Northeast Fisheries Science Center Reference Document 05-17

Modification of Chemically-Defined Medium ASP_{12} for Picoplankter *Aureococcus anophagefferens*, with Limited Comparison of Physiological Requirements of New York and New Jersey Isolates

by

John B. Mahoney

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John B. Mahoney

Postal Address: National Marine Fisheries Serv., Howard Lab., 74 Magruder Rd., Highlands, NJ 07732
E-Mail Address: *John.Mahoney@noaa.gov*

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ABSTRACT

Two enriched sea water media used for initial culturing of the picoplankter *Aureococcus anophagefferens* in this laboratory had varied and moderate at best suitability. Utility of both apparently could be affected greatly by difference in the quality of the sea water they were prepared with. One was especially so affected because, found subsequently, it lacked a supplement essential for the picoplankter. Occasional precipitation made it additionally problematic. To enhance vigor and long-term survival of *A. anophagefferens* in batch culture and to support experimental studies, a proven chemically-defined medium, ASP_{12}, was modified for the species. The addition of selenium to original recipe ASP_{12} permitted good growth of *A. anophagefferens*. All ASP_{12} constituents except vitamins were then tested to determine levels most beneficial for *A. anophagefferens*. The vitamins required by the species, but not minimal concentrations necessary for optimal growth, were determined. Additional nutrients found beneficial for the species by other researchers, and some constituents of other media found to have nutritional benefit for various phytoplankton species, were evaluated and adopted if beneficial. Medium chelation, medium pH, and effects of some plant hormones on growth of the species were assessed. This process ultimately provided a very suitable defined medium for *A. anophagefferens*.

Most testing of medium constituents and physical conditions affecting growth was done with an isolate from Great South Bay, New York. Equivalent testing of some of the constituents and physical factors with an *A. anophagefferens* strain isolated from Barnegat Bay, New Jersey, permitted a limited comparison. The two strains appear physiologically similar in many respects but have differences in tolerances to some of the constituents. Salinity and temperature tolerances determined for the New York strain in this study are in general agreement with those previously reported for the strain by other researchers; comparison of tolerances of New York and New Jersey strains to these factors indicates they are basically similar.

KEY WORDS: *Aureococcus anophagefferens culture, harmful algal blooms, New York Bight*.

INTRODUCTION

Beginning in 1985 and continuing for over a decade blooms of a toxic picoplankter, *Aureococcus anophagefferens*, so-called "brown tides," recurred in New York Bight waters, specifically in eastern Long Island, New York, coastal embayments (Nuzzi and Waters, 1989, 2004), and in the New Jersey Barnegat Bay-Little Egg Harbor system and some contiguous or adjacent bays (Olsen and Mahoney, 2001; Mahoney et al., 2003b). The blooms caused actual or probable serious perturbation to various components of the biota in both epicenters. Deleterious effects of Long Island blooms were well substantiated; their detriment to, e.g., bay scallop (*Argopecten irradians*) was especially severe (e.g., Bricelj and Lonsdale, 1997). This paper describes modification of a classic chemically-defined phytoplankton culture medium for *A. anophagefferens* to support experimental studies, maintain high culture vigor, and enhance its long-term batch culture survival.

Enriched sea water (NW) phytoplankton media, especially when prepared with water from the collection site, can be most suitable for isolation and at least initial culturing (Guillard, 1995). The first isolation of *Aureococcus anophagefferens* (Cosper, 1987), a relatively difficult species to culture, employed 'f/2' NW medium (Guillard and Ryther, 1962). Initial culturing of Cosper's *A. anophagefferens* BT-1 isolate in this laboratory likewise employed 'f/2' medium, prepared with sea water from Middle Atlantic Bight locales, including various eastern Long Island bays where the species bloomed, and various shelf locales off the northeast U.S. Utility of 'f/2' in this laboratory for BT-1 varied batch-to-batch and was moderate at best. This appeared linked to where the sea water was collected, with the most successful batches prepared with Middle Atlantic Bight offshore water; e.g., from the shelf break in the vicinity of the Hudson Canyon, or from Georges Bank in the Gulf of Maine. Sea water from the latter area was the most consistently favorable; it is well known that a sea water batch may be favorable or unfavorable for a phytoplankton species depending on presence or absence of essential nutrients or inhibitory substances. Occasional precipitation made 'f/2' medium additionally problematic. The two problems experienced with this medium were recognized decades ago to be common defects of NW media (Provasoli et al., 1957). *A. anophagefferens* was cultured next in this laboratory in 'K' NW medium (Keller et al., 1987). 'K' medium, developed primarily for culture of oceanic ultraplankton, is a modification of medium 'f' (Guillard, 1975). The recipe changes are: a tenfold increase in chelator concentration; an organic instead of inorganic phosphorus source; lowering of copper concentration; and additions of selenium, ammonium chloride, and a pH buffer. The authors reported this medium to not be prone to precipitation, which they believed was probably due to high chelator concentration. Growth in 'K' medium generally was better than in 'f/2' medium but again medium suitability varied batch-to-batch, from good to unacceptably low. Attempts at this laboratory to make the culture axenic at this time failed, apparently due to high antibiotic intolerance. Marginal health of the culture contributing to the intolerance was suspected.

Provasoli et al. (1957) advocated artificial sea water (AW) media for study of phytoplankton nutritional requirements, and avoidance of natural sea water media limitations due to the complex and variable composition of sea water. Provasoli and colleagues (e.g. Provasoli et al., 1957; Provasoli and McLaughlin, 1963) developed reproducible, non-precipitating AW media

designed to be utilized by a wide variety of marine algae. They expected none would be best for particular phytoplankton species. *A. anophagefferens* growth in various of these media in this laboratory was nil or varied, so clearly none tried was suitable, much less best, in its original formulation. One of these, ASP_{12} (Provasoli, 1964), was selected for modification for *A. anophagefferens* because of its known excellence for a variety of marine algae. For example, besides phytoplankton, Provasoli employed ASP_{12} for multicellular marine algae. ASP_{12} NTA (ASP_{12} with nitrilotriacetic acid as additional chelator) was one of the three most suitable AW media of 20 tested for red seaweed species (Provasoli, 1964). Iwasaki (1967) employed slightly modified ASP_{12} NTA for culture of the *Conchocelis* phase of the seaweed *Porphyra tenera*.

Optimization/enhancement of ASP_{12} for batch culture of *A. anophagefferens* was the primary goal of the study. Comparison of physiology of Long Island and New Jersey *A. anophagefferens* strains became a secondary focus. Testing of various nutrients with New York and New Jersey strains supported limited comparison of their relative efficacy and strain tolerance. Comparison of tolerances of the New York and New Jersey strains to salinity and temperature was of particular interest because of similar or different bloom regulatory importance of these factors in the respective bloom epicenters. The comparison is not conclusive but serves at least to direct attention to the question of applicability of findings from research on Long Island *A. anophagefferens* blooms to New Jersey outbreaks.

METHODS

Initial *A. anophagefferens* study at this laboratory, especially trials of various media, employed the BT-1 culture, isolated in 1986 by E. Cosper from Great South Bay, New York, as received from Cosper. *A. anophagefferens* cultures obtained from the Provasoli-Guillard Center for Culture of Marine Phytoplankton (CCMP) were employed subsequently. Most ASP_{12} optimization/enhancement testing was done with strain CCMP 1784 (= BT-1). CCMP 1794, isolated by R. Andersen from Barnegat Bay, New Jersey, in 1997, and CCMP 1984, an axenic isolate derived from CCMP 1784 in 2000 by M. Berg, were secondary test cultures. CCMP 1784 and CCMP 1794 are non-axenic. Strain designation is used guardedly in this report. Stabile et al. (2000) found CCMP 1784 and CCMP 1794 isolates to be genetically different. However, Stabile (personal communication) considered it not possible to extrapolate the level of genetic difference to physiological difference. How representative test cultures are of dominant strains in blooms in New York Bight coastal waters for over a decade is uncertain. CCMP 1784 and CCMP 1794 are genetically different from most isolates and bloom samples studied by Stabile et al. (2000) with some exceptions: CCMP 1784 was not statistically genetically different from a West Neck Bay, New York, bloom sample, and CCMP 1794 was not statistically genetically different rom a Great South Bay, New York, bloom sample.

Batch culture methodology used in this study primarily followed that developed by Provasoli, McLaughlin, and Hutner at Haskins Laboratories, New York (e.g., Hutner et al., 1950; Hutner and Provasoli, 1951; Provasoli et al., 1957; McLaughlin, 1958); Droop, Marine Station, Millport, Scotland (e.g., Droop, 1958a; Provasoli et al., 1957); and Guillard, Bigelow Laboratory for Ocean Sciences, West Boothbay Harbor, Maine (e.g., Guillard, 1975; Guillard and Keller, 1984;

Guillard and Morton, 2003). Glassware was cleaned by a soak for 24 hours or longer in 1% Micro detergent; rinsing followed by machine wash (final rinse, deionized water); a soak with 10% HCl for at least 12 hours; and a second machine wash. It was then silanized (Schenck, 1984) with Prosil-28 or Glassclad-18, rinsed, and again machine washed. Glassware silanization was repeated as needed, usually after two or three uses; need for this was judged by the amount of beading of water on glassware walls. Culture vessel caps were washed similarly but were not silanized. Plastic pipette tips were cleaned by separate ~24-hour soaks in Micro detergent and 10% HCl, each followed by deionized water rinse.

For reagent solutions and media preparation, laboratory centrally-supplied ~17 megohm resistivity water which had passed through ion exchange and activated carbon columns was further purified by passage through a Barnstead B-Pure system with activated carbon and ion exchange columns. It was then filtered through a 0.2 μm polycarbonate filter, microwave-sterilized in 2-liter Teflon® bottles (Keller et al., 1988), and refrigerated until use. The filtration and microwaving were done primarily to avoid microbial contamination of the preparation water and are not expected to be necessary for medium prepared for routine culturing. The recipe for ASP_{12} was that of Provasoli (1964). Medium constituent chemicals were reagent-grade unless ultra-pure. Ultra-pure chemicals, including sodium chloride, magnesium chloride, vanadium, silver, and nickel, were used in some tests. Reagent solutions rarely were retained to depletion. They were used up to 3-4 months for culture maintenance medium; solutions were remade for test media unless batch preparations were close. Other than phosphorus and silicon stock solutions which were in borosilicate bottles, reagent stock solutions were in Teflon® or polycarbonate bottles; all were refrigerator-stored. Provasoli reported ASP_{12} constituents as percent concentrations. Percent concentrations were retained for some of the tests, but molar amounts were used for most. All constituent levels are reported as molar amounts. Culture vessels for *A. anophagefferens* maintenance and medium enhancement tests were mostly 25 x 150 mm borosilicate screw cap test tubes with polypropylene caps. Media were in 20 ml amounts in the test tubes. Four replicates were used in most tests.

Medium sterilization regime varied. Medium for culture maintenance and some tests (e.g., major salts) was autoclave-sterilized. Droop, in Provasoli et al. (1957) recommended "light autoclaving" (i.e., 15 PSI for 1 minute to reduce the possibility of medium precipitation and avoid having to lower pH to prevent precipitation. He found this satisfactory for media in test tubes. The mild autoclaving regime for the present study was ~3 PSI, 105°C for 10 minutes in an AMSCO (STERIS) Model 3031 autoclave "pure steam" stainless steel system, with the steam generator supplied ~17 megohm resistivity deionized water. For many tests (e.g., for metals and vitamins) the basic growth medium was autoclave-sterilized, and test solutions for post-autoclaving aseptic addition were microwave-sterilized (Keller et al., 1988). Nutrient, etc., solutions to be microwave-sterilized were prepared in 400 ml amounts in 500 ml Teflon® bottles; pH of test solutions was adjusted to ~8 prior to microwaving. During microwaving, solutions were cooled to $\leq 25^{o}C$ between the four timed sessions. For certain tests (e.g., metals screening), basic medium and test solutions were microwave-sterilized separately. Sterilization regimes for particular tests are identified in the text.

Following autoclave-sterilization, media were stored at room temperature for ~12 hours and then refrigerator-stored. After microwave-sterilization of media or test solutions for aseptic additions, they were also refrigerator-stored. Aseptic dispensing of media and test solutions and culture inoculations were done by Eppendorf or Oxford pipettor in a Nuaire biological safety cabinet, using cotton-plugged pipette tips that had been pressure cooker-sterilized in vials. Inoculations were made at least 24 hours after test media preparation, as recommended by Morel et al. (1979). To ensure vigor of the inoculum, stock cultures were transferred regularly, using as inoculum the culture having the greatest cell abundance in the current log phase transfer batch. Special preparation of inocula for certain tests is outlined in the respective sections. Experiment test tubes were randomly inoculated and distributed randomly while spaced uniformly, 20 tubes in 40 place racks for incubation. The term "serial transfer" in this report refers to transfer of culture grown in a specific test medium into the same medium.

