

WASHINGTON SEA GRANT PROGRESS REPORT
for the period 2/1/2007 – 1/31/2008

WSG Project Number: **R/B-48**
Project Title: Slick Forming Algae: Growth, Genetics and Toxicity

Principal Investigator(s) and Affiliation:
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1. ABSTRACT ELEMENTS

OBJECTIVES

The induction of toxic slicks by fresh-water plumes is a widespread phenomenon affecting many geographic areas and many different motile algal taxa. When bioconvection and turbulence are minimized by the presence of a halocline, toxic algal cells are effectively trapped in an aggregated state. We hypothesize that as cells participate in structuring slick formation by moving from a low density to a higher density population they become subject to a new set of physiological constraints (e.g., light levels are altered, osmolarity and nutrient loads shift). These cues drive the slick-confined cells to shift their metabolism and cause genetic sieving of the population to select for cells with optimal fitness. The short-term goal of this project is to determine how the formation of slicks impact: (a) growth response (including cyst formation); (b) toxicity; and (c) genetic identity of the aggregated population. The long term goal of this research is to: (a) provide a reference database for analyzing additional toxic raphidophyte taxa; (b) develop molecular probes for measuring gene expression; and (c) generate algal isolates whose dominant haplotype has been selected in response to specific environmental challenges. The toxic raphidophyte *Heterosigma akashiwo* will be used as our test system.

METHODOLOGY

Identification of genes with highest polymorphic sites: If variability among *H. akashiwo* strains is a critical component in vegetative cell/cyst/bloom management, a reliable method must be developed for their identification. Given the morphological and physiological plasticity of *H. akashiwo*, it might be argued that a genetic approach must be used when documenting strain identities. Cellular DNA is shotgun cloned into 40 KB-insert fosmids, which are then screened by high throughput end sequencing for clones that have chloroplast or mitochondrial signatures. These clones are then individually sequenced by standard shotgun sequencing methods. With respect to chloroplast DNA, sequencing reads from fosmids are often more easily assembled into a whole genome structure because duplication of the large inserts is avoided. Though multiple fosmids are needed to assemble a chloroplast genome (~160 kb), the mitochondrial genome of *H. akashiwo* is smaller (~38 kb) than fosmid size (~45 kb), thus only one fosmid/genome is necessary for generating a final mitochondrial sequence. Fosmid clones are finished to an accuracy of an error rate of less than 1 in 10,000 with minimal or no finishing reads required.

Comparison of *H. akashiwo* strains: *H. akashiwo* DNA is isolated from a selected strain that is maintained within our cultures collection. A *H. akashiwo*-specific primer that allows PCR recovery of the entire sequence of a mitochondrial gene (*cox1*; *nad 2*, *nad4*, *nad5*, and *nad7*) or chloroplast gene (*cfxQ*) is used to amplify the gene of choice. Many genes are processed in this manner. The high throughput PCR is performed in 96-well format and sequencing reactions are accomplished using Big Dye terminator in 386 well format using ABI Gene Amp PCR system 9700. Sequencing is on an ABI 3730X1. Sequence contigs are evaluated and assembled with MacVector using Phred and Phrap, then aligned with clustal. Each SNP is confirmed by inspection of the chromat trace. Maximum Likelihood analyses and Bayesian Inference is performed on the aligned nucleotide data using PAUP* 4b.10 and MrBayes 3.1 respectively.

We maintain approximately 50 *H. akashiwo* strains in our culture facility. These strains originate from the Puget Sound region, and sites in Asia, Europe, New Zealand, North and South America. This collection is continuously increasing in size since strains from under-represented geographic areas are being sought. These strains will serve as our reference data base as sequence data is generated.

