungens*		+
P. pungens	Oroe 5	-	*P. pungens*	708	+
F. cylindrus		-			
PSN SERI					
Species	**Strain**	**Signal**			
P. calliantha	CL 187	-			
P. multiseries	CL 174	-			
P. delicatissima	Al-86	-			
P. delicatissima	AL-47	-			
P. pseudodelicatissima	AL-93	-			
P. pseudodelicatissima	SAL-5	-			
P. pungens	Oroe 5	-			
P. pungens		-			
P. seriata	CCMP 1309	+			
F. cylindrus		-			

* likely misidentified should be *P. delicatissima* based on sequence identity

2.5 Publication III

ELECTROCHEMICAL DETECTION OF TOXIC ALGAE WITH A BIOSENSOR

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Manual and Guides: Microscopic and molecular methods for quantitative phytoplankton analysis, submitted

Introduction

DNA-biosensors are known from various areas. Glucose detection was one of the first application areas developed for biosensors (Clark 1956). Today, biosensors are used in many different areas, such as for the identification of infectious organisms (Hartley and Baeumner 2003) and hazardous chemicals, for monitoring of health relevant metabolites or environmental samples. A new detection method used for the identification of harmful algae was developed using a hand held device (Figure 1A) and biosensors. A first prototype was used to identify the toxic dinoflagellate *Alexandrium ostenfeldii* (Metfies et al. 2005). A second prototype manufactured by PalmSens (Houten, Netherlands) was extensively used to improve the biosensors (Figure 1B). Biosensors can be produced very cheaply for mass production.

Molecular probes - Identification of toxic algae is based on oligonucleotide probes that specifically target ribosomal RNA. Targets for the probes are the small and large subunit rRNA genes in the ribosomes of the cells, whose conserved and variable regions make it possible to develop probes specific for different taxonomic levels (Groben et al. 2004). For the probe development, the ARB software package is used (Ludwig et al. 2004). Theoretical probe specificity is dependent on the number of sequences of the targeted gene available in the databases. If molecular probes are designed from only a few sequences, there is a danger of cross-hybridization to non-targeted species and organisms whose sequences are unknown and not in the database. Prior to the analysis of field-samples, molecular probes were tested

for specificity with cultivated target species and closely related species because *in silico* and *in situ* results can show different specificity signals.

Dipsosable sensor-chip and detection principle - The disposable sensorchip consisting of a carrier material on which is printed a working electrode, where the detection reaction takes place, a reference electrode and an auxiliary electrode (Figure 1B). The working electrode has a diameter of 1mm and is made of a carbon paste. A biotinylated probe is immobilised on the reaction layer of the working electrode via avidin. The nucleic acids are detected on the sensor chip via a sandwich-hybridization (Zammatteo et al. 1995; Rautio et al. 2003). The underlying principle of this method is that one target specific probe, the so-called capture probe, is immobilised via avidin on the surface of the working electrode. If a target nucleic acid is bound to the immobilised probe on the working electrode, the detection of the nucleic acid takes place via a hybridization to a second target specific probe, the so-called signal probe, that is coupled to digoxigenin (Figure 1C) (Metfies et al. 2005). The digoxigenin specific antibody coupled to horseradish-peroxidase is added to the sensor chip. Horseradish-peroxodase catalyses the reduction of hydrogen peroxide to water. Reduced peroxidase is regenerated by *p*-aminodiphenylamin (ADPA), which functions as a mediator. The oxidised mediator is reduced at the working electrode with a potential of −150mV (versus Ag/AgCl) (Figure 1D). An electrochemical signal can only be measured if the target nucleic acid bound to both capture and signal probes and thus present in the sample to be treated. (Metfies et al. 2005)

Materials and methods

Laboratory facilities and equipment

- Fume hood for RNA isolation
- Centrifuge
- Filter, 0.5 µm, ISOPORE™, membrane filters, Millipore, Ireland
- Frit, flask and funnel, Millipore, Ireland
- Mini-Beadbeater™, Biospec products, Biospec products Inc, USA
- Mini-Centrifuge
- Thermoshaker
- Incubator
- Vacuum pump with wash bottle
- Biosensors, Gwent Electronic Materials, Pontypool, UK
- Freezer -80°C

Chemicals and supplier - The chemicals used in this method are listed in Table 1 with their suppliers.

Harvesting of cells - The harvesting of cells can be done by centrifugation, the supernatant will be discarded, or by filtration using a filtration device and a hand pump (Figure 2). A maximum of or 1 x 10^7 cells can generally be processed with the RNeasy Plant Mini Kit. The cells can be frozen for long-term storage by flash-freezing in liquid nitrogen and an immediate transfer to –70 °C. Another possibility is the storage of cells in RNALater from Ambion (Huntingdon, UK).

*Preservation and storag*e - After collecting water samples the algae cells can be stored at room temperature over several days by using RNALater from Ambion, Huntingdon, UK for a later RNA isolation. Please read carefully the instructions for using RNALater.

RNA Isolation with the RNeasy Plant Mini Kit (QIAGEN) (modified protocol)

General handling of RNA – Ribonucleases (RNases) are very stable, active enzymes and are difficult to inactivate; even minute amounts are sufficient to destroy RNA. Use only plastic ware or glassware where you have first eliminated possible RNase contamination. Glassware

should be cleaned with a detergent, thoroughly rinsed, and oven baked at 180°C for four or more hours before use. Always wear gloves while handling reagents and RNA samples to prevent RNase contamination from the surface of the skin or from dusty laboratory equipment. Also change gloves frequently and keep tubes closed whenever possible. Keep isolated RNA on ice when aliquots are pipetted for downstream applications.

RNA-Isolation

1. Add 450 µL Buffer RLT with β-Mercaptoethanol to the cells
2. Pipet the lysate to glass beads and shredder the lysate in a bead beater two times for 20 seconds
3. Pipet the lysate directly onto a QIAshredder spin column (lilac) placed in 2 ml collection tube, and centrifuge for 15 minutes at maximum speed. Carefully transfer the supernatant of the flow-through fraction to a new microcentrifuge tube without disturbing the cell-debris pellet in the collection tube. Use only this supernatant in subsequent steps.
4. Add 0.5 volume (usually 225 µL) ethanol (96–100%) to the cleared lysate, and mix immediately by pipetting. Do not centrifuge. Continue without delay.
5. Apply sample (usually 650 µL), including any precipitate that may have formed, to an RNeasy mini column (pink) placed in a 2 ml collection tube. Close the tube gently, and centrifuge for 15 s at ≥8000 x g. Discard the flow-through.
   Reuse the collection tube in the next step.
6. Add 700 µL Buffer RW1 to the RNeasy column. Close the tube gently, and wait for ca. 45 seconds, then centrifuge for 15 s at ≥8000 x g to wash the column. Discard the flow-through and collection tube.
7. Repeat step 6
8. Transfer the RNeasy column into a new 2 ml collection tube (supplied). Pipet 500 µL Buffer RPE onto the RNeasy column. Close the tube gently, and centrifuge for 15 s at ≥8000 x g to wash the column. Discard the flow-through.
   Reuse the collection tube in step 9.
9. Repeat step 8
10. Add another 500 µL Buffer RPE to the RNeasy column. Close the tube gently, and centrifuge for 2 min at ≥8000 x g to dry the RNeasy silica-gel membrane.

11. To elute, transfer the RNeasy column to a new 1.5 ml collection tube. Pipet 30-50 µL RNase-free water directly onto the RNeasy silica-gel membrane. Close the tube gently, and centrifuge for 1 min at ≥8000 x g to elute.
12. To obtain a higher total RNA concentration, a second elution step may be performed by using the first eluate (from step 11).
13. Measure the RNA concentration

Sandwich Hybridization

A. Coating of Sensor chips

1. The sensor chips are moistened with 50 µL of carbonate buffer (pH 9.6) (Table 3, Figure 3A) and aspirated of with a vacuum pump (Figure 3B, 3C)
2. Incubation over night in a moisture chamber at 4 ºC with 2 µL NeutrAvidin (Pierce, Perbio, Germany) in carbonate buffer (Table 3). Storage of the electrodes during this period in Petri dishes with moist Whatman filters to protect the solutions from evaporation (Figure 3D)
3. Excessive NeutrAvidin is removed by washing the chips in PBS (pH 7.6) (Table 3, Figure 3E). Subsequently the chips are dried with a vacuum pump attached to a wash bottle
4. The sensors are blocked for one hour at room temperature with 20 µL 3 % casein in PBS. The casein is removed by washing with PBS
5. The NeutrAvidin coated electrodes can be stored in a fridge for at least 1 year after incubation with 2 % Trehalose in PBS (pH 7.6). The electrodes are coated with 15 µL of Trehalose solution and dried at 37 °C in an incubator. Before use the electrodes are washed with PBS (pH 7.6) to remove the Trehalose.

B. Immobilization of biotinylated DNA-probe

6. The sensor chips are coated with 2 µL of the biotinylated probe [10 pmol / µL in bead buffer (Table 3) and incubated for 30 minutes at room temperature
7. 50 µL of 1x hybridization buffer are added onto the sensors and directly aspirated of to remove excessive probe
8. In accordance to the coated electrodes can be stored in a fridge for at least 1 year after incubation with 2 % Trehalose on PBS (pH 7.6). The electrodes are coated with 15 µL of Trehalose solution and dried at 37 °C in an incubator. Prior to usage the electrodes are washed with PBS (pH 7.6) to remove the Trehalose.

C. Sandwich Hybridization of immobilized DNA probe, rRNA and dioxigenin labelled DNA probe

9. Fragmentation of rRNA by using a fragmentation buffer (200 mM Tris-Acetate, pH 8.1, 500 mM KOAc, 150 mM MgOA). 10 µL rRNA are added to 2.5 µL fragmentation buffer, heated for five minutes at 94 °C in a thermoshaker (Figure 3F) and subsequently immediately chilled on ice.
10. The hybridization preparation was made up as shown in Table 2. The positive control ensures that the probes are working and the negative control shows the detection of the used compounds without RNA during the measurement. This preparation was heated for four minutes at 94 ºC in a thermoshaker for denaturation and immediately chilled on ice.
11. 2 µL of the hybridization solution are applied onto each sensor in triplicate
12. The chips are incubated for 30 minutes at 46 °C in an incubator, cooled down at room temperature for five minutes

D. Detection

13. The sensors are washed in 1x POP buffer (pH 6.45) (Table 3) to remove excessive RNA.
14. The sensors are incubated for 30 minutes at room temperature with 1.5 µL Anti-Dig-POD [7.5 U/mL in PBST] (Table 3).
15. Sensors are separately washed in 1x POP buffer to remove excessive Anti-Dig-POD and dried with a vacuum pump
16. 20 µL of POD substrate are added onto the electrode (POD substrate contains 1.1 mg N-Phenyl 1,4-phenylenediamine hydrochloride (ADPA) solved in 110 µL ethanol, 250 µL of 100 mM H_2O_2 are added and filled up to 25 mL with 1x POP buffer)
17. The chip is plugged in the hand held device and measured (Figure 4). A summary of the used buffers is shown in Table 3
18.

Formulas for calculating results - A calibration has to be determined for each probe set to find the signal intensity (nA) for 1 ng RNA. For each target species the RNA concentration per cell has to be investigated. Subsequently the cell concentration of the target species in a water sample can be calculated from the electrochemical signals:

Let

nA (probe-signal) = total ng RNA (present in the sample)

then

Number of cells = nA (probe-signal)/ ngRNA (per cell)

Discussion

The electrochemical detection method with the hand held device and biosensors is a rapid method to detect toxic algae in a water sample. Electrodes can be produced in mass. Protocols and electrochemical readings of the handheld device are simple and easy even for a scientific layperson to use and interpret.

Our initial prototype with many manual steps, has now been defined and improved in the EU-project ALGADEC so that nearly all steps are automated with an automated flow and heating chamber for biosensors for the detection of 14 species in parallel, except the initial sampling and filtering step and RNA extraction. The present biosensor consists of a disposable sensor chip with 16 electrodes upon which a redox reaction takes place between the capture probe and the signal probe to yield a flow of electrons for an electrochemical detection that is proportional to the RNA of target captured on the chips and hence proportional to the number of cells in water column. Probes for other toxic algae (e.g. *Alexandrium minutum*, *Gymnodinium catenatum* etc.) were developed for operating with the hand held device and about 14 different toxic algae can be detected, because a negative and a positive control have to be included in the assay. The probes must be reviewed for specificity to new sequence data in defined time intervals, because the current 18S rRNA sequence database is only a small part of the biodiversity and is always upgraded. For each target species, the RNA concentration per cell has to be investigated and a calibration curve has to be developed for each probe set to determine the signal intensity for the different RNA concentrations to be able to relate this to cell numbers in the field sample.

The current detection limit of the hand held device requires a high sampling volume, which can be up to 8-10 litres if the cell counts are expected low. For the isolation of target rRNA a sufficient amount of cells is needed. The detection limit with the hand held device for *Alexandrium ostenfeldii* is ~16 ng/µL, with an average yield of 0.02 ng/cell. This equates to

ca. 800 cells or a sampling volume of 6.4 l to get a detectable amount of rRNA from 250 cells/litre (Metfies et al. 2005).

The manual isolation of RNA is currently the limiting factor of the system, because the concentration and quality needs to be high. Different users could possibly isolate different amounts of rRNA with different qualities from the same number of algae cells. This results in different signal intensities, which cannot be compared to cell counts. An automated RNA isolation, as developed during the ALGADEC-project, will overcome the quality in rRNA extraction efficiency. A validation of probe signals against total rRNA and over the growth cycle of the algae under different environmental conditions is also being conducted to verify the calibration curves to extrapolate to cells/litre.

Acknowledgements

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Table 1. Chemicals and suppliers

Chemical	Supplier
NeutrAvidin™, biotin binding protein	PIERCE, Perbio, Germany
D(+)-Trehalose, ≥ 99.5 % HPLC	Fluka BioChemika, Switzerland
Biotin-labelled probe (18 bases)	Thermo Electron
Digoxigenin-labelled probe (18 bases)	Thermo Electron
Herring-Sperm DNA	Roche
1x PBS	PIERCE, Perbio, Germany
Tween 20	Sigma-Aldrich Chemie GmbH, Germany
N-Phenyl 1,4-phenylenediamine hydrochloride C12H12N2 HCl (ADPA, N-Phenyl-1,4-benzenediamine hydrochloride, 1,1-Diphenylhydrazin-hydrochlorid) ADPA	MERCK KGaA, Germany
Anti-Digoxigenin-POD fab fragments	Roche
Hydrogen peroxide solution H2O2, 30% (w/w)	Sigma-Aldrich Chemie GmbH, Germany
Ethanol	MERCK KGaA, Germany
Sodium hydrogencarbonat NaHCO3	Riedel-de Haën®, RdH, Laborchemikalien, GmbH & CoKG, Germany
NaH2PO4 * H2O	MERCK KGaA, Germany
NaCl	Sigma-Aldrich Chemie GmbH, Germany
Casein	Sigma-Aldrich Chemie GmbH, Germany
Tris (pH 8.0)	Sigma-Aldrich Chemie GmbH, Germany
SDS	Sigma-Aldrich Chemie GmbH, Germany
BSA	Sigma-Aldrich Chemie GmbH, Germany
β-Mercaptoethanol	MERCK KGaA, Germany
RNeasy Plant Mini Kit	Qiagen, Hilden Germany
Whatman filters	Whatman, Brentford, United Kingdom
Glass beads, 212 – 300 µm, 425 – 600 µm	Sigma-Aldrich Chemie GmbH, Germany

Table 2. Hybridization preparation

Detection of the species	Negative control	Positive control
3.5 µL 4x Hybridization buffer	3.5 µL 4x Hybridization buffer	3.5 µL 4x Hybridization buffer
7.5 µL rRNA	1 µL Herring DNA (3480 ng/µL)	1 µL Herring DNA (3480 ng/µL)
1 µL Herring DNA (3480 ng/µL)	1 µL DIG marked DNA probe (1.4 pM/µL)	1 µL Test DNA (36 bases, 1.4 pM/µL)
1 µL DIG marked DNA probe (1.4 pM/µL)	8.5 µL milliQwater	1 µL DIG marked DNA probe (1.4 pM/µL)
1 µL milliQwater		7.5 µL milliQwater

Table 3. Buffers for sandwich hybridization on carbon electrodes

Buffer	Compound	Concentration
carbonate buffer (pH 9.6)	NaHCO3	50 mM
10x PBS (pH 7.4)	NaH2PO4 * H2O	0.5 M
	NaCl (pH 7.4)	1.54 M
"bead buffer"	NaCl	0.3 M
	Tris (pH 7.6)	0.1 M
4x hybridization buffer	NaCl	0.3 M
	Tris (pH 8.0)	80 mM
	SDS	0.04%
10x POP buffer (pH 6.45)	NaH2PO4 * H2O	0.5 M
	NaCl (pH 6.45)	1 M
PBS-BT (pH 7.4)	PBS	1x
	BSA	0.1 % [w/v]
	TWEEN 20 (pH 7.4)	0.05 % [v/v]

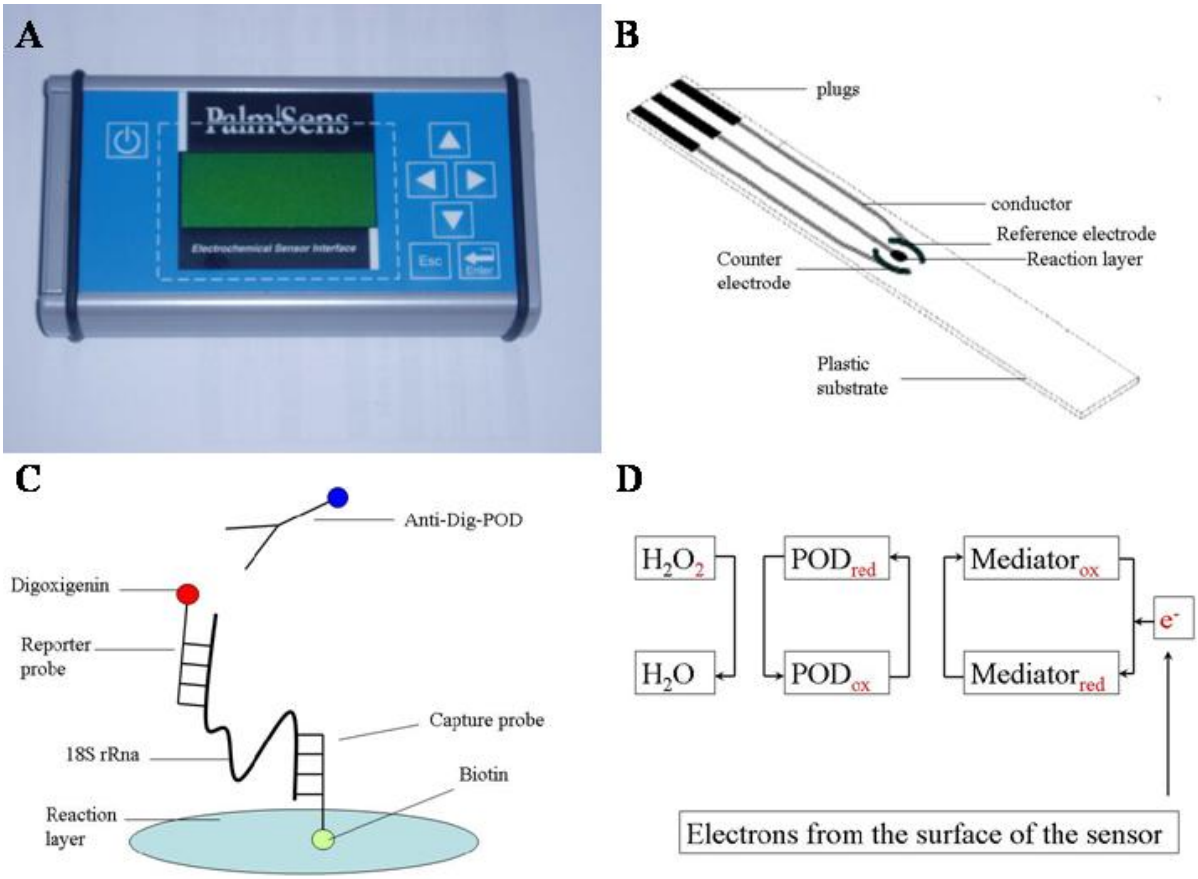

Figure 1. (A) PalmSens device, (B) Sensor chip of original prototype, (C) Sandwich hybridization, (D) Principle of redox-reaction