Culture incubation temperature was routinely 18°C. Light bank illumination from below through a pane of glass (wire reinforced 1/4 inch, pebbled upper surface) provided ~50 μE^{-2} m^{-2} sec^{-1} (measured with a Li-Cor Model 185 quantum radiometer/photometer) from cool white fluorescent lamps. Lighting was computer-programmed for a 12/12 hour light/dark cycle, with dimming control to adjust light intensity and simulate 15-minute dawn and dusk periods. The light/dark cycle was routine for this laboratory's primary light banks and not optimized for *A. anophagefferens*.

Cell abundance or yield of *A. anophagefferens* maintenance and test batch cultures was assessed indirectly by measurement, primarily at 2-3 day intervals, of *in vivo* fluorescence (Brand et al., 1981), using a Turner Designs Model 10 fluorometer. Fluorescence measurements are adequate to estimate cell populations of *A. anophagefferens* cultures (Dzurica et al., 1989). Fluorometer readings were taken 4-5 hours after start of light period. Each test culture was moderately vortex-mixed just before being measured. A single mixing pulse of 3-4 seconds sufficed for CCMP 1784 and CCMP 1794. Additional mixing of CCMP 1984 (e.g., three pulses of 5-6 seconds) was often necessary to disperse cells aggregated in a floc at tube bottom; cell aggregation was most common in older cultures with high cell abundance. Floc formation was not seen in non-axenic cultures.

When growing vigorously, non-axenic CCMP 1784 and CCMP 1794 cultures characteristically reached maximum cell abundance after ~2 weeks of incubation, had a stationary phase of about a week's duration, and then declined; the decline generally was gradual. Axenic CCMP 1984 achieved comparable cell abundance during similar incubation, and generally survived weeks longer than the non-axenic cultures. Prior to disposal, all *A. anophagefferens* cultures were microwave-sterilized.

To assess the CCMP 1784 culture microbial contamination, standard agar plate streaking technique was used at this laboratory to isolate contaminants. Minitek Bacterial Differentiation and supplementary tests performed on contaminant isolates at the NEFSC Milford Laboratory (Kapareiko, internal report) indicated a complex of seven bacterial and one yeast species. The origin of this contaminant burden and whether it changed over time are unknown. CCMP did

not attempt to purify the culture after it was deposited by E. Cosper (Andersen, personal communication). Microbial contaminant diversity of CCMP 1794 was not assessed.

The alga/contaminant relationship in the non-axenic *A. anophagefferens* cultures is only partly known. Provasoli (1964) suggested that associated bacteria might benefit an alga in culture by supplying growth factors. Bacteria growth enhancement of alga growth, as well as bacteria toxicity to alga, have been reported (e.g., Berland et al., 1970; Berland et al., 1972). A certainty is that microbial contaminants in the CCMP 1784 and 1794 cultures eventually seriously stress *A. anophagefferens*, i.e., following long-term incubation (e.g., ~30 days). Apparently, the picoplankter can compete with the contaminants while it is growing vigorously, but eventually it is overwhelmed. Similar process was described by Soli (1963), who found that three diatom species in bacterized unialgal cultures grew abundantly after transfer into fresh medium, but when diatom growth ceased bacteria multiplied rapidly and overwhelmed the diatoms. Incubation of non-axenic *A. anophagefferens* cultures at 10°C prolonged survival. Comparison of culture growth at 10°C with that in parallel culture at 18°C suggested this most likely was due primarily to bacterial growth reduction rather than a slowing of *A. anophagefferens* growth. Because of apparent microbial contaminant influence on culture survival at least, some of the results obtained with non-axenic *A. anophagefferens* test isolates may best be considered presumptive. Provasoli et al. (1957) warned that assays of bacterized cultures cannot provide precise interpretation, particularly when organic substances are in question, but also regarding mineral and trace constituents. Only some of the tests could be repeated when the axenic isolate CCMP 1984, of parent culture CCMP 1784, became available.

Interpretation of test results was made on qualitative comparison of growth response, prolongation of stationary phase, and culture long-term survival. Except for temperature tests, it is assumed that, in addition to or alternative to increased cell abundance, prolongation of culture survival was related to medium composition. Inhibition of microbial contaminants in some cultures by certain enrichments rather than stimulation of *A. anophagefferens* growth is an alternative possibility. Correlation of fluorescence measurements to culture cell abundances with population microscope enumerations could only be done occasionally. Therefore, extrapolation to cell numbers and determination of growth rates are not possible. Fluorescence measurements likely less accurately reflect cell populations in tests of varied physical conditions, e.g., light and temperature (Brand et al., 1981), and when cultures become nutrient-limited. This could be addressed only by conservative interpretation of test results. Methodology details for tests are mentioned in the particular sections for those who may wish to repeat tests to obtain quantitative data. Format for tables in this report follows that of Guillard and Keller (1984), with molar concentrations in written-out exponential form.

Results with axenic CCMP 1984 were generally more reliable than those obtained with the non-axenic isolates, e.g., through greater consistency among test replicates. Although apparently not as serious a complication as bacterial contamination, some results with the axenic isolate also may be problematic due to cell aggregation as the culture aged. Assessed by the unaided eye with the culture vessel held to a light source, CCMP 1984 could form cell floc even when the culture had daily vortex-mixing. Cell aggregation can cause microscope enumeration of *A. anophagefferens* to be highly inaccurate (Mahoney et al., 2003a), and important effect of cell

clumping on fluorometer measurements is a possibility. Mixing prior to fluorometer readings was always at least largely effective in dispersing the floc, but sometimes refractory small clumps visible to the unaided eye remained, and considerable cell aggregation could be present even when cell floc was not apparent to the unaided eye. For example, as seen by the unaided eye, vortex mixing appeared to fully disperse cells of a two-month-old CCMP 1984 culture that had aggregated in a floc at the bottom of the culture tube. Microscope examination of the culture at 400X magnification detected occasional clumps of ~30 to 300 cells, although most cells were solitary. Effect, if any, of greater vortexing of CCMP 1984 on culture cell abundance was undetermined; it did not reduce culture long-term survival. Suggesting cell fragility, *A. anophagefferens* has an exocellular polysaccharide-like layer but lacks a cell wall (Sieburth et al., 1988). Also suggesting fragility, Anderson et al. (1989), when developing an *A. anophagefferens* enumeration protocol, found considerable disruption of cells unless they were suitably fixed. Apparent normal increase of cell abundance of CCMP 1984, as determined by fluorometer assessments through culture incubation, suggests relatively vigorous vortex-mixing at least did not cause major disruption of cells. Perhaps the exocellular polysaccharide-like layer supports structural integrity of live cells. It is assumed that fluorometer assessments provided sufficient accuracy despite the complications. Electronic counting was not attempted. Precluding this with the non-axenic cultures was closeness in size of the picoplankter and culture microbial contaminants. Cell aggregation precluded accurate electronic counting of the axenic culture.

Optimization of ASP_{12} medium for *A. anophagefferens* entailed empirical assessment of requirement and optimum concentration of 27 original medium constituents including macro- and micro-nutrients, vitamins, chelator, and pH buffer (except for buffer tests, the medium never was prepared with more than half original recipe buffer concentration). Provasoli et al. (1957) referred to this process as 'tailoring' a medium for a species. Some alternative or additional nutrients and growth factors, and non-nutritional medium constituents including metals, vitamins, plant hormones, pH buffers, and chelators, also were evaluated. Medium modification was generally cumulative, with indicated change from one test most often incorporated into the basal medium for the next. Modifications were adopted conservatively; e.g., the lowest concentration of a constituent that would support a given growth improvement was selected even when the species would tolerate higher concentrations. Growth inhibition obtained with a constituent concentration, even if just slight or temporary, caused a non-inhibitory concentration to be selected. Notwithstanding incremental changes, intermediate versions of the medium are designated ASP_{12}. Except for chelator tests, simultaneous study of multiple medium constituents, such as was done with major inorganic ions by Droop (1958a), was not done. The final recipe is designated $ASP_{12}A$ (Tables 1 and 2). Abbreviations NW and AW for natural seawater media and artificial seawater media, respectively, were adopted from Harrison et al. (1980).

RESULTS AND DISCUSSION

ASP_{12} BASIC DESIGN CHARACTER

One of the concerns of Provasoli and coworkers in development of AW media (e.g., Provasoli et al., 1954; Provasoli et al., 1957) was avoidance of precipitation of the medium and associated sequestering of various nutrients. For this practical consideration they often lowered the general salinity and reduced concentrations of calcium, magnesium, and phosphorus. ASP_{12}, developed primarily for oceanic dinoflagellates, is an exception to this. Many of the concentrations of ASP_{12} major constituents are close to reported averages in sea water (Goldberg, 1963; Bruland, 1983). These average concentrations accompany test results; the values listed by Goldberg (1963), converted to molar, are those available around the time ASP_{12} was developed. Because ASP_{12} medium was intended for batch culture, some concentrations naturally are greatly in excess of those in sea water.

SELENIUM: PRIMARY ASP_{12} MODIFICATION FOR *A. anophagefferens*

Early trials of various NW and AW media for *A. anophagefferens* in this laboratory showed that if transferred from 'K' medium to ASP_{12}, it would grow reasonably well when the inoculum was in culture maintenance amounts (0.5 ml or 0.75 ml of culture for 20 ml of medium), but not if the inoculum was reduced to test volume (0.2 ml). Culture transfer from 'f/2' medium or ASP_{12} to ASP_{12} had little success even with the larger inoculum. Nutrient carry-over from 'K' medium to ASP_{12} seemed a likely explanation. Comparison of consitituents revealed provision of selenium in 'K' medium, but not in 'f/2' medium. This was suspected to be the major nutritional difference between the media. Growth of some phytoplankton is stimulated by selenium (e.g., Pintner and Provasoli, 1968), and it is essential for others (e.g., Price et al., 1987). Dzurica et al. (1989) concluded that selenium is not a major requirement for *A. anophagefferens*, although the element enhanced its growth. Addition of selenium in 'K' medium level, 10^{-8} M, to ASP_{12} (unmodified except for pH buffer reduction) resulted in its basic suitability for *A. anophagefferens*. Suitability of a proven medium is to be expected unless the species has a nutritional requirement not satisfied by the medium. Selenium obviously is an indispensable enrichment for *A. anophagefferens* growth in ASP_{12}. Subsequently, Cosper et al. (1993) reported that selenium is critical for *A. anophagefferens* growth.

Microwave-sterilized selenium, aseptically added to autoclave-sterilized ASP_{12}, was tested in concentrations of 1 to 5 x 10^{-8} M, with 10^{-8} M increments. The full range was comparably suitable for CCMP 1784 and its axenic isolate CCMP 1984. CCMP 1794 apparently has a slightly greater selenium requirement than the New York strain, and is slightly less tolerant to the highest concentration. When general cell abundance of CCMP 1794 was maximum in the test, yield supported by the lowest and highest selenium levels was approximately 20% lower than with intermediate levels. Selenium addition of 2 x 10^{-8} M is adopted for ASP_{12}A. It is expected that adding selenium to 'f/2' medium would very likely improve its utility for *A. anophagefferens.*

ASP_{12} MAJOR CONSTITUENTS, ORIGINAL, NEWLY ADOPTED OR CONSIDERED

Sodium chloride

Sum of major salts shows ASP_{12} has an open-ocean salinity of 34.4 PSU. Results of salinity tolerance tests in ASP_{12} (discussed below) indicated its salinity could be reduced 2 to 6 PSU. Avoidance of possible medium precipitation through lowering NaCl concentration was the primary concern. The long documented contamination of reagent grade chemicals with impurities, e.g., certain metals (Pintner and Provasoli, 1963; Morel et al., 1979), was additional incentive. NaCl reduction from 4.79×10^{-1} M to 4.28×10^{-1} M had little if any effect on *A. anophagefferens* growth, and the lower concentration is adopted for $ASP_{12}A$. In one instance, however, when *A. anophagefferens* CCMP 1784 was incubated in a batch of ASP_{12} having either the original or reduced NaCl level, the original concentration apparently was slightly more beneficial for long-term culture survival. Substitution of AR grade NaCl and $MgCl_2 \cdot 6H_2O$ with Baker Ultra Pure chemicals resulted in no discernible change in growth, suggesting that any element stimulatory or essential to *A. anophagefferens* not supplied as a medium constituent is introduced in sufficient amounts in other salts, etc. The original NaCl concentration could be retained if medium precipitation is not a problem.

