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Detection of *Heterosigma akashiwo* (Hada) using specific RNA probes: Variability of RNA content with environmental conditions

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ABSTRACT

The raphidophyte *Heterosigma akashiwo*, which forms toxic blooms, causes major economical losses to the fish industry because of the fish kills involved. It is therefore important to be able to detect not only *H. akashiwo* but other toxic phytoplankton species as well, rapidly and accurately to reduce losses by fish kills. With this purpose, DNA sequences from *H. akashiwo* 18S and 28S rRNA gene regions were studied *in silico* to design species-specific probes to be used in a microarray format. Three strains of *H. akashiwo* (AC 265, AC 266 and GUMACC 120) were grown at optimal conditions and transferred into new environmental conditions changing either the light intensity, salinity, temperature or nutrient concentrations, to check if any of these environmental conditions induced changes in the cellular RNA concentration. The aim of this experiment was the calibration of several species-specific probes for the quantification of *H. akashiwo*. Differences on RNA content were not significant ($p < 0.05$) in any of the treatments, therefore the calibration curves were validated. The designed probes are reliable for the detection and quantification of *H. akashiwo* cells in natural waters.

1. Introduction

Heterosigma akashiwo is a bloom-forming raphidophyte distributed worldwide (Tomas, 1978b; Hallegraeff and Hara, 1998). Its survival is poor at low light intensities and temperatures, favouring its coastal distribution in temperate and subtropical waters (Tomas, 1978b). *H. akashiwo* is an euryhaline and eurythermal species (Tomas, 1978a; Smayda, 1998), surviving a salinity range of 2–50, and showing high growth rates at temperatures from 10/14 to 30 °C (Tomas, 1978a; Yamochi, 1989), although inter-strain variability may occur (Fredrickson et al., 2011). This wide range of salinities and temperatures tolerance, makes *H. akashiwo* a successful species as it can outcompete other dominant species such as *Skeletonema costatum* in Hiroshima Bay (Itakura et al., 1996b). *H. akashiwo* may also produce allelopathic substances that inhibit the growth of other phytoplankton species, such as *S. costatum* (Honjo, 1993). Selective grazing may also contribute to its success (Verity and Stoecker, 1982). Moreover, Fukami et al. (1996) observed bacterial assemblages that inhibited the growth of other phytoplankton species contributing to the dominance of *H. akashiwo* from the Uranouchi-Wan inlet, Japan. Diel vertical migration seems to be another ecological advantage of this species, i.e. it can move to phosphate-rich deeper waters at night where it accumulates phosphate that is later used when the cells are back in the illuminated surface waters (Yamochi and Abe, 1984; Watanabe et al., 1988). Vertical migration in *H. akashiwo* is believed to be controlled by the light/dark cycle as well as an internal clock (Takahashi and Hara, 1989). Furthermore, non-favourable periods for growth may be overcome by a benthic stage (Yamochi, 1989; Itakura et al., 1996a).

H. akashiwo has been associated with fish kills because of brevetoxin-like compounds (Khan et al., 1997). Other compounds, which may influence fish mortality, are reactive oxygen species (ROS) and toxic poly-unsaturated fatty acids, the first have been observed in *H. akashiwo* (Twiner and Trick, 2000), and both types in the raphidophyte *Chattonella marina* (Ahmed et al., 1995; Marshall et al., 2003). Furthermore, sub-lethal effects have been observed on the south-eastern oyster (*Crassostrea virginica*) after exposure to *H. akashiwo* (Keppler et al., 2005). Thus, *H. akashiwo* blooms have an important economic impact in fisheries (Yamochi, 1989; Chang et al., 1990, 1993; Taylor, 1993; Taylor and Haigh, 1993) and need to be monitored.

As many microalgal species, *H. akashiwo* is not easy to identify by optical microscopy because it can show great morphological variability (Bowers et al., 2006). The use of fixatives may change

Abbreviations: LS, low salinity; HS, high salinity; LL, low light; HL, high light; LT, low temperature; HT, high temperature; T0, start of the experiment; T1, 24 h; T2, 48 h; T3, 72 h; T4, 96 h; strain 1, AC265; strain 2, AC266; strain 3, GUMACC120.

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morphology, size and colour of the cells making their identification even more difficult (Woelfl and Whitton, 2000). For efficient monitoring of toxic phytoplankton, rapid detection methods are necessary. Molecular probes have become an alternative to the classic microscopy for the identification to species level (e.g. Hiroishi et al., 1988; Miller and Scholin, 1996; Tyrrell et al., 2002). Partial DNA sequences are pooled into databases allowing comparison of genetic variability among strains, which helps improve the specificity of oligo-nucleotide probes (Anderson et al., 2005). One of these alternatives is the use of microarrays, which offers faster means of community analysis (Medlin et al., 2006).

This work is part of the project MIDTAL (MIcroarrays for the Detection of Toxic ALgae), which main objective was to create a universal microarray for the detection and quantification of toxic algae, as well as their toxins. Since one of the target species is *H. akashiwo*, specific DNA probes were designed for this species and spotted onto the microarray chip. The aim of the work presented here was to test if there are important variations in RNA content of *H. akashiwo* under stress conditions, as well as to calibrate the different rRNA probes on the microarray, to be able to estimate cell numbers reliably.