Figure 2. Filtration equipment and filtration device

Figure 3. (A) Applying of buffer onto the electrode, (B) Pump and washbottle, (C) Drying of chips, (D) Petri dish with Whatman filter and electrodes, (E) Washing of chips, (F) Thermoshaker

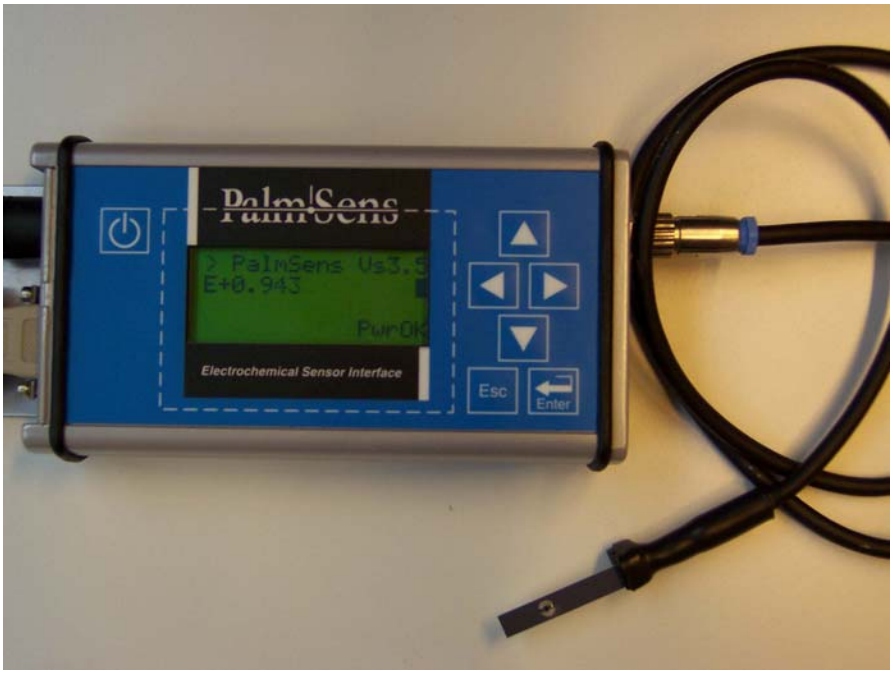

Figure 4. Measuring of chips with hand held device

2.6 Publication IV

EVALUATION OF LOCKED NUCLEIC ACIDS FOR SIGNAL ENHANCEMENT OF OLIGONUCLEOTIDE PROBES FOR MICROALGAE IMMOBILIZED ON SOLID SURFACES

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Abstract

Biosensors and microarrays are powerful tools for species detection and monitoring of microorganisms, e.g., phytoplankton. A reliable identification of microbial species with probe-based methods requires highly specific and sensitive probes. The introduction of LNA (locked nucleic acid) probe technology promises an enhancement of both specificity and sensitivity of molecular probes. In this study, we compared the specificity and sensitivity of conventional molecular probes and LNA modified probes in two different solid phase hybridization methods; sandwich hybridization on biosensors and on DNA-microarrays. In combination with the DNA-microarrays, the LNA-probes displayed an enhancement of sensitivity, but also more false-positive signals. In combination with the biosensor, the LNA probes could show neither signal enhancement nor discrimination of only one mismatch. In all examined cases, the conventional DNA probes showed equal or better results than the LNA probes. In conclusion, the LNA technology may have great potential in methods that use probes in suspension and possible in gene expressions studies, but under certain solid surface-hybridization applications they do not improve signal intensity.

Introduction

LNA (locked nucleic acids) were first presented by Wengel (Koshkin et al. 1998a; Koshkin et al. 1998b) and Imanishi (Obika et al. 1998) and their co-workers. They are a class of bicyclic RNA analogs with exceptionally high affinities and specificities toward their complementary DNA and RNA target molecules (Koshkin et al. 1998b; Singh et al. 1998). They can be substituted for any conventional nucleic acid in any synthetic oligonucleotide. It is possible to enhance the Tm of conventional oligonucleotides by replacing any of the conventional nucleic acid in the oligonucleotides with a LNA (Singh et al. 1998). Thus, the use of LNAs could significantly increased mismatch discrimination (Kauppinen et al. 2003). In modified nucleic acids, a methylene bridge connects the 2'-oxygen and the 4'-carbon (Parekh-Olmedo et al. 2002) and consequently produces higher conformational determination of the ribose and increased local organization of the phosphate backbone in a 3P-endo conformation (Braasch and Corey 2001). Furthermore, LNAs obey Watson-Crick base pairing (Koshkin et al. 1998b) and thus, are easy to implement into standard oligonucleotide synthesis chemistry (Kauppinen et al. 2003). LNAs offer new potentials for use in DNA/RNA oligo recognition based methods because of certain enhanced properties over normal nucleic acids. According to (Kongsbak 2002), they could be used in any hybridization assay as a modified probe or primer to increase specificity and reproducibility. They are used with standard reagents and protocols, have the same solubility as DNA or RNA, low toxicity, can make chimeras with DNA or RNA, are obtainable from industrial companies (Braasch and Corey 2001) and are not affected by nucleases (Vester and Wengel 2004). The only disadvantage is that they are much more expensive than conventional nucleic acids. Because of these enhanced properties, LNAs have been used in many applications since their first introduction, e.g., gene expression profiling (Nielsen and Kauppinen 2002), genotyping assays (Jacobsen et al. 2002a; Jacobsen et al. 2002b), fluorescence in situ hybridization (Silahtaroglu et al. 2003; Silahtaroglu et al. 2004; Wienholds et al. 2005; Kloosterman et al. 2006; Kubota et al. 2006), real-time PCR (Ugozzoli et al. 2004a; Hummelshoj et al. 2005; Sun et al. 2006) DNAzymes (Vester et al. 2004; Vester et al. 2006) and other methods.

Because of these successful applications of LNA-modified probes, their use in species identification in sandwich hybridization and microarray assays should be evaluated. LNA modified probes could possibly overcome problems of low hybridization efficiency and cross

hybridization of probes to closely related non-target species, often separated from the target species by a single base mismatch.

Molecular probes are widely applied for the identification of micro-organisms, e.g., toxic algae. They are applied in combination with a variety of detection techniques: Fluorescence in situ hybridization or FISH (Scholin et al. 1996; Scholin et al. 1997; Simon et al. 2000; Smit et al. 2004; Kim and Sako 2005), sandwich hybridization assays or SHA (Scholin et al. 1996; Metfies et al. 2005) and DNA microarrays (Metfies and Medlin 2005a; Metfies and Medlin 2005b). The small and the large subunit ribosomal RNA genes are the usual targets for molecular probes, because there is a high target number in the cell and they contain more or less conserved regions, making it possible to develop probes that are specific at different taxonomic levels (Groben et al. 2004). Probe specificity is dependent on the number of sequences of the targeted gene available in databases. Cross-reactions can occur with unknown non-targeted species if the target sequence of the probe is designed from a low number of sequences or the group is relatively unknown or unculturable and there are many non-targeted species whose sequences have not yet been determined. Even when a probe is designed from a large database, it is necessary to revise probe sequences frequently because new sequences are added almost daily to databases. Genetic variability has been documented among geographically dispersed strains of the same species (Scholin et al. 1994), making specific probes design even more challenging if global strains have not be sampled. One important problem in probe design and construction is to choose the best sequence from several possibilities that could theoretically identify the target. Excellent in-situ hybridization results of any probe does not always appear to correlate well with in-silico parameters, such as G–C content or melting temperature (Graves 1999). It is not possible to predict which probes will work well under all hybridization conditions. Sometimes probes that work well in dot blot and FISH formats do not work at all in a microarray format (Metfies and Medlin, unpublished).

The identification of phytoplankton, especially of harmful algae species, is important from an ecological and economic point of view. Certain harmful algae have the potential to produce toxins that have the capability to seriously harm, or even kill, other organisms or even humans if intermediaries in the food chain, such as mussels, are consumed. Numerous monitoring programs are established along all coastlines around the world for the detection of harmful algae. The European Union demands the monitoring of the coastlines for toxin-producing

phytoplankton and toxins in mussels by the member states (Directive 91/492d/EC and Commission Decision 2002/225/EC). Cell detection methodology based on light microscopy can be tedious and time-consuming when large numbers of samples need to be processed routinely, and identification of some species may require highly trained personnel and expensive equipment (Tyrrell et al. 2002). Reliable species identification and long-term monitoring are difficult to achieve by traditional methods, because unicellular algae are taxonomically challenging with toxic and non-toxic strains belonging to the same species. In the past decade, a variety of molecular methods have been adapted for the identification of microbial species, which are often lacking in distinct morphological features. Molecular identification is a very useful alternative in the study of natural phytoplankton populations (Guillou et al. 1999b). In our lab, we are working on the development of a molecular probe-based biosensor and a DNA-microarray for the detection of harmful algae and for estimating hidden biodiversity. In particular, we focus on those species that have the potential to harm the environment by the production of potent toxins.

The two solid-phase methods described here: DNA microarrays for phylogenetic analyses and an rRNA-biosensor, are used to measure the abundance of algal species using target specific probes bound to a surface.

rRNA biosensor - The detection method using a rRNA-biosensor was successfully introduced by (Metfies et al. 2005) as a molecular method for the detection and identification of the toxic dinoflagellate *Alexandrium ostenfeldii*. It utilizes sandwich hybridization (SHA) with a capture probe that binds to the target RNA or DNA and a second signal probe that carries the signal moiety and binds near the binding site of the capture probe. A third additional probe, the so-called helper probe, binds near the binding site of the two other probes to modify the secondary structure of the molecule so that the signal probe can easily form its heteroduplex. This region usually consists of approximately 50 bps leaving little for probe manipulation should the probes not work properly. The search for suitable probes is complicated by the relative conservation of the 18S gene at the species level (Gagnon et al. 1996; Ki and Han 2006). More variable genes have not been rigorously evaluated because only hyper-variable regions have been sequenced leaving the majority of the gene unknown and open for non-specific binding. The detection is measured electrochemically by the PalmSens instrument and its PSLite software (Palm Instruments, Houten, Netherlands) and was adapted from the original biosensor presented by Metfies et al. (2005).

Probes for the rRNA biosensor (Table 1) - AOST1 (the signal probe), AOST2 (the capture probe), and their helper oligonucleotide, H3, are 18S-rRNA probes designed by (Metfies et al. 2005) and were tested for specificity with dot blot and SHA. Although normalized signals for *A. ostenfeldii* are significantly higher than the signals from all non-target organisms, there is a low cross hybridization to *A. minutum,* which has 2 mismatches to the capture probe. An improved protocol for the isolation of algal RNA with the Qiagen RNeasy Plant Mini Kit, Hilden only enhances this cross reaction. The recently described non-toxic *Alexandrium tamutum* (Montresor et al. 2004) presents a single mismatch to the capture probe for *A. ostenfeldii*, thus challenging the limits of specificity of this probe.

DNA-Microarray -A DNA-microarray consists of a glass-slide with special surface properties (Niemeyer and Blohm 1999a) and many copies of nucleic acids, e.g., oligonucleotides, cDNAs or PCR-fragments spotted on it (Graves 1999) in a specific pattern . It is a widely used routine tool in many applications because it offers the possibility to analyze a large number of up to 250,000 different targets in parallel without a cultivation step (Lockhart et al. 1996; Graves 1999; Ye et al. 2001). Nucleic acids are fluorescently labelled before hybridization and they are detected afterwards with a microarray scanner (Derisi et al. 1997). Many functional genomic methods benefit from this technology, such as genome expression profiling, single nucleotide polymorphism detection and DNA resequencing (Lipshutz et al. 1999; Kauppinen et al. 2003; Ji and Tan 2004; Yap et al. 2004; Al-Shahrour et al. 2005; Broet et al. 2006; Gamberoni et al. 2006). DNA-microarray technology is also used to differentiate microalgae (Metfies and Medlin 2005a; Ki and Han 2006; Metfies et al. 2006), fish (Kappel et al. 2003) and bacteria (Loy et al. 2002; Peplies et al. 2003; Peplies et al. 2004a; Peplies et al. 2004b; Lehner et al. 2005; Loy et al. 2005a; Peplies et al. 2006).

Probes for the DNA-microarray –Four out of five probes used here (Table 2) were previously evaluated on the DNA-microarray (Metfies and Medlin 2005a). The fifth probe, Crypto B, recognizes all pigmented cryptomonad algae. It could be shown that these probes work specifically with microarrays, but there was potential for enhancement of the signal-to-noise-ratios because these probes gave low signals and thus were good candidates for signal-enhancement with LNAs.

Materials and Procedures

Culture conditions - All algal strains were cultured under sterile conditions in seawater-based media (Eppley et al. 1967; Keller et al. 1987) at 15 °C and 150 µEinstein – 200 µEinstein with a light: dark cycle of 14:10 hours (Table 1).

RNA-extraction - Total RNA was isolated from all algal cultures with the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) with modifications of the protocol to enhance the quality of the RNA. This involved a centrifugation of 15 minutes instead of two minutes to achieve an improved separation of supernatant and cell debris. Buffer RW1 was applied two times to the RNeasy column, incubated for one minute and then centrifuged. The first wash step with buffer RPE was repeated. RNA concentration was measured with a Nanodrop Spectrophotometer (Peqlab, Erlangen, Germany). All of these changes increased the removal of polysaccharides and proteins to improve quality and quantity of the rRNA extracted.

DNA-extraction - The template DNA from the environmental clones was isolated from bacteria by using the Plasmid Mini Kit (Qiagen, Hilden, Germany). DNA from the algal strains was extracted from pure cultures with the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany).

PCR Amplification of 18S rRNA - The entire 18S gene (1800 bp) from the target DNA was amplified with universal specific PCR primers 1F (5'-AAC CTG GTT GAT CCT GCC AGT-3') and 1528R (5'- TGA TCC TTC TGC AGG TTC ACC TAC- 3') without the polylinkers (Medlin et al. 1988). The PCR protocol was: 5 min 94°C, 2 min 94°C, 4 min 54°C, 2 min 72°C, 29 cycles and 7 min 72°C. All PCR experiments were done in a Mastercycler (Eppendorf, Hamburg, Germany). A 250 bp fragment of the TATA-box binding protein-gene (TBP) of *Saccharomyces cerevisiae* was amplified with the primers TBP-F (5'-ATG GCC GAT GAG GAA CGT TTA A-3') and TBP-R-Biotin (5'-TTT TCA GAT CTA ACC TGC ACC C- 3') and used as a positive control in the microarray hybridization experiments. The TBP amplification protocol was: 5 min 94°C, 1 min 94°C,1 min 52°C, 1 min 72°C, 35 cycles, 10 min 72°C. All PCR-fragments were purified with the QIAquick PCR purification (Qiagen, Hilden, Germany) with modifications of the protocol to enhance the quantity of the PCR-fragments. The elution with the elution buffer EB (Step 8) was performed twice with the same

buffer. The concentration of the DNA was measured with a Nanodrop Spectrophotometer (Peqlab, Erlangen, Germany).

Biotin-Labelling of the purified PCR- fragments - For the enhancement of signal intensities the Biotin DecaLabel DNA Labeling Kit (Fementas, St. Leon-Rot, Germany) was used. Labelling of 200 ng of purified PCR-fragment was carried out over night (17 to 20 hours) to maximize biotin incorporation into the PCR-fragments. After labelling the purification was done with the MinElute PCR Purification Kit (Qiagen, Hilden, Germany) with modifications of the protocol to enhance the quantity of the PCR-fragments as above. Concentration of the DNA was measured with a Nanodrop Spectrophotometer (Peqlab, Erlangen, Germany).

Probe synthesis –All probes and helper oligonucleotide probes and positive and negative controls were synthesized from Thermo Electron Corporation, Ulm, Germany. The locked nucleic acids were synthesized from Exiqon (Vedbaek, Denmark). The position of the LNA-residues within the sequence is proprietary information from Exiqon but they were regularly interspersed among normal nucleic acids.

rRNA biosensor

Probe set – A set of two specific 18S-rRNA probes (AOST1 and AOST2, Table 2) was used to assess the impact of LNA-probes on the specificity of probes with the biosensor. The sequence of capture probe AOST2 was redesigned from Exiqon with locked nucleic acids as a shorter oligonucleotide to maintain the identical melting temperature as the conventional probe AOST2. Three different probes, LNA 65, LNA 66 and LNA 67, were synthesized with a biotin-label and were used as signal probes in combination with AOST1. Probe AOST2 has a melting temperature (Tm) of 66 °C, AOST1 of 64.3 °C, LNA 65 and LNA 66 of 65°C and LNA 67 of 60°C. The positive control was not modified with LNAs.

Algal strains and templates - The specificity of the LNA probes using the rRNA biosensor was tested with the target strain *Alexandrium ostenfeldii* (Table 1) and the non-target strains, *Alexandrium minutum* AL3T and *Alexandrium tamutum* SZNB029.

Immobilization of the probes on the sensor chip - The biotinylated capture probes (AOST2, LNA 65, LNA 66; LNA 67) were immobilized on the sensor chips as described by (Metfies et al. 2005). The working electrode was pretreated with Carbonate buffer (50 mM $NaHCO_3$, pH

9.6) following which incubation with NeutrAvidin [0.5 mg/ml] (Pierce Biotechnology, Rockford, USA) for at least 4.5 hours at 4 °C was carried out. Excessive NeutrAvidin was removed from the working electrode by washing the sensor with PBS (BupH phosphate saline pack, Pierce Biotechnology, USA). Subsequently, the working electrode was blocked with 3% [w/v] casein in PBS for 1 hour at room temperature and afterwards washed in PBS. The probes were dissolved at a concentration of 10 µM in bead buffer (0.3 M NaCl/0.1M Tris, pH 7.6) prior to immobilization on the electrodes for 30 minutes at room temperature. All incubation steps were carried out in a moisture chamber to avoid evaporation. Unbound probe was removed from the electrode by washing with hybridization buffer (75mM NaCl/20mM Tris, pH 8.0/0.04% SDS).

Hybridization - Prior to hybridization the total rRNA was fragmented in fragmentation buffer (40mM Tris, pH 8.0/100mM KOAc/30mM MgOAc) for 5 minutes at 94°C. The hybridization mixture for the detection of rRNA contained 1x hybridization buffer (75mM NaCl/20mM Tris, pH 8.0/0.04% SDS), 0.25 µg/µL herring sperm DNA, 0.1 pmol/µL dig-labeled probe AOST1 and rRNA at different concentrations. Negative control and positive controls contain water and Test-DNA, respectively, instead of rRNA. Incubation for 4 minutes at 94°C of the hybridization mixture was carried out to denature the target nucleic acid. Subsequently 2 µL of the mixture was applied to the working electrode and the sensor was incubated for 30 minutes at 46°C. The hybridization was accomplished in a moisture chamber to avoid evaporation. Afterwards, the sensor chips were washed with POP buffer (50mM $NaH_2PO4 \times H2O$, pH 7.6/100mM NaCl).

