MOLECULAR TOOLS FOR MONITORING HARMFUL ALGAL BLOOMS

Review: advances in electrochemical genosensors-based methods for monitoring blooms of toxic algae

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Abstract Harmful algal blooms (HABs), which have expanded worldwide in their occurrence and frequency, are a serious menace to aquatic ecosystems and humans. The development of rapid, accurate and cost-effective detection systems for toxic algal monitoring in aquatic environments is urgently required. Although many efforts have been devoted to develop reliable tools to monitor the entire spectrum of existing toxic algae, a portable semi-automated system that enables HAB monitoring at a low cost is still not available for general purchase. This work reviews the challenges and opportunities in translating the remarkable progress of electrochemical genosensors-based methods towards practical in situ HAB monitoring applications. It is specifically focused on reviewing the optimised methods for a detection system based on a sandwich hybridisation assay (SHA) performed over transducer platforms of different materials, geometries and dimensions and presenting the diverse advantages and disadvantages among them. Probe design and specificity and optimisation of the genosensor in terms of hybridisation conditions and electrochemical signal are discussed as well as their long-term stability and storage and semi-automation attempts. With continuous innovation and attention to key challenges, we expect semi-automatic devices containing DNA-based electrochemical biosensors to have an important impact upon monitoring of serious HAB events.

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Introduction

Frequency, intensity, duration and geographic distribution of harmful algae blooms (HABs) have a serious impact not only on public health but also on the economic stability of different areas, including aquaculture, fisheries and tourism. HABs can introduce hazard toxins, mainly neurotoxins, into humans through any of the links in their food chain, e.g. fish, seafood products and contaminated water (Hallegraeff 1993). Visual confirmation of water discoloration, fish kills and cell counts are the most common methods used to detect these kinds of contamination episodes. However, such methods lack practicality to analyse a high number of samples routinely because they are time consuming, require specialised or trained personnel and expensive equipment in specialised laboratories. Such drawbacks leave no option for mitigation responses. New approaches using rapid, accurate and cost-effective detection systems for toxic algal monitoring in aquatic environments is highly needed. Although considerable progress over the past decade has been made in addressing HAB problems, a portable semi-automated system that enables the monitoring of low concentrations of toxic algae at a low cost is still not available for general use.

As an alternative to the widely applied but impractical light microscopy methods, molecular methods have demonstrated faster and more accurate information to identify phytoplankton (Ayers et al. 2005, Diercks et al. 2008b, 2009; Gescher et al. 2008; Greenfield et al. 2006; Haywood et al. 2007; Ki and Han 2006; O'Halloran et al. 2006; Tyrrell et al. 2002). These methods circumvent the selective step of laboratory cultivation (Giovannoni et al. 1990); and have proven to be of special value for the analysis of extremely

small picophytoplankton samples (Díez et al. 2001; Moon-van der Staay et al. 2001). The major drawback to these approaches is the fact that they can only be used to identify one or a few organisms at a time (De Long et al. 1989). However, other methods in which multiple species can be identified simultaneously have been developed (Metfies and Medlin 2004). Molecular methods can discriminate species in field material. For instance, fluorescence in situ hybridisation (FISH) targeting cellular rRNA molecules is often used to identify harmful species in field samples (Miller and Scholin 1998; Groben and Medlin 2005; John et al. 2005; Anderson et al. 2005; Töbe et al. 2006; Touzet et al. 2010a, b). The FISH method enables the direct visualisation of target cells. However, the time-consuming FISH procedure is inadequate to achieve the high sample throughput needed in routine monitoring programs (John et al. 2003, Touzet et al. 2009). The use of multiple probes at one time is also restricted by the limited number of fluorochromes available.

Real-time quantitative polymerase chain reaction (qPCR), biosensors and microarrays are cell free formats that have overcome the issues associated with FISH whole cell formats. Among them, biosensors have demonstrated special features to be exploited for species detection in both colourimetric (e.g. Scholin et al. 1996) and electrochemical approaches. Common limitations are minimised by the simplicity, high speed in response, sensitivity, accuracy and versatility of electrochemical biosensors (Diercks et al. 2008b; Metfies et al. 2005, Diercks et al. 2011). Electrochemical and chemiluminescence biosensors have the advantage of being capable of directly identifying nucleic acids from complex samples without target purification and amplification steps as compared to other approaches, such as qPCR that does require these steps (Liao et al. 2007).

The DNA microarray or phylochip is another technology with tremendous potential to be applied for the quick analysis of complex samples because it does not require any culture cultivation step. It provides the possibility to analyse more than one target at a time (Ki and Han 2006). Recently developed DNA microarray technology allows the simultaneous analysis of up to 250,000 probes at the same time (Lockhart et al. 1996). From the first DNA microchip devised to study nitrifying bacteria (Guschin et al. 1997), many ribosomal RNA probes for equal number of toxic algae species has been developed, some of them available in microarray format (Metfies and Medlin 2004, Gescher et al. 2008) and others even tested in the field (Metfies et al. 2010, see the papers from the EU MIDTAL project in this volume). Results have been in agreement with those obtained by flow cytometry and FISH hybridisation. The use of rRNA for the identification of organisms with genosensors or microarrays is advantageous as the molecule is present in high numbers in a cell—up to 80 % of the RNA is rRNA in prokaryotes, thus being not necessary amplification of the target sequence. Furthermore, microarrays require

an expensive equipment set-up (the microarray reader and the Bioanalyzer), whereas the genosensors are less expensive to set up and become operational. This work reviews the challenges and opportunities in translating the outstanding progress that has been made in the past decade towards a practical application of the biosensors in toxic algal monitoring from field samples. It is specifically focused on reviewing the optimised methods for a detection system based on a sandwich hybridisation assay (SHA) performed over transducer platforms of different materials, geometries and dimensions and presenting the diverse advantages and disadvantages among them. For example, the genosensors developed, so far, for testing the presence of toxic algae have addressed the optimisation of the fabrication processes, e.g. probe design and specificity, hybridisation conditions and electrochemical signal. However, limitations mainly regarding sensitivity and the necessity of filtering a large volume of water have limited their application in the field. Long-term stability and storage are also reviewed along with the development of a semi-automatic device and limitations of the HABs genosensors. A section dedicated to outlook with future work proposing different practices and optimisation of the fabrication protocols that would benefit from recent developments in surface chemistry approaches and nanotechnology is included. Such practices will enhance substantially the prospects of semi-automatic devices with DNA-based electrochemical biosensors and have a remarkable impact upon a resolution of the HAB monitoring problems.

Review of the methods

Probe design and specificity

Molecular probes used for early and rapid detection of toxic algal species in biosensors are based on the SHA, which requires two probes for each species, i.e. a capture probe and a nearly adjacent signal probe. Only one of them has to be specific for the target species. Targets are the small and large subunit rRNA genes in the cell's ribosomes. Their conserved and variable regions enable the development of probes specific for different taxonomic levels (Groben et al. 2004). Such strands are designed using the probe match function within the ARB software package (Ludwig et al. 2004). Mismatches to non-targeted strands are positioned in the middle of the oligonucleotide during probe design to maximise specificity (Diercks et al. 2008a, b, c, d). For species-specific identification and quantification of toxic algae using a SHA involves the target sequence being immobilised between single-stranded oligonucleotide capture and signal probes by hybridisation (Rautio et al. 2003; Zammatteo et al. 1995).

All synthetic oligonucleotides targets and probes are synthesised commercially, e.g. ThermoFischer Scientific

(Germany) and then diluted in 18 MΩcm ultrapure deionised water (free of DNA and RNA) to have 100 mM stock solutions. Capture probes either have a biotin label at the 5′ end or a thiol label if carbon or gold electrodes are to be used, respectively. Signal probes have a Digoxygenin (DIG) label, normally at the 3′ end, but probes labelled at the 5′ end and at both 5′ and 3′ ends have also been tested.