Comparative analysis of vegetative vs cyst cell function: To understand the impact of strain identity on vegetative cell function and cyst survival, we need a method to measure the molecular capability of *h. akashiwo* as it moves through these various life history phases. qPCR primers were optimized for 30 chloroplast genes representing photosynthesis, energy generation, Calvin cycle, lipid biosynthesis, protein translation etc. Samples of *H. akashiwo* cells during various life history phases are centrifuged for 10 minutes at 5,000 rpm and 4C. 1 ml of TRIzol reagent (Invitrogen) is added to the pellet and the sample is kept at -80C until use. RNA is quantified and then digested with DNase I until a negligible signal is produced when performing real-time PCR using iQ SYBR Green Supermix and a Chromo4 Real-Time PCR Detector (Bio-Rad Laboratories) with 16s ribosomal RNA PCR primers. cDNA from each RNA sample is generated using the iSCRIPT cDNA Synthesis Kit (Bio-Rad Laboratories). For the standard curve, 1ul of combined RNA from all samples are added into a single cDNA synthesis reaction. The combined cDNA is serially diluted and qPCR is performed with 16s rRNA primers to obtain the standard curve needed to normalize the 30 PCR primers and samples. All qPCR reactions are performed in triplicate with an annealing temperature of 60C and a melting curve to check for primer specificity.

H. akashiwo toxicity: The production of reactive oxygen species (ROS) has been proposed as a mechanism that causes toxicity of harmful raphidophyte algae. Hydrogen peroxide was quantified fluorometrically using the Amplex® Red reagent, (AR: 10-acetyl-3,7-dihydroxyphenoxazine. A Victor³V 1420 multilabel plate reader (Perkin Elmer, Waltham, MA, USA) was used to measure fluorescence, with excitation lamp filter F531 and emission filter F595. The luciferin analog 2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one (MCLA) was used in a luminescence assay for superoxide quantitation. Plates containing reactions were read using a Victor³V 1420 multilabel plate reader. The luminescence was measured integrated over 1 second, using no filter.

RATIONALE

Natural processes impacted by global climate change and increasing anthropogenic activity support an increased occurrence of algal blooms. These toxic events impact the survival of eco-cohorts at all trophic levels and compromise the health of coastal ecosystems. The studies proposed here will: (a) address longstanding questions concerning the functional biology of toxic phytoplankton blooms: How does the genetic identity of an algal population impact bloom dynamics (i.e., why do blooms initiated by the same algal species differ in intensity, longevity and toxicity)? How can a photosynthetic HAB-forming organism survive long-term stasis? and (b) provide information that will help in generating policy with respect to coastal ecosystem management.

2. ACCOMPLISHMENTS AND OUTCOMES

Identification of genes with highest polymorphic sites: We have now fully sequenced the complete chloroplast and mitochondrial genomes of two geographically isolated *H. akashiwo* strains (NIES 293: West Pacific and CCMP 452: West Atlantic). The number of single nucleotide polymorphisms (SNPs) observed in comparing these two organelle genomes is high. For the mitochondrial genomes, a total of 505 SNPs were identified between NIES 293 and CCMP 452 (38,724 and 38,641 bp in length respectively). The chloroplast genomes of NIES 293 and CCMP 452 (159,370 and 160,149 bp in length respectively) differ by 150 SNPs, averaging approximately 1 substitution per 1000 bases. In contrast, the mitochondrial genomes have approximately 13 substitutions per 1000 bases - more than a tenfold increase over that in the chloroplast DNA. Mitochondrial SNPs occur almost equally when coding and intergenic regions are compared, whereas chloroplast sequence differences between strains are almost completely confined to intergenic regions.

Identification of strains: Importantly, gene sequence comparison can be used to differentiate among strains. Analysis of a single chloroplast gene (*cfxQ*) in 16 *H. akashiwo* cultures revealed the presence of 5 different strain signatures. Profiling the mitochondrial *nad 7* gene in 45 *H. akashiwo* cultures revealed at least 11 strain clusters, which agree with those seen for the chloroplast genome. Analysis of 5 additional mitochondrial genes in 50 *H. akashiwo* cultures is in progress. These data strongly support the conclusion that *H. akashiwo* strain differences do occur and suggest that observed strain differences are the product of a more ancient radiation event.

