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Accuracy Enhancement of Microscope Enumeration of Picoplankter *Aureococcus anophagefferens*

by

John B. Mahoney, Dorothy Jeffress,
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by

John B. Mahoney[1,4], Dorothy Jeffress[2,5], John Bredemeyer[3,6], and Kari Wendling[1]

Postal Addresses: *[1]National Marine Fisheries Serv., 74 Magruder Rd., Highlands, NJ 07332*
[2]National Marine Fisheries Serv., 212 Rogers Ave., Milford, CT 06460-6499
[3]Suffolk County Dept. of Health Services, Evans K. Griffing County Ctr., Riverhead, NY 11901

E-Mail Addresses: *[4]John.Mahoney@noaa.gov*
[5]Dorothy.Jeffress@noaa.gov
[6]John.Bredemeyer@co.suffolk.ny.us

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ABSTRACT

Two main types of enumeration error were experienced in the use of an immunofluorescence protocol for identification of the picoplankter *Aureococcus anophagefferens* from western New York Bight coastal waters. Microscopist discrimination was affected when cells were too numerous to count reliably or too lightly stained to be identified. This was remedied by sample dilution, and increase of antisera and stain concentration and incubation time. The second type, extensive clustering of *A. anophagefferens* cells in monospecific clumps, and as embeds in floc matrices, was much more problematic. To remedy this, cell disaggregation was sought through mechanical and chemical treatments. A single treatment effective for all samples was not found but various treatment combinations greatly improved enumeration. Varied efficacy of treatment regimes among samples suggests that cell binding strength was variable.

KEY WORDS: *Aureococcus anophagefferens enumeration, harmful algal blooms, brown tide, New York Bight*.

INTRODUCTION

Northeast United States "brown tide" species, *Aureococcus anophagefferens,* cannot be distinguished reliably from similar 2-3 μm diameter picoplankters with phase contrast or epifluorescence microscopy (Sieburth et al., 1988). For more than a decade an immunofluorescence protocol (Anderson et al.,1989) has been the main means to identify and enumerate the species. The method is, or has been, relied on in approximately a half dozen laboratories (Kulis, personal communication). The NMFS James J. Howard Marine Sciences Laboratory used the method in 1997-2001 to determine distribution of *A. anophagefferens* in the western New York Bight and track bloom development in the New Jersey Barnegat Bay-Little Egg Harbor estuarine system. During a 1999 bloom, and subsequently, serious error in enumeration of many samples was experienced, primarily due to tenacious cell clumping. *A. anophagefferens* embedding in floc matrices of amorphous unidentified material, plankton spp., and detrital particles, contributed to clustering error. Enumeration accuracy also was affected when *A. anophagefferens* concentration was high, and when cell staining was insufficient due to high cell number and abundance of particulates. These enumeration problems were not present or were not recognized in samples from the Barnegat Bay-Little Egg Harbor system during a 1997 bloom, or in 1998, a non-bloom year when *A. anophagefferens* maximal abundance was ~10^4 cells ml^{-1}. This paper outlines a series of sample treatments and immunofluorescence protocol changes to overcome or minimize these error sources.

BASIC METHODS

The immunofluorescence protocol for *A. anophagefferens* enumeration as developed by Anderson et al. (1989) is: (1) *A. anophagefferens* in water samples is preserved with 0.6-1.0% glutaraldehyde; samples are stored at 4ºC until processed; (2) When processed, a small aliquot (e.g., 200 μl) is incubated with 1.0 ml of 3% normal goat serum for 40 min. in a 12 x 75 mm test tube; (3) The aliquot is then rinsed with10 ml of phosphate buffered saline (PBS) onto a 0.2 μm pore 25-mm diameter black polycarbonate membrane filter, backed by a 25-mm glass fiber filter, in a 25-mm micro-filtration funnel; the filter is rinsed three times with 10 ml of PBS; (4) One ml of *A. anophagefferens* antiserum (1:3200 dilution) is applied; after incubation for 40 min., the filter is PBS-rinsed as before; (5) A 1:800 dilution of FITC conjugated goat anti-rabbit antiserum is applied, incubated for 20 min., and the filter PBS-rinsed as before; (6) The filter is gently dried, placed on a slide, and covered with a drop of 9:1 glycerine/PBS and a cover slip; (7) Slides are examined at 400X using an epifluorescence microscope with a FITC filter set; *A. anophagefferens* is identified by fluorescent labeling around the cell perimeter, resembling a green ring or halo. Using a cross pattern over the membrane filter, 50 fields are enumerated. Processing of a 200-μl sample aliquot provides an estimated detection limit of 100 cells ml^{-1}. Subsequently, Anderson et al. (1993) recommended 1.0-μm pore polycarbonate membrane filters, and application of glycerine/PBS to the cover slip, rather than the filter.

Protocol modifications made by J. Bredemeyer (unpublished), N. Y. Suffolk County Department of Health Services (SCDHS), and adopted by the James J. Howard Marine Sciences

Howard Laboratory (HL), include doubling the salinity of PBS, from 8.7 to 17.4 PSU, to make it more isotonic with the samples; change of the polycarbonate membrane filter pore size to 0.8 μm; incubation with goat serum on the membrane filter rather than in a test tube; increase of incubation time of goat serum, primary anti-serum and secondary anti-serum to 45, 45, and 30 min., respectively; decrease of PBS rinsing following secondary anti-serum incubation to one rinse; and change of glycerine/PBS ratio to 5:1. Eventual increases in anti-sera concentration and incubation time at HL are discussed below. HL personnel were trained in the immunofluorescence procedure by SCDHS personnel. SCDHS personnel had been trained by the Anderson Laboratory, Woods Hole Oceanographic Institution (WHOI).

At HL, slides routinely were prepared on one day, refrigerator-stored in covered trays, and examined the next day using a Zeiss Axiovert microscope. Prepared slides could be refrigerator-stored for at least several days with no apparent reduction of cell numbers or fluorescent stain brightness. Enumeration of *A. anophagefferens* in each sample was done at least twice, by two microscopists when possible, or by the same microscopist at different times. Initially, 60 fields per count were enumerated; later this was extended to 100 fields per count. If counts were within 20% of each other, they were assumed to be representative and were averaged. If initial counts were not in such agreement, counting was repeated. A cross pattern (as on a clock face:12 to 6, 9 to 3, etc.) was used in counting; in a traverse, the stage was advanced field-to-field in a random manner. Distributing enumeration effort uniformly helps to minimize the effect of non-random cell distribution on the membrane, especially concentration at the periphery (Scientific Committee on Oceanic Research, UNESCO, 1974). During slide examinations, besides cell enumeration, cell staining level and overall cells/background contrast was noted, as well as the presence of *A. anophagefferens* cells in clumps or detrital aggregates. "Clump" refers to aggregation of *A. anophagefferens* cells into a monospecific cell mass. "Embed" refers to aggregation of cells (usually not contiguous) in a matrix of unidentified apparently organic material, other phytoplankton spp., and various particulates. Clumps were identified by their fluorescence and form (they resemble a cluster of grapes). Numbers of embeds and trapped cell numbers; numbers of clumps and approximate observable clump dimensions, including diameter or length and width, were noted. Cells in small clumps, $\leq$10 cells, were counted; counting of cells in larger clumps is considered unreliable. Focusing at different planes was done for each field; this was especially necessary when counting cells in small clumps.