Effect of capture probe density and specificity on signal level and hybridisation efficiency must be empirically tested and optimal conditions defined. Probe theoretical 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, then cross-hybridisation to non-targeted species and organisms whose sequences are unknown and not yet in the database is likely to occur. Specificity of the molecular probes is tested with cultivated target species and closely related species as in silico, in or by means of a computer simulation and in situ results show variability in the signals. In in silico testing, programs, such as Oligo, are used to determine if the probes can bind to each other, fold back on themselves and bind. The melting temperature of the probe from its target is determined. Hybridisation temperatures, usually two degrees below the melting temperature, are selected for initial testing.

Apparatus and materials

Initial voltamperometric measurements performed as described in Metfies et al. (2005) used a glucose sensor from Inventus Biotech (Germany) that was adapted for use as a DNA biosensor with carbon printed electrodes. Later measurements that further optimised these initial tests (see Table 1) were made with an EmStat (Electrochemical Sensor Interface), using the PSTrace software from Palm Instruments BV (The Netherlands) and a boxer connector DSC from Dropsens (Spain). Measurements were carried out with a three electrode cell printed over ceramic substrates, whose total volume was 50 μl. This cell consisted of a DNA-modified working electrode and an on-chip counter and pseudo-reference electrode. Four-millimetre diameter single-wall carbon nanotubes (CNT) DRP-110SWCNT (SWCNT) and screen-printed gold electrodes DRP-220AT (SPAuE) were purchased from Dropsens (Spain). One millimetre Graphite and gold electrodes were purchased from BVT Technologies, Czech Republic. Potentials reported refer to Ag and Ag/AgCl pseudoreference electrode for SWCNT and SPAuE, respectively.

Reagents and solutions

In the hybridisation mixture, a 3.48 mgml^{-1} salmon sperm DNA solution is used as blocking reagent.

Buffer solutions and other reagents needed for the hybridisation are listed in Table 2. All buffers are prepared by diluting analytical grade reagents (Sigma–Aldrich, France) in 18 MΩcm deionised water and stored at 4 °C when not in use. Sulphuric acid, potassium hexacyanoferrate, neutravidin, 6-mercapto-1-hexanol and hydrogen peroxide were supplied by Sigma–Aldrich (France). N-Phenyl-p-phenylenediamine monohydrochloride 99 % (ADPA) was purchased by Acros Organics (Belgium), anti-digoxigenin-horseradish-peroxidase (HRP) fragments were fabricated by Roche (France). All others chemicals were of analytical reagent grade.

Characterisation of the transducer platform, immobilisation of the probes and detection strategy

Gold electrodes must be pre-treated by placing a 50-μl drop of a 0.5 M H$_2$SO$_4$ solution containing 0.01 M KCl on their surface and cycling the potential between 0.0 and +1.2 V around 15 times respectively at a scan rate of 100 mVs^{-1}. The electrodes are then rinsed with water, dried with a vacuum pump and their behaviour in an acidic solution evaluated, being analogous to these reported in the literature for gold wire electrodes (Wang et al. 2001). After the pre-treatment (or stabilisation in the case of carbon electrodes) step, electrodes are then characterised by cyclic voltammetry in a 1 M KCl solution containing 1 mM K$_3$Fe(CN)$_6$ at a scan rate of 50 mVs^{-1}, by applying a potential scan ranging from +0.25 to +0.5 V and they are acceptable if they showed a well-defined reversible oxidation and reduction peaks and high response reproducibility. Carbon paste and CNT electrodes do not require any pre-treatment. However, they were allowed to stabilise in 50 mM NaHCO$_3$ buffer solution (pH 9.6).

The capture specific probe anchors either to the gold electrodes (by overnight incubation in 10 mM thiol-labelled probe) or to carbon paste and CNT electrodes (via a 30-min incubation in 10 mM biotin-labelled probe). In the first case, an additional step consisting of incubation the DNA-modified electrodes in 6-mercapto-1-hexanol (1 mM aqueous solution) was carried out during 1 h in order to avoid unspecific adsorption. In the latter case, the attachment of the biotin-labelled probe is performed through the Neutravidin molecule linker, and the chips were incubated overnight in a 0.5 mgml^{-1} Neutravidine solution (Sigma–Aldrich). When the target sequence hybridises to the immobilised capture probe, a second hybridisation event with a signal probe linked to digoxigenin (Penna and Magnani 1999) occurs.

Detection takes place when an antibody coupled with a HRP enzyme binds to the DIG-labelled signal probe to begin the electrochemical signal amplification. HRP electrochemically converts an inactive substrate to an electroactive product that can be detected amperometrically. The

Table 1 DNA-biosensors developed for the different toxic algal species using electrochemical detection

Species	Working electrode		Current density, (positive/negative) controls	ADPA/H$_2$O$_2$ concentration	References
	Material	Area/mm^{-2}	µAmm^{-2}	mM	
A. minutum	Carbon	1.77	0.66/0.13	0.2/15.7	Diercks et al. 2011
A. ostenfeldii	Carbon	0.79	2.42/0.1	0.2/15.7	Metfies et al. 2005
	Gold	1.77	0.70/0.16	0.2/15.7	Diercks et al. 2008b
	Carbon	1.77	0.86/0.02	0.2/15.7	
	Carbon	1.77	2.65/0.11	30/600	
A. tamerense	Carbon	1.77	1.023	0.2/15.7	This work, Fig. 2
G. catenatum	CNT	12.57	0.84/0.17	0.4/10	Orozco et al. 2011
	Gold	12.57	0.85/0.37	0.4/10	
P. parvum	CNT	12.57	1.48/	0.2/5	Orozco and Medlin 2011
	Gold	12.57	1.66/	0.2/5	
	Gold	12.57	2.15/0.21	0.4/10	
	Graphite	0.79	10.09/0.14	0.4/10	
P. australis	CNT	12.57	1.07/0.45	0.4/10	Orozco et al. 2011

Effect of electrode material and dimensions, and mediator/substrate ratio concentration on the biosensor analytical response

current signal is proportional to the amount of the bound enzyme (and hence to the analyte concentration in a sample). Target sequence and signal probes are assembled over the capture probe-modified chips in a unique step by incubation in a hybridisation mixture (master mix) containing 1 µl DIG-labelled signal probe (1.4 mM), 3.5 ml 4× hybridisation buffer, 1 µl salmon sperm DNA and 7.5 ml milliQ water and either 1 µl target sequence (36–39 bases, 1.4 mM, complementary to the capture and signal probes) for the positive control and 1 µl non-complementary sequence for the negative control. One percent BSA is added to the master mix to facilitate the hybridisation event and minimise the unspecific interactions. Chips are incubated in the hybridisation mixture at 46 °C for 30 min, after which a final incubation in the anti-DIG–HRP enzyme for 30 min completed the biosensor development. The addition of p-

aminodiphenylamine (ADPA) as a redox substrate and application of a fix potential of −0.15 V between the working and pseudo-reference electrode generated a HRP-mediator redox cycle that is detected by the electrochemical sensor as a current (Penna and Magnani 1999). The electrochemical cell of 50 µl contains of 0.2 mM ADPA and 10 mM H$_2$O$_2$ in the POP buffer except otherwise specified. Once the potential is applied, the potentiostat measures the resulting electrochemical current, which can only be measured if the target nucleic acid sequence binds to both the capture and the signal probes and thus it must be present in the sample to be analysed (Metfies et al. 2005).

