

Monitoring for *Heterosigma akashiwo* using a sandwich hybridization assay

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Abstract

Field testing of a ribosomal RNA (rRNA)-targeted sandwich hybridization assay (SHA) for *Heterosigma akashiwo* (Raphidophyceae) in Puget Sound, WA, USA, has showed that the lower limit of detection is well below the level at which cells pose a danger to fish. Moreover, the assay has proven to be both rapid and easy-to-use. Isolates of *H. akashiwo* from Australia, Japan, New Zealand, South Korea, Spain and USA were correctly identified using the SHA, indicating that this diagnostic tool could be deployed globally. Samples containing *H. akashiwo* can be preserved for subsequent SHA analysis using several methods: fixation with acidic Lugol's iodine followed by room temperature storage, collection onto Durapore filters followed by storage at -70°C or, alternatively, the filters are mixed with a lysis solution buffer and the sample lysate stored at -70°C . Additionally, we sought to determine whether the SHA could successfully detect *H. akashiwo* in the presence of clay that might some day be used to mitigate the impacts of natural *H. akashiwo* blooms. Results from preliminary laboratory trials indicate that clay at the maximum proposed dosage rate does not interfere with the assay. Thus, it may be possible to use the SHA as a simple means of following the fate of *H. akashiwo* cells during larger-scale clay mitigation trials.

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1. Introduction

Rapid detection and enumeration of fish-killing algae is crucial for the management of finfish farms. Cell detection methodology based on light microscopy can be tedious and time-consuming when large numbers of samples need to be processed routinely, and identification of some species may require highly trained personnel. In an effort to overcome these limitations

we are developing DNA probe diagnostic tools that provide rapid identification of harmful algal species in near real-time (e.g. Scholin et al., 1996, 1997, 1999; Tyrrell et al., 2001).

This contribution focuses on the fish-killing alga *Heterosigma akashiwo* (Hada) Sournia (Raphidophyceae) that has been responsible for major finfish kills globally (e.g. Rensel et al., 1989; Taylor, 1993; Honjo, 1994; Yamochi, 1987; Hard et al., 2000). The mode of 'toxicity' responsible for these kills is not clear. Neurotoxins, hemagglutinating and hemolysing compounds, as well as the production of reactive oxygen species (ROS) may all play a role in the mortality of finfish (Onoue and Nozouwa, 1989; Ahmed et al., 1995; Oda et al., 1996; Khan et al., 1997). The

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production of brevetoxin-like compounds also leads to concern of possible human health impacts (Khan et al., 1997).

The accurate enumeration of *H. akashiwo* in natural samples can be difficult due to its delicate nature, small size and clumping of cells following some fixation methods (Connell, 2002; Tyrrell et al., 2001). Some raphidophyte species are pleomorphic (Tomas, 1978; Hara and Chihara, 1987; Aizdaicher, 1993) and alterations in morphology may result upon chemical preservation (Thronsen, 1993) further hampering accurate identifications. Live raphidophycean algae may shed their flagella and lose surface details under the microscope (Thronsen, 1993). Furthermore, net tows used to collect *H. akashiwo*, as well as other raphidophytes, often result in the recovery of disrupted cells and cell fragments (Connell, unpublished data). All of these factors can interfere with efforts to detect and enumerate these potentially harmful organisms.

Ideally, samples containing *H. akashiwo* should be analyzed in near real-time to provide adequate warning of an imminent bloom so mitigation options can be employed. Towards that end, we have developed a sandwich hybridization assay (SHA) as a means to rapidly identify and enumerate harmful species (Scholin et al., 1996, 1997, 1999; Tyrrell et al., 2001). This test was developed to be rapid, cost-effective, easy-to-use and require minimal sample handling. The SHA relies on extracted nucleic acids from cell lysates and does not require microscopic observation of intact cells (e.g. Scholin et al., 1996, 1997, 1999; Tyrrell et al., 2001). Two oligonucleotide probes are utilized: first a biotinylated “capture probe” attached to a streptavidin-coated solid support and functions to immobilize target sequences of ribosomal RNA (rRNA) from a crude cell extract. Then the solid support transfers the immobilized rRNA to a second solution containing a fluorescein-labeled “signal probe”, thus forming the “sandwich” hybrid complex. The hybrids are detected using an anti-fluorescein antibody conjugated to horseradish peroxidase that reacts with substrate to produce a blue colorimetric product. The intensity of the blue product is related to the number of target cells present in the original sample. Here, we report on the suitability of the SHA for monitoring *H. akashiwo* in field samples and efforts to determine how these field samples may be archived for future analysis.