Magnesium

In sea water, magnesium exhibits a nearly constant ratio to salinity, and is present in an average concentration of 53.2 mM (Bruland, 1983); Goldberg (1963) reported the average concentration to be 55.53 mM. The ASP_{12} original recipe provides 48 mM magnesium, added as $MgSO_4 \cdot 7H_2O$ and $MgCl_2 \cdot 6H_2O$. Magnesium optimum concentration for *A. anophagefferens* CCMP 1784 was tested, using $MgCl_2 \cdot 6H_2O$, in the range 25 to 70 mM, with 5 mM concentration increments. $Na_2SO_4 \cdot 10H_2O$ was substituted as sulfur source. McLachlan (1964) used a similar procedure for testing effects of magnesium on growth of a variety of phytoplankton species. McLachlan (1973), because of extreme deliquescence of $MgCl_2 \cdot 6H_2O$, recommended titration of a solution of the compound to determine concentration. Instead, this compound and $Na_2SO_4 \cdot 10H_2O$, which is also deliquescent, were dried at 70°C for ~1 hour before weighing for medium preparation. Medium and test magnesium additions were autoclave-sterilized together. Despite the test inoculum being acclimated to 48 mM, magnesium concentrations higher than 45 mM were slightly inhibitory through week 1 of incubation; the inhibition was absent by day 11 and beyond. When general cell abundance was at or close to maximum at day 11 of incubation, yield was highest with 40 mM magnesium; yield with 30, 35, and 45 mM was slightly lower and comparable, and slightly further decreased with 25 mM. Cell abundances, moderately below maximum, were comparable with all Mg test concentrations and had little or no decline during week 3 of incubation. *A. anophagefferens* can flourish with a wide range of magnesium concentrations, but based on slightly greater day-11 cell abundance with 40 mM, and the early incubation partial inhibition with concentrations 45 mM and higher, 40 mM is adopted for $ASP_{12}A$. In a preliminary test in which the same concentrations of magnesium as above were obtained by varying concentrations of both $MgSO_4 \cdot 7H_2O$ and $MgCl_2 \cdot 6H_2O$ while maintaining their ratio, and with sulfur concentration allowed to vary, the more favorable magnesium

concentration range was 35 to 50 mM, with 40 mM slightly superior; week one growth inhibition was absent below 60 mM.

Potassium

ASP_{12} potassium recipe concentration is 9.38 mM, which is close to its reported average concentration in ocean water, 10.2 mM (Bruland, 1983) and 9.72 mM (Goldberg, 1963). Potassium concentration best for *A. anophagefferens* CCMP 1784 was tested in the range 5 to 15 mM, with concentration increments of 1 mM. Medium and test potassium additions were autoclave-sterilized together. Potassium antagonist TRIS buffer (Provasoli et al., 1957), as usual, had half original recipe concentration in the tests. No major yield differences throughout incubation showed that a wide range of potassium concentrations are suitable. Slightly greater yield at 11 to 13 days of incubation when the test general maximum cell abundances were attained, greater uniformity of cell abundance among test culture replicates, plus slightly decreased culture decline through day 17 when the test was terminated, however, suggested 11 mM to be most beneficial. This level is adopted for $ASP_{12}A$. In a separate test for minimum potassium required, levels in the 2.55 to 7.67 mM range resulted in reduced long-term culture survival.

In culture, *A. anophagefferens* cells are in suspension during growth phase, but eventually most sink to the bottom of the culture vessel. Hayward (1970) suggested that loss of monovalent ions from the cells, particularly potassium, may be a contributory factor to similar sedimentation of the diatom *Phaeodactylum tricornutum*. Replicate CCMP 1784, CCMP 1794, and CCMP 1984 cultures were grown for 2 weeks, then half of them were supplemented with 7.3 mM potassium, and all were further incubated. Based on fluorometer measurements taken pre- and post-vortex mixing, periodic through incubation, no difference in cell sedimentation was apparent between potassium-supplemented and unsupplemented cultures. Potassium concentration in the medium apparently is not a determinant of *A. anophagefferens* sedimentation.

Sulfur

Bruland (1983) reported the average concentration of sulfur in sea water to be 28.2 mM; the Goldberg (1963) average is just slightly lower, 27.6 mM. Added as $MgSO_4 \cdot 7H_2O$, the ASP_{12} recipe sulfur concentration is 28.397 mM. Droop (1958a) advised that SO_4 concentration can be reduced if the nutritional need of the organism is satisfied. Sulfur requirement of *A. anophagefferens* CCMP 1784 was tested in the range 0 to 40 mM, with 5 mM concentration increments. The source compound was $Na_2SO_4 \cdot H_2O$, dried at 70°C for ~1 hour before weighing for medium preparation. The medium and $Na_2SO_4 \cdot H_2O$ additions were autoclaved together. The test inoculum was grown in full-strength ASP_{12}. With no sulfur supplementation growth was severely retarded throughout test incubation. Maximum cell abundances maintained in days 11 through 15 of incubation were comparable with sulfur in the range 5 to 20 mM; 10 to 20 mM concentrations provided slightly superior long term (through 28 days) culture survival. Levels of 20, 25, and 30 mM resulted in slight inhibition during week 1; levels of 35 and 40 mM caused slight growth inhibition throughout incubation and shortened long-term culture survival. Based on these results, sulfur concentration is lowered to10 mM for $ASP_{12}A$. Sulfur concentration

might more conveniently be adjusted (only one chemical would have to be dried) by adding $MgSO_4 \cdot 7H_2O$ at 10 mM and lowering $MgCl_2 \cdot 6H_2O$ to 30 mM, but this was not tried.

Calcium

The reported average calcium concentration in ocean water is 10.3 mM (Bruland, 1983) and 9.98 mM (Goldberg, 1963). The ASP_{12} recipe concentration matches the latter value. Optimum calcium concentration for *A. anophagefferens* CCMP 1784 was tested in ASP_{12}, prepared with original recipe chelator and metals concentrations, in a 2.49 to 14.96 mM range with increments of 2.49 mM. Medium and test calcium additions were autoclave-sterilized together. Levels of 2.49 and 4.98 mM were inadequate, permitting only slight initial growth which was followed shortly by culture collapse; 7.47 mM supported moderate culture growth and longer culture survival. The original recipe level, 9.98 mM, basically was suitable. However, slightly higher cell abundance through most of the incubation term (day 4 to day 22) and slightly improved long-term culture survival was obtained with 12.47 to 14.96 mM. Calcium concentration is increased to 12.47 mM for $ASP_{12}A$. The concentration could be lowered to original level without major detriment to *A. anophagefferens* growth if, e. g., medium precipitation is encountered or less chelation is necessary.

Fluoride

Fluoride, a major ion in sea water, with reported average sea water concentration of 68 μM (Bruland, 1983) or 68.5 μM (Goldberg, 1963), is not a constituent of ASP_{12} or a normal constituent of other Provasoli media. It is a component of various media developed by other researchers including: Aquil, 7.14×10^{-5} M, (Morel et al., 1979); EASW, 6.57×10^{-5} M, (Harrison et al., 1980); and 'AK', 10^{-5} M, (Keller et al., 1987). The latter authors reported that fluoride was beneficial for culture of oceanic ultraplankton species. Aquil AW medium, with varied iron and chelator and with the addition of selenium, has been used to culture *A. anophagefferens* (Cosper et al., 1993), indicating the species at least could tolerate fluoride at Aquil recipe level. Effect on *A. anophagefferens* growth was tested with fluoride at 1.052, 2.63, and 5.26×10^{-5} M, and 1.05×10^{-4} M. Microwave-sterilized fluoride solutions were added aseptically to autoclave-sterilized medium. Fluoride at all test levels was beneficial throughout incubation; it increased maximum yield and prolonged culture survival of *A. anophagefferens* isolates CCMP 1784, 1794, and 1984. Growth with fluoride at 5.26×10^{-5} M was slightly superior to that with the lower concentrations, and comparable to that with the highest concentration; 5.26×10^{-5} M is adopted for $ASP_{12}A$. A possible secondary benefit of fluoride supplementation is reduction of cell floc formation in older cultures of axenic CCMP 1984. Unaided-eye observation suggested cell floc to be less prevalent in fluoride-supplemented cultures, and when present it appeared more amenable to disaggregation by vortex mixing.

Carbon

Inorganic carbon average concentration in sea water is 2.3 mM (Bruland, 1983). Other than the organic carbon portion of $Na_2 \cdot glycero \cdot PO_4$, carbon is not a recipe nutrient constituent of ASP_{12} and reliance for carbon in the medium is on diffusion of carbon dioxide into it. McLachlan

(1964) reported that addition of 2 mM of sodium bicarbonate to various AW and NW media, including ASP (Provasoli et al., 1957), enhanced growth of most phytoplankton species tested; concentrations greater than 2 mM were not additionally beneficial and were inhibitory to one test species, the diatom *Skeletonema costatum*. Bicarbonate effect on growth of *A. anophagefferens* CCMP 1784 was tested in the range 1 to 6 mM, with 1 mM increments. Microwave-sterilized bicarbonate was aseptically added to autoclave-sterilized medium. Results indicated that under the culture conditions, bicarbonate supplementation was basically unnecessary for maximum cell abundance and long-term culture survival, but it did enhance growth moderately in ~week 1 of incubation. $NaHCO_3$ concentrations of 1 to 2 mM were approximately equally beneficial for *A. anophagefferens.* Similar to the findings of McLachlan (1964) for *S. costatum*, the higher concentrations were progressively inhibitory. $NaHCO_3$ in concentration of 1 or 2 mM is suggested as an optional supplement for culture of *A. anophagefferens* in $ASP_{12}A$.

ASP_{12} MINOR CONSTITUENTS, ORIGINAL OR CONSIDERED

Silicon

Silicon concentration in sea water ranges <1 to 180 µM, with an average concentration of 100 µM (Bruland, 1983); Goldberg's (1963) reported average concentration is close to this (106.5 µM). ASP_{12} recipe includes 527.8 µM silicon. Provasoli's inclusion of silicon in ASP_{12}, which is required by diatoms, silicoflagellates, and some chrysomonads (although the medium primarily was developed for oceanic dinoflagellates) reflects its intended general utility. *A. anophagefferens* CCMP 1784 requirement for silicon was tested first in silanized borosilicate test tubes, in medium without silicon addition, and with silicon additions in the range 25 to 200 µM, with 25 µM increments. Microwave-sterilized silicon solutions were added aseptically to autoclave-sterilized medium. Growth over a 20-day incubation term was similar throughout the test. Following this, serial transfers were made into the same test regime. *A. anophagefferens* growth during a 33-day incubation again was similar over the silicon supplementation range, but cell abundance of cultures without silicon supplementation was slightly lower, particularly in early incubation. Serial transfer from the second test was made into the test regime with polycarbonate centrifuge tubes substituted for borosilicate test tubes. Cell abundance of silicon-unsupplemented cultures was greatly reduced; silicon in as low as 25 µM concentration (the lowest tested) adequately supported *A. anophagefferens* growth. These results indicate that *A. anophagefferens* requires silicon. Other researchers (LaRoche et al., 1997) concluded that it does not require this element. Silicon in 25 µM concentration is included as an $ASP_{12}A$ constituent, despite the apparent likelihood that the borosilicate culture vessels routinely used would contribute sufficient silicon for cell abundance only slightly lower than that in silicon-supplemented medium.