Cultures of all target species used to validate the probe specificity are usually ordered from known algal collections and grown in enriched seawater, such as K medium (Keller et al. 1987). Total RNA was initially isolated from algal cultures with the RNeasy Plant Mini Kit (Qiagen, Germany), whereas genomic DNA was extracted from pure cultures with the DNeasy Plant Mini Kit (Qiagen, Germany) as defined in Metfies et al. (2005) but later RNA extractions were much improved both quantitatively and qualitatively using TriReagent (Sigma, Orozco et al. 2010).

RNA-fragmentation and hybridisation for sample analysis is carried out in a fragmentation buffer (40 mM Tris, pH 8.0, 100 mM KOAc, 30 mM MgOAc) at 94 °C. The hybridisation mix contained 1× hybridisation buffer (75 mM NaCl, 20 mM Tris, pH 8.0 and 0.04 % SDS), 0.25 µg µl^{-1} herring sperm DNA, 0.1 pmol µl^{-1} DIG-labelled probe and rRNA at different concentrations. To denature the target nucleic acid, the hybridisation mix is incubated for 4 min at 94 °C prior to the application onto the working electrode. The hybridisation 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

Table 2 Buffers used for the SHA on electrode surfaces

Buffer	Composition	Concentration
Carbonate pH 9.6	NaHCO$_3$	50 mM
10× PBS pH 7.4	NaH$_2$PO$_4$.H$_2$O	0.5 M
	NaCl	1.54 M
Bead buffer pH 7.6	Tris	0.1 M
	NaCl	0.3 M
4× hybridisation buffer, pH 8.0	Tris pH 8.0	80 mM
	NaCl	0.3 M
	SDS	0.04 %
10× POP buffer pH 6.4)	NaH$_2$PO$_4$·H$_2$O	0.5 M
	NaCl (pH 6.45)	1 M
1× PBS pH 7.4	PBS	1×
	BSA	0.1 % [w/v]
	Tween 20	0.05 % [v/v]

chamber. Subsequent to the hybridisation the sensor chips are washed with 50 mM $NaH_2PO_4 \cdot H_2O$, pH 7.6 and 100 mM NaCl.

Review of the optimisation of the genosensors

Probe specificity

Probe sets for the identification of 10 toxic algal targets (the species *Gymnodinium catenatum*, *Protoceratium reticulatum*, *Lingulodinium polyedrum*, *Prymnesium parvum*, *Chrysochromulina polylepis*, *Pseudo-nitzschia multiseries*, *Pseudo-nitzschia australis*, *Pseudo-nitzschia seriata* and *Pseudo-nitzschia pungens* and the genus *Pseudo-nitszschia*) have been designed (Diercks et al. 2008c) and their specificity tested using SHA in a microtiter plate with rRNA isolated from laboratory strains of each target species and compared with other closely related species. The microtiter plate assay, a colorimetric assay, has been shown to be a cheap and effective means for testing probe specificity and the 10 probe sets designed were specific in the identification of these toxic algal targets. Specificity of *Alexandrium ostenfeldii* probes were tested with a single electrode DNA-based biosensor (Metfies et al. 2005) against 10 other algal species. Later, a multi-probe electrode capable of testing 14 species simultaneously was developed and tested with *A. ostenfeldii* (Diercks et al. 2008d). *Alexandrium minutum* has been colorimetrically detected by using the SHA in the microtiter plate assay developed by Diercks et al. (2008a) and then used to test a semi-automated rRNA biosensor device (ALGADEC) (Diercks et al. 2011). Probes for *P. parvum*, *G. catenatum*, *P. australis*, *A. ostenfeldii* and *A. minutum* (Diercks et al. 2008c) have been tested and optimised using a biosensor (Orozco and Medlin 2011).

Hybridisation conditions

The hybridisation protocol has been optimised with regard to the sensitivity of the SHA method. Single parameters of the hybridisation protocol, such as ionic strength (NaCl concentration in the hybridisation buffer), hybridisation temperature, effect of the presence of a helper-oligonucleotide and fragmentation of the rRNA prior to the hybridisation, have been optimised one at a time with regard to hybridisation signal efficiency. For example, hybridisation conditions for *A. ostenfeldii* were systematically studied and an optimised protocol was reported by Metfies et al. (2005). 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 probe that binds in close proximity to the target specific probe to relax the

secondary structure of the RNA molecule and open the target site for the capture and signal probe to bind efficiently (Fig. 1). Using the optimised protocol, they observed an optimal signal with an increase in the intensity by a factor of 6.3, with the concomitant decrease in the detection limit of a DNA-based biosensor device ($\sim$16 ngl^{-1}), with respect to a non-optimised protocol (100 ngl^{-1}). Accordingly, they got an average yield of 0.02 ngcell^{-1} (800 cells) for a sampling volume of 6.4 l and a detectable amount of rRNA from 250 celll^{-1}.

Transducer platform and electrochemical signals

Transducer platforms (chips) of different materials, geometries and dimensions have been used for the development of toxic algal electrochemical genosensors, presenting diverse advantages and disadvantages when the analytical performance was interrogated. For example, DNA-based electrochemical biosensors developed with gold electrode surfaces have shown a higher signal/noise ratio response with respect to other materials, such as carbon, CNT and graphite. Such improved response is related to the better blocking of the unspecific adsorption that can be achieved with a gold surface through self-assembled monolayers of alkyl thiols as spacer molecules between the anchored capture probes to ensure their accessibility to the target sequence. In agreement, higher undesirable capacitive currents are recorded by cyclic voltammetry when the SHA is performed over carbon electrode surfaces (Orozco et al. 2010). Gold platforms require an electrochemical pre-treatment to activate the surface before immobilising the capture probe. In contrast, a simpler conditioning step of submerging carbon electrodes in a carbonate buffer is the only requirement if carbon electrodes are used. Time of the assay is independent of electrode material. Gold electrodes require overnight
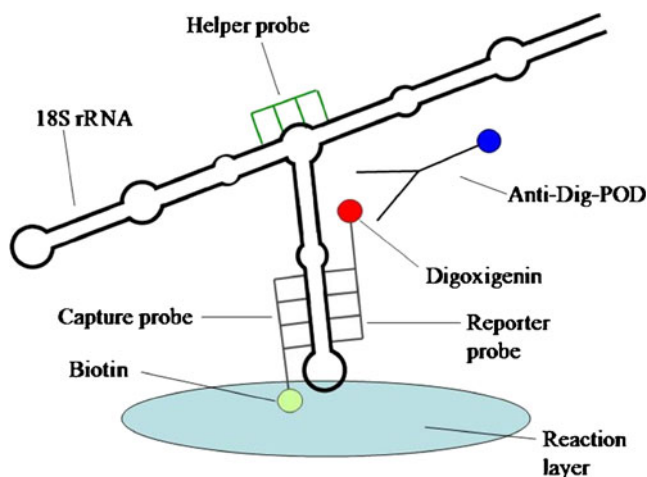

Fig. 1 Scheme showing the relationship between the three probes used in a SHA

incubation in the thiolated capture probe solution, whereas carbon electrodes need overnight incubation in a neutravidin solution to attach the biotinylated capture probe to the electrode. Gold electrodes thus need an additional 1 h post-treatment step in the shorter alkyl thiol solution, as explained above, that slightly increases the analysis time.

