Electrochemical detection of the toxic dinoflagellate *Alexandrium ostenfeldii* with a DNA-biosensor

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Abstract

The steady rise of observations of harmful or toxic algal blooms throughout the world in the past decades constitute a menace for coastal ecosystems and human interests. As a consequence, a number of programs have been launched to monitor the occurrence of harmful and toxic algae. However, the identification is currently done by microscopic examination, which requires a broad taxonomic knowledge, expensive equipment and is very time consuming. In order to facilitate the identification of toxic algae, an inexpensive and easy-to-handle DNA-biosensor has been adapted for the electrochemical detection of the toxic dinoflagellate *Alexandrium ostenfeldii*. The detection of the toxic algae is based on a sandwich hybridisation, which is carried out on a disposable sensor chip. A set of two probes for the species-specific identification of *A. ostenfeldii* was developed. The specificity of the probes could be shown in dot-blot hybridisations and with the DNA-biosensor. The sensitivity of the DNA-biosensor was optimised with respect to hybridisation temperature and NaCl-concentration and a significant increase of the sensitivity of the DNA-biosensor could be obtained by a fragmentation of the rRNA prior to the hybridisation and by adding a helper oligonucleotide, which binds in close proximity to the probes to the hybridisation.

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Keywords: DNA-biosensor; Toxic algae; *Alexandrium ostenfeldii*; Sandwich hybridisation; Helper oligonucleotide; Disposable sensor chip

1. Introduction

The safety and quality of the world’s coastal areas is an important issue because they harbour complex ecosystems and represent an important economic source with regards to tourism, fishery and aquaculture. However, blooms of microalgae that are potentially harmful with respect to the ecosystem, public health or economic aspects frequently affect coastal areas. Harmful algae can be classified into three groups. The first group is not toxic, nevertheless, it has the potential to harm the environment by forming dense blooms that can cause an oxygen-depletion that results in indiscriminate kills of fish and other organisms. Toxic algae are assigned to a second group of harmful algae that threaten the environment by the production of very potent toxins, which can cause both animal and human poisoning.

The third group of harmful algae comprises those microalgae that either harm fish mechanically or by the production of haemolytic substances (Hallegraeff, 1993). There are about 300 species known to have the potential to form algal blooms and approximately 85 of these species are potential toxin producers (Sournia et al., 1991; IOC, 2002). The majority of the known toxic algae species belongs to three divisions: Cyanophyta, Dinophyta and Haptophyta (Fogg, 2002). However, the division Dinophyta contains the largest number of harmful algal species (Taylor, 1985). Among the Dinoflagellates there are about 20 species known to produce potent toxins, for example, saxitoxins that cause the life threatening syndrome paralytic shellfish poisoning (PSP). PSP is caused by the consumption of contaminated shellfish (Hallegraeff, 1993). The dinoflagellate *Alexandrium ostenfeldii* produces PSP-toxins (Hansen et al., 1992) and other toxins. Recently, new toxins, including the spirolides, produced by the marine dinoflagellate *A. ostenfeldii* have been isolated. Mammalian toxicity of spirolides was confirmed by mouse bioassays. Spirolides proved to be highly toxic in intraperitoneal injec-
In the light of the threats by the toxic algae that occur at the coastlines all over the world (Hallegraeff, 1995) numerous monitoring programs have been launched that observe the phytoplankton composition on a regular basis. This is done not only to initiate testing of toxin in shellfish, but also to serve as an early warning system for fish-farms to avoid economic damage caused by the bloom of a toxic algae that has the potential to kill the caged fish. With regard to this the European Union requires that the member states monitor the water at their coastlines for toxin-producing plankton has the potential to kill the caged fish. With the technical background was presented in detail previously to the public in the German patent application DE 10032 042 A1 (Elektrochemischer Einwegbiosensor für die quantitative Bestimmung von Analysenkonzentrationen in Flüssigkeiten). The system is based on two major parts. The first part is a disposable sensor chip and a handheld device for the measurement of the electrochemical signal. The disposable sensor chip consisting of a carrier material harbours a working electrode, on which the detection reaction takes place, a reference electrode and an auxiliary electrode. The working electrode has a diameter of 1 mm and consists of a carbon paste. A biotinylated probe is immobilised in the reaction layer of the working electrode via avidin. The nucleic acids are detected on the sensor chip via a sandwich-hybridisation (Zammatteo et al., 1995; Rautio et al., 2003). The underlying principle of this method is that one target specific probe, here called capture probe, is immobilised 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 hybridisation to a second target specific probe, which is coupled to digoxigenin that is recognized by a digoxigenin specific antibody. The antibody in turn is coupled to horseradish-peroxidase that catalyses the reduction of hydrogen peroxide (substrate of the horseradish-peroxidase) to water. The reduced peroxidase is regenerated by p-aminodiphenylamin (ADPA), that functions as a mediator. The oxidised mediator gets reduced at the working electrode with a potential of ~150 mV (versus Ag/AgCl) (Fig. 1). In this set up it is only possible to measure an electrochemical signal, if the target nucleic acid as the link between the two probes is present in the system.