Nitrogen

Nitrogen concentration in sea water ranges 1 to 45 µM, with an average concentration of 30 µM (Bruland, 1983); Goldberg (1963) reported a moderately higher average concentration, 38.5 µM. ASP_{12} recipe $NaNO_3$ level is 1.176 mM, approximately 39 times the Bruland average. Test $NaNO_3$ concentrations were 1.17, 2.35, and 5.88 x 10^{-4} M, and concentrations in 2.94 x 10^{-4} M

increments from 8.82 to 23.52 x 10^{-4} M. Medium and test nitrogen additions were autoclaved together. Test inoculum was prepared by growing *A. anophagefferens* CCMP 1784 in ASP_{12} having one tenth the recipe level of $NaNO_3$; 200 ml of this depletion medium in a 500 ml Erlenmeyer flask was inoculated with a 20 ml culture; after five days 10 ml of this culture was used to inoculate a second such flask which after incubation served as inoculum. Growth was absent without nitrogen supplementation, indicating the depletion was adequate and atmospheric ammonium diffusion into the medium was not sufficient for growth. 1.17 x 10^{-4} M supported moderate cell abundance through days 9 to 11 of incubation; the cultures then declined with apparent bleaching of cells. Cultures having 2.35 to 23.52 x 10^{-4} M supplements all reached their respective cell abundance maxima by day 11. Cell abundance supported then by 2.35 x 10^{-4} M was below general maximum but considerable; within two days these cultures evidenced pronounced decline with apparent bleaching of cells. $NaNO_3$ concentrations of 5.88 and 8.88 x 10^{-4} M supported cell abundances by day 11 nearly comparable or comparable, respectively, with those associated with higher nitrogen levels; these cultures then began a gradual decline. Cell abundance maxima supported by $NaNO_3$ in 1.17 to 2.35 mM enrichments supported comparable yield maxima which were sustained 2 to 5 days longer than with lower concentrations. The ASP_{12} medium $NaNO_3$ recipe level is suitable for *A. anophagefferens*, but 1.47 mM prolonged maximum yield ~3 days longer and slightly improved long term culture survival. Higher concentrations were not additionally beneficial. $NaNO_3$ concentration is increased to 1.47 mM for $ASP_{12}A$ medium.

NH_4Cl is not a constituent of ASP_{12} medium. It is included in 'K' medium (Keller et al., 1987), which supports growth of *A. anophagefferens*, in recommended maximum concentration of 5 x 10^{-5} M. It was tested as sole nitrogen supplementation for *A. anophagefferens* CCMP 1784 in levels of 2, 3, 4, 5, 6, 8, 10, 15, and 20 x 10^{-5} M. The inoculum was prepared as before. CCMP 1784 did not grow without nitrogen supplementation. Maximum yield, at days 12 to 14, was greater with increasing NH_4Cl concentrations to 10 x 10^{-5} M; the latter concentration also provided the best long-term culture survival. As to be expected, yield was much lower than that supported by $NaNO_3$ in higher concentrations. Partial inhibition in week one of incubation occurred with 8 and 10 x 10^{-5} M NH_4Cl. Concentrations of 15 and 20 x 10^{-5} M resulted in initial growth lag, followed by low-level growth, then severe culture decline in less than two weeks. NH_4Cl in the test concentration range, as supplement to $NaNO_3$ in 1.47 mM concentration, had no apparent effect on cell abundance with 2 to 6 x 10^{-5} M concentrations, but again partial early growth inhibition occurred with 8 and 10 x 10^{-5} M concentrations, and severe general inhibition with the higher concentrations. Results show utilization by *A. anophagefferens* of ammonium at low levels but do not support its addition to $ASP_{12}A$. Comparison of NH_4Cl utilization by CCMP 1784 and CCMP 1794 revealed greater NH_4Cl toxicity to the latter. Tested with 10^{-5} M, 3 x 10^{-5} M, and 5 x 10^{-5} M NH_4Cl, CCMP 1794 grew with the two lower levels but 5 x 10^{-5} M resulted in severe growth lag and delayed maximum cell abundance for approximately a week.

Phosphorus

Phosphorus concentration in sea water ranges 1 to 3.5 µM, with an average concentration of 2.3 µM (Bruland, 1983), and 2.26 µM. (Goldberg, 1963). ASP_{12} recipe provides a total of 79 µM of phosphorus from combined additions of K_3PO_4 and $Na_2 \cdot glycero \cdot PO_4$; the medium has a

nitrogen : phosphorus ratio of ~15 : 1. The two phosphorus compounds initially were tested singly in concentrations (μM) of 1, 3, 7, 10, 12, 15, and in 5 μM increments from 20 to 60 μM. Medium and test phosphorus additions were autoclaved together. CCMP 1784 test inoculum was prepared in ASP_{12} medium having one-tenth original recipe levels of K_3PO_4 and $Na_2 \cdot glycero \cdot PO_4$: 4.7 μM and 3.2 μM, respectively.

Cultures unsupplemented with phosphorus had very slight growth and collapsed in approximately one week. K_3PO_4 in 1 μM concentration supported low level growth and apparent culture survival for approximately two weeks. Three μM supported moderate cell abundance and culture viability through approximately three weeks, although with pronounced decline in the third week. The ample growth response with at least the next to lowest level indicates that autoclaving the complete medium did not make phosphorus unavailable, or at least not the majority of it. Five μM improved general growth and yield, and moderately extended culture viability. Close to maximum yield was obtained with 7 μM in two weeks and culture viability persisted through the full incubation term of 31 days. Concentrations >7 μM provided respective peak yields two days earlier than the lower ones. Levels of 10 and 12 μM, respectively, provided cell abundances slightly lower than maximum or at maximum, respectively. Levels ≥15 μM better maintained the culture long-term. No inhibition was noted to 60 μM, which is well below inorganic phosphorus concentrations (3.22 to 6.44 mM) which Provasoli et al. (1957) reported to be toxic. K_3PO_4 in 40 μM concentration, although levels as low as 15 μM basically were suitable, is selected for $ASP_{12}A$ because this level supported slightly better long-term culture survival.

Initially, the *A. anophagefferens* strains tested did not optimally utilize inorganic phosphorus when previously grown in medium having recipe level of the organic source. Grown in the latter then transferred to inorganic phosphorus-only medium, CCMP 1784 had a slight growth lag that persisted through week one of incubation. CCMP 1794 and especially CCMP 1984 had more severe growth lags, persisting approximately two weeks. No growth lag occurred when the isolates were transferred from medium having only inorganic phosphorus, in which they had acclimated, to the same medium.

At the 1 μM level, $Na_2 glycero \cdot PO_4$ supported slight growth of CCMP 1784 for approximately a week; culture collapse followed rapidly. Concentrations ≥35 μM caused partial inhibition during ~week one. Other than this, response was similar to that with the inorganic compound. For approximately the same amount of phosphorus, the inorganic and organic phosphorus sources also were used equally well by *A. anophagefferens* CCMP 1794 and CCMP 1984. Because the species does not require organic phosphorus this constituent could be eliminated from $ASP_{12}A$ medium. However, the organic phosphorus compound is highly soluble, so besides increasing medium versatility, inclusion of both inorganic and organic phosphorus sources serves to provide ample phosphorus concentration while helping avoid precipitation. No major difference in long-term culture survival was seen when bacterized CCMP 1784 and CCMP 1794 strains were grown with either inorganic or organic phosphorus, which suggests microbial contaminants did not benefit from the organic carbon, or they benefited in such a way that the picoplankter was not caused extra detriment. There is sufficient justification, therefore, for retention of both K_3PO_4 and $Na_2 \cdot glycero \cdot PO_4$. As with K_3PO_4, $Na_2 \cdot glycero \cdot PO_4$ in 15 μM concentration basically

could be adequate but based on slightly better long term culture survival with higher levels, and the reason noted below, 20 μM is selected for $ASP_{12}A$.

With the compounds in equal molar combination, cell abundance supported by ≥3 ≤60 μM phosphorus was similar to that with either compound alone for the same amount of phosphorus. Partial inhibition occurred with >60 μM during the first week of incubation, although CCMP 1784 tolerated concentrations to 120 μM. To lower phosphorus concentration from 79 μM to 60 μM, the concentration chosen for $ASP_{12}A$ medium, $Na_2 \cdot$ glycero $\cdot PO_4$ is reduced most because it was inhibitory in concentrations ≥35 μM, whereas no inhibition by K_3PO_4 was found with all levels tested. The results suggest that $ASP_{12}A$ phosphorus concentration might be ~halved if desired without major detriment to culture growth and survival.

Vitamins

Potential complications in determining phytoplankton vitamin requirements include inability to obtain vitamin-free test media so that the vitamin may appear only stimulatory rather than essential (Droop, 1958b), and the possibility that bacteria in algal cultures may supply vitamins (e.g., Berland et al., 1970). Provasoli et al. (1957) advised that tests of vitamins in bacterized phytoplankton cultures can be indicative but may not be completely reliable. Because of this, primary reliance for vitamin requirements was on tests with the axenic isolate.

ASP_{12} recipe includes vitamin B_{12}, thiamine, and biotin. Because the three *A. anophagefferens* isolates flourished in ASP_{12} over many transfers it is assumed that its vitamin requirement is satisfied by the recipe group under the conditions of culture. Also, enrichment with a mixture of the three recipe vitamins plus 13 others (Vitamins 8; Provasoli et al., 1957) did not increase axenic CCMP 1984 cell abundance. Water for medium preparation for vitamin testing being filtered through activated charcoal cartridges presumably removed vitamins, if any were present. Filtration, microwave sterilization, and refrigerator storage of medium preparation water in a closed Teflon® container until used, eliminated or minimized microbial contaminant presence or growth in it. Microwave-sterilized vitamin additions were added aseptically to autoclave-sterilized medium.

Axenic CCMP 1984, grown in ASP_{12} having full vitamin supplementation then inoculated into medium having only single vitamin additions, grew comparably with all vitamin regimes during two weeks of incubation. Apparently, stored reserve and/or carry-over with the inoculum (the latter introducing amounts ≤ than in a 1 : 100 dilution of recipe vitamin levels) sufficed. Comparably high cell abundance was prolonged post two weeks of incubation by vitamin B_{12} and thiamine. Two serial transfers of these CCMP 1984 cultures into medium with vitamin supplementation limited to vitamin B_{12} showed that the isolate would not grow without thiamine. Transfer of CCMP 1984 inoculum grown with combined vitamin B_{12} and thiamine supplementation to medium having either thiamine or thiamine plus vitamin B_{12} supplementation resulted in comparable cell abundance. Again, apparently stored reserve and/or carry-over of vitamin B_{12} with the inoculum sufficed. Transfer of thiamine-only supplemented culture from this test to medium supplemented with thiamine or thiamine plus vitamin B_{12} resulted in growth only with the combined vitamins. Therefore, CCMP 1984 requires vitamin B_{12} and thiamine.

Similar results were obtained for non-axenic CCMP 1784 and CCMP 1794. The similarity of vitamin test results for the three isolates suggests that the bacterial flora in the bacterized cultures is not supplying the required type or level of vitamin. These vitamins are those most commonly required (vitamin B_{12} more so than thiamine) by marine auxotrophic algae (Provasoli et al., 1957; Provasoli, 1963). Minimum and optimum vitamin B_{12} and thiamine concentrations required were not determined. Doubling of thiamine concentration appears beneficial but this was not confirmed. Utilization of vitamin B_{12} analogues and thiamine moieties (Provasoli, 1964) was not determined.

Biotin elimination did not affect cell abundance. The three isolates grew well through several incubations in medium having only vitamin B_{12} and thiamine supplementation. Biotin replacement after the latter incubations did not result in noticeable change in culture condition. Although included in Table 1 as an $ASP_{12}A$ medium constituent, biotin possibly could be eliminated for *A. anophagefferens* culture. It is retained because it is not detrimental and its utility was not tested with a range of culture conditions. The level of vitamin B_{12} used by Iwasaki (1967) in his modification of ASP_{12}, five times the original recipe level, is adopted for $ASP_{12}A$. This is done as a precaution. This level of vitamin B_{12} and higher are common in other Provasoli AW media (Provasoli et al., 1957). Concentrations of the other two vitamins are original recipe levels. As done in this laboratory, autoclaving of complete $ASP_{12}A$ for routine culturing did not destroy or apparently diminish efficacy of the medium vitamin complement.

Hormones

Bentley (1960) reported substances with biological activity similar to auxins of higher plants, and Mowat, *nee* Bentley (1964), reported gibberelin-like substances in marine phytoplankton. Plant hormones including kinetin, indolacetic acid, and gibberellic acid increased growth of the macroscopic alga, *Ulva lactuca* (Provasoli, 1958). These hormones also increased growth of the *Conchocelis* phase of seaweed *Porphyra tenera* (Iwasaki, 1965). Reviews of the question of whether plant hormones control development of macroalgae concluded either that they do (Bradley, 1991), or that available data are equivocal (Evans and Trewavas, 1991). Effect of 10^{-10}, 10^{-9}, 10^{-8}, and 10^{-7} M concentrations of kinetin, indolacetic acid, and gibberellic acid on growth of *A. anophagefferens* CCMP 1784 was tested. Microwave-sterilized hormone solutions were added aseptically to autoclave-sterilized medium. No *A. anophagefferens* growth inhibition or enhancement resulting from these hormone supplements was apparent.