Cell disaggregation tests were done at HL, initially in consultation with SCDHS. Field samples rather than standardized material were used for the tests. In consequence, test sample character varied. Some of the test samples, collected by SCDHS from various sites in the Long Island Peconic Bay system, were previously counted by SCDHS microscopist J. Bredemeyer. These data provided inter-lab count reference. Some multiple replicate SCDHS Long Island samples were pooled for treatment tests. HL samples collected during a 1999 Barnegat Bay-Little Egg Harbor brown tide also were used in disaggregation tests. SCDHS samples are identified by permanent station number and collection date, HL samples by a dedicated number.

RESULTS AND DISCUSSION

Causes of Enumeration Error; Some Remedies

High *A. anophagefferens* concentration in some New Jersey samples increased count difficulty and, depending on the microscopist, to a greater or less degree contributed to count inaccuracy. Preparations considered "too numerous to count" (TNTC) were encountered for Barnegat Bay-Little Egg Harbor bloom samples in mid-May 1999, and subsequently. This fairly routine problem was addressed in reprocess by reducing the amount of sample filtered and/or diluting the sample. Similarly, when Long Island *A. anophagefferens* samples are enumerated by SCDHS, reprocess is done when counted cells exceed 600. Insufficient staining also was encountered in New Jersey sample preparations, associated with high numbers of *A. anophagefferens* and/or abundance of particulates. The latter apparently sequestered stain. This reduced ability to discriminate *A. anophagefferens* cells. In addition to sample dilution and/or reducing the volume of sample filtered, insufficient staining was remedied by doubling the concentrations of the primary and secondary anti-sera (advised by D. Kulis, WHOI). Another measure, advised by K. Milligan who participated in testing of the immunofluorescence protocol while at the Marine Sciences Research Center, SUNY, Stony Brook, NY, increase of primary and secondary anti-sera incubation an extra 15 min., to 60 min. and 45 min., respectively, also was adopted. These measures greatly improved counting of some samples. For example, counts (cells ml $^{-1}$) in two samples rose from 86,020 and 500,711, to 376,541 and 1,079,024, respectively, increases of 337% and 115%, respectively. SCDHS did not incorporate these changes because the staining problem was deemed less serious in Long Island samples.

Erroneously low *A. anophagefferens* enumeration due to cell aggregation was far more problematic than cell number TNTC or insufficient staining. Cell clustering can greatly compound non-random dispersion of cells on the filter membrane, and is considered a major cause of microscope enumeration error (Scientific Committee on Oceanic Research, UNESCO,1974). Referring to enumeration error caused simply by the presence of diatoms in chains, Holmes and Widrig (1956) reported the difficulty of obtaining accurate estimates of abundance when cells were so clustered. In our study, extensive clumping, with pronounced non-random distribution of *A. anophagefferens* cells was first noted in a May 1999 Little Egg Harbor, NJ, sample; this sample had been stored five days prior to processing. Subsequently, clumps of *A. anophagefferens* cells (sometimes of ~75-100 cells), and cells embedded in matrices of varied composition, were frequently observed in samples from the Barnegat Bay-Little Egg Harbor system, especially during blooms.

The occurrence or degree of *A. anophagefferens* aggregation in nature is undetermined. Cell aggregation was not assessed in unpreserved/unprocessed samples because of unreliability of distinguishing *A. anophagefferens* from other picoplankters using light microscopy. Suggesting that aggregation of live cells in a natural population can occur, at HL clumping in older cultures of an axenic *A. anophagefferens* strain (Center for Culture of Marine Plankton 1984) is common. Rigorous mixing, e.g., 30 sec. or more of vortexing, is required to disperse cell floc in the latter but even then smaller aggregates (~1 mm diameter) often remain. This

clumping was not seen in bacterized strains (CCMP 1784 and 1794 - 1784 is the parent culture of 1984); presumably associated bacterial culture contaminants metabolized the binding material. One of the authors (JB) observed that clumping was more likely in samples from certain Long Island locales than others, raising the question of whether it may be linked to cell metabolism and/or physiological status. Clumping appeared more prevalent in New Jersey samples with higher *A. anophagefferens* concentrations than in samples with lower concentrations (it expectedly would be more noticeable with higher cell concentration). Clumps of preserved *A. anophagefferens* cells were found in some sample preparations soon after collection, e.g., within a day. However, cell clumping can initiate or advance during storage of preserved samples in which cells apparently were in, or close to, single cell suspension a short time earlier. Cell count reductions of 20-30% were found for certain samples reprocessed after an additional week of storage. This suggested cell loss, but when reprocessed with cell disaggregation treatment counts close to the original ones were obtained.

Counts routinely are done of only a relatively small number of microscope fields, i.e., 25-50 by SCDHS, 120-200 by HL, out of approximately 4,000 fields for SCDHS and 6000 fields for HL (field size in the respective laboratories is determined by the Whipple count disc being used). Clumping/embedding lowers the likelihood of a small part of the total filter membrane being representative. It might be thought that accuracy could be increased by greatly increasing the number of fields counted, thereby increasing potential for encountering cell clumps. Fournier (1978) believed the only certain method is to count all the cells on the membrane. These measures would not be practical or sufficient with *A. anophagefferens* due to the large numbers of cells frequently encountered and the need, at least during a bloom, to process many samples. Even great expansion of membrane area counted likely would not be a sufficient solution when cell aggregation is pronounced. Microscope counting of cells embedded in floc matrices is difficult but doable. Estimation of cell numbers in larger clumps (>10 cells) is highly error prone, however. Although cells in surface planes of *A. anophagefferens* clumps are countable, discrimination of cells at all planes would be problematic at best. Another complication is varied clump shape. Clumps that may have formed in nature from cells aggregating in the water column likely would be globular. Cells aggregating during storage in the collection vessel likely formed layered clumps. We suspect we have seen both types but were unable to view clumps adequately; they could not be rotated to view all their dimensions.