2. Material and methods

2.1. Algal strains

The specificity of the probes targeting the 18S rRNA of A. ostenfeldii was tested with the following strains: A. ostenfeldii K0287 and K0324 (Scandinavian Culture Centre for Algae and Protozoa, University of Copenhagen), A. ostenfeldii BAH ME 136 (Biologische Anstalt Helgoland, Germany), Alexandrium minutum AL3T (Gulf of Trieste, Italy, A. Beran), Alexandrium lusitanicum BAH ME91 (Biologische Anstalt Helgoland, Germany), Alexandrium pseudogonyaulax AP2T (Gulf of Trieste, Italy, A. Beran), Alexandrium andersonii 012b, Alexandrium担负 VKY2T (Lagoon of Marano, Italy, A. Beran), Alexandrium affine CCMP112 (Provasoli-Guillard National Centre for Culture of Marine Phytoplankton, USA), Alexandrium tamarense BAH ME182 (Biologische Anstalt Helgoland, Germany), Alexandrium catanella BAH ME217 (Biologische Anstalt Helgoland, Germany), A. tamarense 3141 (Cork Habor, Ireland, W. Higman), A. tamarense SZN01 (Gulf of Naples, 16/06/99), Gymnodinium varians CCMP421.
concentration of 1 pmol/ml at a hybridisation temperature of
hybridisation was carried out for at least 12 h with a probe
published (Lange and Medlin, 2002). In this assay, the
organisms was amplified by PCR using the primer 1F and
1528R (Medlin et al., 1988). The PCR-fragments were pu-
ferred with the Qiaquick PCR-Purification Kit (Qiagen, Ger-
2.3. Isolation of nucleic acids
Total RNA was isolated from algal cultures with the
RNasea Plant Mini Kit (Qiagen, Germany). Genomic DNA
was extracted from pure cultures with the DNeasy Plant
Mini Kit (Qiagen, Germany).
2.4. Dot-blot assay
Prior to the dot-blot analysis, the 18S-DNA of the target
organisms was amplified by PCR using the primer 1F and
1528R (Medlin et al., 1988). The PCR-fragments were pu-
rified with the Qiaquick PCR-Purification Kit (Qiagen, Ger-
many). The dot-blot assay was done according to previous
publications (Lange and Medlin, 2002). In this assay, the
hybridisation was carried out for at least 12 h with a probe
concentration of 1 pmol/µl at a hybridisation temperature of
56 °C.
2.5. Immobilisation of the probes on the sensor chip
Subsequent to an equilibration with Buffer 1 (50 mM
NaHCO₃, pH 9.6), the working electrode was incubated with
NeutrAvidin [0.5 mg/ml] (Pierce Biotechnology, USA) for
at least 4.5 h at 4 °C. Excessive NeutrAvidin was removed
from the working electrode by washing the sensor with PBS
(BuP buffer saline pack, Pierce Biotechnology, USA).
In the following the working electrode was blocked with 3%
w/v casein in PBS for 1 h and washed afterwards in PBS.
Prior to the immobilisation the probe was dissolved at a
concentration of 10 µM in Buffer 2 (0.3 M NaCl/0.1 M Tris,
pH 7.6). The electrode was incubated for 30 min with the
probe at room temperature to immobilise the probe on the
electrode. To avoid evaporation, all incubations have been
carried out in a wet chamber. Excessive probe was removed
by washing the sensor with hybridisation buffer (75 mM
NaCl/20 mM Tris, pH 8.0, 0.04% SDS), 0.25 µg/µl herring
sperm DNA, 0.1 pmol/µl dig-labeled probe and rRNA at dif-
ferent concentrations. To denature the target nucleic acid,
the hybridisation mix was incubated for 4 min at 94 °C prior
to the application onto the working electrode. The hybridi-
sation was carried out with 2 µl of the hybridisation mix
for 30 min at 46 °C. To avoid evaporation the hybridisation
was carried out in a water-saturated wet chamber. Subse-
quent to the hybridisation the sensor chips were washed
with Buffer 3 (50 mM NaH₂PO₄ × H₂O, pH 7.6/100 mM
NaCl).
2.8. Detection
The initial step of the detection reaction was an incubation
of 30 min of the sensor chip with horseradish-peroxidase,
which was coupled to an antibody that recognised Digox-
igenin (Anti-Dig POD). The sensor chip was incubated
with 1.5 µl of the horseradish-peroxidase solution (7.5 U/ml
in PBS, pH 7.6/0.1% BSA [w/v]/0.05% Tween 20 [v/v]).
Prior to the detection, excessive enzyme was removed
by washing the sensor chips with Buffer 3. Subse-
quently to the washing step the sensor chip was connected
to the potentiostat and 20 µl of the substrate solution
(4-aminophenylamine hydrochloride [44 µg/ml]/0.44%
ethanol [v/v]/0.48% H₂O₂ [v/v]/50 mM NaH₂PO₄ × 
H₄O/100 mM NaCl) was applied to the working elec-
trode. The electrochemical signal of the detection reaction
was measured directly after the application of the sub-
strate solution for 10 s at a potential of ~150 mV (versus
Ag/AgCl).
3. Results
3.1. Probe design
For the sandwich-hybridisation two probes that specifi-
cally bind to the 18S-rRNA of A. ostenfeldi (Table 1) have
been designed using the ARB software package (Ludwig
et al., 2004). The probes have at least two mismatches
against all non-target organisms listed in the ARB database.
The overall specificity of the probes was tested by doing a
BLAST search (Altschul et al., 1990) against all public
available sequences. To avoid possible effects of degra-
dation of the target nucleic acid the chosen probes were
located in close proximity to each other. The distance be-
tween the binding sites of the probe set that we present here
for A. ostenfeldi was 50 bp.
Table 1