ASP_{12} ORIGINAL METALS CONSTITUENTS

ASP_{12} includes two metal mixes: PII, consisting of a chelator, ethylenedinitrilotetraacetic acid (EDTA), and iron, boron, manganese, zinc, and cobalt; and SII, consisting of bromine, strontium, rubidium, lithium, molybdenum, and iodine (Table 2). Possibly because it was developed for oceanic dinoflagellates, ASP_{12} has 1.0% addition of PII metal mix, rather than 3.0% more commonly used in various other Provasoli AW media. PI metal mix at 3.0% was best for most species, but some species required additional (Provasoli et al., 1957). (The difference between PI and PII mixes is elimination of copper in the latter). This suggested that greater than recipe

amount might be beneficial for *A. anophagefferens*, although judging from long-term culturing of *A. anophagefferens* in ASP_{12}, PII and SII metals mixes in recipe level were basically adequate.

The possible benefit for *A. anophagefferens* from increased metals levels was approached through testing of individual PII and SII metals rather than the whole mix. It was assumed that this would avoid metals excess and a possible chelation problem. Potential complications in tests of metals requirements include metal contaminants in reagent grade chemicals used in media preparation, and nutrient carry-over with test inocula (e.g., Morel et al., 1979). Pintner and Provasoli (1963) advised that reliable results from tests of trace metal requirements can only be had by virtual elimination of them. To at least partly achieve this, potential growth limitation by individual PII and SII metals was tested by a depletion assay in which *A. anophagefferens* CCMP 1784 was grown in ASP_{12} lacking in-turn supplementation with one, followed by post-incubation serial transfers twice into the same medium, each transfer being 1 : 100 dilutions of the culture volume. Water for ASP_{12} medium preparation was purified by passage through ion exchange columns, but metals removal from the complete medium in this manner (such as used in Aquil medium preparation; Morel et al., 1979), was not done. The ASP_{12} chelator : trace metals ratio, normally 2.7 : 1, was not balanced in the assays. The most extreme balance change was from manganese omission which resulted in a ratio of ~10 : 1. Medium and test metals were microwave-sterilized separately and added aseptically to autoclave-sterilized culture tubes. During autoclaving, the tubes contained deionized water, which was emptied just before medium dispensing. Test inocula were grown in ASP_{12} prepared with normal enrichment levels. Reduction of concentrations of metals by the depletion procedure, but likely not elimination of them, is not considered a major complication because the goal was enhancement of culture vigor and long-term survival rather than determination of absolute nutritional requirements.

Assay results suggested all PII metals could be in greater concentrations. The most important potential growth limiter, evident in the initial screening, was cobalt. Slight growth reduction due to deficiency of iron and manganese was apparent in the first serial transfer. Boron and zinc limitation was not apparent until the second serial transfer. Strontium was the only SII metals mix constituent identified as relatively deficient, although not until the second serial transfer. ASP_{12} bromine recipe concentration of 1.25×10^{-4} M is considerably less than its sea water concentration, 8.4×10^{-4} M (Bruland, 1983) or 8.13×10^{-4} M (Goldberg, 1963), but the depletion assay indicated additional is not needed. Underestimation of trace metal limitation by the assays is a possibility because simultaneous limitation by more than one metal can be more severe than limitation by one metal alone (Murphy and Guillard, unpublished data; Sunda, unpublished data; both cited in Brand et al., 1983).

Tests of optimum concentrations of the individual PII and SII metals identified as potentially growth limiting were made next. The original recipe ASP_{12} chelator concentration was not adjusted when these metals were tested in concentrations higher than original; change of chelator : metals ratio to 1.7 : 1 was the most extreme, but this is only slightly below the lower limit of the range 2 to 3 : 1, which Provasoli et al. (1957) found most useful. Microwave-sterilized metals solutions were added aseptically to autoclave-sterilized medium; *A. anophagefferens* CCMP 1784 inoculations were made ~24 hours later. Some tests were repeated for isolates CCMP 1794 and CCMP 1984. Concentrations of sea water metals constituents in Goldberg

(1963), converted to molar, show values available at the time ASP_{12} was developed, although the medium metal mixes were developed some years earlier (Provasoli et al., 1957) and were partly based on even earlier studies by other researchers. Some of the Goldberg (1963) values are considerably different than those provided two decades later by Bruland (1983). Bruland stated that post-1975, more accurate values of sea water constituents were developed, including concentrations of many trace elements found to be lower by factors of 10 to 1000 than those previously accepted. He considered the primary reasons for this to be major advances in instrumental analysis and analytical chemistry, and elimination or control of contamination during sampling, storage, and analysis.

Average concentration of cobalt in sea water is 0.02 nM (Bruland, 1983). Goldberg (1963) reported a much higher level, 8.48 nM. Cobalt concentration in ASP_{12} is 1.7×10^{-7} M. Cobalt was tested in concentrations of 1.7, 1.95, 2.2, 2.7, and 3.2×10^{-7} M. Based on increased cell abundance and uniformity of growth of CCMP 1784 among eight test replicates, 2.2×10^{-7} M was the most beneficial level and is selected for $ASP_{12}A$. The two higher levels resulted in partial growth inhibition in early incubation. Other testing suggested that cobalt likely is the limiting metal constituent for the species in 'K' medium also.

Average concentration of iron in sea water is 1.0 nM (Bruland, 1983); Goldberg (1963) reported this as 1.79 nM. ASP_{12} recipe iron level is 1.79×10^{-6} M. Tested in multiples of recipe level, two- or threefold increases stimulated higher cell abundances and greater long-term survival of CCMP 1784 and axenic CCMP 1794. Doubling of iron concentration was beneficial for CCMP 1984; the three-fold level was slightly inhibitory although it prolonged culture survival. Iron in 3.58×10^{-6} M concentration is selected for $ASP_{12}A$ medium. The recipe amount in PII metal mix is unchanged, and the additional iron is supplied separately as FeEDTA, primarily to avoid precipitation in the metals mix.

Boron is a major constituent of sea water, having an average concentration of 0.416 mM (Bruland, 1983), comparable to 0.426 mM reported by Goldberg (1963). ASP_{12} recipe boron level is 1.85×10^{-4} M. Tested in concentrations of 1.85, 2.77, 3.7, and 4.62×10^{-4} M, the three higher boron levels increased cell abundance and long-term survival of CCMP 1784, CCMP 1794, and axenic CCMP 1984 to about the same degree. Because there was no apparent additional benefit of the two higher levels, boron concentration of 2.77×10^{-4} M is selected for $ASP_{12}A$.

Average manganese concentration in sea water is 0.5 nM (Bruland, 1983). Goldberg (1963) reported a much greater level, 36.4 nM. ASP_{12} recipe manganese level is 7.28 μM. Tested at recipe level, and with 9.1 and 12.75 μM, the higher levels promoted roughly comparable increase of cell abundance and lengthened culture survival of CCMP 1784, CCMP 1794, and CCMP 1984. Manganese concentration is increased to 9.1 μM for $ASP_{12}A$.

Average zinc concentration in sea water is 6 nM (Bruland, 1983); Goldberg (1963) reported a much greater average concentration, 1.53×10^{-7} M. Zinc concentration in ASP_{12} is 7.65×10^{-7} M. Tested in concentrations of 0.76, 0.92, 1.07, and 1.22 μM, CCMP 1784 long-term culture

survival was enhanced greatly with Zn at the two higher levels. Zn concentration of 1.07 μM is selected for $ASP_{12}A$.

Strontium is a major constituent of sea water, having an average concentration of 90 μM (Bruland, 1983), or 91 μM (Goldberg, 1963). Its ASP_{12} recipe level is 22.83 μM, far less than average sea water concentration. Tested in 22.8, 34.2, 45.6, 57, and 68.4 μM concentrations, the four higher strontium concentrations comparably enhanced long-term survival of CCMP 1784. Strontium concentration of 34.2 μM is selected for $ASP_{12}A$.

In routine culturing of *A. anophagefferens* in ASP_{12} medium (original recipe except for selenium addition and reduced buffer), cobalt apparently would be the only metal in PII metals mix likely to become limiting. PII metals mix modification might be restricted to this metal alone. Motivation to increase levels of the other constituents primarily was to defer potential limitation. However, their increased enrichment improved growth and/or survival of isolate CCMP 1784. The SII metals that the screening did not indicate to be required by *A. anophagefferens* in the medium as prepared are not deleted. Their removal did not improve growth in metals assays; the tests are limited in scope, and only medium modifications that enhanced *A. anophagefferens* growth are considered necessary.

ADDITIONAL METALS CONSTITUENTS

Arsenic and vanadium, found beneficial for *A. anophagefferens* (BT-1 = CCMP 1784) by Dzurica et al. (1989), and various metal constituents of other phytoplankton media (Keating, 1985; Keating, personal communication; Keller et al., 1987; Guillard, 1995), also were assessed for benefit to *A. anophagefferens* in ASP_{12}. Metals concentrations used by Dzurica et al. (1989) or concentrations in various published media were test starting levels. Microwave-sterilized metal test solutions were added aseptically to autoclaved medium.

Average sea water concentration of arsenic is 23 nM (Bruland, 1983); Goldberg (1963) reported this to be 40 nM. Tested in 14, 28, 42, and 56 nM concentrations, 42 nM was most beneficial for *A. anophagefferens* CCMP 1784, especially with regard to yield uniformity and long-term culture survival, and this concentration is adopted for $ASP_{12}A$. Dzurica et al. (1989) reported 10^{-8} M arsenic to be beneficial for this strain.

Average sea water concentration of vanadium is 30 nM (Bruland, 1983); Goldberg (1963) reported this to be 40 nM. Tested with *A. anophagefferens* CCMP 1784, vanadium at 10^{-8} M, the level used by Dzurica et al. (1989), did not appear beneficial. Tested subsequently with axenic CCMP 1984 in 1, 2, and 5 x 10^{-8} M concentrations, vanadium enhanced long-term culture survival. The highest concentration was slightly inhibitory; the intermediate concentration was more beneficial than the lower level and is adopted for $ASP_{12}A$.

Average sea water concentration of aluminum is 20 nM (Bruland, 1983); Goldberg (1963) reported this to be 3.7 μM. Aluminum effect on growth of *A. anophagefferens* CCMP 1784 was tested in concentrations of 3.7, 7.4, 11.1, and 14.8 x 10^{-7} M. Aluminum in 11.1 x 10^{-7} M concentration increased long-term culture survival and is adopted for $ASP_{12}A$. The lower

concentrations were not noticeably beneficial, and the highest concentration was slightly inhibitory.

When preparing $ASP_{12}A$, it was convenient to add solutions of the additional metal constituents individually in 1.0 ml amounts for a liter of medium. Combining them in a mix was not tried. Metals also tested that had no apparent benefit for growth or survival of *A. anophagefferens* CCMP 1784 in ASP_{12} as prepared, and under the culture conditions, include copper, silver, nickel, cadmium, chromium, lead, tin, tungsten, and barium. If *A. anophagefferens* requires or uses any of this group or other metals that were not tested, this need apparently was satisfied by contaminants in the reagents used in medium preparation or metabolic substitution; introduction of metals from the glassware or during autoclave sterilization are other possibilities but considered less likely.