Different protocols designed to improve the electrochemical signal have been tested by Orozco et al. (2010), such as pre-treatment of the gold electrodes and post treatment of the thiolated capture probe anchored over the gold electrodes, with and without a spacer thiol and with and without subsequent blocking of the uncovered electrode with BSA (to prevent unspecific adsorption of the capture probe). A 1.6-fold enhanced current density (1.39 μAmm^{-2}) vs a non-pre-treated ones (0.48 μAmm^{-2}) was achieved with electrochemically activated electrodes. After formation of the DNA-thiol SAM, a post-treatment with mercaptohexanol (a shorter chain thiol) seems to straighten better the thiol-probes, thus serving as spacers between the DNA probe strands to reduce steric hindrance and to force them into an extended conformation more amenable for hybridisation. Such post-treatment allows for a better exposure of the capture probe to the target sequence, producing higher signals (1.66 μAmm^{-2}) and improving reproducibility among devices (SD 0.05, $n=4$). Overall, the gold activated surface, the thiol post-treatment and BSA blockage showed a remarkable positive effect on the intensity of the amperometric signal. Orozco and Medlin (2011) demonstrated a synergetic effect of the mixed DNA-monolayer and BSA that increases almost 2-fold the current density over that achieved these components were not added. Remarkably, each treatment by itself leads to worse results, respectively. Likewise, blocking the surface with BSA effectively avoided unspecific adsorption in CNT electrodes, whereas biosensors fabricated on carbon platforms—using neutravidin (avidin)-enzyme conjugates—without BSA as blocking agent led to poorer results.

HRP enzyme-linked immunomarker coupled to digoxigenin-specific antibody are usually placed at the 3′ end of the signal probe in the SHA format to follow the electrochemical signal. O-phenylenediamine and 2,2 bis(3-ethylbenzenediamine-6-sulphonic acid) are the most commonly used mediators in the reductive catalysis of H_2O_2 to H_2O. However, because they are mutagenic in the Ames test (Voogd et al. 1980 and van der Bos et al. 1981), p-aminodyphenylamine, neither a mutagenic nor carcinogenic mediator of this reaction, was tested as a mediator. Selection of the suitable mediator/substrate concentrations is crucial to obtain optimal electrochemical signals. An optimised signal of (2.15/0.21 μAmm^{-2}) for positive/negative controls was obtained with 0.4 mM ADPA and 10 mM H_2O_2 for $P.$ $parvum$ (Orozco and Medlin 2011). Higher mediator and substrate concentrations (0.8/20)mM led to slightly worst

results under the same experimental conditions. Likewise, lower concentration neither improved the signal, e.g. positive controls of 1.48 and 1.66 μAmm^{-2} were obtained with CNT and gold electrodes, respectively, for $P.$ $parvum$ at 0.2/5 mM mediator/substrate concentrations (see Table 1). These results correlate well with previous mediator/substrate concentration studies with $A.$ $ostenfeldii$ where the positive/negative controls increase from 0.86/0.02 to 2.65/0.11 μAmm^{-2} when a mediator/substrate concentration ratio increases from 0.2/15.7 to 30/600 mM (Diercks et al. 2008b). Table 1 summarises these comparisons. Experiments performed with methylene blue as a mediator led to worst results.

Dimensions of the electrodes have also been shown to have a remarkable effect on the electroanalytical signal. Electrodes of different dimensions have been tested as transducer platform when developing genosensors for different toxic algal species. Whereas a signal of 0.86 μAmm^{-2} was observed by (Metfies et al. 2005) when testing the SHA over a 1.77 mm^{-2} carbon electrode for an $A.$ $ostenfeldii$ positive control, a 2.3-fold enhanced signal (2.42 μAmm^{-2}) was recorded by Diercks et al. (2008b) studying the same species on a 0.77 mm^{-2} electrode of the same material. An even more dramatic improvement of around 5-fold current density was observed when changing from a 12.57 mm^{-2} carbon electrode (2.15 μAmm^{-2}) to a 0.77-mm^{-2} graphite electrode, (10.09 μAmm^{-2}), for a $P.$ $parvum$ positive control (Orozco and Medlin 2011). The lower response reported by Metfies et al. (2005) (2.42/0.1 μAmm^{-2}) for $A.$ $ostenfeldii$ positive and negative controls, respectively anchored on a 0.77-mm^{-2} carbon electrode with respect to the greatly improved 10.09 μAmm^{-2} signal recorder for $P.$ $parvum$ on a graphite electrode of the same dimensions can be attributed to the non-optimised mediator/substrate concentrations tested (see Table 1). The fact that two different species were compared may also contribute to different signal results. However, the overall results suggest that the smaller the electrode surface, the higher the signal recorded. In Orozco and Medlin (2011), it was hypothesised that the higher diffusion of the electroactive species present in solution towards smaller electrode surfaces was responsible for signal improvement.

The working response and performance characteristics of the DNA-based electrochemical sensors are commonly evaluated by amperometry. For example, plotting the estimated current density value against the concentration of the target resulted a calibration curve, in which linear dynamics ranged from 0.0 to 5.2 ngμl^{-1}, with a sensitivity (slope) of 1.41 μAlng^{-1}mm^{-2}, an intercept of 0.16 μAmm^{-2} and a linear regression coefficient of 0.98, $n=26$. The limit of detection was estimated in 0.058 ngμl^{-1} of synthetic $P.$ $parvum$. Standard deviations among results (low than 8 % each point) demonstrated the high reproducibility among

different devices. A calibration curve using RNA extracted from this algal species has to be performed before field samples can be analysed.

Probe orientation and effect of the digoxigenin-enzymatic label

Unlike the common distal orientation of the 5′ thiol (biotin) group respect to the 3′ DIG label, to increase the sensitivity of the SHA genosensors, a different orientation of the capture and signal probes was studied using *P. parvum* and *Pseudo-nitzschia* strands as model targets by Orozco et al. (2011). In this new arrangement, the 5′ thiol (biotin) group was proximal to the 3′ DIG label. It was hypothesised that the proximal arrangements of the probes would have created sufficient steric hindrance to influence both the access of the antibody to the DIG and the communication between the electroactive species and the electrode. However, results showed that the current intensity for *P. parvum* positive control in both distal (1.16 μAmm^{-2}) and proximal orientation (1.13 μAmm^{-2}) were only slightly different. Such minor variation suggests orientation of the capture and signal probes on the electrode surface are not as critical as expected. This observation was confirmed when the signals obtained with this same arrangement (proximal orientation) using *P. australis* over CNT electrodes (1.07 μAmm^{-2}) were comparable to those from *P. parvum* in a distal orientation (1.16 μAmm^{-2}). Yet, a 2-fold increase in background observed from the negative control (0.45 μAmm^{-2} vs 0.2 μAmm^{-2}), which will led to a poorer limit of detection for each species, suggests a certain preference for the distal orientation of the probes. Thus, the probe sets developed and tested in a microtiter plate assay by Diereck et al. (2011) need to be re-evaluated with respect to their orientation because they are not all in the same orientation.

With the purpose of increasing sensitivity and improving analytical performance of the biosensor, two variations of the probe synthesis protocol were interrogated. First, a double DIG-labelled signal probe (at both the 5′ and the 3′ end of the oligonucleotide) instead of the single DIG label at the 3′ end was tested. A decrease in the signal from the positive control and an increase from the negative one were observed when the double DIG-labelled signal probe was amperometrically interrogated, using ADPA mediator and *P. parvum* as target sequence. A second variation consisted of the hybridisation of the *P. parvum* target sequence between a biotin capture probe and a directly HRP-labelled signal probe, thus avoiding the anti-DIG-labelled HRP step. The amperometric response for positive and negative controls was practically indistinguishable and much lower than those obtained with the standardised procedure using the DIG-labelled signal probe. These results suggest that neither the double DIG-labelled signal probe nor the directly HRP-labelled signal improve the analytical performance of the biosensor as expected. Therefore, the standard protocol as originally published by Metfies et al. (2005) with its improvements and optimisations in Orozco and Medlin (2011) give the best performance and sensitivity.

Long-term stability and storage of coated sensors

Having in mind in field toxic algal monitoring, feasibility of mass production along with long-term stability and storage of the biosensors were tested for *A. ostenfeldii* as a target model (Diercks et al. 2008a, d). Capture probes immobilised on both carbon and gold working electrode surfaces were protected by coating the chips with a Trehalose buffered solution. The sensors were stored at 4 °C and hybridised after 4, 6 and 12 months with a target and reporter DNA-strand. Signal intensity after 12 months of storage decreased around 50 and 25 % when compared to the freshly prepared carbon and gold sensors, respectively. Although these results are quite appealing when mass production of the biochips is implemented, a rigorous calibration of them is required before any measurement.