Sequences of the A. ostenfeldii specific probes and the helper oligo H3

<table>
<thead>
<tr>
<th>Probe</th>
<th>Probe sequence</th>
<th>Position in 18S rRNA</th>
<th>$T_m$ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AOST1</td>
<td>CAA CCC TTC CCA AEA GTC AGG T</td>
<td>180</td>
<td>64.3</td>
</tr>
<tr>
<td>AOST2</td>
<td>GAA TCA CCA AGG TTC CAA GCA G</td>
<td>232</td>
<td>66</td>
</tr>
<tr>
<td>H3</td>
<td>GCA TAT GAC TAC TGG CAG CAT C</td>
<td>210</td>
<td>58.5</td>
</tr>
</tbody>
</table>

3.2. Specificity of the probes

The specificity of the probes was tested separately for each probe in a dot-blot-assay. The probes were tested against PCR-fragments of the 18S-DNA of those species that had the lowest number of mismatches and that were the nearest neighbour in a phylogenetic tree (John et al., 2003). Additionally the probes were tested against more distantly related species of the genus Alexandrium, G. varians and P. parkae. The dot blot assay exhibited that both of the probes for A. ostenfeldii are highly specific. It was possible to observe a hybridisation signal for all tested A. ostenfeldii strains, whereas no hybridisation signal could be observed for any of the non-target organisms (Fig. 2).

