論 文 の 内 容 の 要 旨

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論文題目 Development of practical quantification protocols of the marine diatom *Pseudo-nitzschia* spp. for ecological studies
(海産珪藻 *Pseudo-nitzschia* 属の生態研究のための実用的な定量手法の開発)

Conventionally there are two methods of expressing amount of phytoplankton. Number of individuals by species is often used for community composition and amount of components such as chlorophyll and carbon for function of community. The former is essential to study species succession including blooming patterns of specific species. For example, observation of change of cell numbers in relation to environmental parameters is necessary to clarify blooming mechanisms of harmful microalgal species. This is traditionally done by light microscopy (LM). But a problem arises in some new species of phytoplankton as they are classified based on ultra structures visible only by electron microscopy. This is evident in diatoms such as *Pseudo-nitzschia*, a genus which contains some species known to produce toxin causing Amnesic Shellfish Poisoning (ASP). Species in this genus are currently delineated based on morphological features visible only by electron microscopy. As of this year 2009, there are 32 species of the genus *Pseudo-nitzschia*, 11 of which are known to produce ASP causing toxin. The objective of this study is to establish quantification protocols for *Pseudo-nitzschia* for ecological studies and also for monitoring purposes. The protocols developed must be effective, practical and can answer to different situations such as the wide variety of research objectives and availability of facilities among others. In this study, identification groups to facilitate inference of *Pseudo-nitzschia* species under LM and species count under TEM were developed based on morphological characters of species visible under LM. Fluorescence *in situ* hybridization (FISH) method was developed especially using newly designed probes and new fluorescent labels. A novel protocol is proposed based on combination of improved molecular (FISH) and morphological attributes (groups).

Pseudo-nitzschia samples to test protocols were collected by 20 µm mesh size plankton net and/or Van Dorn sampler and/or bucket from Tokyo Bay at Odaiba and/or Chiba sites from April 2008 - December 2009, San Pedro Bay, Philippines from December 2006 - May 2008. Samples were also collected from four other bays in

Japan, and Cat Ba Bay, Vietnam. 12 species among 105 strains in cultures of *Pseudo-nitzschia* were established for development of FISH.

To develop protocol 1, lengths (L) widths (W), ratio of lengths versus widths (L/W), tip and cell shapes among others were collected from original descriptions of all species. L, W and L/W were graphed to establish parameters for groups of species. Morphologically similar species based on these characteristics which are discernable under LM were grouped. Groupings were tested on field samples. Cell counts of Protocol 1 were compared with that of protocol 2. Readjustments of some groups were done after a series of comparisons were made. Protocol 2 was developed to assess results of protocols 1, 3 and 4. Number of striae and fibulae in 10μm, number of poroids in 1 μm, rows of poroids, inner poroid features were examined by TEM for critical species identification. A way to count *Pseudo-nitzschia* species under TEM that will include species occurring in low densities is devised. In protocol 3, FISH method was tested. Hybridization time and temperature, time of fixation, washing conditions, growth conditions of population and proper cell density for counting were experimented to find the optimum conditions using cultures prior to field application. Newly designed probes for FISH including one genus targeted probe and nine species targeted probes, which were based on D1-D3 region of LSU rDNA sequences were designed and used. All probes were tested by cross reaction checks using cultures. Experiments to develop protocol 4 involved examinations of efficacy of 10 fluorescent dyes and selection of combination of dyes that did not exhibit cross talk.

Four protocols were developed. Protocols 1 and 2 are morphologically based. Protocol 3 is molecularly based. Protocol 4, the most advanced of the three protocols is a combination of both morphological and molecular techniques.

Protocol 1 involves counting of morphologically similar species by groups under LM by the following steps: 1. Sample collection; 2. concentration of fixed samples; 3. observation by cell and tip shapes, measuring L, W and comparison to groupings flowchart; 4. identification of groups which the cells belong; 5. counting of cells by groups; 6. acquisition of results of cell number of groups of *Pseudo-nitzschia.*

The seven groups with some species belonging to two groups as follows:
AME (*P. americana* group; contains 3 species): no bulge in the center; L not 10 or more times longer than W.
AUS (*P. australis* group; contains 14 species): bulging central W; with L 10 or more times longer than W; about more than 50μm in L.
CAC (*P. caciantha* group; contains 11 species): straight species with no bulge at the center; elongated with L 10 or more times longer than W.
GAL (*P. galaxiae* group; contains 6 species): L 10 or more times longer than W; less than 50μm in L.
MICRO (*P. micropora* group; contains 2 species): bulging centers; L are not 10 or more times longer than W.
MULTA (*P. multistriata* ; contains 1 species): cells with sigmoid shape and curving tips in girdle view.
SUBC (*P. subcurvata* ; contains 1 species): cells are curved, bulging central W abruptly constricted towards the tip.

Protocol 2 involved observing, classifying and counting species by groups established for LM quantification as in Protocol 1, steps 1 to 5. Then additional steps are as follows: 6. isolation of cells by groups and placing these in tubes designated for each group; 7. verification of species in groups by TEM; 8. counting of all

Pseudo-nitzschia species found in TEM grid; 9. extrapolation of species cell density by multiplying relative abundance with total *Pseudo-nitzschia* cell number from LM; 10. acquisition of results of cell number of *Pseudo-nitzschia* species.