MEDIUM pH AND NON-NUTRITIONAL CONSTITUENTS

pH

Provasoli (1964) recommended a pH range of 7.8 to 8.0 for ASP_{12}. *A. anophagefferens* CCMP 1784 was tested in pH-adjusted medium that 24 hours after autoclave sterilization had, respectively, pH values of 8.0, 8.4, 8.5, 8.6, and 8.8. Levels 8.4 through 8.6 were more favorable than the lowest; the highest pH was unfavorable. A possible explanation for slightly better *A. anophagefferens* growth with higher pH is greater CO_2 absorption into the medium (Provasoli et al., 1957) and somewhat greater EDTA chelation efficiency (Chaberek et al., 1955). Medium precipitation with high pH is a concern (Provasoli et al., 1957), so routinely $ASP_{12}A$ pH was set at 8.4 before autoclaving, which provided ~8.2 after autoclaving. Microwave sterilization resulted in less decrease. Overall, medium pH ranged ~8.2 to 8.3. The species flourishes in nature with pH in the range recommended by Provasoli for ASP_{12}. Water pH at various sites in the New Jersey Barnegat Bay-Little Egg Harbor estuarine system during a major *A. anophagefferens* bloom ranged 7.8 to 8.1 (unpublished data).

pH Buffer

Provasoli et al. (1957) found TRIS (Tris (hydroxymethyl) aminomethane) buffer was inhibitory in a concentration of 24.78 mM, but was not toxic in 8.26 mM concentration for any of the phytoplankton species they tested. Subsequently, Provasoli and McLaughlin (1963) reported TRIS concentration of 8.26 mM to be generally adequate, and that three *Gyrodinium* spp. tolerated concentrations to 49.56 mM. The original recipe ASP_{12} TRIS concentration is 8.26 mM. Decades ago tests at this laboratory of *Heterosigma akashiwo* (initially identified as *Olisthodiscus luteus*) TRIS tolerance showed this concentration is toxic to this species. Thereafter, TRIS in 4.13 mM concentration was adopted. This level has been satisfactory for maintaining medium pH; e.g., no medium precipitation from autoclaving or large pH change during culture incubation, and it apparently was not toxic to any species cultured in this laboratory. *A. anophagefferens* CCMP 1784 tolerance to TRIS in ASP_{12} was tested in multiples of the concentration in 'K' medium (10^{-3} M). In the initial test series *A. anophagefferens* tolerated

TRIS well to 5 mM; 6 mM caused slight early growth inhibition, and after cell abundance peaking, culture decline was greater than with lower levels. The highest concentration, 10^{-2} M, resulted in even greater early inhibition, a short period of low level of growth, then pronounced culture decline. Serial transfers into the same test range showed *A. anophagefferens* tolerance to 7 mM, suggesting the species can acclimate to a higher level. Despite the species tolerance of 5 mM without acclimation, a TRIS level of 4.13 mM is recommended for $ASP_{12}A$ medium. Droop (1958b) and McLachlan (1964) recommended glycylglycine and glycine buffering as a substitute for TRIS. McLachlan (1973) cautioned that glycylglycine can be used only with axenic cultures. This buffering was tried, nevertheless, with CCMP 1784 and CCMP 1794, and the result was overgrowth by bacterial culture contaminants. It was not assessed with the axenic culture.

Chelation

The primary chelator in ASP_{12} is EDTA, added as a component of PII metals mix (Table 2). EDTA provision in ASP_{12} is relatively moderate, being one third the level in various other Provasoli media. Additional EDTA is not provided for trace metals in the second metals mix in ASP_{12}, SII, which except for lithium are in ≤ 5 µM concentrations (Table 2). The ratio of chelator to trace metals in PII mix is 2.7 : 1, within the range of 2 to 3 : 1 found most useful by Provasoli et al. (1957). The range of chelator to metals ratios common in marine phytoplankton media is 0.8 to 2.7 : 1 (McLachlan, 1973).

Provasoli et al. (1957) did not favor EDTA : metals ratios greater than 3 : 1 because of the possibility of metal deficiencies and the active binding of EDTA with calcium and magnesium at pH's greater than 8.0. For increased chelation in ASP_{12} medium, Provasoli employed nitrilotriacetic acid, NTA; the medium so modified is designated $ASP_{12}NTA$. Provasoli et al. (1957) described NTA as a weaker metal chelator and McLachlan (1973) considered NTA less effective than EDTA for most purposes. NTA is provided in 5.23×10^{-4} M concentration in $ASP_{12}NTA$, in contrast to EDTA in 2.7×10^{-5} M. In $ASP_{12}NTA$, therefore, the NTA : trace metals ratio, with the molar total of trace metals in original PII mix used as basis for calculation, is ~52 : 1. (The ratio of NTA to EDTA is ~19 : 1.) An advantage of NTA is that its calcium and magnesium salts are highly soluble, which lessens the likelihood of precipitation and concomitant loss of micronutrients from solution (Provasoli et al., 1957). Also, McLachlan (1973) reported common use of NTA in marine media to prevent precipitation of sodium glycerophosphate at elevated temperatures.

Comparison of growth of *A. anophagefferens* CCMP 1784, CCMP 1794, and CCMP 1984 in ASP_{12} and $ASP_{12}NTA$, with original recipe chelator and metals levels, showed that the latter was superior, indicating ASP_{12} is underchelated for the species. A 10 : 1 EDTA : trace metals enrichments ratio in 'K' medium favored culture of oceanic ultraplankton, but the medium is not recommended for culture of coastal phytoplankton due to the possibility of metal deficiencies (Keller et al., 1987). 'K' medium was moderately suitable for *A. anophagefferens* in this

laboratory. Also, a 10 : 1 chelator : trace metals ratio is used in chemically-defined Aquil medium (Morel et al., 1979), which as modified by Cosper et al. (1993) to include selenium, is likewise suitable for *A. anophagefferens*. It is certain, therefore, that the species can grow in highly chelated medium.

EDTA and NTA were tested singly and in combination, and in a range of chelator : trace metal ratios: 1, 3, 7, and 10 : 1, with the trace metal concentrations in unmodified PII metals mix used for the ratio calculations. Growth of *A. anophagefferens* CCMP 1784 improved with increasing EDTA : trace metals ratios from 1 : 1 to 7 : 1. The highest ratio tested, 10 : 1, resulted in growth retardation in week one of incubation but subsequently was suitable. NTA in 3 : 1 chelator : trace metals ratio was slightly superior to EDTA in the same ratio. In 7 : 1 ratio growth with EDTA and NTA was comparable through day 10 of incubation, but subsequently culture growth and survival was superior with EDTA. Comparable growth of CCMP 1784 was obtained in medium with EDTA : trace metals in 7 : 1 ratio or with Provasoli's combination of EDTA : trace metals in 2.7 : 1 ratio plus 5.23 $x10^{-4}$ M NTA. Similar results were obtained for CCMP 1794 and CCMP 1984.

EDTA as sole chelator in a ratio with PII trace metals of 7 : 1, or EDTA in 2.7 : 1 ratio if coupled with NTA, is optional for $ASP_{12}A$ medium (Table 2); the first regime is the one in current use in this laboratory. In both regimes the concentration of EDTA is adjusted for trace metals increases in PII metals mix, but not for trace metals in SII metals mix (rubidium, lithium, iodine, and molybdenum), the additional iron as Fe EDTA, or arsenic, aluminum, and vanadium enrichments (Table 2). With EDTA as sole chelator, compensation for the Fe EDTA addition would require an EDTA increase from 8.53 x 10^{-5} M to 8.8 x 10^{-5} M to maintain a 7 : 1 chelator : metals ratio; utility of this was not tested. Selenium and molybdenum are influenced little if at all by chelation (Guillard, 1995).

The empirically determined chelation regimes for $ASP_{12}A$ are suitable for *A. anophagefferens* but may not be optimal. Chelator : metals ratios intermediate between 3 : 1 and 7 : 1, and between 7 : 1 and 10 : 1, were not tested. Suggesting an EDTA : trace metals ratio higher than 7 : 1 might be favorable is that the 10 : 1 ratio (the highest tested) was only temporarily growth-retarding. The EDTA correction for trace metal increases may be superfluous for the alternative combined EDTA and NTA chelation, given the large NTA supplementation. Provasoli's NTA level of 5.23 $x10^{-4}$ M in $ASP_{12}NTA$, unquestionably arrived at through exhaustive testing, has proved beneficial in a variety of media (Provasoli et al., 1957). The ratio tests suggest, however, that the concentration likely could be lowered for *A. anophagefferens.* This was not pursued. EDTA was superior to NTA in 7 : 1 chelator : metals ratio, but this may not preclude NTA utility as sole chelator if its level is appropriately adjusted. The effect of different pH's on chelation was not tested. The concentration of TRIS buffer in $ASP_{12}A$ medium, which also acts as a chelator although weaker than EDTA (e.g., Morel et al., 1979), has been halved, with undetermined effect on chelation. Greater efficacy of citric acid than EDTA and NTA was found by Cosper et al. (1993) when *A. anophagefferens* was transferred into medium with selenium enrichment following two serial transfers in medium without selenium. The selenium depletion likely introduced major stress to the picoplankter, so the basis for chelator comparison is not ideal. Moreover, use of citric acid is limited to axenic cultures.

To explore what effect chelation of specific ASP_{12} constituents might have on *A. anophagefferens* growth, various chelators suggested by S. Hutner (personal communication), including ethylene-bis(oxyethylene-nitrilo)tetraacetic acid (EGTA) (which selectively complexes calcium); tripolyphosphate (complexes iron, calcium, magnesium); sulfosalicylate and desferoxamine (complex iron); and triethylenetetramine (complexes copper and nickel), were evaluated as substitutes or supplements to EDTA and NTA. No chelator or combination benefited growth of CCMP 1784 more than EDTA and NTA.

TEMPERATURE, SALINITY AND LIGHT CONDITIONS AFFECTING GROWTH

Described in Chizmadia et al. (1984) and Hunchak-Kariouk et al. (1999), the lagoonal Barnegat Bay-Little Egg Harbor estuarine system -- the New Jersey epicenter for *A. anophagefferens* blooms -- extends ~70 km along the New Jersey coast. It ranges from ~2.0 to 6.5 km in width. Depths are shallow, averaging 1.3 m in the northern half and ≥2.0 m in Little Egg Harbor. The eastern portion, averaging <1 m in depth, is generally shallower than the middle and western portions, which range to ~4.0 m deep. Tidal connections to the Atlantic Ocean are through the Bay Head-Point Pleasant Canal and the Manasquan River at the northern end; Barnegat Inlet at the center; and Little Egg Inlet at the southern end (which also connects to the Great Bay-Mullica River estuary). Tidal exchange is relatively restricted in the central and northern portions and is greater in the southern portion. Primary exchange of ocean and bay water in Barnegat Bay occurs through the Barnegat Inlet. Wind has a dominant role in circulation, which has a complex pattern. Periods of complete vertical mixing occur, particularly when wind velocities are high, but a tendency toward two-layered circulation exists in areas deeper than 1.5 m. Several major freshwater tributaries, the largest of which is Toms River at northern Barnegat Bay, discharge into the system along the northwestern perimeter, whereas only a few smaller creeks discharge along the southwestern perimeter. Numerous storm drains contribute freshwater runoff, either directly or through lagoons and tributaries. Freshwater input from groundwater seepage is also considerable. The proxmity of the ocean causes a moderation of summer and winter termperatures. July and August are the warmest months, with a water temperature high of ~28°C, and January and February are the coldest, with a low of ~1°C. Precipitation is well distributed. Extratropical storms, especially from the northeast, may occur from September to March.

A. anophagefferens temperature and salinity tolerances are of particular interest because of known or potential bloom regulatory importance of these factors in the respective epicenters. Water temperature >25°C truncated blooms of the species in various eastern Long Island embayments (Nuzzi and Waters, 2004), and truncated a major bloom in the New Jersey Barnegat Bay-Little Egg Harbor system (personal observation). Salinity is not considered a primary regulator of *A. anophagefferens* blooms in the Long Island bays because salinity of those waters rarely falls below levels favorable for growth of the picoplankter (LaRoche et al., 1997). Based on a two-year survey (unpublished data), salinity can have primary importance in *A. anophagefferens* bloom regulation in the Barnegat Bay-Little Egg Harbor system. This is particularly true in the northern half, given its restricted tidal exchange and greater freshwater input. Temperature and salinity tolerances of New York and New Jersey *A. anophagefferens* strains (CCMP 1784 and CCMP 1794) are compared to assess physiological differences.

Temperature Tolerances of New York and New Jersey Isolates

Cosper et al. (1989) reported growth rates of Long Island *A. anophagefferens* (presumably BT-1 = CCMP 1784) in a temperature range of 5 to 25°C, tested with 5°C increments, to be greatest at 20 and 25°C. Data in Figure 9 of Cosper et al. (1989) indicates that growth rate at 25°C, although one of the highest, was reduced from the optimum at 20°C, so the optimum temperatures may be ~20<25°C. In this laboratory, CCMP 1784 and CCMP 1794, New York and New Jersey isolates respectively, had similar growth and maximum cell abundance at routine incubation temperature, 18°C. Estimating from the Cosper et al. Figure 9, temperature decrease of 18°C to 10°C resulted in approximate halving of BT-1 growth rate, declining from ~0.7 to ~0.3 doublings day^{-1}. Strain CCMP 1794 yield at 10°C in this laboratory was considerably reduced below that with 18°C, requiring 16 to 18 days versus 10 to 13 days with the higher temperature to achieve a comparable level. Fluorescence measurements may have lessened accuracy in indicating cell population in this type of test (Brand et al., 1981); e.g., if cells incubated at 10°C had less pigment than cells grown at the higher temperature, the apparent yield difference would shrink. CCMP 1784 and CCMP 1794 grew comparably well at 5°C in ASP_{12}. Growth with this temperature was consistent but slow, 8 to 10 weeks being required to achieve yields comparable to those reached in ~2 weeks with 18°C. Data in Figure 9 of Cosper et al. (1989) shows that BT-1 grew at ~0.1 doublings day^{-1} with 5°C. Quantitative comparison of data from the Cosper study and this one is not possible. However, accepting that the BT-1 culture grew seven times faster at 18°C than at 5°C, and considering difference in time to achieve comparable yield with these temperatures in the present study having a factor of ~4 to 5, some similarity of growth response over the temperature range is suggested. Neither Cosper et al. (1989) nor the present study tested *A. anophagefferens* upper temperature tolerance. From field observations (Nuzzi and Waters, 2004; Mahoney, unpublished data) this appears to be >25°C in both epicenters, suggesting similar tolerance of multiple strains.