3.3. Optimisation of the hybridisation protocol

The probes that have been previously shown to be specific for A. ostenfeldii in the dot-blot-assay have been used for a sandwich hybridisation of rRNA on a sensor chip. The hybridisation was carried out according to the protocol described in the Section 2. It was possible to measure A. ostenfeldii rRNA isolated from a laboratory strain with the handheld device. A calibration curve was established for different rRNA concentrations (Fig. 3). The signal intensity was proportional to the rRNA concentration applied to the sensor. However, the calibration curve revealed a detection limit of $\sim 100$ ng/µl rRNA for the handheld device. At this concentration the signal is $\sim 2 \times$ above the signal of the negative control. In average, it was possible to isolate 0.02 ng rRNA from a single cell (data not shown). If it is considered, that 250 cells/l of Alexandrium cells would be the limit for a closure of a muscle-bed, a detection level of 100 ng/µl rRNA would require at least a sampling volume of 40 l to gain enough rRNA for a detection of this cell concentration with the handheld device. Therefore, the existing hybridisation protocol needed to be optimised in order to increase the sensitivity of the method. With this regard, parameters of the hybridisation protocol that were known from literature to influence the result of a hybridisation reaction have been optimised. The quantitative results shown in the following sections are based on measurements from eight independent hybridisations originating from two different RNA isolations.

3.4. Salt concentration and hybridisation temperature

The hybridisation of two single stranded nucleic acids is determined by the salt concentration in the hybridisation buffer and the hybridisation temperature. In the standard protocol 46 °C hybridisation temperature and a salt concentration of 75 mM were suggested as hybridisation conditions. However, a determination of the melting temperature (Breslauer et al., 1986) of the probes for A. ostenfeldii revealed that 46 °C is $\sim 20$ °C below the calculated melting temperature of the probes, which is 64.3 °C for AOST1 and 66 °C for AOST2. Therefore, different hybridisation temperatures and salt concentrations were tested in respect to their effect on the hybridisation signal. Hybridisation signals resulting from hybridisations at 46 °C, 50 °C, 55 °C and 60 °C were compared to each other. The strongest reliable improvement of the hybridisation signal was observed at 55 °C. A hybridisation temperature of 55 °C was discov-
erred to increase the hybridisation signal 2.9-fold. In addition, NaCl-concentrations of 75 mM, 150 mM, 300 mM and 600 mM were tested to optimise the hybridisation signal. The strongest improvement of the hybridisation signal was observed for a NaCl-concentration of 150 mM. At this concentration the hybridisation signal was increased 2.3-fold in comparison to the original salt concentration of 75 mM (Fig. 4).

3.5. Helper oligonucleotides

In previous publications, it has been discussed that the secondary structure of the 16S-rRNA could entail problems in hybridisation experiments (Fuchs et al., 1998; Behrens et al., 2003). It was reported for FISH (fluorescence in situ hybridisation) that the problems caused by the higher-order structure could be overcome by the addition of unlabeled helper oligonucleotides that bind in close proximity to the probes. In this respect, the helper oligonucleotides have a synergistic effect for the probe binding (Fuchs et al., 2000). Here, we present a detection system that targets the 18S-rRNA, which is also known to form intense higher order structures (Behrens et al., 2003). It was examined, if the application of a helper oligonucleotide would also improve the result of the sandwich hybridisation on the sensor chip. With this regard a helper oligonucleotide was designed (Table 1), which has a similar hybridisation temperature like the target specific probes. The binding site of the oligonucleotide is located in-between the two 18S-rRNA targeted probes. However, the sequence of the oligonucleotide is not specific for the target organism. If the helper oligonucleotide was added in a hybridisation experiment it could be observed that the hybridisation signal in the presence of the helper oligonucleotide was 1.8× higher than the signal observed for a hybridisation in absence of the helper oligonucleotide (Fig. 4).