Genosensors for toxic algal monitoring

Much effort has been invested in the development of DNA-based biosensors for detection toxic algae. They range from the detection of only one species using the FISH and SHA formats, both electrochemically and colorimetrically to multiple species detection using multichips microtiter plates or microtiter plates, respectively. For instance, the SHA was first used to optimise the hybridisation conditions for the electrochemical detection of the toxic dinoflagellate *A. ostenfeldii* (Metfies et al. 2005). A colourimetric assay was later developed by using the SHA in a microtiter plate for detecting the toxic dinoflagellate *A. minutum* (Diercks et al. 2008a). With multi-species detection purposes, a multiprobe chip with an array of 16 gold electrodes for the simultaneous detection of up to 14 target species was developed (Diercks et al. 2008d). More recently, the different steps of the fabrication process from the electrochemical point of view, proof of concept with different algal species and evaluation of the influence of the transducer platform geometry and material in the biosensor analytical performance has been elucidated (Orozco and Medlin 2011). With the purpose of increasing sensitivity, probe orientation, the effect of a double DIG label and the use of a directly HRP-labelled signal probe on the electrochemical sensor performance was evaluated (Orozco et al. 2011). Most of these variations in the protocol (Table 1) have led to better electrochemical performance of the biosensors but to date no electrochemical biosensors have been field tested.

Semi-automatic devise

Rapid identification of aquatic microorganisms is of great importance to understand coastal dynamics and processes that can impact marine ecosystems. Simultaneous detection of multiple species is desired because phytoplankton communities consist of different species that vary greatly temporally and spatially. Arrays of electrodes enable, for example, a simultaneous detection of multiple species with different molecular DNA probes. Biosensors, meanwhile, can be used onsite and therefore circumvent the need to return samples into the laboratory. As a first approach towards semi-automatic monitoring of toxic algae, a detection system consisting of the multiprobe electrode and a semi-automated device was developed during the EU-project ALGADEC. The development of a multiprobe electrode for the detection of specific compositions of toxic algae by simultaneous detection of 16 different target molecules was the first step (Diercks et al. 2008d) and later (Diercks et al. 2011) details of the design and adaptation of the semi-automated device for the in situ analysis of toxic algae using the multiprobe electrode was described. The disposable multiprobe electrode was designed by iSiTEC GmbH (Bremerhaven, Germany) with the size of a conventional glass slide and produced by GEM (Pontypool, UK). The multiprobe electrode consisted of a carrier material that contained 16 gold-working electrodes, each with the size of 1.5 mm and a combined counter/reference electrode above the electrode array. The stems of the electrodes were fitted to a typical connecting strip. Valox and ceramic were tested as carrier materials. Multiprobe chips were either hand-spotted or spotted with a non-contact dispenser (Biodot Ltd., UK) from GEM and subsequently coated with Trehalose and dried for storage and shipment. The portable semi-automated device automatically processed the main steps of the probe to target hybridisation and facilitated the electrochemical detection of toxic algae in less than 2 h. The device can be used by laypersons because a manual RNA isolation is no longer required with the development of a lysis protocol. Only one prototype was developed in the lifetime of ALGADEC and was never commercialised. It has design faults that need corrected before commercialisation and field trials can begin. However, the multiprobe chip and the ALGADEC device can be used as a stand-alone system in the field and will contribute to monitoring programs to provide an early warning system for the aquaculture and tourist sectors that are most affected by toxic algal blooms once the design faults in the first prototype can be overcome.

Laboratory and field testing of the genosensor for Alexandrium tamarense

Capture and signal probes were developed for toxic North American clade of *A. tamarense*, along with a helper oligonucleotide to maintain the target site open for hybridisation of the signal probe. Capture probe (AACACTCCCACCAAG CAA) is biotinlyated and is bound to carbon-printed electrodes as described above. Total RNA was extracted using a Qiagen Kit and hybridised to the carbon electrode as described in Metfies et al. (2005). A helper oligonucleotide (TGCACCTCTGTTGGTRRTACATT) was added to the hybridisation solution. The signal probe (AACACTCC CACCAAGCAA) was 3′ DIG labelled. A concentration series was performed with decreasing amounts of RNA hybridised to the Chip, which resulted in a linear decrease of the electrochemical signal (Fig. 2).

Field samples were taken in the Orkney Islands as part of the BMBF TEPS project and RNA extracted. Cell counts were made on the same material by Dr. Malte Elbraechter. RNA was hybridised to the five replicate carbon electrodes (Fig. 3) with the signal probe (G), without the signal probe (G+C), with the helper oligo (S) and without the helper oligo probe (S+C). Certain stations clearly exhibited a higher electrochemical signal than the negative control (compare stations 7 and 14 with a current greater than 250 nA to stations 35 and 47 with a current less than 150 nA. The stations where the measured current was equal to or less than the negative control, these stations did not have any target cells nor were any toxins measured. When stations where the measured current was greater than the negative control were obtained, cell counts were regressed against the current measured and a positive correlation between current and cell counts were obtained (Fig. 4), with the detection limit measured at about 60 cellsl^{-1}. These results show that the genosensor can be applied to field material to provide an early warning system for toxic algae.