CCMP 1784 and CCMP 1794 Salinity Tolerances

A. anophagefferens was found between Portsmouth, New Hampshire and Chesapeake Bay in the salinity range 18 to 32 PSU (Anderson et al., 1993). It was found in a similar salinity range (18.5 to 34.5 PSU) in western New York Bight coastal waters (Mahoney et al., 2003b). Salinity range for optimal growth and bloom development apparently is considerably narrower, however. Cosper et al. (1989) determined that maximal growth of *A. anophagefferens* BT-1 (=CCMP 1784) was with salinity of 30 PSU. Major Long Island blooms of the species were associated with salinities ≥27 PSU (Bricelj and Lonsdale, 1997). Salinity tolerances of strains CCMP 1784 and CCMP 1794 in this laboratory were determined in ASP_{12} by varying concentrations of Na, Mg, Ca, and K, while the ratio of these, and the concentrations of other constituents, were maintained (after Pintner and Provasoli, 1963). Test inocula were acclimated to 27 and 21 PSU, respectively, through five serial transfers over a two month period, prior to parallel salinity tests in the range 17 to 38 PSU, with salinity increments of 1.9 PSU. Salinity values other than whole numbers are an artifact of test design. It is assumed that relatively high increase of yield in the first week after inoculation reflects medium compatibility with the state of *A. anophagefferens*

physiology achieved during inoculum incubation. Results for CCMP 1784 are compared with those reported by Cosper et al. (1989) for BT-1 (= CCMP 1784); the latter study employed Instant Ocean Sea Salts medium with 'f/2' medium nutrient enrichment. Cosper et al. (1989) reported adaptability of *A. anophagefferens* BT-1 (=CCMP 1784) to relatively low and high salinities; BT-1 tolerated from 22 to 35 PSU if given time to acclimate.

CCMP 1784, when high salinity-adapted (27 PSU), had greatest cell abundance in the first week of test incubation in the salinity range 24.7 to 28.5 PSU. Low salinity intolerance following high salinity adaptation, such as severe BT-1 growth inhibition below 28 PSU when adapted to 30 PSU (Cosper et al., 1989), was not found in the present study. The difference in high salinity acclimation levels (27 versus 30 PSU) possibly was a factor in this. Growth of CCMP 1784 lagged temporarily with initially less suitable salinities; Cosper et al. (1989) noted similar growth response. After two weeks, comparably high cell abundance was evident at an expanded range, 22.8 to 34.2 PSU. With another week of incubation, the salinity range for comparably high cell abundance expanded to include 20.9 and 36.1 PSU, and included 38 PSU in week four. The strain did not grow below 20.9 PSU. Growth to comparably high yield in these tests was in a broader salinity range than the range for high growth rates reported by Cosper et al. (1989).

When low salinity adapted (21 PSU), greatest cell abundance of CCMP 1784 in the first week of incubation was in the salinity range 20.9 to 28.5 PSU. Therefore, acclimated at the lower salinity, CCMP 1784 grew optimally without lag at a lower salinity than when acclimated at the higher salinity. As occurred when high salinity adapted, growth lagged temporarily with initially less suitable salinities. After two weeks, comparable high cell abundance was obtained in the range 20.9 to 34.2 PSU, and this level was obtained with 36.1 PSU in week three. With 38 and 19 PSU, at least moderate growth was achieved after two or more additional weeks of incubation; reduced uniformity of cell abundance among the four replicates suggested culture stress, however.

High salinity adapted (27 PSU) Barnegat Bay isolate CCMP 1794 had greatest cell abundance in the first week of incubation in the salinity range 26.6 to 32.3 PSU, a slightly higher range than the similarly adapted New York strain. As with the New York strain, growth lagged temporarily with initially less suitable salinities After two weeks, the salinity range for comparably high cell abundance was expanded to 22.8 to 34.2 PSU, the same range for post two week incubation of high salinity adapted CCMP 1784. Cell abundance of the New Jersey isolate comparable to that with the above salinities was reached in weeks three and four, respectively, with 36.1 and 38 PSU, respectively, the same response as CCMP 1784 when tested similarly. Growth of CCMP 1794 was moderate with 20.9 PSU, and still lower although considerable with 19 PSU. The New York strain, CCMP 1784, similarly adapted, would not grow with salinity level at 19 PSU, even after weeks of incubation.

Low salinity acclimated (21 PSU) CCMP 1794 had greatest cell abundance in the first week of incubation in the salinity range, 20.9 to 32.3 PSU. As the New York strain did when acclimated at the lower salinity, the Barnegat Bay strain grew optimally without lag at a lower salinity than

when acclimated at the higher salinity. Additionally, it had slightly greater high salinity tolerance than the New York strain under the same conditions. Comparably high cell abundance was evident with 20.9 to 36.1 PSU in week two. Comparable cell abundance was obtained with 38 PSU in week three. Again, cell abundance was lower than optimal but considerable with 19 PSU.

The lag in CCMP 1784 and CCMP 1794 growth with salinities higher or lower than those initially favorable may reflect need for an adaptation period, possibly recovery from transfer shock, or both. The test inoculations represented sudden considerable salinity changes, e.g., from 27 to 17 PSU, and from 21 to 38 PSU. The observed lag periods might be shortened or eliminated if the change was gradual, but this was not tested. The ability of the species to withstand much of this change demonstrates robustness. This may or may not have environmental relevance; salinity change of ~10 PSU can occur in the Barnegat Bay-Little Egg Harbor system over days or weeks, but not instantaneously.

Salinity tolerances of CCMP 1784 and CCMP 1794 *A. anophagefferens* strains are basically similar. Both flourish with neretic salinities and have considerable ability to adapt to open ocean salinities or higher, and to lower salinities generally present in coastal bays in the northeast region in late winter and spring. Evidencing the latter in the tests, when both isolates were acclimated to 21 PSU, cell abundance with this salinity was comparable during week one to cell abundance reached with consistently suitable higher salinities. Both strains likewise grew moderately well with 19 PSU when low salinity (21 PSU) adapted. Both strains have unchanged, although different for each, high salinity preference whether acclimated in low or high salinity. Cosper et al. (1989) also found that *A. anophagefferens* BT-1 (=CCMP 1784) has the same high salinity preference whether acclimated in low or high salinity. Comparison of ranges of salinities suitable for growth without lag following acclimation to 21 or 27 PSU, suggests that CCMP 1794 has a slightly broader salinity tolerance than CCMP 1784, with a preference for higher salinity. This distinction disappeared later in incubation, however.

CCMP 1784 and CCMP 1794 Light Requirements

Phytoplankton laboratory light bank illumination commonly is 5 to 10 percent of maximum daylight intensity, e.g., 170 $\mu E\ m^{-2}\ sec^{-1}$ (Guillard and Keller, 1984). Cosper et al. (1989) used 100 $\mu E\ m^{-2}\ sec^{-1}$ for *A. anophagefferens* culture. Illumination at 50 $\mu E\ m^{-2}\ sec^{-1}$ routinely was employed in this study, and this level apparently is adequate for *A. anophagefferens*. The species is well adapted to growth with relatively low light intensities when not nutrient-limited (Milligan, 1992). Growth with lighting levels lower than 50 $\mu E^{-2}\ m^{-2}\ sec^{-1}$ was tested by light attenuation with Kodak neutral density filters variously having percent transmissions of 50, 25, and 10. Culture tubes were incubated atop the filters (filters were sandwiched between two squares of thin glass), on the light bank shelf, in sections of 3-inch ID PVC pipe, open on the bottom (lighting is from below) and light-sealed on top. With the lowest light level, cell abundance of *A. anophagefferens* CCMP 1784 was slightly less than yields with lighting from 25% to full light, which was comparable until day 12 of incubation, when cell abundance with all lighting

levels was comparable. Ability to grow under low light conditions can be particularly advantageous during major blooms of the species. During an intense *A. anophagefferens* bloom in 1999 in the Barnegat Bay-Little Egg Harbor system, Secchi depths in various locales where it was most prevalent ranged only 0.2 to 0.4 m (unpublished data).

RESULTS SUMMARY AND COMMENTS

Modification of ASP_{12} for *A. anophagefferens* entailed increase of concentrations of most original constituents, decrease of concentrations of others, and adoption of additional nutrients found beneficial. The most critical medium modification is the addition of selenium. Changes in concentrations of major ions are relatively modest, with the largest being reduction of magnesium and sulfur levels, and introduction of fluoride. A change of magnesium, calcium, and potassium molar ratio from 5.1 : 1.06 : 1 in ASP_{12} to 3.63 : 1.13 : 1 in $ASP_{12}A$ is due almost entirely to magnesium reduction in the latter. A wide range of magnesium, sulfur, and potassium concentrations support flourishing growth of *A. anophagefferens*; absence of exacting requirement for magnesium and potassium is shared by many phytoplankton species (Provasoli et al., 1957).

Changes in original recipe minor constituents include: considerable reduction of silicon concentration (requirement by *A. anophagefferens* for silicon was found); slight increase of nitrate nitrogen (ammonium is used but is not sufficiently beneficial to warrant inclusion); moderate reduction of inorganic and organic phosphorus constituents (flourishing growth in medium with inorganic phosphorus, K_3PO_4, as sole phosphorus source was obtained); increase of all constituents of PII metals mix and one constituent of SII metals mix; selenium, arsenic, vanadium, and aluminum are newly introduced.

EDTA chelator level is greatly increased, or original level raised only to account for PII trace metals increases, if NTA is provided as second chelator. The two chelator regimes are conducive to excellent growth of *A. anophagefferens.* More study is needed to determine if they can be improved. This could include evaluation of untested EDTA : metals ratios between 3 : 1 and 10 : 1, and simultaneous chelator : trace metals testing. NTA, a less effective chelator than EDTA (Provasoli et al., 1957; McLachlan, 1973), is found basically suitable but less so than EDTA for culturing *A. anophagefferens* in $ASP_{12}A$. This is in contrast to the findings of Dzurica et al. (1989), who reported that NTA and citric acid were suitable chelators for *A. anophagefferens* and growth was slight at best with EDTA. Underchelation is a possible explanation for this because Dzurica et al. (1989) used medium 'f' or 'f/2' chelator to trace metals ratio of 0.9 : 1. An explanation of the seemingly contradictory situation of ASP_{12} being found under-chelated for *A. anophagefferens*, but metals tests suggesting potential deficiencies of some, which resulted in largely precautionary increases to defer limitation, is the emphasis on long-term culture survival. The effects on chelator and metals balance of lowered salinity, increased calcium concentration, and lowering by half of TRIS pH buffer concentration in $ASP_{12}A$ medium, are undetermined. Requirement for vitamin B_{12} and thiamine is established.

Vitamin B_{12} concentration five times original recipe level (after Iwasaki, 1967) is adopted as a precautionary measure; thiamine level is unchanged but doubling the concentration may be beneficial and certainly is not harmful. Biotin apparently is not needed by *A. anophagefferens* but is retained. Effect, if any, on *A. anophagefferens* growth of three plant hormones tested was not apparent.

The final version of the medium $ASP_{12}A$ as prepared in this laboratory is autoclavable, does not precipitate, and retains utility long-term; e.g., with six months' refrigerator storage. Batch-to-batch quality of $ASP_{12}A$ is not perfectly consistent, but it is consistently superior to enriched sea water media prepared in this laboratory. Growth of *A. anophagefferens* with microwave-sterilized or autoclave-sterilized batches is comparable. $ASP_{12}A$ supports *A. anophagefferens* growth over at least a 5 to 18°C range of temperatures, and with relatively low light levels. With the culture conditions in this laboratory, $ASP_{12}A$ routinely provides maximum *A. anophagefferens* yields of 2.0 to 3.0 x 10^7 cells ml^{-1}. Because prolonged culture survival was a primary development criterion for $ASP_{12}A$, it is a conservation medium. In one possibly extreme instance a CCMP 1984 six-month-old $ASP_{12}A$ batch culture was successfully transferred. This is not to say that the medium is sufficient to support, routinely, viability of *A. anophagefferens* for this duration in batch culture, but at least it did not prevent whatever was operative. Acclimation of certain strains of *A. anophagefferens* to $ASP_{12}A$ medium may be required. When transferred into final version of the medium from an earlier version, CCMP 1794 growth was partly inhibited in week one; such lag was not seen when the same strain was transferred from final version to final version. In contrast, axenic CCMP 1984, previously cultured in L1 medium in the NMFS Milford Laboratory, on initial transfer to $ASP_{12}A$ grew without lag and flourished. $ASP_{12}A$ medium may be especially beneficial for bacterized *A. anophagefferens* cultures by delaying microbial contaminant ascendancy or ameliorating stress of such ascendancy to the picoplankter. Multiple laboratories (~10) experienced loss of one *A. anophagefferens* strain (CCMP 1794) at about the same time (Andersen, personal communication). What caused these losses is undetermined, but culturing in $ASP_{12}A$ may be an explanation for the strain's survival solely in this laboratory.