Detection - The sensor chip was incubated for 30 minutes at room temperature with an antibody-enzyme complex directed against the digoxigenin coupled to horseradish-peroxidase (Anti-DIG-POD). 1.5 µL of the antibody-enzyme solution (7.5 U/ml in PBS, pH 7.6/0.1% BSA [w/v]/0.05% Tween 20 [v/v]) was added onto the electrode. Excessive enzyme was removed by washing the sensor with POP buffer; subsequently the sensor chip was inserted into the PalmSens (Palm Instruments BV, Houten, Netherlands), 20 µL of the substrate solution (4-aminophenylamine hydrochloride [44 µg/ml]/0.44% ethanol [v/v]/0.048% H_2O_2 [v/v]/50mM $NaH_2PO_4 \times H_2O$/100mM NaCl) was applied to the working electrode and an electrochemical signal was generated that was directly measured for 10 seconds at a potential of -147 millivolt (versus Ag/AgCl) after 8 seconds of equilibration.

Experimental setup - The LNA probe and the AOST probe experiments were carried at four different temperatures: 46 °C (normal hybridization temperature), 55°C, 60 °C and 65°C. Each LNA probe and the AOST2 probe were tested using the rRNA of the target and non-target species at each temperature. A hybridization experiment contained three replicates for detection of target RNA, and a negative and positive control. Unclear results were repeated to verify the data. The mean value of the signals was calculated and the standard derivation was determined with the following formula:

$$\frac{\sqrt{\frac{n\sum x^2-(\sum x)^2}{n(n-1)}}}{\sqrt{n}}$$

Microarray

Probe set DNA microarray - The five probes evaluated in this publication target the 18S-rRNA: one for the super kingdom of Eukarya and one for each of these four major phyla of algae: the Chlorophyta, Bolidophyta, Prymnesiophyta and Cryptophyta. The probe lengths of the conventional probes varied from 16-20 base pairs (Table 3). Euk1209, Chlo 02, Boli 02, Prym 02 and Crypto B were processed by Exiqon with two different locked nucleic acid modifications, LNA2 or LNA3 varying in the number of LNAs/probe and the length and in the methylation of Cytosine. The positive control was not modified with LNAs.

Algal strains and templates - The tests of the LNA probes using the microarray-format were carried out with PCR-fragments amplified from two uncultured, environmental clones and two algal strains (Table 3) as target strains. Four strains from the genus *Alexandrium* (*A. catenella* BAHME217, *A. ostenfeldii* BAHME 136, *A. ostenfeldii* AOSH1 and *A. minutum* Nantes) were used as non-target strains.

Microarray production - The probes for the microarray had a C6/MMT aminolink at the 5'-end of the molecule and were spotted onto epoxy-coated “Nexterion Slide A” slides (Peqlab Biotechnologie GMBH, Erlangen, Germany). The oligonucleotides were diluted to a final concentration of 1µM in 3x saline sodium citrate buffer and printed onto the slides with the pin printer VersArray ChipWriter Pro (Bio-Rad Laboratories GmbH, München, Germany) and split pins (Point Technologies, Inc., Colorado, USA). The probes were immobilized on the slides with a baking procedure of 30 min. at 60°C and stored at -20°C.

Standard hybridization protocol - The hybridization solution contained a hybridization buffer (1M NaCl/10 mM Tris, pH 8/ 0,005% Triton X-100/ 1 mg/ml BSA/ 0.1 µg/µL HS-DNA), the biotin-labeled PCR-fragment in a final concentration of 11.25ng DNA per µL and the positive control, the 250 bp PCR-fragment TBP from *S. cerevisiae* with biotin-labeled primers in a final concentration of 4.7ng DNA per µL. First, 1 hour pre-hybridization was carried out at 58C with 2xSTT buffer. The hybridization solution was denatured for 5 min at 94°C and for even dispersal of hybridization solution between the chip and the coverslip, a volume of 30 µL was injected under a Lifter Slip cover slip (Implen, München, Germany). The slides were hybridized as follows: 1 hour hybridization in a humid chamber with the hybridization solution at a hybridization temperature of 58°C, washing afterwards with 2x and 1x saline sodium citrate (2 × SSC/10 mM EDTA/0.05% SDS; 1 × SSC/10 mM EDTA) for 15 min each. In all microarray hybridization experiments, the chip contained four replicates of each probe in four individual arrays. These hybridizations were done four times with the perfectly matched targets. For the non-target hybridizations, the hybridizations were repeated twice.

Staining - The bound PCR-fragments were stained subsequently with Streptavidin-CY5 (Amersham Biosciences, Stadt, Germany) in hybridization buffer at a final concentration of 100 ng /ml. The staining took place for 30 min. at room temperature in a humid chamber. Excess staining moieties were removed by washing twice with 2x saline sodium citrate for 5 min. and once with 1x saline sodium citrate for 5 min.

Scanning and quantification of Microarrays - The fluorescent signals of the microarrays were scanned with a GenePix 4000B scanner (Molecular Devices Cooperation, Sunnyvale USA) and the obtained signal intensities were analyzed with the GenePix 6.0 software (Molecular Devices Coperation, Sunnyvale USA). The signal to noise-ratio was calculated with a formula according to (Loy et al. 2002) and all ratios were normalized on the signal of the TBP positive control. The mean value of the signal-to-noise-ratios was calculated as above.

Assessment

rRNA Biosensor - The PalmSens was adapted for the biosensors using a control chip with a fixed resistance of 2682 nanoampere (nA). In this study, an amperometric detection technique was used with measurement duration of 10 seconds. At the recommendation of Palm Instruments, the time equilibration of 8 seconds was programmed into measuring method,

which means a total measurement duration of 18 seconds, 8 seconds longer than with the Inventus Biotec GmbH potentiostat used by Metfies et al (2005). The redox-reaction goes to completion and then signals decrease over the measurement time because of the limited substrate amount. Consequently, the signal intensity is lower after 18 seconds than after 10 seconds. Compared to the signals measured by Metfies et al (2005), all the signals presented in this study are about 600 nA lower for the AOST probes than those in Metfies et al. (2005).

The hybridization temperature for both *Alexandrium ostenfeldii* probes was optimized in the present assay to 46°C (Figure 1A). This is around 20 °C below the calculated Tm of AOST probes. Hybridization reactions can be carried out at a Tm 25°C below its theoretical calculation because the rate of DNA annealing is maximal at 20-25°C below its melting temperature. Hybrids formed from completely homologous nucleic acids will be thermally stable under these conditions (Howley et al. 1979). However, if hybridizations are performed at temperatures significantly below the theoretical Tm, the probes could cross hybridize to non-target nucleic acids. The AOST probes gave a signal for *Alexandrium ostenfeldii* of 680 nA and also showed high cross hybridization signals for *A. minutum* at 605 nA. However, *A. tamutum*, having only one mismatch to AOST2 was not detected by the AOST probes, thus it is possible to discriminate target from non-target with a single base pair mismatch. All three LNA probes showed almost no signals at 46°C for the different species (Figure 1A). Only LNA 66 showed a weak signal for *A. ostenfeldii*. Also the positive control signals were about twofold lower for LNA 65 and about 2.7 x lower for LNA 66 and LNA 67 than for the AOST probes, which can be explained by the suboptimal hybridization temperature for the LNA probes and their melting temperature. It seems that LNA probes do not have the same hybridization properties as conventional probes in this method.

Metfies et al 2005 showed that a temperature of 55°C results in higher hybridization signals than at 46°C but at this temperature, all probes were non-specific (Figure 1B). Only LNA 67 gave very low signals for all species similar to the signals at a hybridization temperature of 46°C. Probes AOST1/AOST2, LNA 65 and LNA 66 have a Tm of about 65 °C; LNA 67 has a Tm of 60°C. A hybridization temperature of 55°C should be the optimum temperature for the first three probes. We maintained uniform temperatures and salt concentrations in the washing buffers in order to compare the performance of the LNAs against optimal conditions for the unmodified probes. At hybridization temperature of 60°C (Figure 1C) the AOST probes were specific for *A. ostenfeldii* and showed no signals for the other species, but the

signal intensity was lower than at 46 °C. All three LNA probes detected *A. ostenfeldii* and *A. minutum*. The AOST probes detected all three species at a hybridization temperature of 65°C (Figure 1D), but the signals for *A. ostenfeldii* and *A. minutum* were quite low and there was a high signal for *A. tamutum* similar to the signals obtained at 55°C. LNA probe 65 was specific at 65°C and detected only *A. ostenfeldii*. This was the only specific signal that we detected. LNA probes 66 and 67 showed only low signals for *A. ostenfeldii* and *A. minutum* but high signals for *A. tamutum*. The properties of the LNA probes should enhance the signal intensity at higher temperatures and discriminate the mismatches but we obtained exactly the opposite results. All three LNA probes show non-specific signals at 46°C, 55°C and 60°C for *A. ostenfeldii*.

For the use on an rRNA biosensor the probes were also tested for long term stability (data not shown). Probes without LNAs are stable over a year. During the experiments with LNA probes on the biosensors, it was observed that the LNA probes were unstable after immobilization after only a few weeks of storage.

Microarray

Probe development/design - For this hybridization study, previously published and microarray tested probes were used. They all target higher taxonomic levels, so it is challenging to design probes to achieve better specificity and sensitivity that can recognize all taxa belonging to the target group. The selected probes are working moderately well but do not show sufficient sensitivity for use in routine applications and monitoring of phytoplankton because cell counts in field samples are often not high and taxonomic groups with low abundance cannot be detectable.

Validation of results in the hybridization protocol - The results of the microarray hybridization (Figure 2) with specific PCR-fragments indicated that both LNA probes showed increased signal intensity. LNA2 performed the best, except for CryptoB, the probe for the Cryptophyceae, where LNA3 had the highest result. Signal enhancement varied from approx. 4.5-fold higher results in the Cryptophyceae and Bolidophyceae to 8.5-fold higher signals in the Chlorophyceae.

Validation of results using non- target hybridizations - In comparison to the above results, signals of the hybridization of the conventional and LNA-modified probes with non-target

algae species (Figure 3) demonstrated that the conventional probes worked specifically with weak cross hybridization with non-related species. All probes, both conventional and LNAs, showed positive enhanced signals with the Eukaryotic probe, as they should but there was no pattern to the enhancement and these data are not presented. All LNA probes showed cross hybridization signals with non-target DNA. Hybridizations with 27 other *Alexandrium* strains all showed the same tendency (data not shown).

Increase of hybridization temperature to enhance the discriminative potential of the LNAs was already tested with the biosensor and the LNA modified probes did not perform as conventional oligonucleotides. Thus the microarray protocol was not modified any further. Even though the results from the hybridizations with target DNA using standard protocols are promising with increase in signal-to-noise-ratios, in the hybridization with non target DNA, the LNA probes show an unacceptable lack of pecificity. For further clarification, the mismatches of the probes to the sequences of the four *Alexandrium* strains are shown in Table 4. The differences span from 2 to 9 base pairs. Theoretically, it is impossible for these DNAs to bind to these probes.

Discussion

In this study, we tested and evaluated the use of LNA probes in two solid-phase hybridization methods. Although there have been many publications on enhancement of probe or hybridization signals with LNA modified probes, there has been no rigorous testing of these probes using known target sequences. We found that LNA probes showed no signal enhancement in the sandwich hybridization method using the rRNA biosensor. Only one of the tested LNA probes showed specific signals at a hybridization temperature of 65°C. Using the microarray, the LNA probes could enhance the sensitivity and gave higher signals than the conventional probes using only target DNA but unfortunately, unspecific binding with non-target DNA also was enhanced. These results were surprising because in other methods the LNA modified probes show great potential and an ability to enhance the signals and to improve specificity, accuracy and sensitivity in the whole method (Silahtaroglu et al. 2004; Wienholds et al. 2005; Kloosterman et al. 2006; Kubota et al. 2006; Sun et al. 2006). Results from other methods using LNA probes cannot be easily compared to the results presented in this study, because of the different experimental setups, such as *in situ* hybridizations in tissues (fluorescence in situ hybridization (FISH), in situ hybridization). In FISH experiments,

the LNA probes using human-specific repetitive elements were very efficient (Silahtaroglu et al. 2003; Silahtaroglu et al. 2004). To evaluate the potential possibilities and abilities of LNA probes, more experiments with more methods are necessary. A comprehensive and ultimate evaluation of the potential of LNA probes cannot be conducted here because only a small subset of probes were tested in two different solid phase based hybridization techniques with the use of our standard hybridization protocols. It is likely that the increased signals seen in these studies result from non-sepcific binding which cannot be documented because the target and non-target sequences are unknown. The standard protocols developed for our unmodified probes on multiprobe chips at specific hybridization temperatures are appropriate for monitoring of phytoplankton. By choosing other salt concentrations in combination with other hybridization temperatures, the signals of the LNA probes could be different. Further optimization experiments are only appropriate for the use of only one LNA probe at a time, because different LNA probes can have different hybridization temperature optima. Additionally to unspecific binding, other problems occurred using LNA probes. For example, the biosensors for the monitoring of the toxic algae are prepared in advance of application. Because of this, the probes on the biosensors have to be stable and need to give the same signals after several months of storage. With the LNA probes, this application was not possible.

Signal enhancement of both methods, biosensors and microarrays, has been achieved by changing substrate concentration for the biosensor and by reducing the background noise with the help of other blocking solutions. In the case of the microarrays, signal enhancement can be accomplished by using labelling kits that incorporate multiple labels to a target.

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Table 1. Algae cultures

Species	Strain	Culture medium	Temperature	Origin
Alexandrium minutum	AL3T	K	15 °C	Gulf of Trieste, Italy, A. Beran
Alexandrium minutum	Nantes	K	15 °C	Atlantic Ocean, France
Alexandrium tamutum	SZNB029	K	15 °C	Gulf of Naples, Italy, M. Montresor
Alexandrium ostenfeldii	AOSH 1	K	15 °C	Ship Harbour, Nova Scotia, Canada, A. Cembella
Alexandrium ostenfeldii	CCMP 1773	K	15 °C	Limfjordan, Denmark, Hansen
Alexandrium ostenfeldii	BAH ME 136	K	15 °C	Biologische Anstalt Helgoland, Germany
Prymnesium parvum f. patelliferum	PLY 527	K	15 °C	Plymouth Culture Collection, UK
Rhinomonas reticulate	PLY 358	IMR	15 °C	Plymouth Culture Collection, UK
Alexandrium catenella	BAH ME 217	IMR	15 °C	Biologische Anstalt Helgoland, Germany

Table 2. Sequences of the probes, the helper oligonucleotide H3, positive and negative control for the biosensor

Probe name	Probe sequence	Target	Source
Signal probe: AOST1	CAA CCC TTC CCA ATA GTC AGG T	*Alexandrium ostenfeldii* CCMP 1773	(Metfies et al. 2005)
Capture probe: AOST2	GAA TCA CCA AGG TTC CAA GCA G	*Alexandrium ostenfeldii* CCMP 1773	(Metfies et al. 2005)
Capture probe: LNA 65	AAT CAC CAA GGT TCAA	*Alexandrium ostenfeldii* CCMP 1773	Exiqon
Capture probe: LNA 66	AGG TTC CAA GCAG	*Alexandrium ostenfeldii* CCMP 1773	Exiqon
Capture probe: LNA 67	CCA AGG TTC CAAG	*Alexandrium ostenfeldii* CCMP 1773	Exiqon
Helper oligonucleotide: H3	GCA TAT GAC TAC TGG CAG GAT C	*Alexandrium ostenfeldii* CCMP 1773	(Metfies et al. 2005)
Test DNA (positive control	CTGC TTG GAA CCT TGG TGA TTC ACCT GAC TAT TGG GAA GGG TTG		(Metfies et al. 2005)

Table 3. Probe Sequences for the microarray

Probe name	Probe sequence	Target	Source
Euk 1209	GGGCATCACAGACCTG	All Eukaryotes 18S	(Lim et al. 1993)
Chlo 02	CTTCGAGCCCCCAACTTT	Chlorophytceae HE001005.53*	(Simon et al. 2000)
Boli 02	TACCTAGGTACGCAAACC	Bolidophyceae HE001005.51*	(Guillou et al. 1999a)
Prym 02	GGAATACGAGTGCCCCTGAC	*Prymnesium parvum* f. *patelliferum* PLY 527**	(Simon et al. 2000)
Crypto B	ACGGCCCCAACTGTCCCT	*Rhinomonas reticulata* PLY 358**	Medlin, unpublished
Positive control (PC)	ATGGCCGATGAGGAACGT	*S. cerevisiae*, TBP	(Metfies and Medlin 2005a)
Negative control (NC)	TCCCCCGGGTATGGCCGC		(Metfies and Medlin 2005a)

*Environmental clone from EU FP5- Project PICODIV, ** Plymouth Culture Collection, UK

Table 4. Mismatches of the probes to the *Alexandrium* strains in base pairs (bp)

	A. catenella BAHME217	***A .ostenfeldii BAHME136***	***A.ostenfeldii AOSH1***	***A. minutum Nantes***
Chlo 02	3 bp	2 bp	2 bp	2 bp
Boli 02	9 bp	8 bp	8 bp	5 bp
Prym 02	5 bp	5 bp	5 bp	5 bp
Crypto B	3 bp	3 bp	3 bp	3 bp

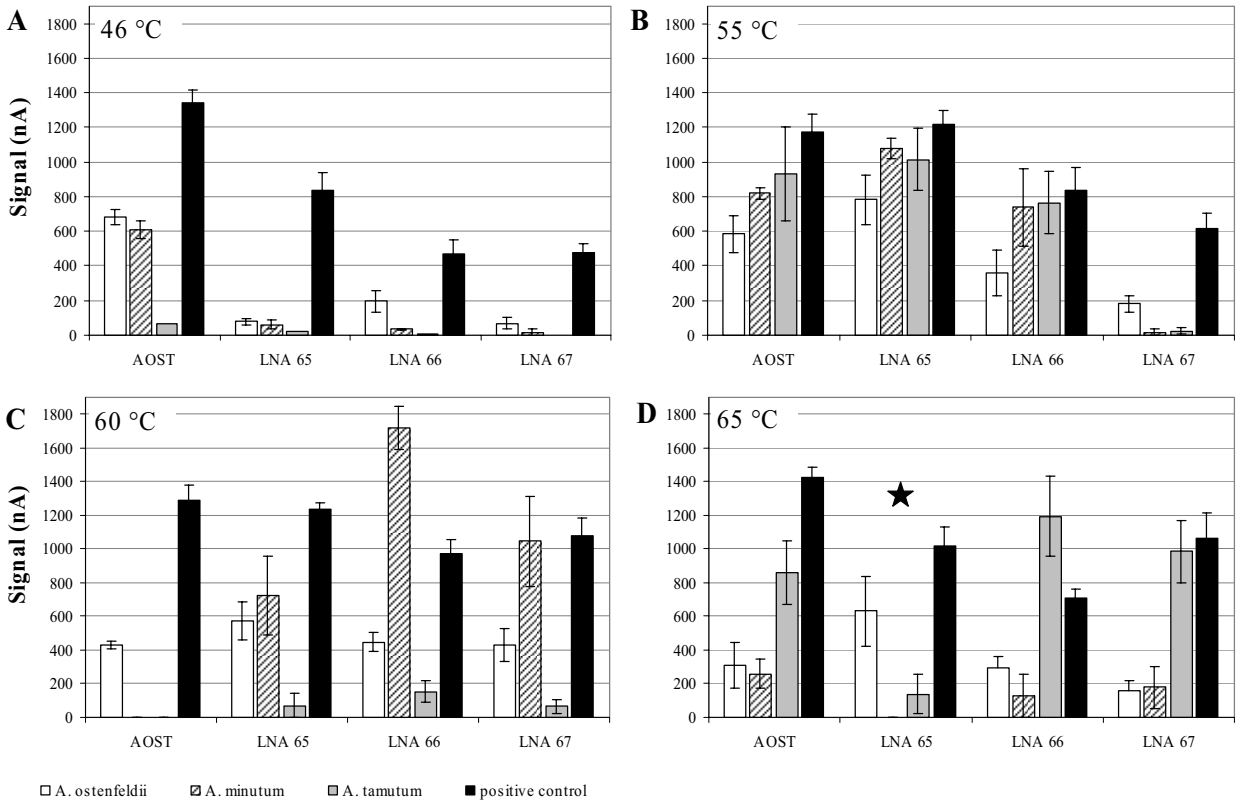

Figure 1. Signal intensity of the rRNA-biosensor. Four different probes were tested at four different hybridization temperatures and with three different species. (A) 46°C, (B) 55°C, (C) 60°C, (D) 65°C. The concentration of the rRNA for all tested species was 450 ng/µL. The asterisk marks the only specific LNA probe.