Counting of cells in a single plane in larger clumps obviously would consider an inadequate fraction of aggregated cells. Illustrating how this could affect enumeration, approximate length and width of four *A. anophagefferens* clumps (variously having slightly bulbous sides or incomplete corners) were measured with an ocular micrometer. Cells in a single surface plane in the four clumps were counted, providing a total of 388. If these clumps are assumed roughly cylindrical, with depth approximating diameter (assuming cylindrical shape is conservative because a cuboidal shape would contain more cells), the estimated cell total would be 3019. The two estimates vary by a factor of 7.78.

According to Lund et al. (1958), the accuracy of results needed for estimating algal populations is not normally very large; algal population estimates with an accuracy of $\pm$ 50%

may suffice for investigations concerned with generations, i.e., change in abundance of 100%. Greater accuracy than this is needed for a species that causes highly detrimental effects. Bricelj et al. (2001) reported that *A. anophagefferens* concentrations as low as 3.5 x 10^4 cells ml^{-1} significantly reduce northern quahog, *Mercenaria mercenaria*, feeding, and clearly *A. anophagefferens* enumeration can be subject to serious error when its cells are aggregated. Single cell suspension is essential for accurate counting of microorganisms (Nebe-von-Caron et al., 2000).

The material causing tenacious cell binding of *A. anophagefferens* or its embedding in floc matrices has not been identified. Hypotheses explaining mucilage formation in the sea assume accumulation of colloidal and gel-like polysaccharide from phytoplankton exudates (Alldredge and Crocker, 1995, in Najdek et al., 2002). Larger aggregates (defined as clouds 0.5-5 m) may be formed directly by the coagulation of gels entrapping plankton cells and other particles (Degobbis et al., 1993, in Najdek et al., 2002). *A. anophagefferens* has an exocellular layer of organic material, probably mucopolysaccharide, which is sometimes copious (Sieburth et al.,1988). If this excreted material has adhesive properties it could be the binding material in clump formation, and it possibly is the material in which cells were seen to "embed" in field samples. Middlebrook and Bowman (1964) remarked that purification of cultures of algae having mucopolysaccharide capsules is particularly difficult because bacteria stick tenaciously to them. At HL, cultures of an axenic strain of *A. anophagefferens* (CCMP 1984) regularly form an extensive concentration of cells in a mucus-like material at culture tube bottoms. Evidenced by resistence to dispersion of cells by vigorous vortex mixing, this material has binding strength. Whether degree of binding might be based on amount or quality of the binding material, and whether cell binding strengthens or weakens over time, likewise is unknown. Therefore, disaggregation of *A. anophagefferens* was approached empirically, using a variety of treatment options employed in various similar microbiology/cytology applications.

Cell Disaggregation Protocol Development

To achieve disaggregation of *A. anophagefferens* we assumed high integrity of the original cell complement despite prolonged storage. Glutaraldeyhde is an excellent fixative for phytoplankton in general, and is known to maintain cells for long periods. Anderson et al.(1993) found that counts of *A. anophagefferens* in glutaraldehyde-preserved field samples were constant over an extended period of time (six months). Cell numbers of glutaraldehyde-preserved phytoplankton, assessed by epifluorescence microscopy, were unchanged after a year in storage (Booth et al., 1993).

Sample mixing was examined first. Routine *A. anophagefferens* immunofluorescence protocol mixing procedure, i.e., inverting the sample vials 50 times, was insufficient when cells were aggregated. Anderson et al. (1993) reported no difference in counts whether samples were shaken vigorously or mixed gently; apparently *A. anophagefferens* cells in the samples they compared were not aggregated. Vortex mixing (suggested by J. Bredemeyer) was tested as a replacement. Cause for caution about vigorous mixing was that *A. anophagefferens* lacks a cell wall (Sieburth et al., 1988); and Anderson et al. (1989) reported fragility of *A. anophagefferens*

cells unless fixed for several weeks. Kulis (personal communication) expressed concern that vortexing might disrupt cells. At HL vortexing was optimized with the particular mixer in use, a Vortex-Genie with variable power setting, and an on-off switch activated by pressure on the mixing head. Mixing was done by repeatedly pulsing the mixer long enough to get a vortex, and presumably considerable shear force. Madrigal et al. (1993), in addition to other treatments, recommended shear force through agitation to separate cells for flow cytometric analysis. Long-term glutaraldehyde-preserved *A. anophagefferens* cells proved relatively robust, and a 100 pulse vortexing (this took about 60 sec.) eventually was adopted as standard. Longer-term vortexing (as a sole treatment) was not additionally beneficial. Pulsed vortexing is assumed to be more effective for cell disaggregation than continuous vortexing but relative efficacy was not determined. Different mixer power settings were tried and a moderate setting ("3.5") was adopted - approximately one third power. Higher power than this ("4") resulted in cell loss in two test samples. Eventually, multiple vortexing applications as described were used during sample cell disaggregation treatments. Although an improvement over sample vial inversion (data not shown) vortex mixing, as is shown in Table 1, did not adequately remedy cell aggregation.

Detergent in combination with other treatments commonly is used to lyse cells, e.g., for harvesting of subcellular constituents (Buetow, 1973). However, McDaniel et al. (1962) used detergent (ARKO Hospital and Laboratory Detergent) to separate bacteria from mucoprotein-capsulated blue-green algae to which they were strongly attached, and (Kutkuhn (1958) had general success using detergent (unspecified) to remedy phytoplankton cell aggregation due to centrifuge compaction. R. Guillard (personal communication) recommended Aquet detergent (Bel-Art Products) for cell disaggregation. Results with Aquet, tested at 0.5, 1.0, 1.5 and 2.0 % with ~3-10 min. exposure, varied with concentration and sample (data not shown). For some samples enumeration was improved; at minimum, clump size was reduced. In two of four samples 1.0% had no more effect on cell separation and cell count than 0.5%, but in another the higher concentration was additionally beneficial and there was very dramatic benefit in the remaining sample. For the latter sample, when treated with 0.5% Aquet, large clumps and embeds and uneven staining persisted, whereas 1.0% Aquet provided better cell dispersion, greatly reduced cell aggregation, and improved staining. Aquet at 2.0% lowered count levels with some samples so 1.0 %, the concentration recommended by Guillard as a starting level, was adopted. Combination of Aquet and vortexing was more beneficial than Aquet singly. Long-term Aquet treatment (17, 26, 41 and 47 hr, respectively) showed some additional cell disaggregation but at the cost of reduced cell staining. Limited trials were made of some other detergents: FL-70 (Fisher SF 105-1) reduced clumps and improved enumeration, but apparently was disruptive to cells and filter membranes. Micro-90 (International Products Corp.) reduced cell aggregation in one test, but caused clogging of filter membranes in two other tests. BRIJ 35 (Sigma P 1254) was less effective than Aquet.