2. Materials and methods

2.1. Field sites

Sampling locations are shown in Fig. 1. M1: NMFS Manchester Field Station—47°575'N latitude, 122°545'W longitude; C1: Olalla Creek—Colvos Passage 47°421'N latitude, 122°539'W longitude. Additionally, a massive bloom of *H. akashiwo* in Hood Canal (HC), WA (Connell, 2001), was used as a sample of opportunity.

2.2. Sampling methods

Samples were taken from a bridge for the site C1. M1 samples were collected from a pier and HC samples from a boat. Net tows were taken using a 20 µm mesh net. The net was allowed to descend approximately 2 m then pulled straight up. An integrated water sample was collected over 3 m depth with a non-flexible tube and the sample was pooled into a single bottle (bottle sample) for each sampling site. All field samples were collected during daylight hours and stored on return to the laboratory (overnight) in an illuminated 12 °C incubator (near ambient temperature for the samples) prior to sample analysis the following morning. Dominant phytoplankton species were identified from live net tow samples using a Zeiss Axiovert 135 light microscope. Determination of *H. akashiwo* cell numbers were from the bottle samples by light microscopy using a Palmer–Maloney counting slide. An aliquot was removed from the bottle samples and fixed with acidic Lugol's iodine prior to cell enumeration. Four replicate counts of the entire Palmer–Maloney chamber were made from each aliquot, then averaged to estimate *H. akashiwo* cell numbers. All cell counts and dominant species identification were done just prior to or during the SHA sample processing.

2.3. Sandwich hybridization assay

The SHA was performed using reagents pre-dispensed in 96-well microtiter plates (Saigene Corp., Seattle, WA). For sites C1 and M1, 400 ml of bottle sample were filtered onto a 5 µm pore size, 25 mm Durapore membrane (Millipore). Filters were placed into 1 ml of lysis buffer and processed as previously described using an automated robotic workstation