Analysis of strain-specific vegetative cell vs cyst function: The metabolic program of a strain will give insight to its fitness – and thus survival potential. Historical data shows definitively that *Heterosigma* vegetative cells and cysts display strain-specific survival capabilities when subject to different environmental stresses. We now have generated the first molecular data by which that fitness can be quantitated. Preliminary analysis of the metabolic programs for *H. akashiwo* strains as they progress from the vegetative state through cyst induction has now been accomplished using quantitative PCR (qPCR). We monitored RNA levels of 30 genes representing a spectrum of metabolic functions (see above). Cells were sampled at 0 time (immediately before cyst induction conditions were imposed) and after the cells remained for 14 days in the dark and cold induction condition. In the vegetative state *H. akashiwo* strain CCMP 452 was able to maintain a level of RNA synthesis that was far greater than that measured for NIES 293). As cells progressed through cyst formation CCMP 452 was able to maintain RNA synthesis for most genes whereas NIES 293 had only a selected few genes that were expressed (e.g., *acpP*, *psbA*, *cfxQ*, 23sRNA). Both strain reduced RNA levels substantially (~100 fold) during cyst biogenesis. Taken together, one may hypothesize that the fitness of a strain (i.e., a high metabolic capacity) may influence the capability of a cell to reproduce, its ability to form viable cyst seed bank and survive long-term stasis thus be able to more efficiently initiate a new bloom event. Special Note: We are continuing on the work (in conjunction with our NSF AToL grant) to sequence the chloroplast and mitochondrial genomes of the toxic raphidophyte *Chattonella japonica*. The complete chloroplast genomes have now been completed for the pelagophytes *Aureoumbra laguensis* and *Aureococcus anophagefferens*. These three algae are recognized as destructive HAB formers. Our strain identification technique may be applied to these organisms.

Toxicity: The production of reactive oxygen species (ROS) has been proposed as a mechanism that causes toxicity of harmful raphidophyte algae. Adaptive explanations for ROS production by phytoplankton include the suggestion that these cytotoxic oxygen-based molecules are generated to support the reductive assimilation of iron when this element limits cell growth. Iron appears to be increasingly limiting in coastal waters due to anthropogenic activity. For this reason we hypothesized that prolonged iron limitation would lead to an evolutionary shift in *Heterosigma akashiwo* populations, selecting for cells having increased ROS production and therefore elevated toxicity potential. Under prolonged iron stress, a selective event was observed. Contrary to expectations, the resulting cultures appeared to have a lessened ability to respond to stress by increasing ROS levels. Significantly, our results show that hydrogen peroxide is produced in *H. akashiwo* cultures predominantly through indirect, light-driven reactions of extracellular ‘photoROSogenic’ substances excreted by the algae. Moreover, the bacterial population that co-exists with the algae influences this indirect route of ROS production. When bacteria were added to *H. akashiwo* cultures, a decrease in extracellular hydrogen peroxide concentrations was observed. Our data suggest that simple, direct, physiological explanations may be insufficient to explain raphidophyte toxicity. Instead, an approach that examines the alga within its explicit ecological context, including abiotic and bacterial processes, may be better suited to elucidate the causes and mechanisms by which raphidophytes manifest toxicity.

3. IMPACTS

a) A technology for genetically fingerprinting algal strains has been developed. This technology will be applicable to a broad range of HAB-producing organisms. The method, when optimized, should allow us to fingerprint in all phases of algal life history – including both vegetative and cyst stages.

b) With respect to the destructive HAB-former *Heterosigma akashiwo*, our strain identification technique will generate the first data that profiles the genetic diversity of *H. akashiwo* populations in Puget Sound (Black, Deadato and Cattolico) for cells that are in the free swimming state and slick-aggregated state (Nishizai, Grunbaum Cattolico) as well as in the cyst stage (Tobin, Grunbaum and Cattolico).

c) Our data concerning the production of reactive oxygen species clearly demonstrates why the experimental approach previously used by several colleagues failed to detect the significant generation of this product by *H. akashiwo*. We show that ROS production is a stress response and can, given the ability of *H. akashiwo* to form massive aggregates on surface waters, certainly impact eco-cohort survival.

d) A recent paper in Harmful Algae has received much attention. This work discusses the genetic drift that occurs in all laboratory-maintained cultures. Our paper warns of the problems of directly extrapolating laboratory findings to field situations.

(e) We now have available PCR probes for 30 chloroplast and mitochondrial genes that can be used by investigators world-wide for assessing the metabolic status of *H. akashiwo*.