3.6. Fragmentation of the rRNA

As an alternative strategy to overcome the problems caused by higher-order structures it was tested if a fragmentation of the RNA would positively influence the hybridisation signal. By the generation of smaller sized nucleic acids a fragmentation of the RNA could avoid higher-order structures and antagonise negative effects on the hybridisation efficiency caused by steric hindrance because of the size of the target nucleic acid. For fragmentation, the RNA...
was incubated at 94 °C over periods of 5 min, 10 min and 35 min. It could be observed that a short incubation of the RNA over a period of 5 min resulted in an increased signal intensity of 5.7× compared to the non-fragmented RNA (Fig. 4). However, the signal decreased if the incubation was extended to 10 min and falls even below the signal intensity of the non-fragmented RNA if it is incubated for 35 min (data not shown).

### 3.7. Combination of the optimised hybridisation conditions

In the previously described experiments various parameters that influence a hybridisation have been optimised. It was expected that a combination of parameters that antagonise the negative influence of the secondary structure on hybridisations like temperature, helper oligonucleotide and fragmentation of the rRNA, would have a synergistic effect on the signal intensity. However, it was observed that only the combination of the helper oligonucleotide with the fragmentation step had a weak synergistic effect in comparison to the fragmentation alone (Fig. 4). This led us to the conclusion that the optimal hybridisation protocol consists of a fragmentation step with an incubation of 5 min at 94 °C, a hybridisation temperature of 46 °C and the addition of a helper oligonucleotide that binds in close proximity to the target specific probe (Fig. 4). With the optimised protocol it was possible to increase the signal intensity 6.3× in comparison to the standard protocol.

### 3.8. Application of the detection system

The specificity of the optimised protocol was examined in accordance to the dot-blot assay. The rRNA isolated from three different strains of *A. ostenfeldii* and the nearest neighbours in the phylogenetic tree (John et al., 2003) were used for the sandwich hybridisation on a sensor chip. The results of the hybridisation reveal that the normalised signal of the rRNA isolated from different strains of *A. ostenfeldii* is significantly higher than the signal of all non-target organisms (Fig. 5). However, there is a weak cross hybridisation of the probes with *A. minutum* (AL3T) and *A. lusitanicum* (BAH ME91), which are the nearest neighbours of *A. ostenfeldii* in a phylogenetic tree analysis (John et al., 2003).

### 4. Discussion

Here, we present a chip-based electrochemical biosensor for the identification of the toxic dinoflagellate *A. ostenfeldii* via a sandwich hybridisation on molecular level. The biosensor is an inexpensive, easy and rapid technology for the identification of phytoplankton. Electrochemical readings of the handheld device and the protocols are unambiguous and even for a scientific layperson easy to use and interpret. Therefore, this system has great potential to serve as an alternative to conventional methods that are time consuming and expensive. In this publication, the sandwich hybridisation is based on two probes that specifically target the 18S-rRNA of *A. ostenfeldii*. Probes that target rRNA sequences are routinely applied for the detection of microalgae e.g., in combination with fluorescent *in situ* hybridisation or dot-blot-assays (Guillou et al., 1999; Simon et al., 2000). The use of rRNA for the identification of organisms with the DNA-biosensor is advantageous because the molecule is present in high numbers in a cell. In prokaryotes up to 80% of the RNA is rRNA (Woese, 1987). This circumvents an amplification of the target sequence, which is part of other DNA-biosensor protocols (Baeumner et al., 2003; Hartley and Baeumner, 2003; Litaker et al., 2000).