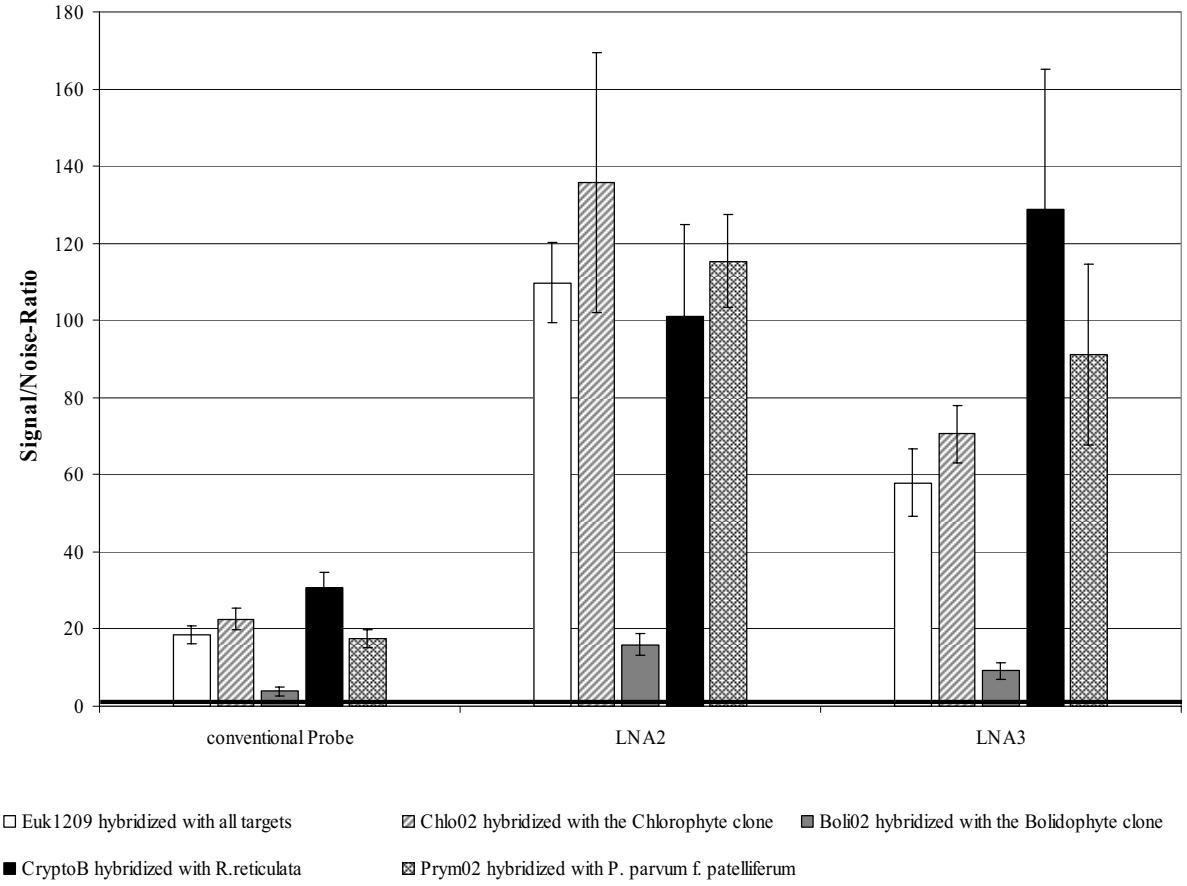

Figure 2. Signal/Noise-Ratios of all fifteen probes in comparison from hybridization with specific PCR-fragments for each set of probes. The black line represents the value of 2 for the signal-to-noise ratio, defining the threshold for a true signal.

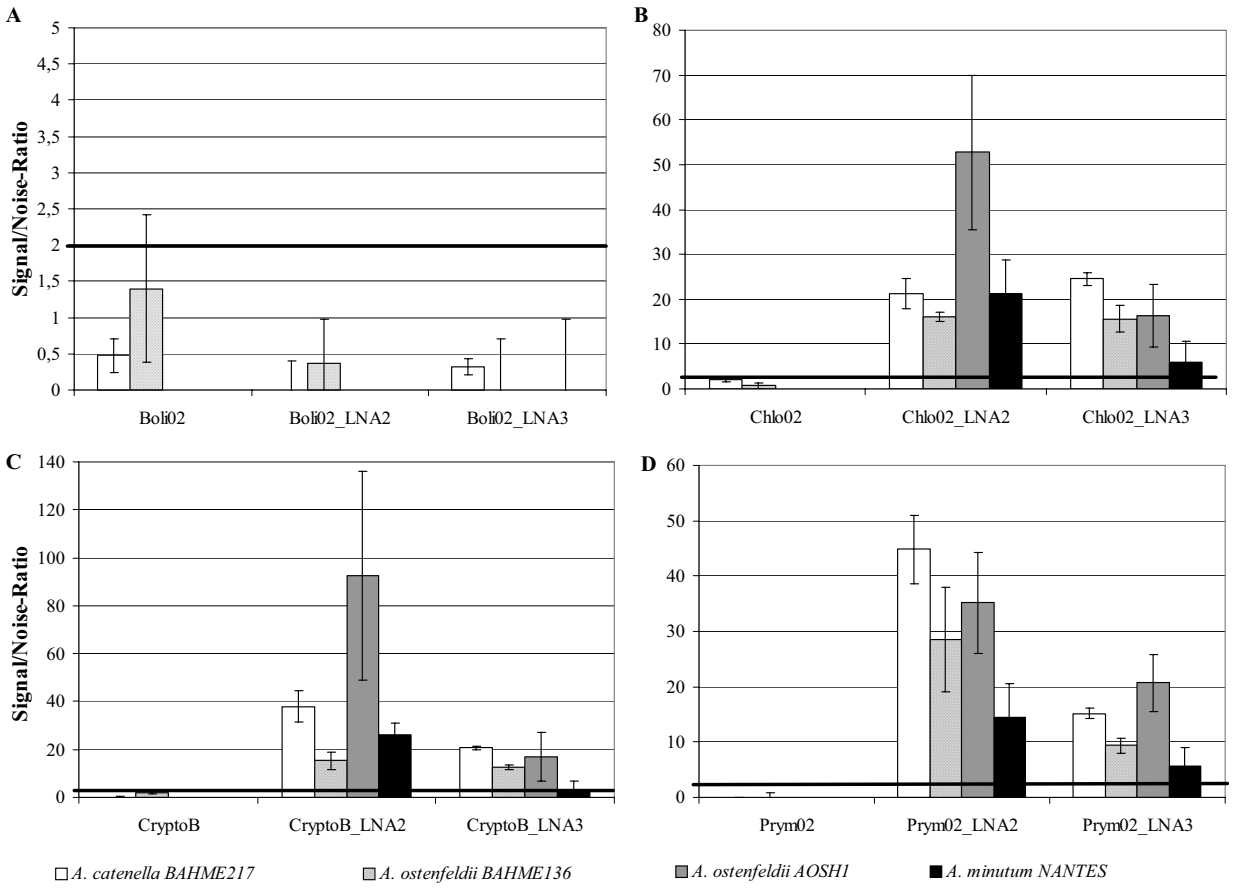

Figure 3. (A) Signal/Noise-Ratios of the set of three Boli02 probes in comparison from hybridization with unspecific PCR-fragments from the genus *Alexandrium.* (B) Signal/Noise-Ratios of the set of three Chlo02 probes in comparison with hybridization with unspecific PCR-fragments from the genus *Alexandrium.* (C) Signal/Noise-Ratios of the set of three CryptoB probes in comparison with hybridization with unspecific PCR-fragments from the genus *Alexandrium.* (D) Signal/Noise-Ratios of the set of three Prym02 probes in comparison with hybridization with unspecific PCR-fragments from the genus *Alexandrium.* The black line represents the value of 2 for the signal-to-noise ratio, defining the threshold for a true signal.

2.7 Publication V

DEVELOPMENT AND OPTIMIZATION OF A SEMI AUTOMATED rRNA BIOSENSOR FOR THE DETECTION OF TOXIC ALGAE

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Abstract

In order to facilitate the monitoring of toxic algae, a multiprobe chip and a semi-automated rRNA biosensor for the *in situ* detection of toxic algae were developed. Different materials for the electrodes and the carrier material were tested using single electrode sensors and sandwich hybridization that is based on species specific rRNA probes. The biosensor consists of a multiprobe chip with an array of 16 gold electrodes for the detection of up to 14 target species. The multiprobe chip is placed inside an automated hybridization chamber, which in turn is placed inside a portable waterproof case with reservoirs for different solutions. A peristaltic pump moves the reagents into the flow cell containing the multiprobe chip. For use of the device by layperson, a lysis protocol was successfully developed and manual rRNA isolation is no longer required. Only water sample filtration has to be done manually. The stand-alone system was evaluated using isolated total rRNA from algae cultures and field samples. The device processed automatically the main steps of the analysis and completed the electrochemical detection of toxic algae in less than two hours in comparison to other routine monitoring methods that need at least a day for analysis.

Introduction

Coastal areas are an important economic source for fishery aquaculture and tourism. Aquaculture is an increasingly important industry world-wide as a source of food and employment. Planktonic algae are critical food for shellfish and fish and thus, in most cases, marine phytoplankton blooming is a natural phenomenon and beneficial for aquaculture and wild fisheries operations. Marine phytoplankton blooming is regarded as a sudden increase in the population and can be activated by suitable growth conditions and cell concentrations can reach up to 10^4-10^5 L-1 (Masó and Garces 2006). However, algal blooms can also pose a threat, because about 80 or even more algal species produce potent toxins that can find their way through the food chain via shellfish (e.g. oysters, mussels) and fish to humans (Hallegraeff 2003). Among the toxic algae, the marine dinoflagellate *Alexandrium* includes a number of species producing saxitoxin and potent neurotoxins, which are responsible for paralytic shellfish poisoning (Penna 1999). Also certain Pseudo-nitzschia ssp. produce a neurotoxin, which causes amnesic shellfish poisoning (Scholin et al. 1999; Masó and Garces 2006). World-wide monitoring programs have been introduced to observe phytoplankton composition. Monitoring of toxic algae by means of traditional methods, namely light-microscopy, can be time-consuming if many samples have to be routinely analyzed. Reliable species identification requires trained personnel to carry out the analysis and expensive equipment (Tyrrell et al. 2002; Ayers et al. 2005), because unicellular algae are taxonomically challenging and some of them have only few morphological markers. Various molecular methods are used up to date for the identification of phytoplankton, such as whole cell fluorescent *in situ* hybridization (Anderson et al. 2005; Hosoi-Tanabe and Sako 2005; Kim and Sako 2005), PCR-based assays (Penna 1999; Guillou et al. 2002) and sandwich hybridization assays (Tyrrell et al. 2002; Ayers et al. 2005). A rapid and potential method for the detection of toxic algae was introduced by Metfies et al. (2005) using sandwich hybridization on a biosensor and molecular probes that specifically targeted the rRNA of toxic algae. Electrochemical biosensors combine biochemical recognition with signal transduction for the detection of specific molecules (Gau et al. 2005). Single electrode sensors as well as arrays are known from various sectors like clinical diagnostic and environmental monitoring. Biosensors have been applied for the detection of biochemical substances as well as of micro-organisms like bacteria (Berganza et al. 2006; Lermo et al. 2006; Taylor et al. 2006). Phytoplankton communities consist of different species and the temporal and spatial variability in composition in the sea is substantial. The simultaneous detection of multiple

species can be accomplished using arrays of electrodes with different molecular probes. There are examples for on-site monitoring of toxic algae, such as the environmental sampling processor (Doucette et al. 2006; Silver 2006). However, molecular techniques for the monitoring of harmful algae usually require transportation of samples to specialised laboratories. The same applies to conventional methods. As a consequence, results are usually obtained within five working days after receiving the sample and therefore preventive measures are not always possible.

In this regard, a system with two major parts was developed during the EU-project ALGADEC: a multiprobe biosensor with the aim to detect specific compositions of toxic algae simultaneously in combination with a hand-held device for the *in situ* analysis. The hybridization method involves a capture probe, immobilised on the working electrode surface of a biosensor that binds to rRNA isolated from the target organism as well as a second digoxigen-labelled probe that also binds to the rRNA but carries the signal moiety. An antibody-enzyme complex directed against digoxigenin is added and incubated. A redox-reaction takes place after substrate addition and the resulting electrical current can be measured with a potentiostat.

We present here the testing of all components in the biosensor and the optimisation of the protocol for *in-situ* analysis of toxic algae.

Materials and Methods

Probe sets - One set of capture and signal 18S-rRNA probes (AOST1 and AOST2, (Metfies et al. 2005), Table 1), specific for *Alexandrium ostenfeldii*, was used to compare the performance of carbon sensors and gold sensors (Gwent Electronic Materials (GEM), UK). Another set of 18S-rRNA probes (AMINC and AMINC NEXT), specific for *Alexandrium minutum*, was developed previously (see publication II) and used for the experiments using different lysis buffers and the adaptation of the multiprobe chip to the semi-automated device. The probes and the positive controls were synthesized from Thermo Electron Corporation, Ulm, Germany.

Single electrode chips

Immobilization of probes on carbon sensors - The immobilization of the biotinylated capture probe (AOST2) on single electrode carbon sensors used in this study was done according to a previous protocol (Metfies et al. 2005). All incubation steps were carried out in a moisture chamber to avoid evaporation. The surface of the carbon working electrode was pretreated with carbonate buffer (50 mM $NaHCO_3$, pH 9.6) that was followed by an incubation with NeutrAvidin [0.5 mg/ml] (Pierce Biotechnology, Rockford, USA) for at least 4.5 hours at 4 °C. Subsequently, the sensor was washed with PBS (BupH phosphate saline pack, Pierce Biotechnology, USA) to remove excessive NeutrAvidin. For blocking, the working electrode was incubated with 3% [w/v] casein in PBS for 1 hour at room temperature and afterwards the sensors were washed in PBS. Prior to the application on the electrodes, the probes were diluted in bead buffer (0.3 M NaCl/0.1M Tris, pH 7.6) to achieve a concentration of 10 µM. For the immobilization of the probes on the electrodes, the sensors were incubated for 30 minutes at room temperature. Unbound probe was removed from the electrode by a washing step with hybridization buffer (75mM NaCl/20mM Tris, pH 8.0/0.04% SDS).

Immobilization of probes on gold sensors - The immobilization of thiolated probes on single electrode gold sensors was done according to a modified protocol that was first introduced by Carpini et al. (2004) (Carpini et al. 2004). Prior to the immobilization of the probes onto the gold working electrode the thiolated probes were dissolved at a concentration of 10 µM in 0.5 mol/L phosphate buffer. The gold working electrode surface was incubated with a probe for at least 16 hours at room temperature. During all incubation steps, the sensors were stored in a moisture chamber to protect the solutions from evaporation. In order to minimize the non-specific interaction between the gold surface and the probes, a post treatment with 6-mercapto-1-hexanol (MCH; 1mmol/L aqueous solution) was carried out for 1 hour. Excessive probe and MCH were removed by washing the sensor with 2x saline sodium citrate buffer.

Storage of coated sensors - The sensors were coated with 2% [w/v] Trehalose in PBS and dried for approximately 30 minutes at 37°C. Afterwards coated sensors can be stored at 4°C.

Hybridization of test DNA on single electrode sensors - The hybridization mixture for the detection of test-DNA contained 1x hybridization buffer (75mM NaCl/20mM Tris, pH 8.0/0.04% SDS), 0.25 µg/µL herring sperm DNA, 0.1 pmol/µL dig-labelled probe AOST1

and 0.1 pmol/µL test-DNA (positive control) as target for the probes. The negative control contains no test-DNA. Denaturation of the target nucleic acid was carried out by incubating the hybridization mixtures for 4 minutes at 94°C. 2 µL of the mixture was applied to the working electrode and the sensor was incubated for 30 minutes at 46°C. The biosensors were stored in a wet chamber during hybridization to prevent evaporation. Subsequently, the sensors were washed with POP buffer (50mM $NaH_2PO4 \times H2O$, pH 7.6/100mM NaCl).

Electrochemical detection with single electrode sensors - An antibody-enzyme complex directed against the digoxigenin coupled to horseradish-peroxidase (Anti-DIG-POD, 7.5 U/ml in PBS, pH 7.6/0.1% BSA [w/v]/0.05% Tween 20 [v/v]) was applied onto the single electrode sensor and incubated for 30 minutes at room temperature. Unbound antibody-enzyme complex was removed by washing the sensor with POP buffer and the sensor was inserted into the measurement device, PalmSens (Palm Instruments BV, Houten, Netherlands). 20 µL of substrate solution (4-aminophenylamine hydrochloride (ADPA) [44 µg/ml]/0.44% ethanol [v/v]/0.048% H_2O_2 [v/v]/50mM $NaH_2PO_4 \times H_2O$/100mM NaCl) was added to the working electrode and the resulting electrochemical signal was directly measured for 10 seconds at a potential of -147 millivolt (versus Ag/AgCl) after 8 seconds of equilibration. All experiments were carried out in triplicate, the mean value of the signals was calculated and the standard derivation was determined with the following formula:

$$\frac{\sqrt{\frac{n\sum x^2 - (\sum x)^2}{n(n-1)}}}{\sqrt{n}}$$

<u>*Multiprobe chips*</u>

Spotting of multiprobe chips - Multiprobe chips were either hand-spotted or spotted with a non-contact dispenser (Biodot Ltd., Chichester, UK) from GEM. Hand-spotted chips were covered with 10 µL of thiolated capture probe (10 µM in 0.5 mol/L phosphate buffer) and incubated as described above. 10 µL of MCH solution were added and incubated for one hour, subsequently, unbound probe and MCH were removed by washing the sensor with 2x saline sodium citrate buffer. The multiprobe chips were blocked with 10 µL of 5% [w/v] BSA and washed again with 2x saline sodium citrate buffer. Multiprobe chips were biodotted by immobilising 0.05 µL thiolated capture probe per electrode and adding of 0.05 µL of MCH

after incubation. Wash steps and blocking of the surface was carried out as previously described. The multiprobe chips were subsequently coated with 10 µL 2% [w/v] Trehalose in PBS buffer and dried for storage and shipment.

Hybridization mixture and electrochemical detection - The hybridization mixture using test-DNA (positive control), antibody solution and substrate solution for the multiprobe chip were prepared as described above. A volume of 10 µL hybridization mixture and antibody solution was applied each time onto the chip to cover the whole electrode array. Electrochemical detection was carried out by placing the multiprobe chip into a substrate reservoir that harboured the substrate solution. The electrochemical signals were measured using a multiplexer, which can measure eight electrodes simultaneously, and the PalmSens detector (Palm Instruments BV, Houten, Netherlands).

Semi automated Device

Culture conditions - The *Alexandrium minutum* strain AL3T was cultured under sterile conditions in seawater-based media K (Keller et al. 1987) at 15 °C and 120 µEinstein with a light: dark cycle of 14:10 hours. Prior to the experiments, the cells were counted using the Multisizer 3 Coulter Counter (Beckman Coulter GmbH Diagnostics, Krefeld, Germany).