Because combined vortexing and Aquet was only partially effective, a protocol developed by Velji and Albright (1986) to free bacteria associated with particulates, was adapted for *A. anophagefferens*. Their procedure includes fixation with an aldehyde, treatment with a sequestering/deflocculating agent (tetra sodium pyrophosphate), and ultrasound. In their study, this treatment combination provided more random dispersion of bacteria on the filter membrane,

and increase in cell number enumerated. The pyrophosphate concentration they recommended for sea water samples, 0.001M, lowered *A. anophagefferens* cell enumeration by approximately half, so 0.0001M was adopted. Pyrophosphate was effective at the lower level, and a large margin of safety was considered necessary. The 0.001M pyrophosphate treatment time of 15 min.Velji and Albright (1986) recommended eventually was at least doubled for our treatment with 0.0001M. This treatment minimum of 30 min. was extended incrementally, but usually not past 60 min., for samples in a batch because 15 were processed sequentially. Sonication was approached carefully to avoid cell disruption. Velji and Albright (1986) used their BioSonik II sonicator, with a 4-mm probe, at a power level of 100 W for 30 sec.. At HL, sonication was optimized for *A. anophagefferens* with a Misonix Model XL2015 sonicator having a 12.7-mm probe. Sonication of 10 ml samples was done in polystyrene Coulter Counter cups with the probe immersed to about half sample depth. To minimize heat build-up in the sample, sonication was pulsed for 0.05 second per second. Power setting was tested incrementally, and power setting "4" was adopted, although the cells generally could tolerate the next level of sonication. Sample-to-sample difference in what sonication power level cells could tolerate was apparent. Various exposure times were tested, with 70 sec. (this includes non-pulse time) adopted finally. Results of longer sonication varied, from a net loss to a gain of cells available for enumeration. Presumably, if most cells initially were in single suspension these might be especially vulnerable to disruption by prolonged sonication, whereas many cells in clumps and embeds could be partially shielded temporarily, and then disaggregated. Nebe-von-Caron et al. (2000) described optimal recovery of aggregated bacteria cells with a particular regime of sonication amplitude and time, and cell destruction when exposure time was five times the optimal. *A. anophagefferens* sonication term maximum tolerance is far less; as little as ~15% increase of exposure term beyond apparent optimal could result in destruction of cells.

Table 1 shows results of tests of vortex mixing, pyrophosphate treatment and sonication on a group of 10 Long Island samples. In these tests vortexing was done for ~30 sec.; pyrophosphate treatment for ~15 min.; and sonication for 30 sec.. The data illustrate that vortex mixing as sole treatment was inadequate. Pyrophosphate treatment coupled with vortexing was sufficient to recover (within 20%), or exceed, the SCDHS counts in tests 5, 7, 8, 9. In tests 1, 2, 3, 6, 10, this treatment was inadequate. The latter group had the higher cell levels, which suggests a link between cell concentration and treatment adequacy. Sonication combined with vortexing and pyrophosphate was effective (within 22%) in restoring, or exceeding, the SCDHS counts in all tests. For most samples SCDHS/HL count differences were less than 50%, but for two samples (tests 9,10) increases were slightly over 100%. Because the various treatments in these tests did not incorporate the more effective final treatment times (mentioned above) HL count accuracy might be questioned. However, cell aggregation likely was not serious in these samples; it was seen pre-treatment in only two samples of the group. Furthermore, if *A. anophagefferens* cell excretion is stress-associated, as has been shown for other phytoplankton (Myklestad, 1995), it should be noted that the test samples were collected in December when *A. anophagefferens* would be advantaged due to its ability to grow at low temperature (5.0^{o}C) (Cosper et al., 1989), absence of heat stress, a presumably ample nutrient supply, and reduced competition from other phytoplankton including picoplankon that typically are summer dominants. Cell levels in most of these samples were only ~3.0 x 10^{5} cells ml^{-1} or lower,

whereas levels encountered during a spring/early summer bloom often were 10^6 cells ml^{-1} or greater. In the Barnegat Bay-Little Egg Harbor system, cell aggregates were common in bloom samples but not in winter samples. Besides showing utility of certain treatments, the varied results in these tests were an early indication that cell aggregation alleviation could differ sample to sample. That is, a treatment regime that apparently could disaggregate cells, or at least reduce aggregation below our level of detection, in one sample could be far less effective with another sample. This suggests cell binding strength difference. The Long Island test samples were collected at different times from sites with different water qualities, e.g., salinity, nutrient level. It is likely that the cells in these samples at the time of collection had varied health and metabolic state, and possibly varied type and amount of cellular excretion.

SCDHS processed the Long Island samples used for the tests discussed above (Table 1) 3-7 weeks after collection in December 1999. HL reprocessed these samples 12-19 weeks after collection. In the time between the two processings cells had so aggregated that mechanical and chemical treatments were required to disperse them. How effective were the treatments? A long standing enumeration guideline is that 100 cells are counted to give a 95% confidence interval of the estimate within $\pm$ 20% of the mean value, and 400 cells for $\pm$ 10% of the mean (Lund et al., 1958); this is still commonly accepted (Throndsen, 1995). Of the HL cell counts only the test 4 count was under 400 cells, all others were 500 to over a thousand. Assuming a 10% confidence interval, considering just HL counts, the maximum percent count difference in tests 1-4 is 14; for tests 6-8 the maximum percent count difference is 30; and percent differences for tests 9 and 10 are 52 and 55, respectively. SCDHS cells counted ranged approximately 200 to 400, with the majority in the 300 to 400 range. With confidence interval for half of the SCDHS counts at, and the other half greater than, $\pm$ 10% of the mean, differences between HL and SCDHS cell levels would be additionally lessened. The general agreement between the SCDHS and HL post-sample treatment counts suggests that the mechanical and chemical treatments applied were highly effective, and that cell aggregation was not a serious problem when SCDHS processed these winter samples. *A. anophagefferens* cell aggregation in Long Island samples during other seasons remains to be assessed.

