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# Detection and quantification of the toxic microalgae *Karenia brevis* using lab on a chip mRNA sequence-based amplification

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ABSTRACT

Now and again, the rapid proliferation of certain species of phytoplankton can give rise to Harmful Algal Blooms, which pose a serious threat to marine life and human health. Current methods of monitoring phytoplankton are limited by poor specificity or by the requirement to return samples to a highly resourced, centralised lab. The Lab Card is a small, microfluidic cassette which, when used in tandem with a portable Lab Card Reader can be used to sensitively and specifically quantify harmful algae in the field, from nucleic acid extracts using RNA amplification; a sensitive and specific method for the enumeration of potentially any species based on their unique genetic signatures. This study reports the culmination of work to develop a Lab Card-based genetic assay to quantify the harmful algae *Karenia brevis* using mRNA amplification by the Nucleic Acid Sequence Based Amplification (NASBA) method. *K. brevis* cells were quantified by amplification of the *rbcL* gene transcript in nucleic acid extracts of *K. brevis* cell samples. A novel enzyme dehydration and preservation method was combined with a pre-existing reagent Gelification method to prepare fully preserved Lab Cards with a shelf-life of at least six weeks prior to use. Using an internal control (IC), the Lab Card-based *rbcL* NASBA was demonstrated for the quantification of *K. brevis* from cell extracts containing between 50 and 5000 cells. This is the first demonstration of quantitation of *K. brevis* using IC-NASBA on a Lab Card.

## 1. Introduction

Nucleic acid sequence amplification is a state of the art analytical tool for the identification and enumeration of microorganisms in the ocean including the specific recognition of species that pose a threat to human and animal health. However, this can require the retrieval of samples from remote or hazardous off-shore locations, prior to analysis in a centralised lab, leading to delayed results, increased risks and high economic costs. Accordingly, there is an increasing demand for autonomous systems that can make accurate (laboratory standard) nucleic acid-based measurements *in situ*, using portable or deployable self-powered instrumentation (McQuillan and Robidart, 2017). These systems could be particularly useful for the measurement and surveillance of harmful algal blooms (HABs), which result from the rapid proliferation of certain species of phytoplankton, and which can occur stochastically and offshore (Backer et al., 2015; Berdalet et al., 2015). The incidence of HABs has been increasing globally for some time (Hallegraeff, 1993) and there is a demand for new tools to provide a more reliable early warning of their occurrence (Sellner et al., 2003).

Existing lab-based methods for the measurement of phytoplankton including harmful algae are often based on various forms of nucleic acid analysis (molecular methods) (Coyne et al., 2005; Rinta-Kanto et al., 2005; Penna et al., 2007), flow cytometry (Dubelaar et al., 2007; Buskey and Hyatt, 2006) or microscopy (Karlson et al., 2010). In addition, multispectral satellite imaging can be used for the indirect (based on radiance/ocean colour) measurement of phytoplankton *in situ* (Stumpf, 2001; Carvalho et al., 2010), but is non-specific and limited by a low spatial resolution and high limit of detection (LOD). Deployable flow cytometers such as the CytoBuoy (Dubelaar and Gerritzen, 2000), CytoSub (Thyssen and Denis, 2011) and Imaging FlowCytobot (Campbell et al., 2010; Campbell et al., 2013) can measure phytoplankton cells directly in seawater, but these instruments cannot always reliably discriminate HAB species from morphologically similar, benign cell types. Nucleic acid amplification has the principle advantages of high sensitivity and specific recognition and discrimination of the target species based on unique genetic signatures, even from a complex, mixed

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species sample. For example, a number of nucleic acid-based techniques have been demonstrated for the selective enumeration of *Karenia brevis* (Gray et al., 2003; Casper et al., 2004), a bio-toxin producing microalga which periodically gives rise to HABs known colloquially as "red tides", and which pose a severe threat to wildlife, and can cause respiratory irritation in humans (Flaherty and Landsberg, 2011; Landsberg et al., 2009; McHugh et al., 2011; Pierce and Henry, 2008; Rolton et al., 2014; van Deventer et al., 2012). Specifically, *K. brevis* cells can be quantified from genetic extracts of seawater samples using Nucleic Acid Sequence Based Amplification (NASBA) of the *rbcL* gene transcript (Casper et al., 2004). This encodes a large subunit of the *K. brevis* RuBisCo enzyme, which catalyses the first step in $CO_2$ fixation and has been employed as a proxy for the detection and analysis of phytoplankton groups in environmental samples (Pichard et al., 1997). NASBA is an RNA amplification technique which features a practical LOD of 10 cells per litre of processed water (Loukas, 2016) and, due to the highly labile nature of mRNA, it preferentially measures viable, transcriptionally active cells over dead cells (Compton, 1991).

The potential to use NASBA for the quantification of *K. brevis* from nucleic acid extracts of cell samples *in situ* has been demonstrated using a miniature Lab on a Chip (LOC), known as a 'Lab Card', and a small, battery powered 'Lab Card Reader' (Tsaloglou et al., 2013), developed as part of the European LABONFOIL project (www.labonfoil.eu). Although the Lab Card Reader does not feature nucleic acid extraction capability, it could be combined with any of the large number of portable 'extraction' devices currently under development (Chen et al., 2007; Kim et al., 2009) such that all steps required for *in situ* nucleic acid analysis could be performed in the field. The structure and composition of a Lab Card and Lab Card Reader has been described in detail by Tsaloglou et al. (Tsaloglou et al., 2013), and summarised here, shown in Fig. 1. The Lab Cards are disposable (single use) plastic, credit-card-sized microfluidic assemblages featuring two micro-chambers (approx. 10 μL in volume) in which (i) RNA sample is mixed with heat-tolerant NASBA reagents (a pH-buffered solution of co-factor ions and nucleotides) and denatured before passing to a second chamber where (ii) the mixture is combined with heat-sensitive enzymes and incubated at a stable, low temperature (41 °C) to achieve the amplification of the target sequence. The 'Lab Card Reader' is a small, portable instrument, which features peristaltic pumps and disc valves to actuate the Lab Card microfluidics, heaters to control the temperature of each micro-chamber and a fluorescence optics module which measures in real-time the emission of multi-wavelength fluorescence. An autonomous workflow can be configured and initiated *via* an interface with a Tablet computer.