The two morphology based quantification protocols described above, although limitations abound, may still be used in unique circumstances. Protocol 1 maybe used in cases when only rough identification of *Pseudo-nitzschia* is required such as in preliminary identification during field surveys. Species verification by TEM may be done at a later time. Protocol 2 is the most accurate method in species identification. Counting is possible. But picking up about 10^4 cells and acid washing is time consuming. Results will be acquired only after several days. Protocols 1 and 2 will be useful for bays with less diverse composition of *Pseudo-nitzschia e.g.* San Pedro Bay more so when bays have occurrence of high densities of *Pseudo-nitzschia* but low species diversity scattered in different groups. A bloom of similar composition just described will also find this method helpful.

The seven groups in Protocol 1used other characteristic aside from L and W such as general shape of the cell and tip and ratio of L/W. This is more useful than the currently practiced counting of *Pseudo-nitzschia* in two groups delineated only by 3µm of W since W varies in many strains within species. Species are spread into seven groups thus there is higher possibility to infer species correctly under LM. Results however will be expressed as "groups" of *Pseudo-nitzschia* only. Results of Protocols 1 and 2 correlated for *P. multistriata* very highly significant r=0.982928, AUS significant r=0.730685 and CAC very highly significant r=0.785045 with samples from Odaiba Site in Tokyo Bay.

General steps for protocol 3 are as follows: 1. counting of number of *Pseudo-nitzschia* by LM; 2. Filtration of amount of seawater to achieve at least 300-600 cells/ml of *Pseudo-nitzschia*; 3. processing of samples by FISH method using single colored probe; 4. observation and counting in epifluorescent microscope noting cellular size and shape by groups established from protocol 1 to check if cross reactions occur; 5. acquisition of results of cell number of *Pseudo-nitzschia* species. Only one probe out of five probes by Miller and Scholin, frD1 for *P. fraudulenta* was found to be a good probe for the same species in Tokyo Bay. On the contrary designed probes in this study worked well. Three out of nine designed probes had cross reactions, however by calculations the cell number in each species were obtainable. The nine species-specific probes were for the following species: *P. americana, P. brasiliana, P. caciantha, P. calliantha, P. delicatissima, P.galaxiae, P. multistriata, P. multiseries* and *P. pungens* respectively. A probe for the genus *Pseudo-nitzschia,* P-n2 could catch the species or strains in the bay that were not designed probes.

Protocol 4, the final protocol named group guided multicolour FISH (g-MFISH) is the result of development of three previous protocols. This provides a novel technique of simultaneous observation of both morphological and molecular attributes in a species. This follows the steps described in protocol 3. Except that application of probes involves uniformly labelling members of one group, described in Protocol 1 with one dye color. Members of another group were separately labelled with another dye color. One sample aliquot was applied with probes of member species from different groups. This protocol combines differences of sizes and shapes that reflect morphological variations and the different colors reflect molecular variations in each species. As the case in Tokyo Bay, nine species with developed probes fitted to a three-colored system *i.e.* three species in three separate groups. The following settings of Olympus IX71 epifluorescence microscope were used to observe the following

fluorescence: NIBA3 (green bandpass) for viewing FITC or AF488 (green dye), Filter Cy3 (red band pass filter) for viewing Cy3 or AF568 (red dye) and WU2 (blue UV long pass) for viewing AF350 (blue dye). Cross reaction problems particularly with *P. pungens* and *P. multiseries* were solved with this strategy. Another advantage of this method from ordinary FISH is the decrease in time and materials spent in processing samples. One limitation was that the third dye either exhibits cross talk or had lower percentage recovery (Alexa Fluor 350).

Correlations of data by protocols 2 and 3 for total *Pseudo-nitzschia* and cells reacting with genus probes were always at very highly significant r= 0.986799, for example in Odaiba Site of Tokyo Bay. There is always possibility to find species which could not be detected in the efforts of group component species analysis. Sometimes species will be found in a research area that was not encountered before. Therefore observation of difference between total cell number detected by specific probes and detected by genus probe is important. Correlations of data for species *P. calliantha* significant r=0.589945, *P. fraudulenta* significant r=0.612067, *P. multistriata* very highly significant r=0.98769, and *P. multiseries* significant r= 0.681602 with samples from the same site were also achieved. Except for some probe cross reaction and non reaction problems, results of Kamaishi, Okirai, Ofunatu, Kobe and Cat Ba Bay samples by the three protocols described above matched accordingly. Thus probes can trace species in a bay when these are designed based on the same species or strains in the same bay. Protocols 3 and 4 have an advantage of getting results in a shorter time than Protocol 2. The process takes only about five hours to finish.

Application of g-MFISH or FISH using single colored probes will depend on the variability, number and composition of expected *Pseudo-nitzschia* species in the bay. If there is less or equal to five species in separate groups with no cross reaction of probes among species, a single color of the probe will suffice. Observation by groups should be made to ensure that no cross reaction of unexpected species occurred. Protocol for g-MFISH is useful if simultaneous enumeration of all *Pseudo-nitzschia* species is necessary in a bay with diverse *Pseudo-nitzschia* species concentrated in three groups or less with probes cross reacting to species within groups such as *Pseudo-nitzschia* species composition in Tokyo Bay.

G-MFISH can be applied to ecological studies of individual species with similar difficulty of identification as *Pseudo-nitzschia* such as many other diatoms *e.g. Skeletonema* or dinoflagellates *e.g. Alexandrium.* Data verification should still be made using electron microscopy. Future studies may be directed to improving probe specificity by searching for sequences in other domains of the LSU and advancing g-MFISH by testing other dyes or developing new dyes specific for phytoplankton. This strategy may also be applied to designs at automation of species enumeration.