4. PERFORMANCE MEASURES

Measure 1: Economic and societal benefits derived from the discovery and application of new sustainable coastal, ocean, and Great Lakes products from the sea.

Actual (reporting period covered by this report):

Fish farms in our state (and world-wide) are severely impacted by the presence of *H. akashiwo*. Selected *H. akashiwo* strains impact eco-cohort survival with different efficiencies (e.g., ROS production; fish egg survival). Information on “who is there” with respect to *H. akashiwo* strains would be advantageous to the aquaculturist. Using selected genes we show that significant genetic divergence exists between the West Pacific (NIES 293) and West Atlantic (CCMP 452) *H. akashiwo* strains. We have begun to create a genetic fingerprint reference library. To date, we have assessed 50 *H. akashiwo* cultures using the mitochondrial *nad7*, and the chloroplast gene *cfxQ*. At least 11 different fingerprint signatures have been established.

Anticipated (12-month period following this reporting period):

We intend to continue generating the *H. akashiwo* genetic fingerprint reference library, both by including more gene analysis and expanding the number of clones curated. Work will then focus on restructuring the chloroplast and mitochondrial probes to make them highly *H. akashiwo*-specific. This background work will be done so that PCR data generated from in mixed assemblages of in water and sediment samples will give clean, fingerprint data for our targeted alga. Studies will initiate in which vegetative cells from natural blooms in Puget Sound will be cultured and strain identified.

Measure 2: Cumulative number of coastal, marine, and Great Lakes issue-based forecast capabilities developed and used for management.

Actual (reporting period covered by this report):

A model in which the parameters of bacterial load, *H. akashiwo* reproductive rate, and iron levels on *H. akashiwo* ROS production is in development (Lakeman and Cattolico).

Anticipated (12-month period following this reporting period):

The swimming behavior of both vegetative cells and cysts (as they form and reactivate to the vegetative phase) will be used in models for predicting the efficiency of aggregate formation in the presence of a halocline (Nishitaki, Grunbaum and Cattolico) and the distribution of cysts in sediments (Tobin, Grunbaum and Cattolico).

Measure 3: Percentage/number of tools, technologies, and information services that are used by managers (NOAA and/or its partners and customers) to improve ecosystem-based management.

Actual (reporting period covered by this report):

We have shown that *H. akashiwo* strain identity does matter – impacting swim speed (thus slick formation in response to a halocline), cyst generation (thus seed bed size); cyst survival (potential for bloom induction) and toxicity (bloom impact).

We present new data on the physiological and ecological parameters that impact *H. akashiwo* toxicity (ROS biosynthesis)

We have generated the first available data concerning how cells metabolically survive in long-term stasis.

We have initiated the establishment of a reference library that genetically identifies *H. akashiwo* strains.

Anticipated (12-month period following this reporting period):

In the next 12 months we anticipate continuing our genetic fingerprinting studies, and to generate the genetic tools that will allow identification and analysis of field collected samples of *H. akashiwo* with high specificity.

5. RELEVANT PUBLICATIONS

Published

Lakeman, M., Von Dassow, P., and R. A. Cattolico (2009)

The strain concept in phytoplankton ecology. (*Harmful Algae* 8: 746-758).

Cattolico, R. A., Jacobs, M., Zhou, Y., Chang, J., Duplessis, M., Lybrand, T., McKay, J., Ong, H., Sims, E., and G. Rocap (2008)

Chloroplast genome sequence analysis using a fosmid cloning approach: Analysis of *Heterosigma akashiwo* CCMP452 (West Atlantic) and NIES 293 (East Pacific) strains. (*BMC Genomics*: 9:211doi:10.1186/1471-2164-9-211); PhD thesis.

In review

Lakeman, M.B., Overman, R. and Cattolico, R.A. (2009)

The role of stress and selection in levels of reactive oxygen species associated with the toxic raphidophyte *Heterosigma akashiwo* (submitted to *Aquatic Biology*) (ML:PhD thesis)

In final draft (will be submitted by the end of June, 2009)

Ong, H., Wilhelm, S., Gobler, S., Bullerjahn, G., Jacobs, M., Rocap, G., and R. A. Cattolico (2009).