Because $ASP_{12}A$ is a conservation medium for *A. anophagefferens* the concentrations of various medium constituents likely could be lowered without major detriment to at least *A. anophagefferens*' short-term growth if a more dilute medium is required. Shown by the salinity tolerance tests, proportionate reduction of magnesium, potassium and calcium and sodium chloride to provide a salinity of 25 or 27 PSU does not diminish the medium's suitability. Nitrogen likely could be lowered safely to about original recipe level and phosphorus concentration could be reduced drastically. A moderate reduction of metals and chelator likely would be feasible.

Sensitivity of *A. anophagefferens* to silver and copper has been reported to be relatively high; these metals at ~10 ppb (9.27 x 10^{-8} M and 1.57 nM, respectively) inhibited *A. anophagefferens* growth by 50% (Steele et al., 1989). However, the concentration ranges of silver and copper in sea water are only 0.5 to 35 pM and 0.5 to 4 nM, respectively (Bruland, 1983), so it is unlikely that

the species normally would encounter these inhibitory levels in nature. Moreover, tested in this laboratory, silver was not inhibitory in concentrations to 2.78 x 10^{-8} M, and the species tolerated three times the 'K' medium level of copper, 3 x 10^{-8} M, the latter being the highest concentration tested. Also, comparison of metals enrichments of three media suitable for culturing the species, including $ASP_{12}A$, 'K' medium (Keller et al., 1987), and Aquil medium (Morel et al., 1979) modified by Cosper et al. (1993) to include selenium, shows these have widely disparate concentrations and proportions of metals enrichments. Total level of chelatable trace metals in unmodified Aquil is only 4.8 x 10^{-7} M in contrast to 1.27 x 10^{-5} M in 'K' medium (mostly iron). Comparison of metals proportions reveals 'K' medium has 3.2 times more iron than $ASP_{12}A$, but the latter has 10 and 13.3 times more manganese and zinc, respectively, than 'K' medium. This suggests *A. anophagefferens* may not be especially intolerant of at least some toxic metals and relatively high levels of essential metals and can flourish with a wide concentration range of many required metals. During culture testing it has grown after a lag of several weeks, suggesting acclimation to an inhibitory condition or at least survival without cell division until the inhibitor concentration lessened.

$ASP_{12}A$ suitability for a multiplicity of *A. anophagefferens* isolates is certain. A group of 11 *A. anophagefferens* Long Island isolates, routinely cultured in L1 NW medium (Guillard and Morton, 2003), were evaluated for growth in $ASP_{12}A$ by R. Andersen at CCMP, Bigelow Laboratory. Cultures included CCMP 1785, a 1987 isolate from Great South Bay; CCMP 1706 and CCMP 1707, isolates from West Neck Bay in 1995; CCMP 1790 from Great South Bay in 1995; CCMP 1847, 1848, 1849, 1851, 1852, and 1853, isolates from the same 1998 collection time and site in Great South Bay; and CCMP 1850, an isolate from another Great South Bay site in 1998. All isolates grew well in $ASP_{12}A$ through an initial transfer followed by two serial transfers, which together represented a ~1 : 16,000 dilution of L1. Viability of all strains in $ASP_{12}A$ was supported for at least three months; most strains apparently were viable after 4.5 months. Overall $ASP_{12}A$ suitability for these *A. anophagefferens* cultures was ~equal to that of L1 medium (Andersen, personal communication). Although "tailored" for *A. anophagefferens* $ASP_{12}A$ retains general utility. Suitability of $ASP_{12}A$ for 28 other phytoplankton species in a wide range of classes was tested at CCMP (Andersen, personal communication). Increase of silicon concentration of $ASP_{12}A$ would be necessary for species that require more than the low level in this medium. The medium supported excellent growth of the following CCMP cultures: 732, 736, 1168, 1175, 1278, 1283, 1320, 1333, 1420, 1485, 1493, 1674, 1675, 1768, 1954, 1994, 2057, 2064, 2070, 2112, 2199, 2424, 2495, and 2579. These included, for example, the diatom *Skeletonema costatum*; a chlorophyte, *Dunaliella tertiolecta*; the dinoflagellates *Alexandrium tamarense* and *Prorocentrum lima;* a haptophyte, *Emiliania huxleyi*; a pelegophyte, *Pelegomonas calciolata*; and a raphidophyte, *Heterosigma akashiwo.* CCMP 1722 grew weakly in the medium, and CCMP 1153, 1475, and 2164 did not grow in it. Trial of $ASP_{12}A$ at the NMFS Milford Laboratory (Wikfors and Alix, personal communication) showed the medium is suitable for a diatom, *Nitzschia* sp. (with 1.06 x10^{-4} M silicon added); a dinoflagellate, *Prorocentrum minimum*; a cryptophyte, *Rhodomonas* sp.; a prasinophyte, *Tetraselmis chui*; a prymnesiophyte, *Pyymnesium parvum*; and a rhodophyte, *Porphyridium cruentum*. For decades, ASP_{12} medium has had proven success for a wide variety of algae. The suitability of $ASP_{12}A$ for

the above group of phytoplankton indicates that its "tailoring" for *A. anophagefferens* has not interfered, at least not greatly, with its general utility.

Information on the relative physiology of *A. anophagefferens* strains which bloom in New York and New Jersey locales -- perhaps multiple strains in each region -- has not been available. Consequently, application of aspects of the information on Long Island blooms to New Jersey blooms, the latter having relative paucity of information developed, may or may not be sound. Some growth features of a Long Island isolate and the only New Jersey *A. anophagefferens* culture isolated are compared, despite uncertainty that the *A. anophagefferens* cultures employed in this study were consistently representative of the natural populations. Comparison of growth responses of the New York and New Jersey strains in this study to most of the medium constituents is not possible, because too few tests were done for both. Rather than offering conclusions, perhaps the main benefit of this limited comparison is to highlight the subject.

The two isolates appear physiologically similar in some respects, but differences in tolerance to some of the medium constituents tested are apparent. Requirement for selenium is shared; the New York isolate and its axenic version appear to tolerate higher concentration of selenium than the New Jersey strain. All isolates benefited from fluoride with no tolerance difference in the range tested. NH_4Cl is utilized as sole nitrogen source by New York and New Jersey isolates; in higher concentrations tested, NH_4Cl is more toxic to the latter. The axenic New York isolate was not tested for NH_4Cl toxicity, so possible amelioration by bacterial contaminants rather than higher New York strain tolerance cannot be ruled out. All isolates utilize inorganic or organic phosphorus equally well. Growth lag before use of inorganic phosphorus solely (when previously grown with combined inorganic and organic phosphorus) was greater with the New Jersey isolate and the axenic New York culture than with the non-axenic New York culture. New York and New Jersey strains require the same vitamins. Bacterized New York and New Jersey strains had positive growth response to iron in the highest level tested. This level was slightly inhibitory to the axenic New York isolate, which benefited from a lower-level increase. All isolates had positive growth response to increases of boron and manganese, with no apparent inhibition with the levels tested. Salinity tolerances of the New York (CCMP 1784) and New Jersey (CCMP 1794) strains basically are similar. The finding by Cosper et al. (1989) that *A. anophagefferens* retains the same high salinity preference, whether pre-test acclimated in low or high salinity, is confirmed for CCMP 1784 and also found for CCMP 1794 in this study. Both CCMP 1784 and CCMP 1794 grew at 19 PSU, and at the highest salinity tested, 38 PSU. Comparison of ranges of salinities suitable for early incubation growth following pre-test acclimation to 21 or 27 PSU suggests that CCMP 1794 high salinity preference may be slightly greater than that of CCMP 1784. Direct comparison of published data on temperature tolerance of CCMP 1784 (Cosper et al., 1989) with that determined for CCMP 1794 is not possible due to test design difference. However, in this laboratory both grew comparably well at the normal incubation temperature 18°C, which is close to the reported most favorable temperature range, 20 to 25°C (Cosper et al., 1989). Ability to grow at winter water temperature, 5°C, considered a potentially important factor in eastern Long Island *A. anophagefferens* bloom occurrence, shown for the New York isolate (Cosper et al., 1989), was shown for the New Jersey isolate as well.

The latter steadily increased in cell abundance throughout over two months' incubation at 5°C. These limited and somewhat mixed results are unsatisfying but serve to underscore the need for physiological comparison of *A. anophagefferens* bloom dominants from different regions.

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Table 1. Original composition of ASP_{12} medium and as modified for *Aureococcus anophagefferens* in $ASP_{12}A$. Values are mol liter^{-1}.

Constituent	ASP_{12}	$ASP_{12}A$
NaCl[1]	4.79 E-1	4.28 E-1
KCl[1]	9.4 E-3	1.1 E-2
$MgSO_4 \cdot 7H_20$	2.8 E-2	None
$MgCl_2 \cdot 6H_2O$[1,2]	1.97 E-2	4.0 E-2
Ca (as Cl^-)[3]	1.0 E-2	1.25 E-2
$Na_2SO_4 \cdot 10\ H_2O$[1,2]	None	1.0 E-2
$NaNO_3$	1.2 E-3	1.47 E-3
K_3PO_4	4.7 E-5	4.0 E-5
$Na_2 \cdot$ glycero $\cdot PO_4 \cdot 5H_2O$	3.2 E-5	2.0 E-5
$Na_2SiO_3 \cdot 9H_2O$	5.3 E-4	2.5 E-5
PII metals mix[4]	10.0 ml	
PII metals mix (modified)[4]		10.0 ml
SII metals mix[4]	10.0 ml	
SII metals mix (modified)[4]		10.0 ml
FeEDTA	None	1.79 E-6
Selenium (as H_2SeO_3)	None	2.0 E-8
Fluoride (as NaF)	None	5.26 E-5
Arsenic (as $NaAsO_2$)	None	4.0 E-8
Aluminum (as $Al_2 (SO_4)_3$)	None	1.11 E-6
Vanadium (as $NaVO_3$)	None	2.0 E-8
Vitamin B_{12}	1.48 E-10	7.4 E-10[5]
Biotin	4.1 E-9	4.1 E-9
Thiamine	2.96 E-7	2.96 E-7
Tris (hydroxymethyl amino methane)[1, 6]	8.26 E-3	4.13 E-3
Optional addition:		
$NaHCO_3$	None	1-2 E-3

[1]dry salt additions; all other constituents added as solutions
[2]chemicals dried at 70° C for one hour before weighing.
[3]prepared from $CaCO_3$
[4]constituents are listed in Table 2.
[5]concentration five times original, modification by Iwasaki (1967)
[6]set ph at 8.4 before sterilization

Table 2. Composition of PII and SII metal mixes used in ASP_{12} medium and as modified for *Aureococcus anophagefferens* in $ASP_{12}A$. Note optional chelation. Values are mol liter^{-1}.

Constituent	ASP_{12}	$ASP_{12}A$
PII Metals Mix		
Na_2EDTA	2.7 E-5	8.53 E-5
OR		
Na_2EDTA		3.29 E-5[1]
PLUS		
NTA		5.23 E-4[2]
Iron	1.79 E-6	1.79 E-6
Boron	1.85 E-4	2.77 E-4
Manganese	7.28 E-6	9.1 E-6
Zinc	7.65 E-7	1.07 E-6
Cobalt	1.7 E-7	2.21 E-7
SII Metals Mix		
Bromine	1.25 E-4	1.25 E-4
Strontium	2.28 E-5	3.42 E-5
Rubidium	2.34 E-6	2.34 E-6
Lithium	2.88 E-5	2.88 E-5
Molybdenum	5.21 E-6	5.21 E-6
Iodine	7.9 E-8	7.9 E-8

[1]Concentration compensated for additional manganese, zinc and cobalt.
[2]Original ASP_{12}NTA concentration.

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