![Signal intensity vs. strain](image-url)
4.1. Probes

The probes were designed using the ARB software package (Ludwig et al., 2004). The specificity of the probe set was determined theoretically by comparing the sequences with the sequences in the local ARB database and by doing a blast-search against all public available sequences (Altschul et al., 1990). In practice, the specificity of the probes was shown in a dot-blot-assay and with the DNA-biosensor itself. In accordance to the theory, with both methods the signal for the target species was always significantly above the signal for any non-target organism. To test the specificity of the probes those species have been tested that had the lowest number of mismatches to the target species and were the closest relatives in the phylogenetic tree. It was set aside to test more distantly related species because those species with the lowest number of mismatches would have the highest probability to result in an unspecific signal. High numbers of mismatches antagonise probe binding. Therefore, more distantly related species with higher numbers of mismatches are not very likely to bind to the probe. It is unlikely that a probe, which does not bind to a close relative of a target species, would bind to more distantly related species, which would have more mismatches. However, a weak cross hybridisation was observed with the biosensor for *A. lusitanicum* and *A. minutum*, which are the next neighbours of *A.ostenfeldii* in the phylogenetic tree. The main goal of the biosensor is the detection of a toxic algae. *A. minutum* and *A. lusitanicum* are synonyms for the same species (IOC, 1994) with the potential to cause paralytic shell fish poisoning (Chang et al., 1997; Hold et al., 2001). Even if a signal would be only derived from *A. minutum* it would still account for a serious threat. However, it has to be considered that the number of sequences available in the databases reflects only a small amount of the number of species present in the environment. Therefore, it is important to re-evaluate the specificity of rRNA targeted probes on a regular basis in respect to the continually growing number of algal 18S rRNA sequences in public databases e.g., in the ribosomal database project (RDP) (Maidak et al., 2001). The probes for the sandwich hybridisation have been chosen with regard to the distance of their binding sites in the 18S-rRNA molecule. rRNA is highly susceptible to degradation. To avoid effects of degradation on the success of the sandwich hybridisation, it was intended to choose a specific probe set with binding sites in close proximity to each other. A sandwich hybridisation based on probes that are located at a bigger distance to each other are probably more vulnerable to effects of degradation because it is very likely that a larger molecule is fragmented, which would prevent the two probes from binding to the same molecule.

4.2. Optimisation of the hybridisation protocol

A preliminary hybridisation protocol had to be optimised with regard to the sensitivity of the method. Single parameters of the hybridisation protocol like NaCl-concentration in the hybridisation buffer, hybridisation temperature, addition of a helper-oligonucleotide, or a fragmentation of the rRNA prior to the hybridisation were optimised separately in regard to the hybridisation signal. However, a combination of the optimised parameters resulted only in a weak synergistic effect if the fragmentation was combined with an addition of the helper oligonucleotide. The weak synergistic effect of the optimised parameters could be explained in the way that the optimised hybridisation parameters aim all to break up the higher-order structures of the target molecule. It is likely that the secondary structure was already almost dissolved if a single optimised hybridisation parameter was applied and therefore, leaves only little scope to further increase the signal intensity by combining two optimal factors. However, with the optimised protocol it was possible to increase the signal intensity by a factor of 6.3. As a consequence, the detection limit of the device is decreased accordingly. If the detection limit of the device was 100 ng/µl with the standard protocol, it is ~16 ng/µl with the optimised protocol. With an average yield of 0.02 ng/cell this equals 800 cells or a sampling volume of 6.4 l to get a detectable amount of rRNA from 250 cells/liter.

4.3. Limitations of the system

Besides the good performance of the device for the identification of laboratory strains, it has to be determined, how the device performs in field tests. This means in particular, that it has to be examined, if it is possible to correlate signal intensities with cell counts from water samples. Currently, the system is limited by the manual isolation of rRNA. It is possible that differently experienced users could isolate different amounts of rRNA from the same number of algae cells, resulting in signal intensities that do not reflect the real situation in terms of cell counts. In order to ensure reliable and reproducible results it is desirable to automate the isolation of rRNA. Also, the system has to be adapted to the wide variety of toxic algae.

5. Conclusion

A DNA-biosensor was adapted to the electrochemical detection of the toxic dinoflagellate *A.ostenfeldii* and the sensitivity of the DNA-biosensor was increased by an optimisation of the hybridisation conditions. The electrochemical DNA-biosensor presented in this publication is a proof of principle for a DNA-handheld device that possesses the potential to serve as a quick and easy method for the identification of toxic algae. The device could facilitate the work that must be done in the course of monitoring toxic algae by eliminating the need to count algae and reduce the need for mouse bioassay.
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