Fig. 2 Concentration series of decreasing amounts rRNA hybridised to the *Alexandrium tamerense* 18S probe and measured with the genosensor as in Metfies et al. (2005)
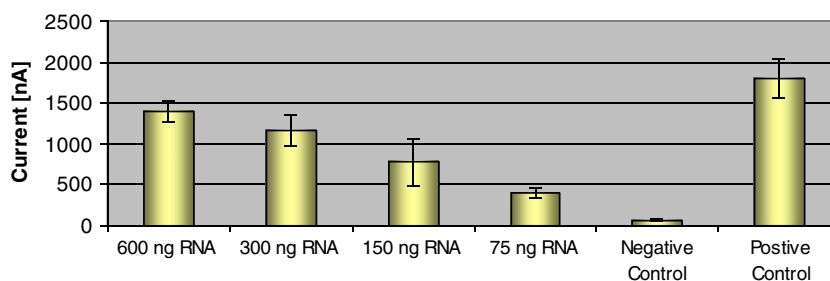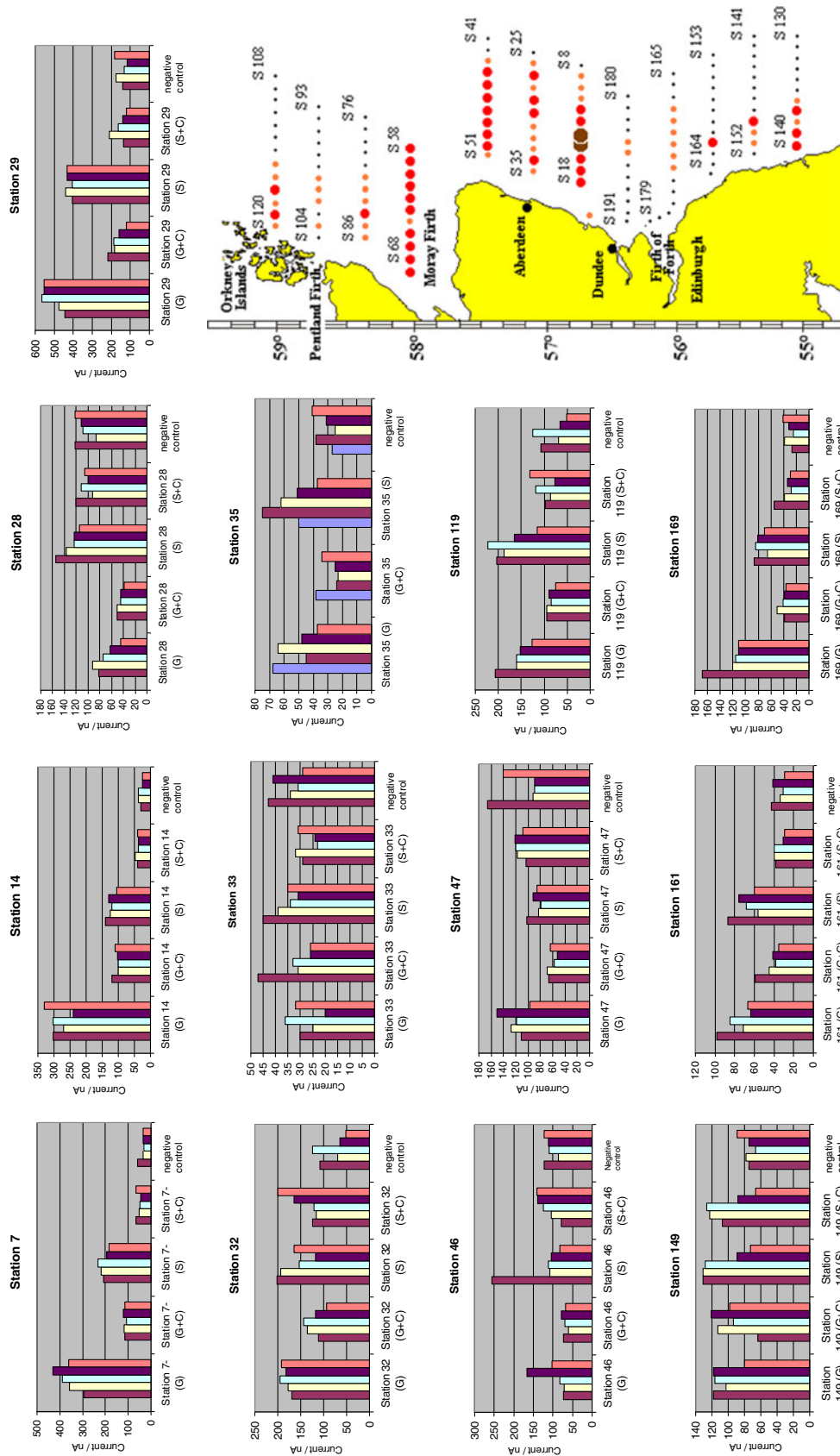
Fig. 3 Detection of rRNA in natural samples taken in different stations near the Orkney Islands during the BMBF project TEP in 2000. A map of the zone is included in the *right side* of the figure and the size of the dots indicates the relative amount of toxins recovered in the samples
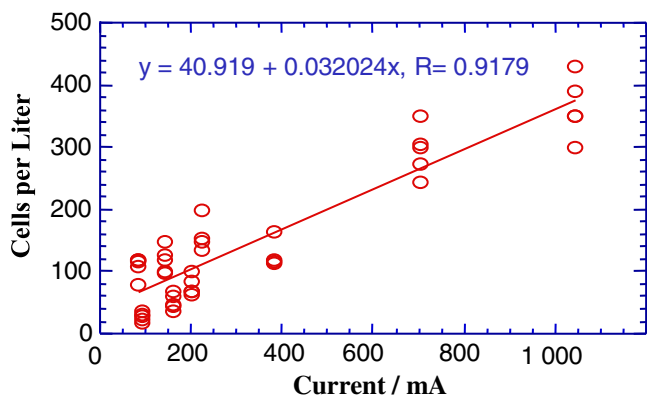
Fig. 4 Comparison of biosensor signals with cell count made on the same sample

Other SHA devises that use chemiluminescent detection have been field tested by the Scholin and Anderson laboratories (Anderson et al. 2005; Scholin et al. 1997) and this system is currently operating in a buoy system for in situ measurements (Scholin et al. 2009) but is very expensive. The SHA detection method has also been adapted to a microarray format Anderson et al. (2006) using a fibre optic detection method.

Limitations

One of the more important limitations for HABs monitoring is the impossibility of detecting extremely low concentration of toxic algae as cell densities are used to be relatively low. Filtration of large volume of samples is a common practice when identification and quantification of toxic alga is required. Another big issue is the very limited number of existing phytoplankton probes and the fact that they must be validated for each region where they can be present. Accuracy of cell counts is currently the limiting factor of all systems as manual isolation of RNA should be performed by trained molecular personal that ensures reliable quantities and qualities of rRNA from the sample. It is common that differently experienced users isolate different amounts of rRNA from the same number of algal cells, resulting in signal intensities that do not reflect the real situation in terms of cell counts. Besides the good performance of the device for the identification of laboratory strains, how the device performs in field tests has also to be determined. In this context, not only automation of the isolation of rRNA but also adaptation of the system based on the type of species, reliability and reproducibility of the results is a prevailing necessity.

Outlook and future prospects

Although there are some few research groups worldwide that are devoted to developing reliable tools to monitor the whole spectrum of toxic algae that threatens the aquatic ecosystems, they have made considerable progress over the past decade. Some efforts have been directed towards the design of synthetic DNA probes that specifically recognise the DNA of more than 10 species (Diercks et al. 2008c). So far only six probes have been tested with SHA formats over different electrodes with an electrochemical detection (Metfies et al. 2005; Diercks et al. 2008b; 2011; Orozco and Medlin 2011; Orozco et al. 2011). Material and dimensions of the transducer platforms have been studied and the signal optimised from both the hybridisation (Metfies et al. 2005) and the electrochemical point of view (Orozco and Medlin 2011; Orozco et al. 2011). Further decreases in the electrode dimensions (micrometric dimensions) will dramatically improve the signal/noise ratio (Orozco et al. 2012). Control of the surface chemistry and coverage of the electrode transducer have enhanced performance of electrochemical DNA biosensors. Recent advances and progress in the development of new surface chemistry approaches based on novel dithiol-based ternary SAM interfaces have lead to a highly efficient surface blocking chemistry and target accessibility, and hence to highly sensitive detection of target nucleic acids (Campuzano et al. 2012; Orozco et al. 2012). In an analogous manner, sensitivity of toxic algae genosensors will improve by controlling the surface chemistry with ternary DNA–SAM interfaces by maximizing the hybridisation efficiency and minimizing of unspecific adsorption events. Different mediators of the electrochemical reaction, such as methylene blue and ADPA have been interrogated (Metfies et al. 2005; Diercks et al. 2008b; 2011; Orozco and Medlin 2011; Orozco et al. 2011). However, other mediators should be considered to improve sensitivity. Towards in situ monitoring a semiautomatic device has successfully been attempted. Yet, for the reliable, timing and practical monitoring of HABs further improvement not only in their efficiency, performance, sensibility, detection limits, but also in versatility, sophistication and automation have to be achieved and field tested.

Different analysis methods, most of them complementary, are commonly required for identification and quantification of pathogens that circumvent the inherent disadvantages among them. The development of molecular methods holds great promise for HABs monitoring. The major advantage of such methods is associated with the design of oligonucleotides able to specifically detect different species and the development of antibodies to detect toxins. Design of molecular probes for the detection of toxic algae has to be based on the populations living in a specific area. Specific probes for several algal taxa have been developed recently; however, still only a small percentage of all toxic algal species is covered. To expand the design of probe sets for the species-specific identification of toxic algal species is still required.