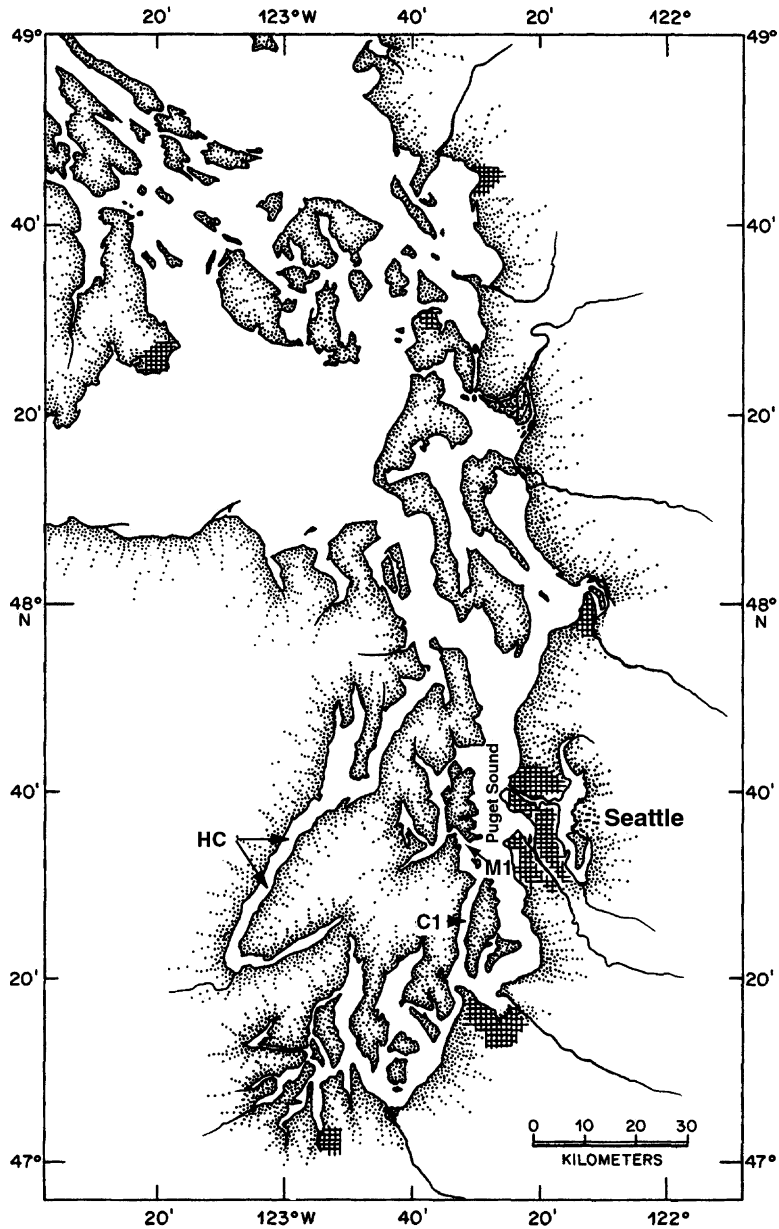


Fig. 1. Sampling locations in Puget Sound and Hood Canal. M1: Manchester, C1: Clovos Passage and HC: Hood Canal.

(Scholin et al., 1999, Tyrrell et al., 2001). The optical density (OD) of the colorimetric product of the assay was determined using a Dynatech Laboratories Inc. (Chantilly, VA) Model # MR5000 96-well plate spectrophotometer. Based on filtering a 400 ml whole

water sample to dryness and adding material collected to 1 ml of lysis buffer, the linear range for the assay is from approximately 300–100,000 cells l^{-1} . For the HC samples, 10–400 ml of sample were filtered depending on the density of the *H. akashiwo* bloom.

2.4. Archiving samples

Three methods were examined for preserving *H. akashiwo* for later SHA analysis:

- (1) *H. akashiwo* was preserved with acidic Lugol's and stored at room temperature in the dark. Two aliquots (~50,000 cells each) were removed, filtered then processed using the SHA.
- (2) Cell lysates were prepared by collecting ~50,000 *H. akashiwo* cells onto filters, then 1 ml of lysis buffer was added and incubated at 85 °C for 5 min. The tube containing the membrane and lysis buffer was mixed by finger tapping prior to incubation, after 2.5 min of incubation, and at the completion of the incubation. Cell lysates were stored at -70 °C until processed using SHA.
- (3) Approximately 50,000 *H. akashiwo* cells were collected onto membranes, then immediately placed into a -70 °C freezer until processed using SHA.

2.5. Clay mitigation trials

The purpose of this experiment was to determine whether the SHA could be used to monitor the presence and abundance of *H. akashiwo* before, during and after clay mitigation. Four types of clay that have been tested by others for use in potentially mitigating the impacts of harmful algal blooms were evaluated (Sengco et al., 2001). These were Wyoming bentonite (WB-B; Wyo-ben Inc.), kaolinite (H-DP; J.M. Huber Co.), kaolinite (H-35; J.M. Huber Co.) and phosphatic (IMC-P2; IMC Phosphates Co.). Clay was suspended in distilled water (25 g l⁻¹) then added to filtered seawater (with or without approximately 5000 *H. akashiwo* cells) and brought to a final volume of 50 ml with a final concentration of 0.25 g l⁻¹ of clay (the final concentration of clay is reported to be most effective at removing *H. akashiwo* from a water column; see Sengco et al., 2001). The entire sample was then filtered onto Durapore membranes and processed using the SHA. Clay in seawater without *H. akashiwo* cells served as a negative control and cells alone served as a positive control. Approximately 1000 homogenized *H. akashiwo* cells were in each final SHA sample well.