The modified Velji and Albright (1986) protocol could be very effective in restoration of single cell suspension, but refractory cell aggregates in some post-treatment sample preparations indicated need for additional treatment. Aquet detergent previously had proved to have benefit so was tested (1.0%; 15 min. exposure) on aliquots of the same sample, in combination with vortexing for ~30 sec., pyrophosphate for ~15 min., and sonication times that varied from 30 to120 sec.(Table 2). Aquet addition resulted in considerable increases in enumeration levels in six of seven tests. The seventh test showed the least increase in cell level with Aquet treatment. Presumably the 90 sec. sonication term used in this test alone was sufficient to disaggregate cells. This was noticeable but less so with test six (80 sec. sonication). Sonication for 120 sec. was the only term over 90 sec. tested and resulted in pronounced cell destruction (not shown). A 90 second sonication term was beneficial for this sample but cell loss was experienced with previous test samples when sonication term was over 70 sec. It is considered preferable to retain an apparently safe sonication term, and employ Aquet as an additional disaggregation treatment. It should not be concluded from these results that the four-treatment combination necessarily

will provide dramatic enumeration change; rather simply that it is effective. A disadvantage of the four-treatment combination is that it results in clearing of the black polycarbonate filters. To minimize this, filters were flushed with 5.0 ml of PBS immediately after sample aliquot placement on the filter. The clearing reduced contrast but did not prevent accurate identification and enumeration. In all but occasional preparations cells showed adequate to bright staining. Necessity to verify some counts by reprocessing treated samples revealed generally detrimental effect of prolonged chemical treatment. Lowered cell enumeration was obtained for four of five samples reprocessed six days after treatment. It probably is best to assume that treated samples are no longer useable more than one day after initial process. If aliquots of a treated sample are diluted (we used 3:7/2:8 sample/diluent, routinely), or reprocessed $\leq$ a day after treatment, cell disruption likely will be avoided.

Reprocessing of HL samples progressed from those collected during non-bloom, to bloom initiation, and finally bloom, conditions. Cell aggregation in the sample collection ranged from none or minimal to very cell-aggregated. Cell disaggregation protocol development reflects this, with treatment options added, and exposure times lengthened, as increasingly more problematic samples were encountered. Combined vortexing, Aquet, pyrophosphate, and sonication treatment sufficed until bloom samples were processed. Refractory aggregates in these necessitated more treatment. Enzyme preparations including hyaluronidase (Sigma H 2126) (recommended by S. Merlin, Becton Dickinson Biosciences, personal communication) and Accumax, a commercial multi-enzyme product for dissociating cell clumps (Innovative Cell Technologies, Inc.) were evaluated. Hyaluronidase was tested at 1, 2, 3, and 4 x 10^{-4} %. The lowest concentration was approximately the concentration Merlin (personal communication) employed to suppress clumping of human and rat intestinal epithelial cells during flow cytometry analysis. These cells, as reported for *A. anophagefferens* (Sieburth et al., 1988), secreted large amounts of mucopolysaccharide. The concentration Merlin employed was used as a starting and lowest level for *A. anophagefferens* disaggregation because separation of preserved cells in formed clumps likely would be more demanding than clumping suppression (for extrapolation an *A. anophagefferens* level of 10^6 cells ml^{-1} was assumed). Initial tests showed hyaluronidase at 10^{-4} % to be very beneficial, raising enumerated cell levels substantially (22-69%) in five of seven samples. Results of tests with higher concentrations were varied, with 2 or 3 x 10^{-4} % usually more effective than the lowest concentration, and the highest concentration not additionally beneficial. Concentrations of 2 and 3 x 10^{-4} % were incorporated in the final treatment regime. In combination with the above treatments, Tween 80 (Sigma P 4780), a surfactant commonly used as a dispersing agent, was tested at 0.5, 1.0, 1.5 and 2.0 %; 1.0 % was most effective most often and this level was adopted. The higher concentrations were beneficial and/or non-disruptive with some samples but apparently disrupted cells in others. Interestingly, sonication, vortexing, Tween 80 and detergent, sodium dodecyl sulfate, are all employed in an immunofluorescence protocol for detection of *Cryptosporidium* and *Giardia* cysts in surface fresh water (LeChevallier et al., 1991). Another enzyme preparation, Accumax, was tested for *A. anophagefferens* disaggregation at 0.5, 1.0, 2.0, 3.0 and 4.0 %. Either 0.5 or 1.0% was beneficial; concentrations $\geq$ 2.0% resulted in lowered cell counts. As with the other options, it could not replace combined treatments. Compared with combined treatments, 1.0% Accumax treatment of 15 test samples with just vortex mixing provided cell counts lower for 9, equivalent

for 3, and higher for 3 (not shown). In routine sample processing it was employed at this level with one of the treatment combinations. When beneficial in sample processing, Accumax could raise enumeration level considerably, e.g., for several samples increases of 23-45%.

Although beyond the scope of this study, enzyme treatment of clumped *A. anophagefferens* deserves additional exploration. The mechanical and chemical treatment options adopted are considered to be at or close to their safe limits for *A. anophagefferens* but may not be sufficiently effective. Increased cell disaggregation with modest increase of pyrophosphate concentration is a possibility, if serious contrast reduction can be avoided. Also, a detergent more effective as a disaggregant might be found. However, testing for more effective enzyme treatment could be the most profitable approach. Considerable breakdown of macromolecules in natural waters is through the action of extracellular enzymes, principally those of bacteria (Price and Morel, 1990, in Leppard, 1995).

Cell Disaggregation Protocol

(1) 10-ml samples in 15-ml screw-cap polypropylene centrifuge tubes first are vortexed (100 pulses, or about 60 sec). (2) Desired dilutions, usually sample:diluent 2:8 or 3:7, are made; dilution depends on the general anticipated cell level, e.g., stage of bloom development, and the number of required treatment combinations. Diluent is bay water filtered with a 0.2-µm pore membrane, and having glutaraldehyde addition at the usual level for sample preservation. (3) All subsamples, usually three, are treated with 0.0001M pyrophosphate, 1.0 % Aquet, and 1.0 % Tween 80; the latter requires vortex mixing to dissolve. (4) One subsample additionally is treated with 2×10^{-4} % hyaluronidase; the third subsample additionally is treated with hyaluronidase at 3×10^{-4} % and 1.0 % Accumax. (5) Subsamples are revortexed, and incubated for 30 min. or until process of a series is complete, usually not exceeding 60 min. (6) Each subsample is again vortexed immediately before being decanted into a polystyrene 20 ml Coulter Counter sample cup, sonicated (at HL, a Misonix Model XL2015 / 5 % pulse / power setting “4" / 70 sec.), and returned to the sample tube. (7) The aliquot to be filtered is immediately dispensed on the filter, and rinsed with 5.0 ml of PBS. It is desirable to have two people working together at this stage. (8) The Anderson et al. (1989, 1993) protocol, is then followed as outlined previously with the changes already noted.