The main drawback of molecular methods in HABs monitoring is related with the very low and variable concentrations of DNA that can be found in a certain sample. As an

alternative, or complementary, the development of new identification and detection systems based on biosensors and arrays of sensors is today a hot topic in environmental research. Among biosensors, those that use electrodes as transducer platforms to get an electrochemical signal offer a simple, rapid and reliable alternative for the development of low-cost mass-produced devices (Orozco et al. 2010). Biosensors features, such as sensitivity, moderate robustness and versatility, allow their integration into multisensor probes, flow systems and portable measurement systems. Material and geometry of the electrodes is a key point to be considered to obtain better functionality, improve sensitivity and decrease detection limits. Until now screen-printed electrodes of millimetric dimensions are the most widely used for the development of DNA-based electrochemical biosensors. However, the enhanced voltammetric response of ultramicroelectrode arrays (UMEAs), in terms of mass transport, current density and faradaic/capacitive current ratio (Orozco et al. 2010), makes them superior candidates to be explored for this purpose.

Filtration of larger volume of sample that preconcentrates the cells is commonly used to overcome the limitation of very low amounts of RNA that can be present in a sample of lower volume with fewer cells. However, this procedure is slow and tedious, so that other alternatives of preconcentration have to be implemented. Sample preconcentration by magnetic particles or signal amplification with gold nanoparticles have been used to improve the sensitivity of some biosensors (Castañeda et al. 2007). Selectively modified magnetic particles entrap the biological material in a preconcentration step and the formed complex is later magnetically captured for its detection at the surface of an electrode. Electrodes modified with gold nanoparticles have the added advantage of having higher surface area respect to unmodified electrodes (Orozco et al. 2007). The increased area expects to augment the capture places for the controlled immobilisation of the DNA receptors selective to toxic algae. Different configurations in which gold nanoparticles have been applied in the development of genosensors of improved properties have been reported (e.g. Castañeda et al. 2007). The major analyte accessibility, better electron transference and faster kinetics have promoted the increase on sensitivity. However, and although the synergic effect of using gold nanoparticles and UMEAs has been used to improve the properties of an enzymatic biosensor (Orozco et al. 2009), this combination is as yet unexplored for the development of genosensors. Such arrangements would dramatically improve the sensitivity of toxic algae genosensors and reduce the need for high volume of sample required for valid identification and quantification.

A prototype of a portable semi-automated electrochemical biosensor system that enables the electrochemical detection of microalgae from water samples in less than 2 h, without the need of expensive equipment has been developed, but is still not commercially available. As a high concentration and quality of the RNA is required and cell counts should not be compared with those using any other enumeration technique, it is necessary a trained molecular scientist for the manual isolation of RNA. Calibration curves are also necessary. Validation of probe signals against total rRNA over the growth cycle of the target microalgae under different environmental conditions has to be conducted to verify these calibration curves. Future research is directed to overcome these difficulties and so an autonomous biosensor can be combined with the state-of-the-art in situ measurement systems for the reliable and high-resolution monitoring of marine phytoplankton in the oceans. With continuous innovation and attention to key challenges, we expect that semiautomatic devices containing DNA-based electrochemical biosensors to have a remarkable impact upon resolution of the HABs.

References

Anderson DM, Kulis D, Erdner D, Ahn S, Walt D (2006) Fibre optic microarrays for the detection and enumeration of harmful algal bloom species. Afr J Mar Sci 28:231–235

Anderson DM, Kulis DM, Keafer BA, Gribble KE, Marin R, Scholin CA (2005) Identification and enumeration of *Alexandrium* spp. from the Gulf of Maine using molecular probes. Deep-Sea Res II 52:2467–2490

Ayers K, Rhodes LL, Tyrrell J, Gladstone M, Scholin CA (2005) International accreditation of sandwich hybridization assay format DNA probes for micro-algae. New Zeal J Mar Fresh 39:1225–1231

Campuzano S, Kuralay F, Wang J (2012) Ternary monolayer interfaces for ultrasensitive and direct bioelectronic detection of nucleic acids in complex matrices. Electroanalysis 24:483–493

Castañeda MT, Merkoçi A, Pumera M, Alegret S (2007) Electrochemical genosensors for biomedical applications based on gold nanoparticles. Biosens Bioelectron 22:1961–1967

DeLong EF, Wickham GS, Pace NR (1989) Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. Science 243:1360–1363

Diercks S, Metfies K, Medlin, LK (2008a) Colorimetric detection of the toxic dinoflagellate *Alexandrium minutum* using sandwich hybridization in a microtiter plate assay. Harmful Algae 7:137–145

Diercks S, Metfies K, Medlin LK (2008b) Development and adaptation of a multiprobe biosensor for the use in a semi-automated device for the detection of toxic algae. Biosens Bioelectr 23:1527–1533

Diercks S, Metfies K, Medlin LK (2008c) Molecular probe sets for the detection of toxic algae for use in sandwich hybridisation formats. J Plankton Res 30(4):439–448

Diercks, S, Metfies, K, Jäckel, S, Medlin, LK (2008d) Development and adaptation of a multiprobe biosensor for the use in a semi-automated device for the detection of toxic algae. Biosens Bioelectron 23:1527–1533

Diercks S, Gescher C, Metfies K, Medlin LK (2009) Evaluation of locked nucleic acids for signal enhancement of oligonucleotide probes for microalgae miniaturised on solid surfaces. J Appl Phycol 21:657–668

Diercks-Horn S, Metfies K, Jäckel S, Medlin LK (2011) The ALGADEC device: a semi-automated rRNA biosensor for the detection of toxic algae. Harmful Algae 10:395–401

Díez B, Pedrós-Alió C, Massana R (2001) Study of genetic diversity of eukaryotic picoplankton in different oceanic regions by small-subunit rRNA gene cloning and sequencing. Appl Environ Microbiol 67:2942–2951

Gescher C, Metfies K, Medlin LK (2008) The ALEX CHIP—development of a DNA chip for identification and monitoring of *Alexandrium*. Harmful Algae 7:485–494

Giovannoni SJ, Britschgi TB, Moyer CL, Field KG (1990) Genetic diversity in Sargasso Sea bacterioplankton. Nature 345:60–63

Greenfield DI, Marin R, Jensen S, Massion E, Roman B, Feldman J, Scholin CA (2006) Application of environmental sample processor (ESP) methodology for quantifying *Pseudo-nitzschia australis* using ribosomal RNA-targeted probes in sandwich and fluorescent in situ hybridisation formats. Limnol Oceanogr Meth 4:426–435

Groben R, Medlin LK (2005) In situ hybridisation of phytoplankton using fluorescently-labelled rRNA Probes. In: Zimmer EA, Roalson E (ed) Methods in Enzymology. Elsevier, San Diego, 395, 299–310

Groben R, John U, Eller G, Lange M, Medlin LK (2004) Using fluorescently labelled rRNA probes for hierarchical estimation of phytoplankton diversity. Nova Hedw 79:313–320

Guschin DY, Mobarry BK, Proudnikov D, Stahl DA, Rittmann BE, Mirzabekov AD (1997) Oligonucleotide microchips as genosensors for determinative and environmental studies in microbiology. Appl Environ Microbiol 63:2397–2402

Hallegraeff GM (1993) A review of harmful algal blooms and their apparent global increase. Phycologia 32:79–99

Haywood AJ, Scholin CA, Marin R, Steidinger KA, Heil C, Ray J (2007) Molecular detection of the brevetoxin-producing dinoflagellate *Karenia brevis* and closely related species using rRNA-targeted probes and a semiautomated sandwich hybridisation assay. J Phycol 43:1271–1286

John U, Cembella A, Hummert C, Ellbrächter M, Groben R, Medlin LK (2003) Discrimination of the toxigenic dinoflagellates *Alexandrium tamarense* and *A. ostenfeldii* in co-occurring natural populations from Scottish coastal waters. Eur J Phycol 38:25–40

John U, Medlin LK, Groben R (2005) Development of specific rRNA probes to distinguish between geographic clades of the *Alexandrium tamarense* species complex. J Plank Res 27:199–204