3. Results and discussion

The objectives of this research were to: (1) determine if the SHA could detect *H. akashiwo* in field samples; (2) determine whether quantitative estimates based on the SHA agree with those based on light microscopy; (3) evaluate methods for archiving field samples for later analysis by SHA; (4) examine the potential of the SHA to monitor the effectiveness of removal of *H. akashiwo* from the water column by clay flocculation; finally, (5) assess the suitability of the assay for routine monitoring.

3.1. Detection of *H. akashiwo* in field samples

H. akashiwo was detected in low to moderate numbers (Figs. 2 and 3) at both field stations regularly over the course of this investigation. The C1 station frequently had large amounts of natural sediment, especially on outgoing tides. The sediment did not appear to interfere with results of the SHA. All samples that contained *H. akashiwo* as determined using light microscopy also tested positive using the SHA. In general, there was good agreement between the density of *H. akashiwo* estimated by light microscopy and by SHA (Figs. 2 and 3). However, there were large differences in cell number estimates at station M1 on 5 and 18 July and for station C1 on 18 July. On 13 September, at stations M1 and C1 approximately 5000 cells l⁻¹ *H. akashiwo* cells were detected by SHA and not by light microscopy counts.

Discrepancies in cell density estimates may be related to SHA false positive reactions, cell count error (light microscopy counts) or variations in *H. akashiwo* rRNA content. It is unlikely that the few differences observed were due to SHA false positives since the myriad of non-target organisms, detritus, etc. present in those samples were also found in many other samples that did not contain *H. akashiwo*, and those samples showed no SHA reactivity. Consequently, there is something very unique about the few samples where the SHA values exceeded those expected based on light microscopy observations.

One of the major problems associated with using light microscopy to verify or refute results of the SHA is the fact that *H. akashiwo* is relatively fragile, quite pleomorphic and its morphology distorts readily following fixation. In turn, microscopy-based cell counts

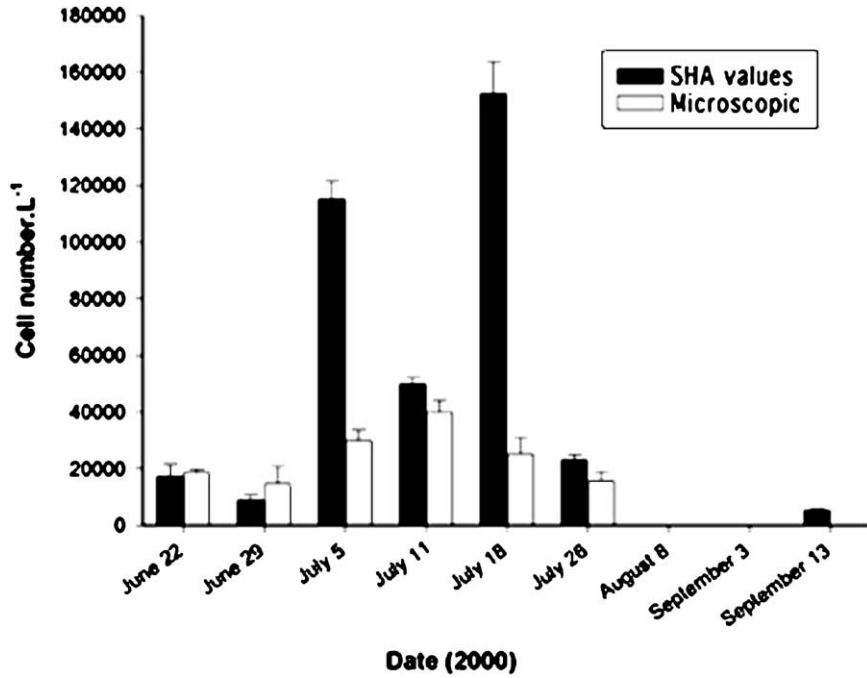


Fig. 2. *H. akashiwo* cell numbers estimated by SHA and light microscopy for Station M1 from 22 June to 13 September 2000.