Assessment of Cell Disaggregation Protocol

Use of a heterogenous collection of field samples for disaggregation tests rather than standardized material complicated protocol development. However, use of diverse test samples revealed that varied combinations of treatments were best instead of a single protocol. The treatment regime that resulted in the highest cell number is assumed most effective for the particular sample. The highest cell number obtained in the treatment series is assumed to be the most accurate. Enumeration obtained when cell aggregates were not detected in a post-treatment preparation is assumed to approximate best the actual level. Cell aggregates were not detected in most of the post-treatment preparations. Especially because microscope scan for cell aggregates is a qualitative check, not a quantitative measure, absolute accuracy is not claimed. Cell

enumeration with the Anderson et al. (1989, 1993) protocol routinely is done with counts of 50 fields, and this is generally accepted as sufficient. However, scan of even 200 fields for cell aggregates may not be sufficient to determine presence or absence of such. Nevertheless, there must be some practical limit to microscope observation when many samples must be processed. Finding a small clump or two of cells in 200 fields of several thousand possible fields suggests that more exist in the preparation, but the associated count error is undetermined. Likewise, certainty about absence of cell aggregates in the preparation when none were visually detected was not attained. In some instances, judging from post-treatment enumeration level increase, aggregates apparently were present although visually undetected. Low abundances of cell aggregates (e.g., one per 100 fields) were seen in some samples even after the most rigorous treatment. The treatment regime that was necessary to disaggregate cells in some samples, without discernible cell disruption, apparently could cause cell disruption in some other samples. The latter suggests an accurate count may not be unattainable for a minority of samples, i.e., ones that may have an aggregation of relatively fragile cells.

For some samples, post-treatment counts were relatively unchanged from the original; cell clustering likely was never serious in these. For many samples, especially bloom samples, disaggregation treatments resulted in considerable increase in cell counts. Table 3 shows enumeration changes for representative samples from the Barnegat Bay-Little Egg Harbor system, following application of the *A. anophagefferens* disaggregation protocol. In bloom initiation samples cell numbers were generally low, and effects of treatments were relatively unimportant. Cell aggregation was not detected visually in these. Severe cell aggregation was encountered first in some bloom development samples, and continued through bloom duration. Generally, sample treatment to restore single cell suspension was effective, presumably to a high degree. For most of the Table 3 examples, treatment resulted in at least 100% higher cell level, and for the sample showing greatest change (660) the increase was over 35 times. Unlike the relatively slight enumeration change (~50% or less) with treatment of those Long Island samples that initially had cell abundance estimates ~ $\leq 200 \times 10^3$ cells ml^{-1} (Table 1), some Barnegat Bay-Little Egg Harbor samples with initial counts at this cell level showed post-treatment dramatic count increases (e.g. Table 3, 645, 660, 683). Illustrating varied degree of cell binding, resulting benefit of sample reprocess soon after initial process, with dilution and additional mixing but no other treatment, for sample 606 was none, slight for sample 660, intermediate for sample 677, and almost as much benefit as full cell disaggregation treatment for sample 643.

SUMMARY AND CONCLUSIONS

The focus of this study was to achieve increased accuracy of microscope enumeration of *A. anophagefferens* populations in the western New York Bight. Especially during post-initiation bloom development in this area cell aggregation had rendered enumeration of many water samples highly inaccurate.

Whether or not *A. anophagefferens* aggregates in nature in this area has not been determined, but a certainty is that it can to a high degree in glutaraldehyde-preserved water

samples within a day of collection. The question of importance of this factor in other areas has not been resolved. Long Island winter samples reprocessed in this study had considerable cell aggregation after prolonged storage (12-19 weeks), but apparently not when initially processed 3-7 weeks after collection. Possible cell aggregation in Long Island samples in other seasons, e.g., the Spring growth season, was not assessed.

To correct enumeration of HL samples, restoration of single cell suspension was sought. A single totally effective cell disaggregant was not found, instead varied combinations of mechanical and chemical treatments provided best results sample-to-sample. The reported *A. anophagefferens* disaggregation protocol is a compromise between effectiveness and need to not disrupt cells, given apparent wide sample variability in terms of cell treatment tolerance and type of cell aggregation. Preserved *A. anophagefferens* cells can withstand a variety of disaggregation treatments if they are properly adjusted.

Treatment combinations can largely or completely restore single cell suspension, although refractory cell aggregates remained in a minority of samples even after the most stringent regime. Presumably, cell binding was strongest in these.

The sample reprocessing and present treatment protocol satisfy the goals of this study, but the treatment regime might be further refined/simplified. For example, could vortexing be eliminated when sonication is employed? Could a more suitable surfactant be found to replace the two used in this study? Enzyme treatment appears deserving of further exploration.

RECOMMENDATIONS

If a microscope scan of *A. anophagefferens* preparations reveals cell aggregation, reprocess with disaggregation measures should be considered. Because the protocol we report was developed for a particular sample set and may not be universally appropriate, treatment should be appropriate for the samples being processed. Part or all of our protocol could be employed, depending on such factors as cell concentration, cell binding strength, and likely sample time in storage.

Multiple treatments to disaggregate cells greatly extends the time and effort required to process a given number of samples, so this could be limited to when most necessary. Accurate population enumeration may not be necessary in routine monitoring, e.g., to simply determine presence or absence of the species in high abundance, whereas it is required when assessing bloom development, and levels for detrimental effects on the biota. Non-bloom samples, i.e., when cell numbers are $\leq 200 \times 10^3$ cells ml^{-1}, may not require treatment beyond vortexing.

Treating a percentage of non-bloom samples should be considered as a check on count accuracy. Careful general microscope exam of slide preparations can suggest when additional sample treatment is required, although this should not be considered an absolute gauge.

Extra caution is recommended regarding a possible secondary pulse in the fall, an overwintering population, and when a bloom may be initiating. Collection of multiple samples is recommended at least during bloom development and duration.

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LITERATURE CITED

ANDERSON, D. M., D. M. KULIS AND E. M. COSPER. 1989. Immunofluorescent detection of the brown tide organism *Aureococcus anophagefferens.* Pp. 213-228 *in* Cosper, E. M., V. M. Bricelj, and E. J. Carpenter (eds.). *Novel Phytoplankton Blooms*: *Causes and Impacts of Recurrent Brown Tides and Other Unusual Blooms*. Springer-Verlag, Berlin.