Total rRNA-extraction - The RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) was used to isolate the total RNA from *Alexandrium minutum* with modifications of the protocol to enhance the quality and quantity of the RNA by removal of polysaccharides and proteins content. For the achievement of an improved separation of supernatant and cell debris, the centrifugation step of two minutes was extended to 15 minutes. The washing buffer RW1 was applied twice to the RNeasy column, incubated for one minute and centrifuged. The first wash step with buffer RPE was repeated. RNA concentration was measured with a Nanodrop Spectrophotometer (Peqlab, Erlangen, Germany).

Fragmentation of total rRNA from Alexandrium minutum - Total rRNA from *Alexandrium minutum* was fragmented in fragmentation buffer (40mM Tris, pH 8.0/100mM KOAc/30mM MgOAc) for 5 minutes at 94°C prior to hybridization.

Testing of different combinations of lysis buffer and hybridization buffers - Two different lysis buffers and hybridization buffers were tested for the determination of the optimal lysis properties and hybridization signals on the multiprobe chip. Lysis buffer 1 (Table 2) was prepared after Kingston (1998) (Kingston 1998) and the second lysis buffer RLT was taken from the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). In combination with the two lysis buffers two different hybridization buffers were tested (Table 2). The 4x hybridization buffer was described by Metfies et al. (2005) and the second hybridization buffer, named sample buffer, was introduced by Scholin et al. (1999) (Scholin et al. 1999). The experiments were carried out using 400,000 cells of *Alexandrium minutum* and 450 µL of the lysis buffers. 600 µL of 4x hybridization buffer and sample buffer were added to the different lysis solutions, respectively. Cell debris was removed by filtration through a 0.45 µm filter (Millipore, USA). Detection probe AMINC NEXT and fragmentation buffer were added to the lysis-hybridization solutions, incubated for 5 minutes at 94 °C and applied onto multiprobe chips with immobilised capture probe AMINC. Negative and positive controls were prepared as described above and total rRNA was isolated from the same cell counts of *A. minutum* and also hybridised for comparison of the signals.

Hybridization and analysis in semi-automated device - The hybridization mixture was prepared as described above, but the amount was amplified. Multiprobe chips consisted of an immobilised AMIN probes on all 16 working electrodes. The adjustment of the device was conducted using Test-DNA as target of the probes for *A. minutum*. Hybridization with different concentrations of target rRNA from *A. minutum* followed instead of the target-DNA. Final adjustments of hybridization mixture and the lysis buffer 1 were carried out using 500,000 cells of *A. minutum*.

Results

The signals of the electrochemical detection are measured with negative values, but for simplification of analysis, the signals are multiplied by –1 unless otherwise noted.

Sensor design using single electrode sensors

Comparison of electrochemical signals of carbon and gold sensors - In order to determine the most efficient and cost effective material for the working electrodes on the sensors, two

different materials, carbon and gold were tested to compare signal intensity and the effectiveness of probe immobilization (AOST2). Additionally the signals were compared to the signals shown by Metfies et al. (2005) with carbon sensors from a different manufacturer. The achieved signals for the positive controls detected on electrodes with different materials and sensors from different manufacturers are comparable being in the range of ~1500 nano ampere (nA) (Figure 1). However, the signal intensity of the negative control for the different surface materials varied highly. The carbon sensor from Metfies et al. (2005) showed the lowest signal with 78 nA, whereas for the carbon sensor from GEM a signal of 190 nA was achieved. The gold sensor showed a very high signal of 611 nA. Therefore the immobilization protocol for gold sensors was optimized to reduce the background noise of the gold sensors.

Optimization of immobilization protocol for gold sensors - The optimization of the immobilization protocol was carried out by adding a surface blocking step to the protocol subsequent to the immobilization of the probe (AOST2) and the treatment with MCH. Two different blocking reagents, casein and bovine serum albumin (BSA), known from the literature for their blocking properties were examined for their attributes to reduce the background noise of the gold surface. As a control, gold electrodes with no blocking were hybridized. The blocking with 3% casein in PBS was accomplished at room temperature for 1 hour, and could reduce the signal of the negative control to 281 nA but also reduced the signal of the positive control to 1168 (Figure 2). Different concentrations of BSA, 3%, 5% and 10% in 4x hybridization buffer, were applied to the gold sensors and incubated for 1 hour at 46°C. All treatments reveal a decrease of signal of the negative control regardless which concentration of BSA is used, but 3% BSA and 5% BSA showed the strongest improvement. Additionally the signals of the positive control of the gold sensors blocked with 5% BSA and 10% BSA increased about 200 nA. In consideration of these results, the 5% BSA blocking solution was chosen for the further experiments.

Long term stability of sensors - Long term stability of carbon and gold sensors was tested by coating the sensors with Trehalose after immobilization of the probes (AOST2) onto the working electrode. The sensors were stored at 4 °C and hybridised with target-DNA and the detection probe (AOST1) after 4, 6 and 12 months. Signal intensity decreased from freshly prepared carbon sensors with 1416 nA to 798 nA for carbon sensors stored over 12 months at 4 °C (Figure 3). Also the signals for gold sensors decreased from 1711 nA to 1282 nA.

Optimization of the substrate concentrations - The enhancement of signals intensity was examined using carbon sensors (GEM) and different concentrations of substrate (POD) by varying the concentration of the mediator 4-aminophenylamine hydrochloride (ADPA) and of the hydrogen peroxide (H_2O_2). Figure 4 shows that an increase of signal was achieved from 1530 nA of normal POD substrate to 3971 nA of 6.6 mg ADPA and 600 mM of H_2O_2 by increasing concentrations of ADPA and H_2O_2, simultaneously. The highest signal was obtained with 6.6 mg of ADPA and 600 mM of H_2O_2, however, also the signal of the negative control increased from 38 nA to 203 nA.

Development of a multiprobe chip

Design of the multiprobe chip - A disposable multiprobe chip was designed from iSiTEC GmbH (Bremerhaven, Germany) with the size of a conventional glass slide and produced by GEM (UK). The multiprobe chip consisted of a carrier material that contains 16 gold working electrodes, each with the size of 1.5 mm and a combined counter/reference electrode above the electrode array (Figure 5). Working and counter/reference electrodes are encircled with a dielectric layer. The stems of the electrodes fit to a typical connecting strip.

Signal transmission between working electrodes - Every second working electrode (e.g. WE 2, 4, 6) of a multiprobe chip with plastic carrier material was spotted by hand with thiolated probe. Signals were detected only for the spotted working electrodes (Figure 6); non-coated electrodes gave signals from 62 nA to 129 nA. There was no signal transmission between the electrodes. The signals are in average 3x lower than the signals for the single electrode sensors because of the smaller diameter of the electrodes.

Selection of carrier material for multiprobe chips - Two different carrier materials (plastic and ceramic) were chosen for comparison of spotting properties and signal intensities. Additionally two variations of the ceramic were tested, a plain ceramic material and ceramic with a hydrophobic polymer. Figure 7 shows the signal intensity for the different carrier materials. Plastic showed signals from 716 nA up to 1099 nA with a mean signal of 913 nA, the ceramic with hydrophobic polymer signals from 728 nA to 1324 nA with a mean of 937 nA whereas the plain ceramic showed the lowest signals from 421 nA to 1296 nA with a mean of 602 nA. The plastic material showed a higher stability of probe drops during spotting, whereas the plain ceramic cannot be spotted with a biodot because of the hydrophilic

properties of the ceramic (data not shown). Ceramic with a hydrophobic polymer underneath the working electrodes shows good stability of probe drops (data not shown). During the experiments the plastic material was found to be difficult to cut into the correct size and spotted with probes because of material plasticity.

Development of a semi-automated device

Development of lysis protocol - The current protocol using a kit for total RNA isolation requires trained personnel and simplification is crucial for the use of the semi-automated device. Two different lysis buffers were tested for their lysis properties and the signal formation in combination with two different hybridization buffers. For comparison of the signals negative and positive controls as well as hybridization with target rRNA were carried out (Figure 8). The signals of all 16 electrodes were averaged out for the different experiments and compared. All experiments with lysis/hybridization buffer combinations and total rRNA showed similar signals. 4x hybridization buffer in combination with lysis buffer 1 achieved the highest mean signal with 554 nA, whereas in combination with RLT buffer from the Qiagen Kit, the lowest signal (365 nA) were detected. Sample buffer in combination with RLT buffer showed a similar signal of 518 nA to the 4x hybridization buffer/lysis buffer 1 combination. Sample buffer with lysis buffer 1 achieved a mean signal of 462 nA.

Development and adjustment of semi-automated device - A semi-automated portable device, named ALGADEC, was developed by iSiTEC GmbH (Bremerhaven, Germany) and the Alfred Wegener Institute (Bremerhaven, Germany) during the EU-Project ALGADEC (Figure 9). The device contains reservoirs for antibody, substrate and washing buffers as well as a flow cell unit for hybridization. The flow cell unit and an additional inlet for applying the samples can be heated and cooled to the required temperatures during the analysis procedure. A peristaltic pump moves the reagents through the flow cell and finally into the waste reservoir (Figure 10). The main steps of the analysis process can be executed automatically in the measurement device. A flow chart was developed for the varying processes (e.g., hybridization, wash steps, antibody incubation and measurement) and pump times were adapted. Adjustment of the semi-automated device was conducted using multiprobe chips with the probe set for *Alexandrium minutum* and Test-DNA as target for the probes. The disposable multiprobe chip was inserted into the flow cell unit before analysis was started. During measurement of the electrochemical reaction, the signals from the working electrodes

with probes are recorded by a microcontroller unit. Process data can be visualized with special software programmed by iSiTEC GmbH if a PC is connected to the system. Graphic results and the measured values are stored on the hard disc. The portable ALGADEC device can be operated as a stand-alone system with a built in keypad, display, power supply and memory card. A waterproofed case protects the system and allows its use under adverse conditions.

Hybridization of target RNA on multiprobe chips - Hybridizations with two different concentrations of target rRNA from *A. minutum*; a negative and a positive control were carried out in the semi-automated device. The measurements were started when washing buffer was still present in the flow cell unit. After approximately 150 seconds of measurement, substrate buffer arrives in the unit and was pumped continuously through it. Redox-reaction takes place and the signals decreased; however, saturation of the reaction was observed after approximately 500 seconds. The highest signals were found for the positive control with a mean signal of 265 nA and for all electrodes after 500 seconds of measurement (Figure 11, A). At the same measurement point, signals for the negative control (Figure 11, B), high RNA concentration (Figure 11, C) and low RNA concentration (Figure 11, D) were observed from 104 nA, 201 nA and 106 nA, respectively.

Hybridization of dissolved cells on multiprobe chips - 500,000 cells from *Alexandrium minutum* were dissolved in lysis buffer, mixed with hybridization solution and analyzed in the device. Both analyses display higher signals at the beginning of the measurements (Figure 12, A+B), than the experiments described above. However, the mean signals of all 16 electrodes of the analyses at 500 seconds were found to be 158 nA and 148 nA, respectively.

Discussion

Design of sensors and comparison of immobilization protocols - Two materials for sensors were tested and an immobilization protocol for gold sensors was developed and tested. Immobilization of probes to gold surfaces was already established (Carpini et al. 2004; Mannelli et al. 2005) and the described methods were adapted to the gold sensors with some modifications. The signal formation of a gold or carbon covered surface was similar and efficient. Signal comparison of long term stability tests showed that the signals of carbon and gold sensors decreased over several month of storage about 45 % and 26 %, respectively, but

stored gold sensors performed better and achieved higher signals. Long term storage enables the production and coating of sensors in advance of use. Experiments with higher substrate concentrations revealed the potential of enhanced signals. A substrate concentration of 2.2 mg ADPA and 200 mM of H_2O_2 would be sufficient for a twofold signal increase. However, the immobilization protocols for the different sensors have advantages and disadvantages concerning costs. One advantage of the carbon sensors is the lower price of the carbon paste in comparison to the gold paste. Gold sensors have the benefit that the coating with expensive NeutrAvidin can be omitted given that thiolated DNA probes bind directly to the surface of the gold. Because the gold sensors required fewer immobilization steps in the protocol, thus reducing manufacturing costs and produced higher values during long term-storage, the gold sensors were chosen over the carbon sensors for the further development of a biosensor.

Development of multiprobe chip - A multiprobe chip was designed from iSiTEC GmbH with 16 gold electrodes, that can detect 16 different target species. The chip was developed with the size of a conventional glass slide, which offers the possibility to use automated dispensing systems for the spotting of probes. Furthermore, the chips are easy to handle because of their size and can be stored in standard boxes. The size of the working electrodes was reduced in comparison to the single electrode sensors to decrease the electrode area and consequently the amount of reagents needed for analysis. Signal transmission between the electrodes was assessed and only background noise was determined. Different carrier materials for the electrodes were investigated for signal formation and probe spotting properties. Plastic material showed high signals and can be spotted with probes by hand, however, the signals from electrodes spotted with probes by hand were irregular and unacceptable. Automated spotting with a dispenser requires accurately cut chips and the plastic material was easily bent, making this material unsuitable. To overcome this problem, the ceramic carrier material was chosen for the multiprobe chips. It could be accurately cut and thus be spotted with an automated dispenser. The experiments with the ceramic chips showed lower signals than the plastic material because the hydrophilic surface hampered the spotting. Finally the addition of hydrophobic polymer overcame this last problem. However, during the manufacturing of these chips, difficulties using the automated dispenser for probe spotting occurred and the multiprobe chips for our device tests presented here were hand-spotted. We anticipate that in the production of these chips for commercial use that all spotting difficulties encountered here will be overcome.

Development of a semi-automated device - The methods described in the first part of our study involve the isolation of total rRNA from the algal cells. The proposed use of our device by inexperienced users meant that we needed to simplify the rRNA extraction method. A lysis protocol was successfully developed to circumvent manually rRNA isolation. The combination of our 4x Hybridization buffer with lysis buffer 1 resulted in high signals and can be inexpensively produced. Thus, all required steps for the automated the detection of toxic algae were achieved. A portable device was developed during the EU-project ALGADEC, which can be used as a stand-alone system in the field (e.g., on ships or shores) as well as in the laboratory. The device is easy to handle even for laymen and sample analyses with all required steps can be performed automatically in less than two hours. Only the water sample has to be filtered by hand by the user and placed in the inlet of the device. Data are stored in the microcontroller unit or, if attached to a PC, can be analyzed directly. Multiprobe chips and the ALGADEC device were tested using isolated RNA and cells from *Alexandrium minutum* and the data were compared. The signals for comparison were chosen after 500 seconds of measurement because saturation of the reaction was observed. Hybridizations with two different concentrations of target rRNA, high and low, from *A. minutum* were carried out. Clearly distinguishable signals were determined for low and high concentration of rRNA; a low rRNA concentration resulted in signals in the range of the negative control and was consequently at the detection limit of the probes for *A. minutum*. A high rRNA concentration gave mean signal of 201 nA. When compared to hybridization signals for dissolved cells of *A. minutum* decreased signals (mean signal 150 nA) can be observed. The isolated rRNA with a high quality originated from about 260,000 cells, whereas the filtered cell lysate of 500,000 cells contained also the proteins and polysaccharides, which can disturb the hybridization immense. Additionally a field sample with *Pseudo-nitzschia* cells from the Orkney Islands, United Kingdom, was tested with a multiprobe chip coated with the genus probe for *Pseudo-nitzschia* (data not shown). The analysis revealed a strong positive signal for *Pseudo-nitzschia*. Hence, the semi-automated device in combination with multiprobe chips can also be successful used for the analysis of field samples.

Forthcoming research - The sensitivity of the system has to be optimized and the detection limit must be reduced, because when a cell count of the toxic algal cells is reached, then the fisheries are closed. We must have a detection limit far less than this number to meet monitoring requirements. To meet these requirements, several adaptations must be made. The spotting of the multiprobe chips with probes has to be automated to achieve a regular signal

formation. We plan to spot different probes, i.e. species onto the chip, thus chips specific for different geographic areas can be developed. Several specific probe sets for toxic algae have been developed and need to be adapted to the chips. Furthermore, the sensors must be calibrated for each probe set to convert the electronic signal into concentration of toxic cells with the help of the software.

Conclusion

A multiprobe chip with 16 gold electrodes was designed and adapted for the use in a sandwich hybridization assay. Furthermore, a portable semi-automated device was developed that automatically processed the main steps of the analysis and facilitated the electrochemical detection of toxic algae in less than two hours. The device can be used by laymen because a manual RNA isolation is not longer required with the development of a lysis protocol. The proof of principle was presented here. The multiprobe chip and the ALGADEC device can be used as stand-alone system in the field and will contribute to monitoring programs to provide an early warning system for the aquaculture and tourist sectors who are most affected by toxic algal blooms.

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Table 1. Sequences of capture and detection probes and positive control for *Alexandrium ostenfeldii* and *A. minutum*

Probe name	Probe sequence	Target	Citation
Detection probe: AOST1	CAA CCC TTC CCA ATA GTC AGG T	*Alexandrium ostenfeldii* 18S	Metfies et al. (2005)
Capture probe: AOST2	GAA TCA CCA AGG TTC CAA GCA G	*Alexandrium ostenfeldii* 18S	Metfies et al. (2005)
Test DNA AOST (positive control	CTGC TTG GAA CCT TGG TGA TTC ACCT GAC TAT TGG GAA GGG TTG		Metfies et al. (2005)
Capture probe: AMIN C	GAA GTC AGG TTT GGA TGC	*Alexandrium minutum* 18S	Publication I
Detection probe: AMIN C NEXT	TAA TGA CCA CAA CCC TTC C	*Alexandrium minutum* 18S	Publication I
Test DNA AMIN (positive control)	GCA TCC AAA CCT GAC TTC GGA AGG GTT GTG GTC ATT A		Publication I

Table 2. Contents of lysis buffer 1 and hybridization buffers

Buffers	Chemical
Lysis buffer 1, pH 11	4 M guanidin-isothiocyanat
	25 mM sodium citrate
	0.5 % Sarcosyl [w/v]
4x Hybridization buffer, pH 8	0.3 M NaCl
	80 mM Tris
	0.04% SDS
Sample buffer, pH 7.5	100 mM Tris
	17 mM EDTA
	5 M Guanidine isothiocyanate
	8.35% Formamide

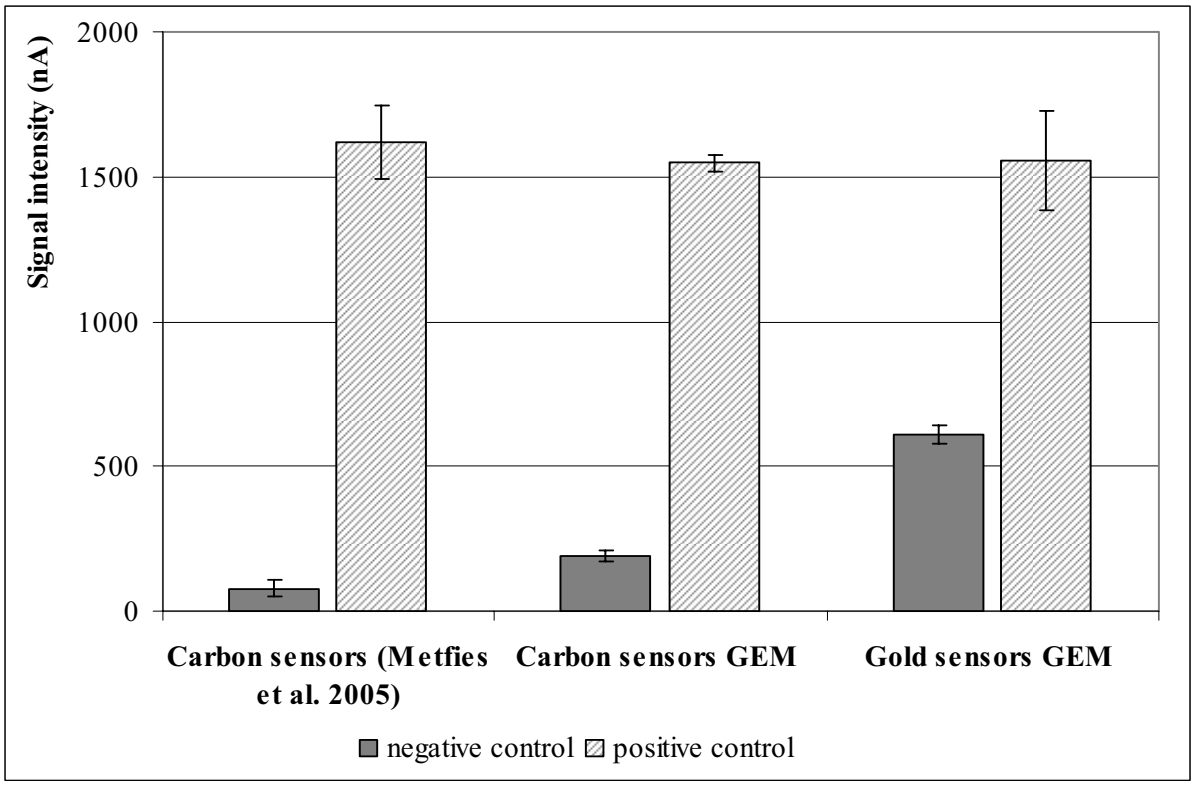

Figure 1. Comparison of signal intensity of carbon and gold sensors was done using probes AOST2 and AOST1 and test-DNA