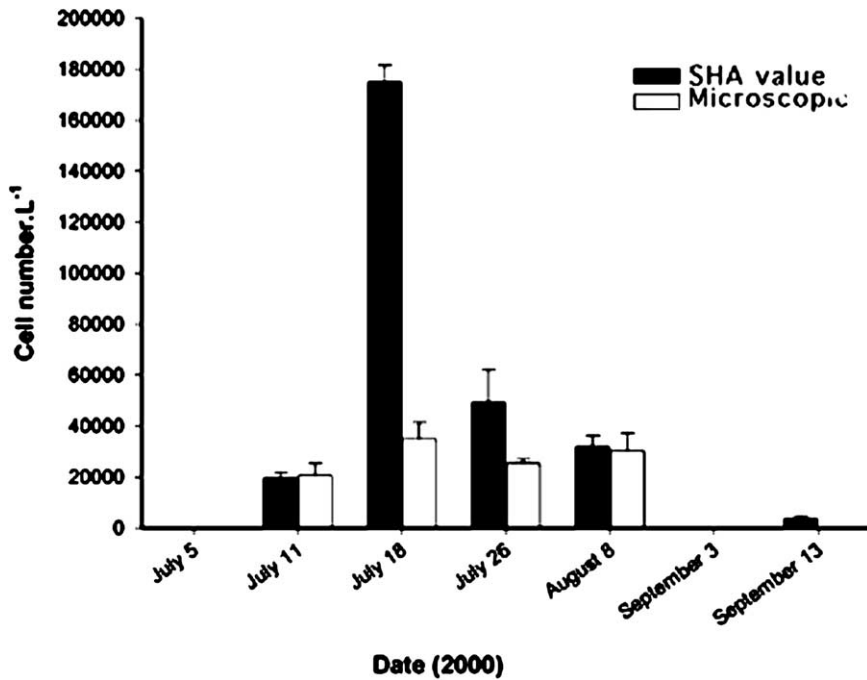


Fig. 3. *H. akashiwo* numbers as determined by SHA and light microscopy for Station C1 from 5 July to 13 September 2000.

are sometimes very difficult. This was especially evident when bottle samples and net tows were compared. *H. akashiwo* was never observed in net tows using light microscopy if the cell number in the corresponding bottle sample was below 30,000 cells l⁻¹; cells were either destroyed or distorted beyond recognition in the net tows. Additionally, as discussed in greater detail later, raphidophytes tend to clump together when fixed with Lugol's iodine. This can lead to under or over estimates of cell numbers using either light microscopy or the SHA, but this problem is particularly relevant for the light microscopy counts where only small volumes of fixed material were examined. Vigorous mixing of the Lugol's fixed material was needed to reduce clumping, but this action effectively destroyed the morphology of cells and rendered them unsuitable for light microscopy identification.

Grazing of *H. akashiwo* by ciliates and zooplankton may also affect the apparent correlation between SHA-based cell abundance estimates and those obtained using light microscopy. It is possible that molecules indicative of *H. akashiwo* that are present within the digestive system of ciliates and zooplankton could be released during sample homogenization and subsequently be detected using the SHA. At the same time, however, intact, free-swimming cells would not be visible microscopically (e.g. see Scholin et al., 1999). Indeed, large numbers of ciliates and zooplankton dominate samples for both M1 and C1 on 18 July and on 5 July for station M1.