Analyses of the complete chloroplast genome sequences of *Aureococcus anophagefferens* CCMP1984

and *Aureoumbra lagunensis* CCMP1507; (will be submitted to J. of Phycology. (H.O; Post-doctoral fellow)

Lakeman, M.B., and R.A. Cattolico, R.A. (2009)

Ecological control over indirect reactive oxygen species production by the toxic raphidophyte *Heterosigma akashiwo* (will be submitted to Aquatic Biology) (ML:PhD thesis)

B. Theses and dissertations:

Lakeman, M. (fall quarter, 2010)

Rapid intraspecific evolution in harmful phytoplankton populations: *Heterosigma akashiwo* as a model system (In final development).

Michael Nishitaki (~ 2011)

Analysis of the swimming behavior of *H. akashiwo*

C. Book chapters:

D. Book or Monograph:

E. Paper in Proceedings

Karol, K. Jacobs, M., Zhou, Z. Sims, E. Gillett, W., and R.A. Cattolico (2009). Organellar genome evolution: comparative analysis of complete mitochondrial and chloroplast genome sequences from geographically distinct strains of *Heterosigma akashiwo*. (Proceedings of the Seventh International Chrysophyte Symposium). (KK; Post-doctoral fellow); in press.

F. Proceedings or Symposia:

G. Technical reports:

H. Advisory publications (e.g. handbooks, manuals, guides):

I. Magazine articles:

J. Media Placements:

I have been interviewed by local newspapers (e.g., Seattle Times, PI) and by local TV for the work I am doing concerning the use of algae for Biofuels. During these interviews and also interacting with local government representatives (e.g., The Puget Sound Regional Council), I have emphasized the support that Sea Grant has given that has fostered the basic research on algae and that this support makes applied work possible.

K. Other publications (e.g., videos, DVDs, software, websites):

6. PRESENTATIONS - inc. Conference (Poster or Oral), Seminar & Public

Oral presentation: Michael Nishitaki, Rose Ann Cattolico and Danny Grunbaum (2009).

Swimming, stability and gyrotaxis: predicting bloom-formation in a marine alga. Society for Integrative and Comparative Biology. Oral presentation (mn)

Oral presentation: Michael Nishitaki, Rose Ann Cattolico and Danny Grunbaum (2008) Predicting bloom formation from cell-level swimming behavior in a marine alga. Western Society of Naturalists. Oral presentation (mn).

Oral presentation: Rose Ann Cattolico, Jean Chang, Christopher Gobler, Han Ong, Elizabeth Sims, Gabrielle Rocap, Steven Wilhelm, Yang Zhou, and Michael Jacobs (2008). The stramenopile chloroplast genome: new evolutionary and functional insights. Seventh International Chrysophyte Symposium (Connecticut)

Oral presentation: Michael A. Jacobs, Kenneth G. Karol, Elizabeth H. Sims, Will Gillett, Rose Ann Cattolico. (2008) Comparison of complete mitochondrial genomes of two geographically and temporally distinct isolates of *Heterosigma akashiwo*. Seventh International Chrysophyte Symposium (Connecticut) – Oral presentation (rac).

Oral presentation: Michael Nishitaki, Rose Ann Cattolico and Danny Grunbaum (2008). Ocean motion: motility and aggregation in a marine alga, *Heterosigma akashiwo*. Northwest Algal Symposium. Oral presentation (mn)

Oral presentation: Edward Theriot, Robert Andersen, Matt P. Ashworth, Rose Ann Cattolico, Stefano Draisma, Robert K. Jansen, , Mathew Julius, Hiroshi Kawai, Han Ong Gabrielle Rocap Elizabeth Ruck and Cai Zhengqiu, (2008)
Assembling the Tree of Life Conference.

Special note:

Four abstracts will be presented at the Plant Physiology/Phycology meetings in July, 2009;

Five abstracts will be presented at the HAB meetings in November, 2009

7. PATENTS AND COPYRIGHTS

US Provisional Patent Appl. #61/038,428: High-lipid producing alga for use in mass culture 3/21/2008

8. NEW BUSINESSES OR JOBS CREATED

Chloe Deodato and Thien Vo are working as technicians in this laboratory to support this project.