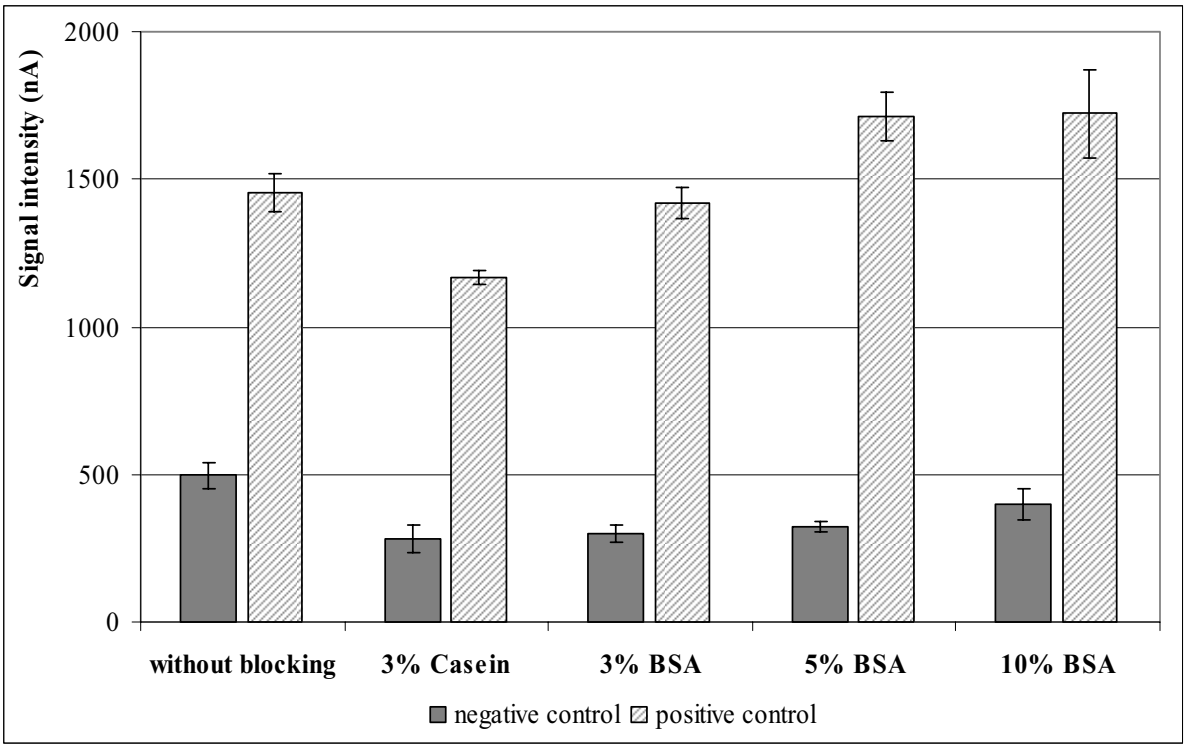

Figure 2. Reduction of background signal by using casein and bovine serum albumin as blocking solutions on gold sensors coated with the thiolated probe AOST2

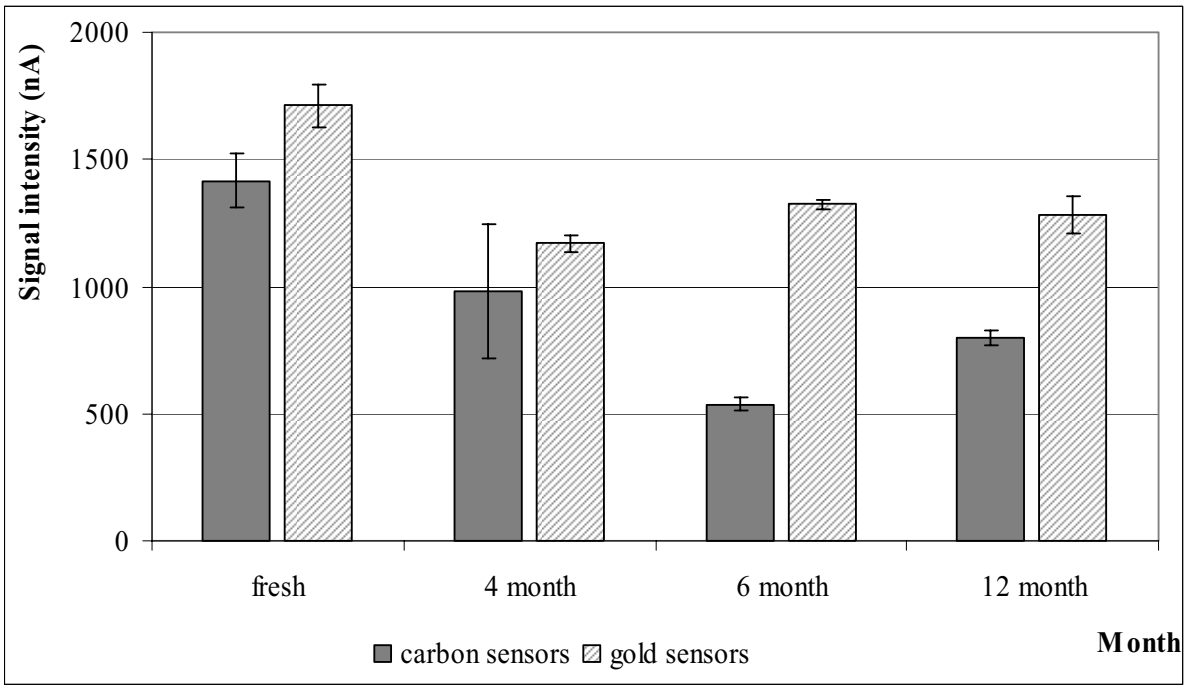

Figure 3. Long term stability of carbon and gold sensors after coating with 2% Trehalose and storage at 4°C over indicated intervals

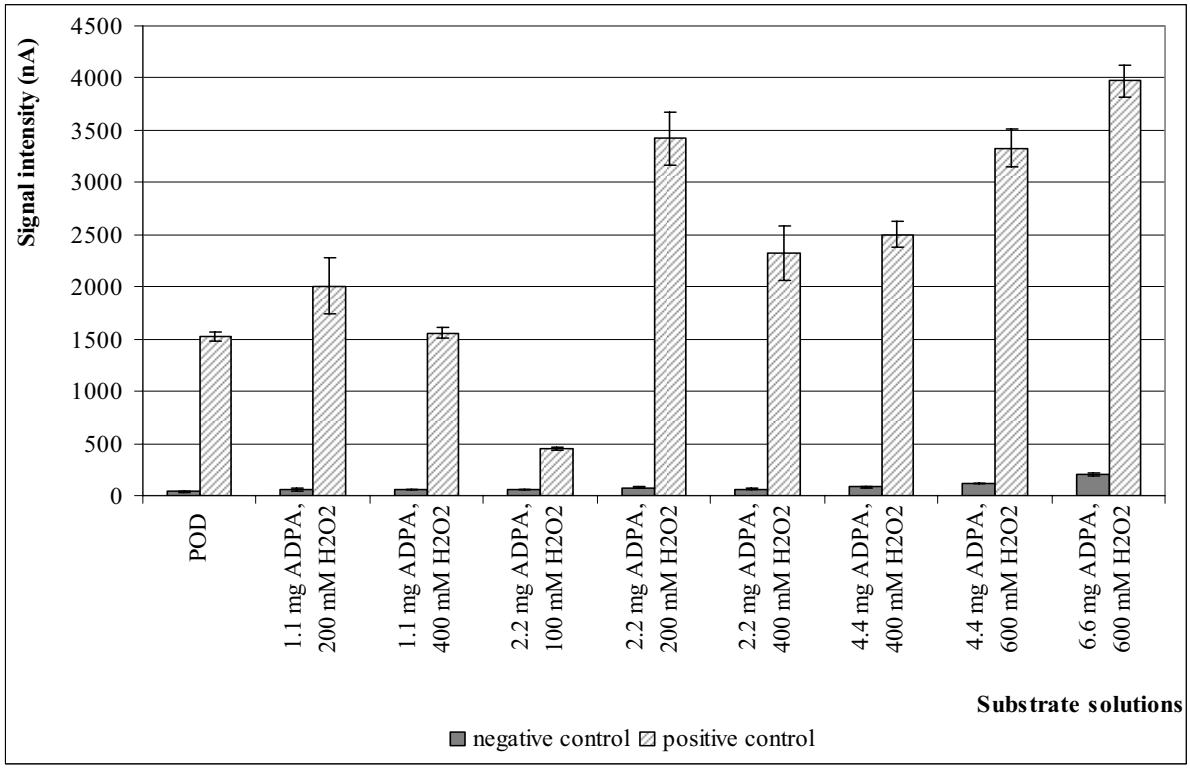

Figure 4. Signal enhancement by varying substrate concentrations

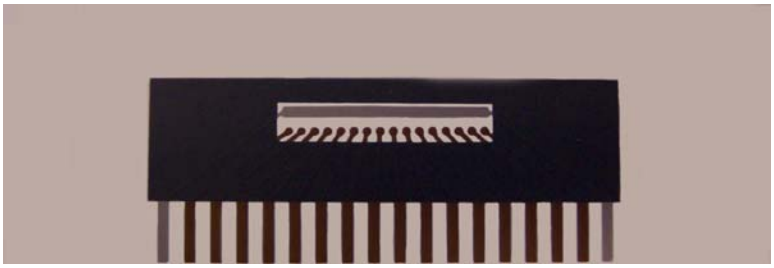

Figure 5. Multiprobe chip with 16 gold working electrodes

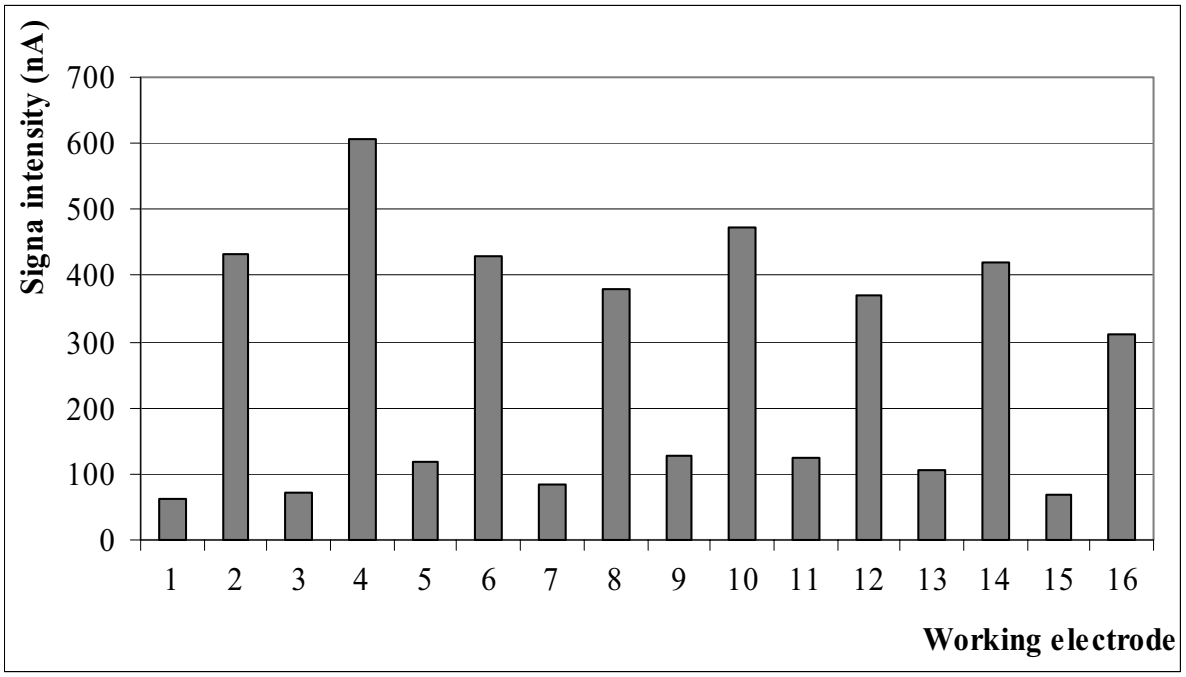

Figure 6. Signal transmission between working electrodes with every second electrode coated with thiolated probe AOST2

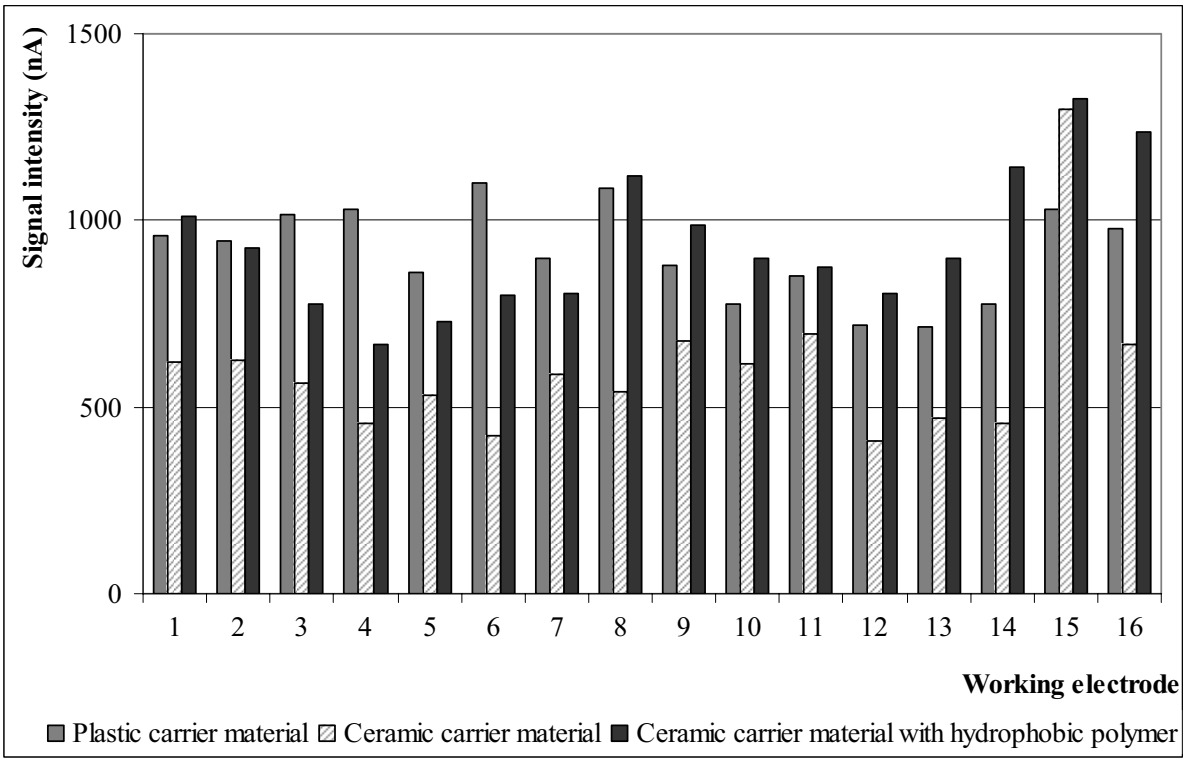

Figure 7. Comparison of hand-spotted multiprobe chips with different carrier materials

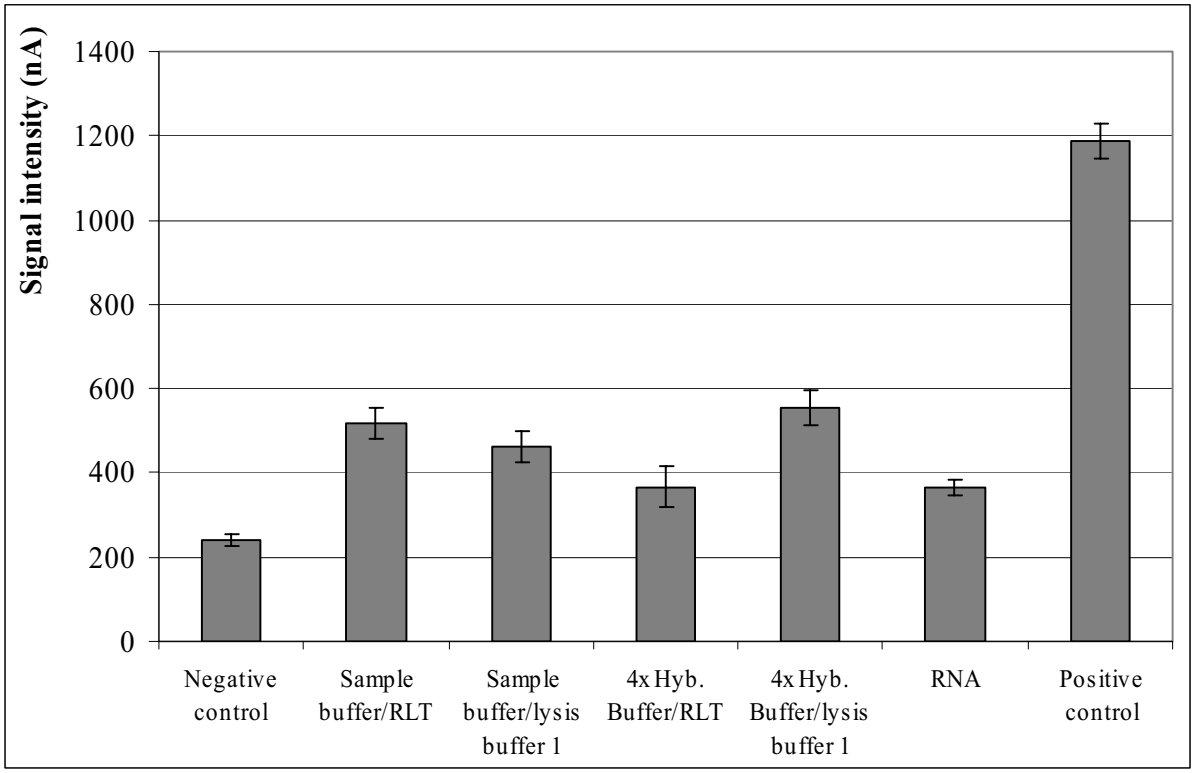

Figure 8. Determination of optimal signal formation using two different lysis and hybridization buffers and probes for *Alexandrium minutum*