Clearly, additional work is required to determine the relationship between cell number estimates generated by light microscopy and those provided by the SHA. In that regard, adequate fixation and subsequent identification of *H. akashiwo* using light microscopy proved to be very problematic. Potential discrepancies associated with samples that contain high numbers of grazers that may have fed on *H. akashiwo*, also calls into question whether the SHA is indeed detecting the rRNA-targeted as cells transition from free-swimming to ingested. In either case, observing samples using light microscopy does not provide an adequate basis for validating results of the SHA since the target species may be obscured from view for a variety of reasons. Therefore, future SHA validation studies should include application of an independent, cell-free nucleic acid-based test such as the PCR assay described by Connell (2002). An assay such as

that can be used to detect *H. akashiwo*-specific DNA, and that result should be coincident with a positive response of the SHA.

An alternative explanation for the discrepancies in estimates of cell numbers seen in Figs. 2 and 3 could relate to the physiological status of *H. akashiwo*. Previous work on cultures showed that the quantitative response of the SHA towards a constant number of *H. akashiwo* vary by a factor of two depending on the growth state of the cells (Tyrrell et al., 2001). In this study, the field samples show six to seven-fold differences in cell numbers. It is possible that the variation in rRNA content for wild *H. akashiwo* may vary more extremely than that observed in laboratory cultures, or that this effect is also superimposed on the problems of accurately enumerating cells collected in natural populations as noted previously. Addressing all of these possibilities requires intensive sampling of natural *H. akashiwo* blooms from background levels through rapid growth, maintenance and termination phases.

3.2. Sample archival

The *H. akashiwo* SHA was designed around the requirement to detect cells at background (or 'pre-bloom') concentrations. Fish growers in Puget Sound, WA have determined that they will use 100,000 cells l⁻¹ as their target to become concerned and intensify monitoring efforts. When cell numbers reach 100,000 cells l⁻¹, reduced amounts of water sample need to be filtered. For example, during mid September 2000 a large *H. akashiwo* bloom was observed in Hood Canal. Although the SHA proved very useful for rapidly screening many samples and for determining the geographic extent of that bloom, the amount of water used for the assay had to be reduced in many cases to as low as 10 ml because of difficulty in filtering the material. Even with such a reduced volume, many of the samples saturated the assay and made quantitative estimates of cell densities impossible. Under such circumstances it is best to create a dilution series of sample lysates so that more accurate estimates of cell densities are possible. Since it is not always possible to know what samples may saturate the assay a priori it is advisable to archive replicates of each sample so that subsequent analyses (like a dilution series) remain an option. Archiving of field

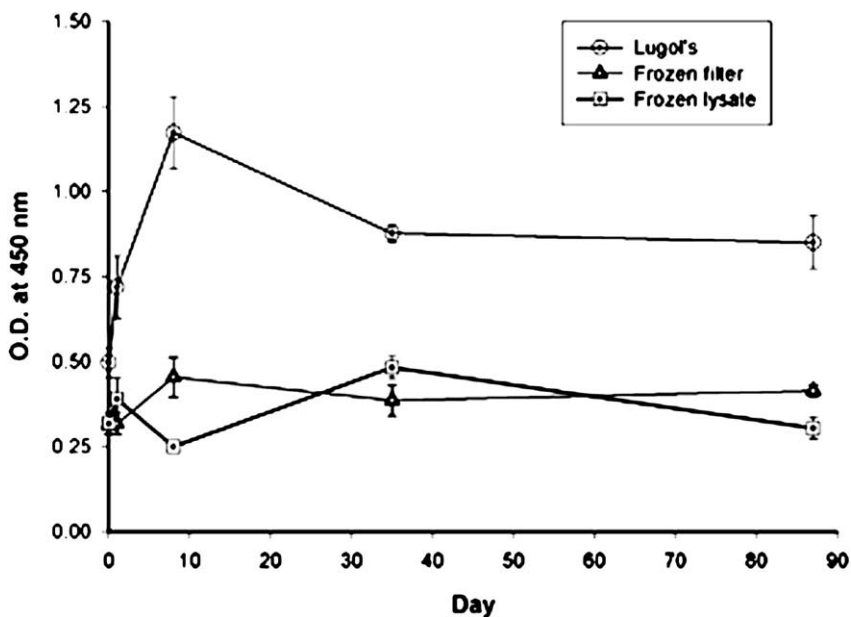


Fig. 4. Stability of samples preserved with acidic Lugol's iodine vs. frozen after filtration or as a sample lysate over 87 days.

samples is also required when samples are collected at remote sites or at locations where the SHA cannot be performed on-site. Also, when sample numbers exceed the throughput capacity for the robotic workstation (Scholin et al., 1997) samples must be archived and processed later. For all of these reasons, we initiated tests to assess the stability of samples that were collected and preserved in a fashion consistent with what might be done in a monitoring program.