9. LIST ALL STUDENTS SUPPORTED BY OR AFFILIATED WITH THIS PROJECT

Student Name: Michael Lakeman

Department: Biology

Major/Degree field: Population biology/ algal toxicology

Major Professor: R.A. Cattolico

Student Type (Ph.D.):

Dissertation/Thesis title: (approximate) Impact of physiological cues on *Heterosigma akashiwo* strain identity and toxicity

Date of graduation (actual or anticipated): ~ 2010

Total support or affiliation period : continual (EPA Star, NIH Molecular and Cellular Training Grant; TA ship; Departmental Fellowship).

Type of support: use of growth chambers, access to large library of *H.akashiwo* cultures that is maintained via a curator, availability of chemicals and general laboratory supplies as well as use of Coulter Counter, publication costs, attendance at meetings.

Current employment if applicable: Boeing Company

Student Name: Michael Nishitaki,

Department: Biology

Major/Degree field: Population biology

Major Professor: R.A. Cattolico and D. Grunbaum

Student Type (Ph.D.):

Dissertation/Thesis title: (approximate) Slick formation in the toxic raphidophyte *Heterosigma akashiwo*

Date of graduation (actual or anticipated): ~ 2011

Total support or affiliation period: continual

Type of support: use of growth chambers, access to large library of *H.akashiwo* cultures that is maintained via a curator, availability of chemicals and general laboratory supplies as well as use of Coulter Counter.

Student Name: Megan Black

Department: Biology

Major/Degree field: Population biology

Major Professor: R.A. Cattolico

Student Type (Ph.D.):

Dissertation/Thesis title: (approximate) *Heterosigma akashiwo*: Strain identification and population profiles

Date of graduation (actual or anticipated): ~ 2012

Total support or affiliation period: continual

Type of support: use of growth chambers, access to large library of *H.akashiwo* cultures that is maintained via a curator, availability of chemicals and general laboratory supplies as well as use of Coulter Counter.

Student Name: Elizabeth Tobin

Department: Oceanography

Major/Degree field: Cell stasis

Major Professor: D. Grunbaum

Student Type (Ph.D.):

Dissertation/Thesis title: (approximate) Analysis of *Heterosigma akashiwo* resting cell formation with emphasis on swimming behavior

Date of graduation (actual or anticipated): ~ 2012

Total support or affiliation period : continual

Type of support: use of growth chambers, access to large library of *H.akashiwo* cultures that is maintained via a curator, availability of chemicals and general laboratory supplies as well as use of Coulter Counter.

10. INTERACTIONS

Collaboration with Dr. Danny Grunbaum. A significant portion of research done by co-mentored students takes place in my laboratory.

11. OUTREACH AND INFORMATION/TECHNOLOGY TRANSFER

BumSoo Han: Student who was in my laboratory as a visiting scholar for one year. Working with him concerning publishing his data on HAB algae in Korean waters.

Undergraduates:

Undergraduates are an integral part of my research commitment. I continue to be invested in training minority students and am particularly interested in helping young women pursue science as a career. Each student is working on a bench oriented research topic. Often, they work in pairs, with a graduate student, post-doctoral fellow or technician as a supervisor.

Husen Husen: Husen was looking at lipid biosynthesis in *H.akashiwo*. Lipid production is reported to affect *H.akashiwo* toxicity.

William Hardin: Worked on genetically “bar-coding” toxic algal strains. Has recently completed BA (biochemistry).

Matt Munch: Impact of physiology on algal growth.

Amy Sage: Recovery, identification and growth rate of field-collected algal strains.

Victor Tran: Also working on lipid production (both quantitative and qualitative analysis) in *H. akashiwo*.

Thien Vo: Algal culture development; impact of physiology on algal growth.

Cathy Walsh: Qualitative and quantitative identification of bacteria in laboratory-maintained algal cultures.

The following students are work-study helpers: **Athena Bautistia and An-thuyet Nguyen**

12. FUTURE ACTIVITIES

Work will continue to determine how different *H.akashiwo* strains impact successful slick formation and cyst production in Puget Sound. Interaction with both fish and shell fish aquaculturists is anticipated.