ANDERSON, D. M., B. A. KEAFER, D. M. KULIS, R. M. WATERS, AND R. NUZZI.. 1993. An immunofluorescent survey of the brown tide chrysophyte *Aureococcus anophagefferens* along the northeast coast of the United States. *J. Plank. Res*.15:563-580.

BOOTH, B., J. LEWIN, AND J. R. POSTEL. 1993. Temporal variation in the structure of autotrophic and heterotrophic communities in the subarctic Pacific. *Progr. in Oceanogr*. 32: 57-99.

BRICELJ, V. M., S. P. MACQUARRIE, AND R. A. SCHAFFNER. 2001. Differential effects of *Aureococcus anophagefferens* isolates ("brown tide") in unialgal and mixed suspensions on bivalve feeding. *Mar. Biol*. 139:605-615.

BUETOW, D. E. 1973. Nuclei and chromatin from *Euglena gracilis*. Pp. 15-24 *in*: Hellebust, J. A. and J. S. Craigie (eds.). *Handbook of Phycological Methods*, *Physiological and Biochemical Methods*. Cambridge University Press, Cambridge.

COSPER, E. M., W. DENNISON, A. MILLIGAN, E. J. CARPENTER, C. LEE, J. HOLZAPFEL, AND L. MILANESE. 1989. An examination of the environmental factors important to initiating and sustaining "brown tide" blooms. Pp. 317-340 *in* Cosper, E. M., V. M. Bricelj, and E. J. Carpenter (eds.). *Novel Phytoplankton Blooms*: *Causes and Impacts of Recurrent Brown Tides and Other Unusual Blooms*. Springer-Verlag, Berlin.

FOURNIER, R. O. 1978. Membrane filters for estimating cell numbers. P. 190 *in* Sournia, A. (ed.). *Phytoplankton Manual. UNESCO Monographs On Oceanographic Methodology, No. 6.* Paris

HOLMES, R. W., AND T. M. WIDRIG. 1956. The enumeration and collection of marine phytoplankton. *J. Conseil* 22: 21-32.

KUTKHUN, J. H. 1958. Notes on the precision of numerical and volumetric plankton estimates from small-sample concentrates. *Limnol. Oceanogr.* 3:69-83.

LeCHEVALLIER, M.W., NORTON, W.D., AND R. G. LEE. 1991. Occurrence of *Giardia* and *Cryptosporidium* spp. in surface water supplies. *Appl. and Environ. Microbiol.* 57:2610-2616.

LEPPARD, G. G. 1995. The characterization of algal and microbial mucilages and their aggregates in aquatic ecosystems. *Sci. Total Environ.* 165:103 131.

LUND, J. W. G., C. KIPLING, AND E. D. LE CREN. 1958. The inverted microscope method of estimating algal numbers and the statistical basis of estimations by counting. *Hydrobiologica* 11:143-170.

MADRIGAL, L., S. LYNCH, C. FIEGHERY, D. WEIR, D. KELLEHER, AND C. O'FARRELY. 1993. Flow cytometric analysis of surface major histocompatibility complex class II expression on human epithelial cells prepared from small intestinal biopsies. *J. Immunol. Methods* 158: 207-214.

McDANIEL, H. R., J. B. MIDDLEBROOK, AND R. O. BOWMAN. 1962. Isolation of pure cultures of algae from contaminated cultures. *Appl. Microbiol.* 10: 223.

MIDDLEBROOK, J. B. AND R. O. BOWMAN. 1964. Preparation of axenic cultures of algae by use of a French press. *Appl. Microbiol.* 12: 44-45.

MYKLESTAD, S. M. 1995. Release of extracellular products by phytoplankton with special emphasis on polysaccharides. *Sci. Tot. Environ.* 165: 155-164.

NAJDEK, M., D., DEBORRIS, D. MIOKOVIC, AND I. IVANCIC. 2002. Fatty acid and phytoplankton composition of different types of mucilaginous aggregates in the northern Adriatic. *J. Plank. Res.* 24: 429-441.

NEBE-VON-CARON, G., STEPHENS, P. J., HEWITT, C. J., POWELL, J. R., AND R. A. BADLEY. 2000. Analysis of bacterial function by multi-color fluorescence flow cytometry and single cell sorting. *J. Microbil. Methods.* 42: 97-114.

SCIENTIFIC COMMITTEE ON OCEANIC RESEARCH, Working Group 33. 1974. *A Review*

of Methods Used for Quantitative Phytoplankton Studies. UNESCO Technical Papers In Marine Science, No. 18. 27 p.

SIEBURTH, J. McN., P. W. JOHNSON, AND P. E. HARGRAVES. 1988. Ultrastructure and ecology of *Aureococcus anophagefferens* gen. et sp. nov. (Chrysophyceae): the dominant picoplankter during a bloom in Narragansett Bay, Rhode Island, Summer 1985. *J. Phycol.* 24: 416-425.

THRONDSEN, J. 1995. Estimating cell numbers. Pp. 63-80 *in* Hallegraeff, G. M., D. M. Anderson, and A. D. Cembella (eds.). *Manual on Harmful Marine Algae.* No. 33. UNESCO, IOC.

VELJI, M. J., AND L. J. ALBRIGHT. 1986. Microscopic enumeration of attached marine bacteria of seawater, marine sediment, fecal matter, and kelp blade samples following pyrophosphate and ultrasound treatment. *Can. J. Microbiol.* 32: 121-126.

TABLE 1. *A. anophagefferens* enumeration (cells ml $^{-1}$) of Long Island, NY, samples at the James J. Howard Marine Sciences Laboratory (HL) following various disaggregation treatments. Results after process with all disaggregation treatments combined are compared with prior enumeration by the Suffolk County, NY, Department of Health Services (SCDHS). SCDHS processed samples collected on 12-2-99 after 50 days storage and samples collected on12-29-99 after 23 days storage. HL reprocessed these after 84-136 days storage. Treatments: Vort : vortexing; Pyro: sodium pyrophosphate; Son: sonication. A minimal amount of aggregated cells was observed in two of the Vort/Pyro/Son-treated samples: three clumps of 2-3 cells in one and an embed of 6 cells in another.