Keller MD, Selvin RC, Claus W, Guillard RRL (1987) Media for the culture of oceanic ultraphytoplankton. J Phycol 23:633–638

Ki J-S, Han M-S (2006) A low-density oligonucleotide array study for parallel detection of harmful algal species using hybridisation of consensus PCR products of LSU rDNA D2 domain. Biosens Bioelectron 21:1812–1821

Liao JC, Mastali M, Li Y, Gau V, Suchard MA, Babbitt J, Gornbein J, Landaw EM, McCabe ERB, Churchill BM, Haake DA (2007) Development of an advanced electrochemical DNA biosensor for bacterial pathogen detection. J Mol Diag 9:158–168

Lockhart DJ, Dong H, Byrne MC, Follettie MT, Gallo MV, Chee MS, Mittmann M, Wang C, Kobayashi M, Horton H, Brown EL (1996) Expression monitoring by hybridisation to high-density oligonucleotide arrays. Nat Biotechnol 14:1675–1680

Ludwig WSO, Westram R, Richter L, Meier H, Yadhukumar BA, Lai T, Steppi S, Jobb G, Förster W, Brettske I, Gerber S, Ginhart AW, Gross O, Grumann S, Hermann S, Jost R, König A, Liss T, Lüßmann R, May M, Nonhoff B, Reichel B, Strehlow R, Stamatakis A, Stuckmann N, Vilbig A, Lenke M, Ludwig T, Bode A, Schleifer K-H (2004) ARB, a software environment for sequence data. Nucleic Acids Res 32:1363–1371

Metfies K, Medlin LK (2004) DNA-microchips for phytoplankton. The fluorescent wave of the future. Nova Hedw 79:321–327

Metfies K, Huljic S, Lange M, Medlin LK (2005) Electrochemical detection of the toxic dinoflagellate *Alexandrium ostenfeldii* with a DNA-biosensor. Biosen Bioelec 20:1349–1357

Metfies K, Gescher C, Frickenhaus S, Niestroy R, Wichels A, Gerdts G, Knefelkamp B, Wiltshire K, Medlin LK (2010) Contribution of the Class Cryptophyceae to phytoplankton structure in the German Bight. J Phycol 46:1152–1160

Miller CA, Scholin PE (1998) Identification and enumeration of cultured and wild Pseudo-nitzschia (Bacillariophyceae) using species specific LSU rRNA-targeted fluorescent probes and filter-based whole cell hybridization. J Phycol 34:371–382

Moon-van der Staay SY, De Wachter R, Vaulot D (2001) Oceanic 18S rDNA sequences from picoplankton reveal unsuspected eukaryotic diversity. Nature 409:607–610

O'Halloran C, Silver MW, Holman TR, Scholin CA (2006) Heterosigma akashiwo in central California waters. Harmful Algae 5:124–132

Orozco J, Medlin L (2011) Electrochemical performance of a DNA-based sensor device for detecting toxic algae. Sensors and Actuators B: Chemical 153:71–77

Orozco J, Suárez G, Fernández-Sánchez C, McNeil C, Jiménez-Jorquera C (2007) Characterization of ultramicroelectrode arrays combining electrochemical techniques and optical microscopy imaging. Electrochim Acta 53:729–736

Orozco J, Jiménez-Jorquera C, Fernández-Sánchez C (2009) Gold nanoparticle ultramicroelectrode arrays for biosensing: a comparative assessment. Bioelectrochemistry 75:176–181

Orozco J, Jiménez-Jorquera C, Fernández-Sánchez C (2010) Ultramicroelectrode arrays: a promising analytical tool for environmental monitoring. Sensors 10:475–490

Orozco J, Baudart J, Medlin L (2011) Evaluation of probe orientation and effect of the digoxigenin-enzymatic label in a sandwich hybridisation format to develop toxic algal biosensors. Harmful Algae 10:489–494

Orozco J, Jiménez-Jorquera C, Fernández-Sánchez C (2012) Electrochemical performance of self-assembled monolayers at gold nanoparticle-modified ultramicroelectrode arrays architectures. Electroanalysis 24:635–642

Penna A, Magnani M (1999) Identification of *Alexandrium* (Dinophyceae) species using PCR and rDNA-targeted probes. J Phycol 35:615–621

Rautio FBKB, Lahdenpera J, Breinstein A, Molin S, Neubaure P (2003) Sandwich hybridisation assay for quantitative detection of yeast RNAs in crude cell lysates. Microbial Cell Factories 2:4

Scholin CA, Buck KR, Britschig T, Cangelosi G, Chavez FP (1996) Identification of *Pseudo-nitzschia australis* (Bacillariophyceae) using rRNA-targeted probes in whole cell and sandwich hybridisation formats. Phycologia 35:190–197

Scholin C, Miller P, Buck K, Chavez F, Harris P, Haydock P, Howard J, Cangelosi G (1997) Detection and quantification of *Pseudo-nitzschia australis* in cultured and natural populations using LSU rRNA-targeted probes. Limnol Oceanogr 42:1265–1272

Scholin C, Doucette G, Jensen S, Roman B, Pargett D, Marin R II, Preston C, Jones W, Feldman J, Everlove C, Harris A, Alvarado N, Massion E, Birch J, Greenfield D, Vrijenhoek R, Mikulski C, Jones K (2009) Remote detection of marine microbes, small invertebrates, harmful algae, and biotoxins using the environmental sample processor (ESP). Oceanography 22:158–167

Töbe K, Eller G, Medlin LK (2006) Automated detection and enumeration for toxic algae by solid-phase cytometry and the introduction of a new probe for *Prymnesium parvum* (Haptophyta: Prymnesiophyceae). J Plank Res 7:643–657

Touzet N, Keady E, Raine R, Maher M (2009) Evaluation of taxa-specific real time PCR, whole-cell FISH and morphotaxonomy analyses for the detection and quantification of the toxic microalgae *Alexandrium minutum*s (Dinophyceae), Global Clade ribotype. FEMS Microbiol Ecol 67:329–341

Touzet N, Davidson K, Pete R, Flanagan K, McCoy GR, Amzil Z, Maher M, Chapelle A, Raine R (2010a) Co-occurrence of the West European (Gr.II) and North American (Gr.I) ribotypes of *Alexandrium tamarense* (Dinophyceae) in Shetland, Scotland. Protist 161:370–384

Touzet N, Farell H, Ní Rathaille A, Rodriguez P, Alfonso A, Botana L, Raine R (2010b) Dynamics of co-occurring *Alexandrium minutum* (Global Clade) and *A. tamarense* (West European) (Dinophyceae) during a summer bloom in Cork Harbour, Ireland. Deep-Sea Res II 57:268–278

Tyrrell JV, Connell LB, Scholin CA (2002) Monitoring for *Heterosigma akashiwo* using a sandwich hybridisation assay. Harmful Algae 1:205–214

van der Bos ES, Doelen AA, van Rooy N, Schuurs AHWM (1981) 3,3′, 5,5′-Tetramethylbenzidine as an Ames test negative chromogen for horse-radish peroxidase in enzyme-immunoassay. J Immunoass 2:187–204

Voogd CE, Vanderstel JJ, Jacobs J (1980) On the mutagenic action of some enzymeimmunoassay substrates. J Immunol Methods 36:55–61

Wang Q, Li NQ, Wu YH (2001) Direct electrochemistry of cytochrome c at a metallothioneins self-assembled gold electrode. Electroanalysis 13:149–154

Zammatteo N, Moris P, Alexandre I, Vaira D, Piette J, Remacle J (1995) DNAprobe hybridisation in microwells using a new bioluminiscent system for the detection of PCR-amplified HIV-1 proviral DNA. J Virological Meth 55:185–197