Figure 9. Semi-automated portable ALGADEC device

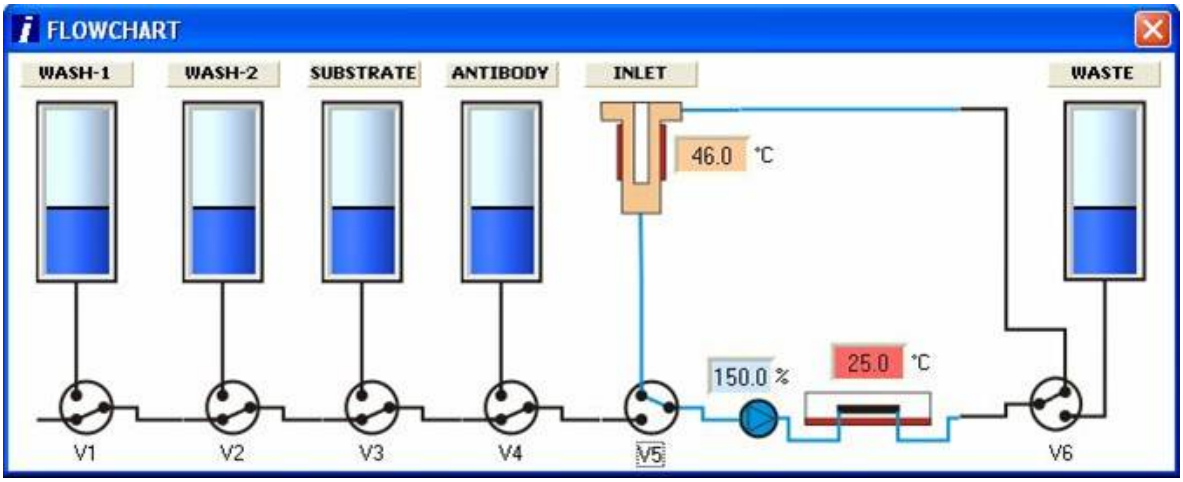

Figure 10. Flow chart of semi-automated device

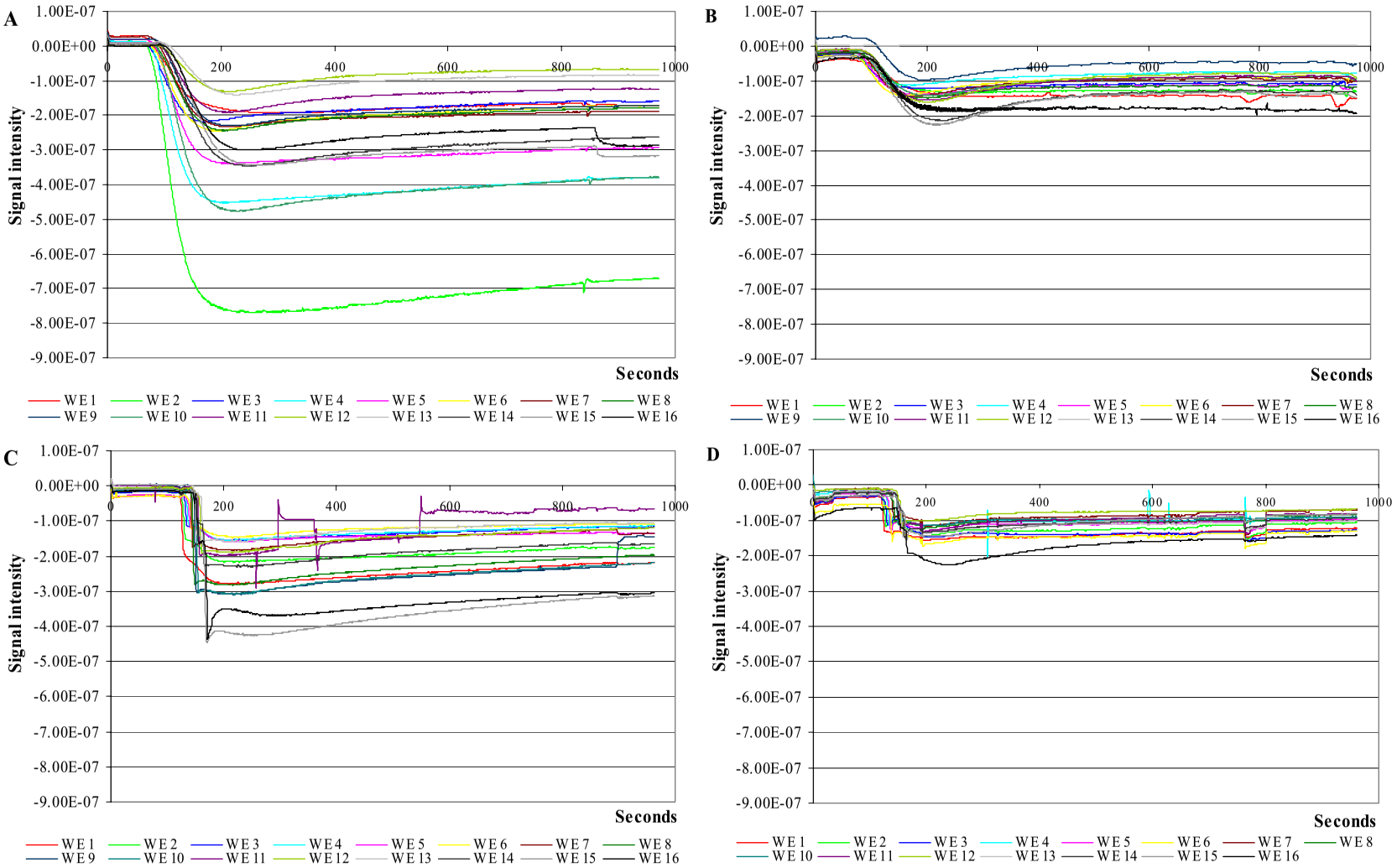

Figure 11. Hybridisation of different targets onto the multiprobe chips in the semi-automated device. (A) Positive control, (B) Negative control, (C) Target RNA with a final concentration of 7.32 µg, (D) Target RNA with a final concentration of 4.95 µg

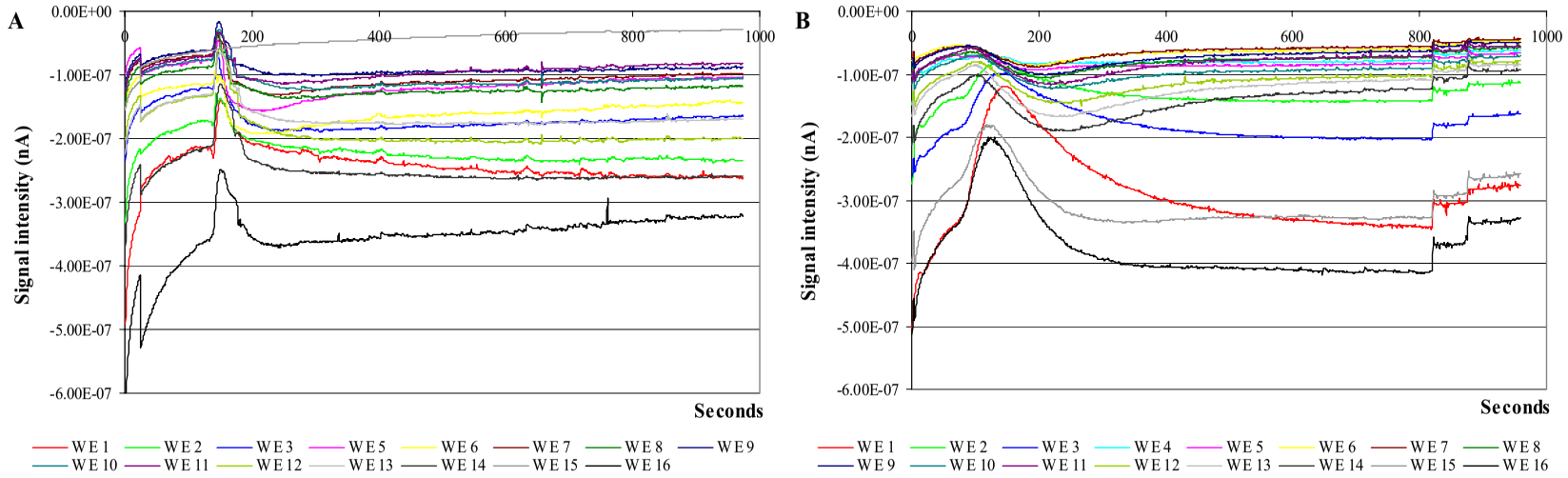

Figure 12. Hybridisation of lysed cells onto the multiprobe chips in the semi-automated device. (A+B) 500,000 lysed cells of *Alexandrium minutum*

3. Synthesis

3.1 Colorimetric assay for the detection of toxic algal species

The composition of phytoplankton communities in Europe includes several genera of toxic algal species, such as *Alexandrium*, *Dinophysis*, *Gymnodinium* and *Pseudo-nitzschia* (Simon et al. 1997; John et al. 2003; Moita et al. 2003; Chepurnov et al. 2005). Monitoring programs aim at the rapid and reliable detection of harmful algae in coastal areas and shellfish and fish farms. Morphological identification and enumeration of harmful species using standard microscopy procedures are time-consuming and a broad taxonomic knowledge is required. For example *Alexandrium minutum* is characterized by minute details of its thecal plates and thus, is difficult to distinguish from other species of the same genus (Taylor et al. 1995). Molecular technologies and molecular probes, that target the large or small subunit ribosomal RNA (rRNA), are rapidly improving the detection of phytoplankton, because the applications are based on the discrimination of the genetic differences of the different species. Down to the present day the development molecular probes is limited to only a small percentage of the different toxic algal species.

In **Publication I** the detection of the toxic dinoflagellate *Alexandrium minutum* was conducted by the use of molecular probes in sandwich hybridization. Two probes are needed in the sandwich hybridization format (Zammatteo et al. 1995; Rautio et al. 2003), and at least one of the probes has to be specific for the target. The so-called capture probe is immobilized on solid surfaces as in combination with DNA biosensors (Metfies et al. 2005) and binds to target RNA or DNA. A signal moiety is covalently bound to a second probe, which binds in close proximity to the binding site of the capture probe. A commercially available PCR ELISA Dig Detection Kit was adapted for the use of sandwich hybridization. A set of two 18S ribosomal RNA probes for the species-specific identification was developed for *A. minutum.* The capture probe was biotin-labelled and the signal probe digoxigenin-labelled for the application in the sandwich hybridization. Probe specificity was successfully demonstrated with the microtiter plate assay; because the signals for all *A. minutum* strains were always significantly higher than the signals for the non-target species. It was also pointed out that *Alexandrium* species with a single mismatch in the target sequence were not detected. For the application of the assay as a monitoring method, achieved signals need to be correlated to cell numbers. Bloom development in the field is expected to correspond most

closely to optimum growth conditions (Ayers et al. 2005). Hence, total rRNA concentration per cell of *A. minutum* was determined at optimum growth conditions for three different strains and a mean concentration of 0.028 ng rRNA per cell was found. Similar findings were achieved from Metfies et al. (2005) for *A. ostenfeldii* and for different growth conditions of *A. minutum* (personal communication L. Carter, Westminster University, UK). Consequently, the calculation of the cell numbers from the isolated rRNA concentration is possible. A standard calibration curve for *A. minutum* was investigated for the assay, resulting in a good correlation of signal with rRNA concentration. Thus, cell numbers can be calculated from the signal intensity of the assay. Natural water samples were spiked with different algal cells to evaluate the potential of the microtiter plate assay for the monitoring of field samples. The results demonstrate that the molecular assay was capable of detecting *A. minutum* cells at different cell counts in the presence of a complex background. However, in comparison to a pure culture with the same number of cells, lower signals were achieved for the spiked samples. One reason for the different hybridization signals could be the composition of the sample, because large amounts of sediment were observed at the sampling station. Sediment seems to disturb the RNA isolation and the isolation protocol needs to be modified. Nevertheless, the adaptation of the commercially available PCR ELISA Dig Detection Kit was successful and the experiments with spiked natural samples present a promising proof of principle.

In this study a method for the detection of the toxic dinoflagellate *Alexandrium minutum* using 18S rRNA probes and a microtiter plate assay was established. This method has the potential to be a fast and reliable method for the detection of toxic algae by eliminating the need for manual algae counts. Furthermore, a rapid assay was developed for the routine testing of probe specificity at both the clade and target sequence level much in the same way that dot blots are used to screen for specificity of FISH probes.

The microtiter plate assay developed in **Publication I** was applied for further species-specific identification of toxic algal species in **Publication II**. Probe sets for the toxic species *Gymnodinium catenatum*, *Protoceratium reticulatum, Lingulodinium polyedrum, Prymnesium parvum, Chrysochromulina polylepis*, *Pseudo-nitzschia multiseries, P. australis, P. seriata and P. pungens* were designed from a database consisting of more than 3000 published and unpublished algal 18S rRNA sequences. Single probes for some species had already been developed and tested for specificity by means of dot blot and FISH (Simon et al. 1997; Töbe

et al. 2006). Hence, for these only a second probe was designed to complete the sandwich hybridization and the combination of both of the probes needed to be tested for specificity. A probe set for the genus of *Pseudo-nitzschia* species was previously designed and needed to be adapted. The large and the small subunit ribosomal RNA genes are the targets for the different probe sets. The sandwich hybridization in the microtiter plate assay was applied for the specificity tests. Target rRNA was isolated from laboratory strains of the target species and closely related species. A total of ten probe sets for different toxic algal species were designed and tested and eight probe sets proved to be highly specific. Two probe sets with the target species *Gymnodinium catenatum* and *Prymnesium parvum* detected one non-target species in addition to the target species, respectively the non-toxic *G. impudicum* and *P. nemamethecum*. The specificity of the probes is dependent on the number of sequences of the targeted gene available in databases. However, even if a probe is designed from a large database, such as the used database, it is almost impossible to avoid the occurrence of some false-positive results with a monostringent hybridization approach. The *in silico* prediction of the stability of mismatched probe-target hybrids is difficult and influenced by many factors, such as the number of mismatches, the nature of the mismatching nucleotides, the position of the mismatches in the probe target site, and possible stacking interactions of nucleotides adjacent to the mismatches (Loy et al. 2005b). The protocol applied in this study using specific hybridization temperatures is optimized for the microtiter plate assay as well as for the biosensor described in **Publication V**. However, specific identification of target organisms is still possible with the probes sets for the species described above. The probe set for *Gymnodinium catenatum* presents significant high signals for only 10,000 cells, whereas for the same signal intensity at least 250,000 cells of the non-target species *G. impudicum* are needed, thus a misinterpretation of signal is unlikely. The detection of *P. nemamethecum* in brackish waters with the probe set for *P. parvum* cannot be ruled out but seems to be unlikely, because the majority of *P. parvum* blooms have been recorded in brackish waters (Edvardsen and Paasche 1998) and there have not been any reports of blooms caused by *P. nemamethecum* (West et al. 2006).

The ten probe sets presented in this study are valuable tools for identifying and monitoring different toxic algae and can be adapted to the multiprobe chip and the semi- automated biosensor presented in **Publication V**. Furthermore, the specific capture probes can also be adjusted to other molecular methods using ribosomal RNA probes, such as the DNA-microarray technology, real-time PCR or FISH.

In summary, a commercially available PCR ELISA Dig Detection Kit was successfully adapted for the detection of the toxic dinoflagellate *Alexandrium minutum* by application of ribosomal 18S probes and sandwich hybridization. The mean concentration of total rRNA per cell of was determined and a standard calibration curve for different RNA concentrations and thus cell counts was investigated for the assay. Additionally the assay was able to detect *A. minutum* cells at different cell counts in the presence of a complex background. This represents the potential to serve as a fast and reliable method for the detection of toxic algae by eliminating the need to count algae manually. Furthermore, the assay showed the specificity of 10 additional probe sets for the toxic algal species *Gymnodinium catenatum*, *Protoceratium reticulatum, Lingulodinium polyedrum, Prymnesium parvum, Chrysochromulina polylepis*, *Pseudo-nitzschia multiseries, P. australis, P. seriata and P. pungens* and the genus *Pseudo-nitzschia*.

3.2 Assessment of signal enhancement

DNA-biosensors are commonly used in clinical diagnostic (e.g., glucose detection), environmental monitoring (e.g., hazardous chemicals), identification of infectious organisms and forensics. Biosensors are also commonly used for the rapid identification of aquatic microorganisms. The identification of the toxic dinoflagellate *Alexandrium ostenfeldii* using a biosensor was presented by Metfies et al. (2005). The advantage of biosensors is displayed by the *in situ* use and therefore the circumvention of sample transport to the laboratory. Therefore, this technique is suitable for the application in monitoring programmes, because of the simple use and analysis. Another potential method is the DNA-microarray-technology, that enables the simultaneous analysis of up to 250,000 probes (Lockhart et al. 1996) and can be used as a method to analyse samples from complex environments (Metfies and Medlin 2004; Medlin et al. 2006). However, the reliable identification of harmful algal species with probe-based methods requires highly specific and sensitive probes and high quality nucleic acids.

In **Publication III** the method for the electrochemical detection of toxic algae is presented, the steps were described in detail and visualized for easy application by inexperienced users. Several modifications were established to the protocol described by Metfies et al. (2005). First of all, different manufacturers were located for the production of the single electrode

sensors and the measurement device for the improvement of the biosensors. In addition, the total RNA isolation protocol from Qiagen (Hilden, Germany) was modified to increase the removal of polysaccharides and proteins to improve quality and quantity of the extracted rRNA. The improved quality of the rRNA led to an enhanced signal intensity of the electrochemical measurements. The improved method was tested with spiked field samples in an intercalibration workshop (Godhe et al. 2007). In **Publication IV** a higher signal was also determined for undesired cross-hybridization of *A. ostenfeldii* probes to *Alexandrium minutum* in comparison to the data previously presented by Metfies et al. (2005). This represents no drawback of the probe detection of *A. ostenfeldii*, because also *A. minutum* is a toxic species and needs monitoring. On the multiprobe chip, presented in **Publication V**; a combination of probes will facilitate the differentiation of both species. The introduction of locked nucleic acids (LNAs) promises an enhancement of both specificity and sensitivity of molecular probes (Kongsbak 2002). LNAs have shown their potential in many applications, such as gene expression profiling, genotyping assays, fluorescence *in situ* hybridization and real-time PCR (Jacobsen et al. 2002b; Nielsen and Kauppinen 2002; Silahtaroglu et al. 2003; Ugozzoli et al. 2004b). Many publications describe the enhancement of probe or hybridization signals with LNA modified probes, but there has been no rigorous testing of these probes using known target sequences. The specificity and sensitivity of conventional molecular probes and LNA modified probes were compared in **Publication IV** by application of sandwich hybridization on biosensors and on DNA-microarrays. Three different species, *A. ostenfeldii*, *A. minutum* and *A. tamutum*, were tested with conventional and LNA modified probes on the biosensor. In addition to the cross-hybridization signal for *A. minutum* also *A. tamutum* was tested for signal formation, because of only one mismatch in the 18S rRNA sequence to the capture probe. Kauppinen et al. (2003) suggested that the use of LNAs could significantly increase mismatch discrimination (Kauppinen et al. 2003). Previously, several probes were successfully adapted to the DNA-microarray but, because of low signal intensities, an enhancement of the signal-to-noise-ratios using LNAs was evaluated. The present study revealed that the LNA probes showed neither signal enhancement using the rRNA biosensor, nor discrimination of only one mismatch. The DNA probes showed equal or better results in all experiments using the biosensor, whereas LNA probes could enhance the sensitivity of the microarray and gave higher signals than the conventional probes. However, unspecific binding with non-target DNA was also enhanced. In conclusion the LNA probes do not improve signal intensity under at these solid surface-hybridization applications. Other potential application for signal enhancement of the biosensor could be the variation of

substrate concentration and the reduction of background noise with blocking solutions. Signal enhancement in case of the microarray can be accomplished by using labelling kits that incorporate multiple labels to a target or also the reduction of background noise.