For quantitative analyses, frozen filters or frozen lysates are the preferred methods for sample archiving (Fig. 4). Analysis of Lugol's preserved samples initially did not yield consistent results with some tests suggesting an increase in signal whereas others a decrease given a constant sample volume. The variation in signal strength associated with the latter is likely related to target cells clumping following fixation and the fixed cells adhering to the glass container walls. The majority of variation is seen in the first few days after fixation and decreases with time. This decrease is consistent with light microscopy observations that revealed a reduction of clumped cells approximately 1 week after fixation. Vigorous mixing of fixed cells prior to removing a sub-sample for the SHA greatly reduced the variation between sampling points. Thus,

Lugol's preserved samples will not provide as accurate results as those samples processed from fresh material, frozen filters or frozen lysates, but nonetheless should be useful for qualitative analyses. A standard curve must be prepared for Lugol's preserved material because of the difference in signal between the fresh and Lugol's preserved samples.

3.3. Effects of clay on the SHA

Clay flocculation has gained increasing attention as a means of removing harmful algal species from the water column (Shirota, 1989a,b; Sengco et al., 2001). To measure the effectiveness of the clay as a removal agent, cell numbers must be observed before, during and after clay addition. As noted earlier, obtaining accurate counts for raphidophytes in natural samples can be very difficult and this problem is compounded when clay is present. This raises the question as to whether *H. akashiwo* densities can be estimated accurately while applying clay. We, therefore, initiated preliminary tests to ascertain the effectiveness of the *H. akashiwo* SHA under such conditions. Re-suspended clay was added to samples that contained *H. akashiwo* cells. Results of these trials are shown in

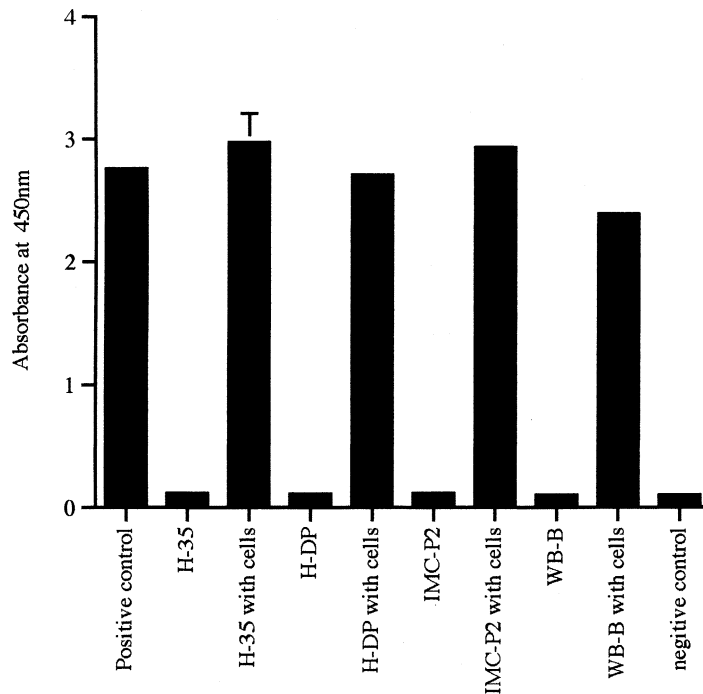


Fig. 5. SHA signal comparison for the presence and absence of clay with and without *H. akashiwo*. Clay types are Wyoming bentonite (WB-B), kaolinite (H-DP), kaolinite (H-35) and phosphatic (IMC-P2).