2. Materials and methods

2.1. Algal strains

Three strains of *H. akashiwo* were used in these experiments. Two of them were isolated in France: strain AC265 isolated in 1992 in the Bay of La Rochelle, and strain AC266 isolated in 1996 in the pond Etang de Diana in Corsica, both obtained from Algobank, Caen, France. The other strain, GUMACC120 was provided by the Göteborg's University Marine Culture Collection, Sweden. They are referred as strain 1, strain 2 and strain 3, respectively. Once in the laboratory, all strains were grown in f/2 (Guillard, 1983) Si-modified medium with salinity 31, at 16 °C and 100 μmol photons m^{-2} s^{-1}.

2.2. Experimental design

A stock culture of each strain was grown in f/2 Si-modified medium salinity 31, 16 °C and under 100 μmol photons m^{-2} s^{-1} irradiation. Fresh media was added regularly to keep cultures in exponential phase.

To test the effect of different stress conditions, the three strains were inoculated separately in 200 ml tissue culture flasks with vented caps. Initial volume in each flask was 200 ml, with 20 ml of initial *H. akashiwo* culture and f/2 modified according to the conditions applied making up the rest of the volume.

Control cultures were grown at the same conditions as the initial cultures. Salinity, light intensity, temperature and nutrient depletion were tested in parallel, changing one parameter per set of cultures. For salinity stress, the strains were inoculated in flasks containing f/2 at 25 and 37 for lower (LS) and higher salinity (HS) than the control conditions. Low light intensity (LL) was 15 μmol photons m^{-2} s^{-1} and high light intensity (HL) was 365 μmol photons m^{-2} s^{-1}. Temperatures were set at 11 °C for low temperature (LT) and 21 °C for the high one (HT). Phosphorus-depletion was carried out by using modified f/2 medium without phosphate. Nitrogen-depletion was carried out in modified f/2 medium without nitrate. All conditions were carried out for all three strains separately and in triplicates. It should be noted that the nutrient depleted treatments did contain at the beginning of the experiment some N or P since a 10% (by volume) of culture with f/2 medium was used for inoculation.

All sets of conditions were run at the same time to use inocula from the same starting cultures. The day of cells inoculation was considered as time zero (T0). Subsamples were taken from the initial cultures at T0 for cell counts, chlorophyll concentration and RNA concentration.

Subsamples of the cultures at each different condition were taken after 24 h of inoculation (T1), after 48 h (T2) and after 72 h (T3). For these, 10 ml were taken from each flask and mixed in sets of three, to have 3 replicates of a 30 ml mix of the three strains (Fig. 1). Strains were not mixed before, to ensure having cells from

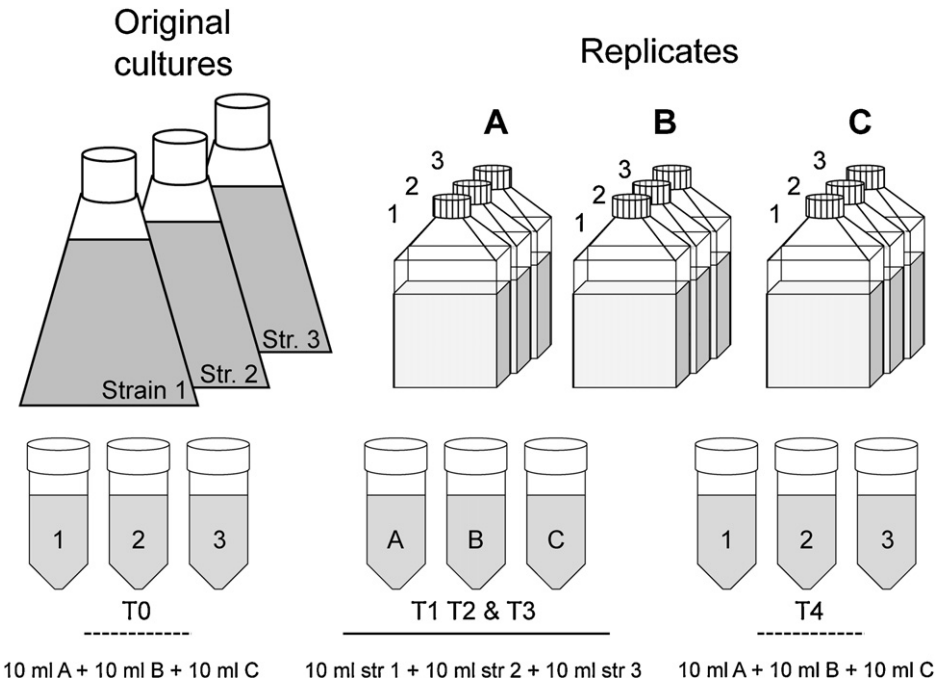

Fig. 1. Schema of the experimental design for each condition tested, from the initial culture of each strain to the replicates and the way subsamples were pooled together at each sampling time (T0 = time 0, start of the experiment, T1 = 24 h, T2 = 48 h, T3 = 72 h, T4 = 96 h, Strain 1 = AC265, Str. 2 = AC266 and Str. 3 = GUMACC120). In T0 and T4 replicates were pooled by strain, whilst in T1, T2 and T3 replicates were pooled together to get a mix of the three strains in each final tube.