Test No.	Station No.	Date Collected	Vort	Vort/ Pyro	Vort / Pyro/ Son	SCDHS enumeration	HL all treatments/ SCDHS, % +,-
1	090110	12-2-99	40,900	52,867	223,398	285,950	-22
2	090130	12-2-99	59,946	71,203	461,862	499,732	-8
3	090160	12-2-99	99,856	221,674	459,235	521,518	-22
4	090200	12-29-99	7,023	13,583	21,506	18,723	14
5	090280	12-29-99	6,099	199,599	204,625	166,804	22
6	090120	12-2-99	102,682	112,280	438,246	311,481	40
7	090280	12-2-99	57,597	199,599	336,440	228,760	47
8	090110	12-29-99	47,268	231,172	318,814	236,930	34
9	090190	12-2-99	48,187	333,663	321,648	150,494	113
10	090150	12-2-99	119,711	184,241	665,981	328,842	102

TABLE 2. Effect of 1.0% AQUET detergent combined with vortexing (30 sec.), pyrophosphate (0.0001M, 15 min.) and varied sonication time on enumeration of *A. anophagefferens* (cells ml^{-1}) in aliquots of the same sample. Microscope observations of cell aggregates in the preparations are noted.

		Treatment		Treatment	
		Vort/ Son/ Pyro	Presence of cell aggregates	Vort/ Son/ Pyro/ Aquet	Presence of cell aggregates
Test No.	Son time (sec.)				
1	30	64,636	multiple embeds	242,514	none
2	40	53,988	"	225,036	one embed/one clump
3	50	37,825	"	193,586	one embed
4	60	26,127	"	270,292	none
5	70	64,378	"	235,766	three embeds
6	80	96,068	"	215,910	single embed
7	90	206,798	single embed	248,848	single embed

Table 3. Enumeration changes for representative *A. anophagefferens* bloom initiation and development samples following full disaggregation protocol treatment. Microscope scan for cell aggregates showed: none (none); many clumps (clmp-m); few clumps (clmp-f). "R"signifies processing repeated, with sample dilution and additional mixing, soon after initial processing. If a sample was "R" reprocessed the highest count obtained was used for the ratio calculation.

Sample No.	Initial process date	Initial population estimate	Presence of cell aggregates	Reprocess date/post treatment	Revised population estimate	Presence of cell aggregates	Ratio of revised to initial population estimates
Bloom initiation:							
544	4-8-99	214	none	11-29-00	752	none	3.5
546	4-8-99	107	none	11-29-00	1,613	none	15
Bloom development:							
592	5-19-99	129,160	clmp-m	8-27-01	$2.3x10^6$	clmp-f	17.8
594	5-19-99	274,656	clmp-f	8-27-01	787,169	none	2.8
606	5-26-99	130,218	clmp-f				
606R	7-26-99	102,251	clmp-f	1-16-02	276,434	none	2.1
643	6-30-99	270,278	clmp-m				
643R	7-5-99	$1.6x10^6$	clmp-f	5-1-01	$1.9x10^6$	clmp-f	1.2
645	6-10-99	81,084	clmp-f	11-15-01	796,976	none	9.8
660	8-2-99	47,051	clmp-m				
660R	8-4-99	85,926	clmp-m	4-25-02	$2.8x10^6$	clmp-f	35.6
661	8-2-99	16,242	none				
661R	8-9-99	11,668	none	12-28-00	29,089	none	1.8
677	6-30-99	322,900	clmp-m				
677R	7-5-99	$2.6x10^6$	clmp-f	8-8-01	$4.2x10^6$	none	1.6
678	6-30-99	444,489	clmp-m	8-8-01	$3.8x10^6$	none	8.5
683	6-23-99	15,068	clmp-f	8-8-01	201,812	none	13.4

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Northeast Fisheries Science Center
National Marine Fisheries Service, NOAA
166 Water St.
Woods Hole, MA 02543-1026

MEDIA MAIL

Publications and Reports of the Northeast Fisheries Science Center

The mission of NOAA's National Marine Fisheries Service (NMFS) is "stewardship of living marine resources for the benefit of the nation through their science-based conservation and management and promotion of the health of their environment." As the research arm of the NMFS's Northeast Region, the Northeast Fisheries Science Center (NEFSC) supports the NMFS mission by "planning, developing, and managing multidisciplinary programs of basic and applied research to: 1) better understand the living marine resources (including marine mammals) of the Northwest Atlantic, and the environmental quality essential for their existence and continued productivity; and 2) describe and provide to management, industry, and the public, options for the utilization and conservation of living marine resources and maintenance of environmental quality which are consistent with national and regional goals and needs, and with international commitments." Results of NEFSC research are largely reported in primary scientific media (*e.g.*, anonymously-peer-reviewed scientific journals). However, to assist itself in providing data, information, and advice to its constituents, the NEFSC occasionally releases its results in its own media. Those media are in four categories:

NOAA Technical Memorandum NMFS-NE -- This series is issued irregularly. The series typically includes: data reports of long-term field or lab studies of important species or habitats; synthesis reports for important species or habitats; annual reports of overall assessment or monitoring programs; manuals describing program-wide surveying or experimental techniques; literature surveys of important species or habitat topics; proceedings and collected papers of scientific meetings; and indexed and/or annotated bibliographies. All issues receive internal scientific review and most issues receive technical and copy editing.

Northeast Fisheries Science Center Reference Document -- This series is issued irregularly. The series typically includes: data reports on field and lab studies; progress reports on experiments, monitoring, and assessments; background papers for, collected abstracts of, and/or summary reports of scientific meetings; and simple bibliographies. Issues receive internal scientific review, but no technical or copy editing.

Resource Survey Report (formerly *Fishermen's Report*) -- This information report is a quick-turnaround report on the distribution and relative abundance of selected living marine resources as derived from each of the NEFSC's periodic research vessel surveys of the Northeast's continental shelf. There is no scientific review, nor any technical or copy editing, of this report.

The Shark Tagger -- This newsletter is an annual summary of tagging and recapture data on large pelagic sharks as derived from the NMFS's Cooperative Shark Tagging Program; it also presents information on the biology (movement, growth, reproduction, etc.) of these sharks as subsequently derived from the tagging and recapture data. There is internal scientific review, but no technical or copy editing, of this newsletter.

OBTAINING A COPY: To obtain a copy of a *NOAA Technical Memorandum NMFS-NE* or a *Northeast Fisheries Science Center Reference Document*, or to subscribe to the *Resource Survey Report* or the *The Shark Tagger*, either contact the NEFSC Editorial Office (166 Water St., Woods Hole, MA 02543-1026; 508-495-2228) or consult the NEFSC webpage on "Reports and Publications" (*http://www.nefsc.noaa.gov/nefsc/publications/*).

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