Fig. 5. The addition of clay has a minimal effect on the SHA and does not appear to depress the signal for a given number of target cells. Therefore, the assay shows promise as a means of determining *H. akashiwo* cell numbers during clay mitigation experiments.

3.4. Routine monitoring for *H. akashiwo* using the SHA

Work done this far has shown the SHA to be a potentially useful means of monitoring for *H. akashiwo* in natural samples (this study; Rhodes et al., 2001). Prospects of deploying this assay globally rests heavily on the genetic similarity of target sequences from isolates representing geographically distinct populations. Previous work has proved that sequences of nuclear large subunit ribosomal DNA (LSU rDNA, Tyrrell et al., 2001) and the nuclear internal transcribed spacers (ITS, Connell, 2000) from globally distributed *H. akashiwo* isolates examined to date are identical, except for one isolate (York River, VA, USA). Consistent with those observations, *H. akashiwo* isolates,

collected from Australia, Japan, New Zealand, South Korea, Spain and USA, all react positively with the probes used in the existing SHA (Tyrrell et al., 2001). Consequently, integration of the SHA into a global monitoring program is possible and may be achieved in two ways. Firstly, the assay could be used as a confirmatory test for samples that have been examined by light microscopy and believed to contain a species of concern. The phytoplankton monitoring team of the Cawthron Institute, Nelson, New Zealand, uses this approach. Secondly, the assay could be used as a tool to screen many samples rapidly, and when *H. akashiwo* is detected that prediction could be confirmed using traditional methods. Regarding the latter, examination of live material is preferable. The characteristic helical swimming motion of *H. akashiwo* together with knowledge of the various morphologies makes identifications reliable, though not necessarily easy nor always strictly quantitative. If the SHA proves to be accurate and robust, then routine confirmation of the SHA results may not always be required.

The SHA in its present format is semi-automated and is relatively portable and easy-to-use. The assay takes just over an hour to examine eight samples for *H. akashiwo*. Of this time only 15 min is 'hands-on'. An experienced taxonomist would take a minimum of 15 min to examine one slide and as stated earlier it can be extremely difficult to perform accurate counts for this species. Additional equipment that is required to perform the SHA are: filter manifold and vacuum pump, heating block to produce lysate; 96-well plate spectrophotometer (rough estimates can also be obtained by using a visual reference, much like pH paper). At the time of this writing, the cost of the robotic processor is approximately USD \$7500 and per sample analytical costs are approximately USD \$5. The assay system can and has been used aboard ships when AC power is available.

There are many ways that the SHA could be refined to improve its portability and reduce sample handling requirements. The chemistry of the SHA is amenable to being placed onto a solid format similar to a pregnancy dipstick test and the assay could be carried out manually. An alternative approach would be to place the assay onto an autonomous instrument that would be moored at monitoring sites. Such an instrument is under development now and is referred to as the Environmental Sample Processor (ESP, Scholin et al., 1998). The ESP can collect water samples for archiving of DNA and toxin samples, apply probes in whole cell formats, and can perform the entire SHA protocol in situ then send the data back to a shore-based station for interpretation. This instrument could be especially useful for monitoring sites that can only be reached infrequently.

In summary, the SHA detects *H. akashiwo* at densities far below the level of concern and is a relatively simple and rapid method. Frozen (-70°C) filters and sample lysates are the preferred archiving strategies for storing samples for future analysis, but Lugol's preserved samples are also suitable for qualitative analysis. The effect of clay and other sediments on the SHA is minimal, so this diagnostic tool may be useful for determining the efficacy of removing *H. akashiwo* from the water column by applying clay (Sengco et al., 2001). Additional studies are required to examine the levels of rRNA in *H. akashiwo* cells in naturally occurring populations, particularly during a bloom, and to assess the whether or not grazers that

have consumed *H. akashiwo* also contribute to a positive SHA reaction.

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