In addition to the Lab Card system, Tsaloglou et al. (Tsaloglou et al., 2013) have reported that *K. brevis rbcL* mRNA can be amplified on a Lab Card in a multiplexed reaction featuring a co-amplified 'internal control' construct of known concentration. This could theoretically enable accurate quantitation of *rbcL* without the need to run multiple standards in parallel or replicate reactions, using a method invented by Weusten et al. (Weusten et al., 2002a; Weusten et al., 2002b) and adapted for the real time quantification of *K. brevis* by Casper et al. (Casper et al., 2005) and Patterson et al. (Patterson et al., 2005). Furthermore, the reactions could be performed on a Lab Card using NASBA reagents that were pre-prepared and preserved using a proprietary Gelification technology, however wet enzyme mixtures had to be prepared and added manually. In the present study, we report the development of fully pre-prepared and preserved (enzymes and reagents) Lab Cards that can perform all stages of *rbcL* NASBA in an autonomous workflow and we demonstrate for the first time the use of the system to quantify *K. brevis* from RNA extracts of samples containing a range of *K. brevis* cell concentrations spanning two orders of magnitude.

## 2. Materials and methods

### 2.1. Karenia brevis culture

*K. brevis* (Strain CCMP2228) was obtained from the Provasoli-Guillard National Centre for the Culture of Marine Phytoplankton (CCMP), and maintained in static cultures at 19 ± 1 °C in L1 medium (Guillard and Hargraves, 1993), with a 12 hour photoperiod using cool fluorescent light. Cell counts were performed using a Sedgewick Rafter counting chamber (Fisher Scientific, UK) and an inverted microscope after fixation and staining in Lugol's Iodine.

### 2.2. The Lab Card system

Full details of the Lab Card system and Lab Card Reader instrument have been described in previous work by Tsaloglou et al. (Tsaloglou et al., 2013), but a brief overview of the system is illustrated in Fig. 1. The Lab Cards are fabricated by injection moulding Cyclic Olefin Copolymer (COC) to form a series of microfluidic channels (approx. 250 μm wide × 450 μm deep) and two reaction chambers (approx. 10 μL volume). The COC is laminated between self-adhesive polypropylene film to seal the features. Luer connectors are used to add RNA sample and recover waste. When inserted into a Lab Card Reader,

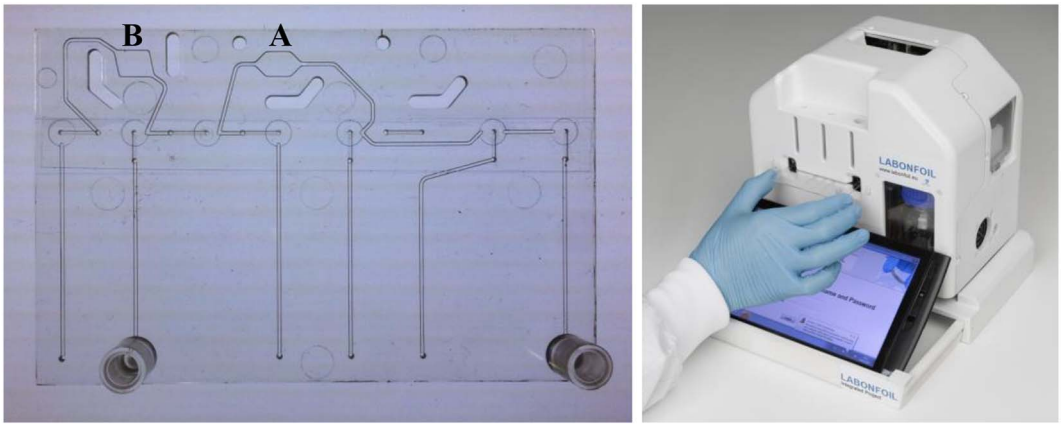


**Fig. 1.** An overview of the Lab Card system, adapted from Tsaloglou et al. (Tsaloglou et al., 2013). Lab Cards are credit-card sized, injection moulded COC cartridges featuring microfluidic channels and micro-chambers (left panel). Lab Cards are inserted into the Lab Card Reader (right panel). Once inserted, peristaltic pumps actuate the movement of an RNA sample into the first Lab Card chamber (A), where it re-hydrates a mixture of preserved (Gelified) NASBA reagents and oligonucleotides. Heating units on the Lab Card Reader raise the temperature of the chamber to 65 °C for 5 min, after which the fluid is moved into a second Lab Card chamber (B), were it re-hydrates and activates a mixture of preserved NASBA enzymes. This chamber is heated to 41 °C for 90 min during which the RNA sample is amplified resulting in the emission of fluorescence from molecular beacons. Amplification is measured in real-time based on the fluorescence emission using a two channel optics module. The process is configured and controlled *via* an interface between the Lab Card Reader and a tablet computer.