In summary, the total rRNA isolation protocol was improved and the hybridization procedure for the electrochemical detection of toxic algal species was described in detail and illustratively visualized for easy application by inexperienced users. Furthermore, locked nucleic acid probes were tested with known target sequences and the specificity and sensitivity was compared to signal formation of conventional molecular probes. The hybridization signals for both of the tested solid surface methods could not be enhanced and the conventional DNA probes showed equal or better results.

3.3 Detection of toxic algal species using multiprobe chips and a semi-automated device

The increasing demand for fast monitoring techniques emerges from poisoning incidences and economic losses, which cannot be foreseen because of sample analysis taking up to five working days. In addition, the statutory method for shellfish flesh analysis, the mouse-bioassay, induces ethical problems. HPLC as well as traditional light microscopy methods are time-consuming and need high trained personnel. Furthermore, the samples have to be transported to specialized laboratories for analysis. A potential tool for bloom formation and thus potential shellfish contamination is provided by the *in situ* investigation of coastal water for the occurrences of different toxic algae. There are examples for on-site monitoring of toxic algae, such as the environmental sampling processor (Doucette et al. 2006; Silver 2006).

In order to facilitate the *in situ* monitoring of toxic algae, a multiprobe chip and a semi-automated rRNA biosensor for the in situ detection of toxic algae were developed and evaluated in **Publication V**. Simultaneous detection of different species can be accomplished using arrays of probes, such as microarrays (Metfies and Medlin 2004). A multiprobe chip with an array of 16 gold electrodes for the simultaneous detection of up to 14 algal target species was designed in this study. For a simplification of sensor handling the standard size of a conventional glass slide was chosen and thus can be stored in standard boxes. Different materials for the electrodes and the carrier material were tested in order to achieve a

multiprobe chip that can be coated with probes automatically. The mass production and coating of sensors in advance of use are able to decrease the costs; consequently the probes have to be stable on the electrode surface and need to give same signals after several months of storage. Long-term storage experiments showed that the sensors are stable over a year, however a signal decrease of 26 % was observed. In order to facilitate a cost efficient method the size of the working electrodes was reduced in comparison to the single electrode sensors used from Metfies et al. (2005). Hence, the electrode area is decreased and the amount of reagents needed for analysis is reduced.

So far, all monitoring methods demand high trained personnel for sample analysis. Experienced users are needed for the isolation of total rRNA from the different algal species for analysis using the biosensor. Scholin et al. (Scholin et al. 1999) reported the use of crude cell homogenates for the detection of *Pseudo-nitzschia* in sandwich hybridization assays. For the use of the biosensor by layperson an adaptation of analysis and hybridization procedures was required. An easy to use lysis protocol was successfully developed, thus manual rRNA isolation is no longer necessary, only water sample filtration has to be performed manually. For the simultaneous detection of several toxic species, a multiprobe chip with 16 working electrodes was generated. An automated, portable device, which is easy to handle even for laypersons, was designed and extensively tested in combination with the multiprobe chip and molecular probes for *Alexandrium minutum*. Isolated RNA and cells from *Alexandrium minutum* were analyzed with the device and the data was compared. It was observed that after 500 seconds of measurement a saturation of the reaction takes place. Clearly distinguishable signals were determined for low and high concentration of rRNA and when compared to hybridization signals for dissolved cells of *A. minutum*, decreased signals were observed. The signal variations can be explained by the quality of the analyzed sample. The isolated rRNA had a high quality, whereas the crude cell lysate contains still proteins and polysaccharides, which can disturb the hybridization. During a demonstration of the device to mussel farmers, a field sample containing *Pseudo-nitzschia* cells was analyzed and resulted in a strong positive signal for *Pseudo-nitzschia*. Hence, the device is able to contribute to monitoring programs to provide an early warning system for the aquaculture and tourist sectors, which are affected by toxic algal blooms the most. The probes presented in **Publication I** and **Publication II** can be adapted to the use on the multiprobe chip, thus area chips for different regions in Europe can be developed.

In summary, a stand-alone, semi-automated system in combination with multiprobe chips was developed. A multiprobe chip with 16 gold electrodes was designed and adapted for the use in a sandwich hybridization assay. Long-term stability of the sensors was examined and the sensors found to be stable over a year. A lysis protocol was adjusted and manual RNA isolation is not longer required. Analysis of different concentrations of isolated total rRNA and crude cell lysates revealed clearly distinguishable signals, but lower signals for the cell lysate. The analysis of all samples was performed in less than two hours with the semi-automated device in comparison to other routine monitoring methods that need at least a day for analysis.

4. Future Research

Two potential monitoring methods for toxic algal species were presented in this study. However, both methods can be improved through several measures. The microtiter plate assay presented in **Publication I** was applied for natural water samples and showed a signal reduction in comparison to experiments using pure laboratory cultures. Sediment seems to disturb the RNA isolation; additionally it was observed that total rRNA cannot be isolated from diatoms, such as *Pseudo-nitzschia* spp., using a conventional Kit but can be using a phenol-chloroform method (**Publication II**). An improvement of the existing protocol has to be examined and the development of an independent system without RNA isolation, such as method described by Scholin et al. (1999), should be included in further experiments. The RNA concentration per cell has to be determined for every target species at optimum growth conditions, because this corresponds most closely to bloom development in the field (Ayers et al. 2005). Subsequently, calibration curves allow the correlation of signal to cell numbers. Detection limits of each probe set for the different toxic species have to be identified. Final test of the microtiter plate assay should include field samples.

The sensitivity of the semi-automated system presented in **Publication V** has to be optimised and probes developed in **Publication I** and **Publication II** need to be adapted to the multiprobe chip to allow the development of chips specific for different geographic areas. For this requirement, the probes have to be dispensed onto the multiprobe chips automatically to achieve a regular signal formation. Recommended action cell concentrations are emphasized for the different harmful algal species (Rensel and Whyte 2003), thus the different probes have to be examined for their detection limit. The detection limit needs to be below the allowed cell numbers limits to meet monitoring requirements. Furthermore, the sensors have to be calibrated for each probe set to convert the electronic signal into concentration of toxic cells with the help of the software. Subsequently, field samples need to be tested for evaluation. A total of 17 different probe sets can be applied to the multiprobe chips, however, about 97 toxic species are known today (Moestrup 2004) and the number is increasing. Consequently, further experiments should include the development of new probe sets and their adaptation to the biosensor. The biosensor presented in this study is a prototype and has to be improved in terms of system integration and maintenance for commercial purpose. Furthermore, the device could also be integrated into buoy systems for continuous analysis of

coastal waters. Finally, the biosensor can also be adapted for several other fields, such as the detection of microbial pathogens in water or for clinical diagnostics.

5. Summary

This doctorial thesis aimed at the development and evaluation of fast and reliable monitoring methods using molecular technologies. The detection of harmful algal species in coastal areas and shellfish farms is an important requirement of monitoring programs, because of their responsibility for poisoning of consumers through ingesting contaminated seafood and for fish and shellfish kills. Current monitoring methods include the statutory application of the mouse-bioassay for the monitoring of toxin contamination of shellfish, toxin determination using HPLC and standard light microscopy. The methods are time-consuming, expensive and require high trained personnel. Molecular technologies using probes can improve the detection of phytoplankton.

The first potential method for the detection of harmful algae is presented by an assay that is based on the discrimination of the genetic variation of the different species. The PCR ELISA Dig Detection Kit is commercially available and was adapted for the detection of the toxic dinoflagellate *Alexandrium minutum* using sandwich hybridization. Sandwich hybridization requires two probes for each species, a capture probe and a nearly adjacent signal probe. A set of two probes for the species-specific identification was designed and were found to be highly specific. The mean concentration of total rRNA per cell was determined from three different strains of *A. minutum* and found to be 0.028 ng. A standard calibration curve for different RNA concentrations and thus cell counts was established for the assay. Spiked water samples were used to evaluate the assay and the standard curve. The results demonstrated the ability of the assay to detect *A. minutum* cells at different cell counts in the presence of a complex background. The assay has the potential to be a fast and reliable method for the detection of toxic algae by eliminating the need to count algae manually. The microtiter plate assay was applied for further species-specific identification of the toxic algal species *Gymnodinium catenatum*, *Protoceratium reticulatum, Lingulodinium polyedrum, Prymnesium parvum, Chrysochromulina polylepis*, *Pseudo-nitzschia multiseries, P. australis, P. seriata and P. pungens* and the genus *Pseudo-nitzschia*. Probe sets were designed to target the large or the small subunit ribosomal RNA genes. The specificity of the different probes sets was tested with ribosomal RNA isolated from laboratory strains of the target species and closely related species. Eight probe sets proved to be highly specific in the assay. Two probe sets, GCAT and PRYM 694, detect one other species, in addition to the target species. The designed probe sets

are valuable tools for the monitoring of toxic algae and can also be adapted to the semi-automated biosensor. The microtiter plate assay is an effective and fast method to test probes for use in a sandwich hybridization formats, similar to the way that dot blots are used to screen for specificity for FISH probes.

The detection of toxic algae can also be electrochemically achieved using a biosensor and sandwich hybridization. Biosensors can measure on-site and sample transport to the laboratory is unnecessary. The protocol introduced by Metfies et al. (2005) using a biosensor for the detection of *Alexandrium ostenfeldii* was modified and illustrated for easy application by inexperienced users. The modifications included the adaptation of single electrode sensors and the measurement device from different manufacturer as well as the total RNA isolation protocol. Improved quality of the rRNA led to an enhanced signal intensity of the electrochemical measurements. An enhancement of both the specificity and sensitivity of molecular probes can also be achieved by introduction of locked nucleic acids (LNAs). The specificity and sensitivity of conventional molecular probes and LNA modified probes were compared in two different solid phase hybridization methods; sandwich hybridization on biosensors and on DNA-microarrays. Conventional molecular probes and LNA probes that target *Alexandrium ostenfeldii* were examined for signal formation in combination with the biosensor. In addition to *A. ostenfeldii* also *A. minutum* and *A. tamutum* were tested for cross-hybridization. However, signal enhancement for *A. ostenfeldii* could not be observed. Furthermore, the LNA capture probes could not discriminate only one mismatch in the 18S rRNA sequence of *A. tamutum*. In addition, the conventional probes showed a higher cross-hybridization signal for *A. minutum* in comparison to the data previously presented by Metfies et al. (2005), because of the higher quality of the rRNA. However, both species, *A. ostenfeldii* and *A. minutum* are toxic and need to be monitored. In combination with the DNA-microarrays, the LNA-probes displayed an enhancement of sensitivity, but also more false-positive signals. In summary, the conventional DNA probes showed equal or better results than the LNA probes. LNA technology could not improve signal intensity under certain solid surface-hybridization applications.

In addition to the microtiter plate assay and the single electrode assay, a multiprobe chip and a semi-automated rRNA biosensor for the *in situ* detection of toxic algae were developed. The design of the multiprobe chip with an array of 16 gold electrodes was conducted by testing different materials for the electrodes and the carrier material with the help of single electrode

sensors. The multiprobe chip can detect up to 14 target species using the previously designed molecular probes. An easy to handle, automated, portable device was designed and extensively tested in combination with the multiprobe chip and molecular probes for *Alexandrium minutum*. A peristaltic pump moves the reagents from the reservoirs into the hybridization/fluidic chamber, thus the main steps of the analysis are processes automatically. Furthermore, a lysis protocol was successfully developed for use of the device by inexperienced staff and manual rRNA isolation is no longer required. The device was evaluated using isolated total rRNA and cells from algae cultures and clearly distinguishable signals were determined. The stand-alone system can analyse samples in less than two hours and can be applied in the field. Thus, the device and the multiprobe chip have the potential to serve as an early warning system for the aquaculture and tourist sectors.

6. Zusammenfassung

In dieser Dissertation wurden schnelle und verlässliche Monitoring-Methoden mit Hilfe molekularer Techniken entwickelt und bewertet. Aufgrund der Vergiftungen von Konsumenten durch kontaminierte Fische und Meeresfrüchte, sowie von Fisch- und Schalentiersterben, ist der Nachweis von schädlichen Algenarten in Küstenzonen und in Zuchtgebieten für Meeresfrüchte und Fische eine wichtige Voraussetzung für Monitoring-Programme. Die derzeitigen Monitoring-Methoden beinhalten den gesetzlich vorgeschriebenen Maus-Bioassay für die Überwachung von kontaminierten Meeresfrüchten, den chromatographischen Toxin-Nachweis sowie die Standard-Lichtmikroskopie. Die beschriebenen Methoden sind zeitaufwendig, teuer und verlangen die Erfahrung von geschultem Personal. Der Nachweis von Phytoplankton kann durch den Einsatz von molekularer Techniken und Sonden deutlich vereinfacht und verbessert werden.

Ein auf der Unterscheidung von genetischer Variation der verschiedenen Arten basierender Assay stellt eine erfolgsversprechende Nachweismethode für schädliche Algen dar. Ein handelsüblicher PCR ELISA Dig Detection Kit wurde für den Nachweis der toxischen Dinoflagellate *Alexandrium minutum* durch Sandwich Hybridisierung angepasst. Für jede Spezies werden eine Fänger-Sonde und eine benachbarte Signal-Sonde in der Methode der Sandwich Hybridisierung verwendet. Folglich wurde ein Satz von zwei Sonden für die artspezifische Identifikation entworfen und als höchstspezifisch nachgewiesen. Anschließend wurde die durchschnittliche Gesamt-rRNA-Konzentration pro Zelle anhand von drei

verschiedener Stämme von *A. minutum* erfasst und auf 0.028 ng ermittelt. Für den Assay wurde eine Standard-Kalibrierungskurve für verschiedene RNA- Konzentrationen und den korrespondierenden Zellzahlen erstellt. Eine Bewertung des Assays und der Standard-Kalibrierungskurve wurde mit Hilfe Algen-beimpfter Wasserproben durchgeführt. Die Ergebnisse zeigen eindeutig, dass der Assay in der Lage ist, eine verschiedene Anzahl an *A. minutum* Zellen in einem komplexen Hintergrund nachzuweisen. Der beschriebene Assay hat das Potential einer schnellen und verlässlichen Methode für den Nachweis von giftigen Algen, wodurch eine aufwendige, manuelle Zellzählung vermieden werden kann. Zusätzlich wurde der Mikrotiterplatten-Assay für die artspezifische Identifikation der toxischen Algenarten *Gymnodinium catenatum*, *Protoceratium reticulatum, Lingulodinium polyedrum, Prymnesium parvum, Chrysochromulina polylepis*, *Pseudo-nitzschia multiseries, P. australis, P. seriata and P. pungens* und der Gattung *Pseudo-nitzschia* verwendet. Sonden-Sätze wurden entwickelt, die an die große oder kleine Untereinheiten der ribosomalen RNA-Gene binden. Die Spezifität der verschiedenen Sonden-Sätze wurde mittels isolierter ribosomaler RNA von betrachteten Zielarten und nah verwandter Arten getestet. Acht der Sonden-Sätze erwiesen sich in dem Assay als höchstspezifisch. Zwei Sonden-Sätze, GCAT und PRYM 694, weisen zusätzlich zur Zielart auch eine andere Art nach. Die entwickelten Sonden stellen ein wertvolles Werkzeug für das Monitoring von toxischen Algen dar und können auch für den halbautomatischen Biosensor verwendet werden. Der Mikrotiterplatten-Assay ist eine effektive und schnelle Technik für die Überprüfung der Sondenspezifität für Sandwich Hybridisierungs-Formate, vergleichbar mit der Ermittlung der Spezifität von FISH-Sonden durch Dot Blots.

Der Nachweis von toxischen Algen kann auch elektrochemisch mit Biosensoren und Sandwich Hybridisierung erfolgen. Biosensoren können auch vor Ort für Messungen verwendet werden, folglich entfällt der Transport von Proben ins Labor. Metfies et al (2005) präsentierte ein Protokoll für die Detektierung von *Alexandrium ostenfeldii* mit Hilfe eines Biosensors, welches nun modifiziert und bildlich dargestellt wurde, um den Zugang für Laien zu vereinfachen. Die Modifizierungen beinhalten die Anpassung von Einzelelektroden und eines Messgerätes von unterschiedlichen Herstellern, sowie des RNA-Isolationsprotokolls. Die erhöhte Qualität der rRNA führte zu verstärkten Signalintensitäten der elektrochemischen Messung. Eine Verstärkung von Spezifität und Sensitivität von molekularen Sonden kann auch durch die Verwendung von Locked Nucleic Acids (LNAs) erreicht werden. Die Spezifität und Sensitivität von herkömmlichen molekularen Sonden und LNA-modifizierten Sonden wurde mit Hilfe von zwei verschiedenen Festphasen-Hybridisierungsmethoden, der

Sandwich Hybridisierung auf Biosensoren und den DNA-Mikroarrays, verglichen. In Kombination mit dem Biosensor wurden die herkömmlichen molekularen Sonden und die LNA Sonden mit *Alexandrium ostenfeldii* als Zielart auf ihre Signalbildung untersucht. Zusätzlich zu *A. ostenfeldii* wurden auch *A. minutum* und *A. tamutum* auf Kreuz-Hybridisierung überprüft. Es konnte jedoch keine Signalverstärkung für *A. ostenfeldii* beobachtet werden. Des Weiteren war mit den LNA-Fänger-Sonden eine Unterscheidung von nur einer Fehlpaarung in der 18S-Sequenz von *A. tamutum* nicht möglich. Darüber hinaus wurde im Vergleich zu den Daten, die von Metfies et al (2005) vorgestellt wurden, eine erhöhte Kreuz-Hybridisierung für *A. minutum* mit den herkömmlichen Sonden festgestellt. Dieses Phänomen kann durch die verbesserte Qualität der rRNA begründet werden. Jedoch sind beide Arten toxisch und müssen überwacht werden. Die LNA-Sonden zeigen in Kombination mit den DNA-Mikroarrays eine erhöhte Sensitivität, jedoch zusätzlich mehr falsch-positive Signale. Zusammengefasst zeigen die herkömmlichen Sonden gleiche oder bessere Ergebnisse als die LNA-Sonden. Die LNA-Technologie konnte unter diesen Festphasen-Hybridisierungsanwendungen nicht die Signalstärke erhöhen.

Zusätzlich zu dem Mikrotiter-Platten Assay und der Einfachelektroden-Anwendung, wurden ein Mehrfach-Sonden-Chip und ein halbautomatischer rRNA Biosensor für den vor Ort Nachweis von giftigen Algen entwickelt. Das Design des Mehrfach-Sonden-Chips mit einer Reihe von 16 Goldelektroden wurde durch den Test von verschiedenen Materialien für die Elektroden und des Trägermaterials mit Hilfe von Einfachelektroden bestimmt. Der Mehrfach-Sonden-Chip kann mit den vorher entwickelten molekularen Sonden bis zu 14 Arten nachweisen. Ein praktisches, sowie automatisiertes und tragbares Gerät wurde entwickelt und ausgiebig in Kombination mit dem Mehrfach-Sonden-Chip und molekularen Sonden für *Alexandrium minutum* getestet. Eine Peristaltikpumpe befördert die Lösungen aus den Vorratsbehältern in eine Hybridisierungs- bzw. Strömungskammer, wodurch alle wichtigen Schritte der Analyse automatisch ausgeführt werden. Zusätzlich wurde erfolgreich ein Lyse-Protokoll für die Anwendung des Gerätes von Laien entwickelt, welches eine manuelle rRNA-Isolation erübrigt. Das Gerät wurde anhand isolierter Gesamt-rRNA und Zellen aus einer Algenkultur bewertet und eindeutige, unterscheidbare Signale wurden ermittelt. Dieses autonome System kann Proben in weniger als zwei Stunden analysieren und auch im Freiland angewendet werden. Somit weisen das Gerät und der Mehrfach-Sonden-Chip Potential für die Anwendung als Frühwarnsystem im Aquakulturbereich und dem Tourismussektor auf.

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