

Appendix 1. Algal Bioassay Methodology to Determine Pond Fertilization Requirements

The algal bioassay method described below is essentially identical to the procedure described by Knud-Hansen et al. (in press). This paper discusses more extensively the scientific justifications and applications of algal bioassays in aquaculture, and examines each aspect of the methodology in more detail. The method as described below emphasizes the use of locally available materials.

Pond Water Collection

Several liters of subsurface pond water are collected in a prerinsed container or bucket. It is better to collect the water soon after dawn, before ambient N, P, and C are stripped from the water column by phytoplankton through photosynthetic activity. The method described here uses 500-ml pondwater subsamples for individual treatment determinations, but this volume and the corresponding nutrient spike volumes can be scaled up or down if more convenient. Larger sample volumes may be preferable, however, in that they reduce potential sources of variability from contamination and/or bottle effects (Mitchell and Malthus, 1984).

Nutrient Spikes

Nutrients are added to pondwater samples in the form of concentrated solutions called spikes. The spike volume and nutrient concentrations are designed to approximate reasonable input rates of N, P, and/or C, and can be made with analytical reagents or directly from chemical fertilizers (Table A1.1). Spikes can be made with either distilled (D) or deionized (DI) water, which can often be found in grocery stores or is available at automobile service stations for filling car batteries. Spike concentrations do not have to be analytically precise, but care must be taken to avoid unwanted contamination of algal nutrients. All containers should be prerinsed with D/DI water, and solutions should be kept covered and refrigerated when not in

Algal Nutrient	Spike Source	Spike Source Concentration (mg l ⁻¹)	N,P, or C Concentration in Spike (mg l ⁻¹)	Spike Volume (ml)	Increased N, P, or C Concentration in Spiked Sample (mg l ⁻¹)	Nutrient Fertilization Equivalent ¹ (kg ha ⁻¹ wk ⁻¹)
N	NH ₄ Cl	575	150	10	3.0	30
N	Urea	325	150	10	3.0	30
N	NaNO ₃	910	150	10	3.0	30
P	K ₂ HPO ₄	280	50	10	1.0	10
P	TSP	230	50	10	1.0	10
P	MSP	75	50	10	1.0	10
C	CaCO ₃	1000	1000 ²	10	20 ²	200 ²
C	NaHCO ₃	845	1000 ²	10	20 ²	200 ²
Initial	D/DI water	-	-	20	-	-
Control	D/DI water	-	-	20	-	-

¹ Loading rate assumes a 1.0-m deep pond.

² Carbon spikes and fertilization given in units of alkalinity (mg l⁻¹ of CaCO₃).

Table A1.1. Spike sources, volumes, and approximate concentrations used in the algal bioassay procedure. Controls and spike solutions are made with distilled or deionized (D/DI) water. Spike volumes are appropriate for 500ml pondwater samples, but can be scaled accordingly (e.g., 1 ml of nutrient spike would be appropriate for a 50ml pondwater sample).

use to prevent contamination.

Thoroughly mix the pondwater sample, and twice rinse nine (9) 1-l clear plastic, screw-capped water bottles with the pond water. Almost any translucent-glass or plastic containers may also be used. Transfer 500-ml pondwater subsamples into each of the nine plastic bottles. Each bottle should be clearly labeled indicating the nutrient spike to be applied: N, P, C, N+P, N+C, P+C, N+P+C, initial, and control.

Add the appropriate spike(s) to each of the nine pond water subsamples according to Table A1.1. For example, the N+P flask would receive 1 ml of the N spike and 1 ml of the P spike. The initial and control treatments receive D/DI water spikes instead of nutrient spikes. Each bottle is then mixed. The water in the initial treatment is immediately filtered (see below), while the remaining eight treatments are incubated to evaluate algal responses to nutrient enrichment. Bottles should be loosely capped to allow limited air exchange while preventing airborne contamination.

Incubation of Culture Bottles

Since the response variable of algal production only needs to be examined relatively and not absolutely (see discussion below), the two primary incubation criteria are that 1) culture bottles are in a light/temperature environment conducive to algal growth; and 2) all culture bottles are placed in identical environmental conditions during the incubation period.

Incubation should be done under artificial lighting or indirect sunlight. If bottles are placed under direct sunlight, photoinhibition will reduce photosynthetic activity and water samples could quickly overheat, killing the phytoplankton (Fogg, 1975). Outdoor exposure under a white cotton cloth with sufficient lateral ventilation is preferable to complete shade (Guttman, 1991). In areas with low nightly temperatures, bottles should be taken indoors at sunset and returned outdoors after sunrise. Guttman (1991) also demonstrated that swirling the vessels at least twice daily enhanced the algal response in cultures where limiting nutrient(s) were supplied in the spike. A two- to three-day incubation period is sufficient for most ponds, although clear water ponds may require up to four days incubation due to the initially low algal standing stock in the culture vessels.

Algal Biomass Comparisons

Bioassay results are primarily the comparisons of algal growth in the initial and control bottles with the seven nutrient-spiked pondwater samples. Algal growth during the incubation period can be measured by various physical (e.g., weight, optical density) or biochemical (e.g., fluorescence, ATP, radioactive carbon uptake) analytical techniques. However, the relatively high algal productivities and densities found in fertilized culture ponds enable algal biomass to be easily and effectively analyzed through visual comparisons of the nine filters after individually filtering each spiked pondwater sample.

The filtering apparatus can be easily created out of local materials. For example, cut a circular section out of a lid from a wide-mouthed jar or bottle with a screw-on top (Mason jars already have

lids with removable center sections). Replace the sectioned part of the lid with a circular filter and a screen/ plastic mesh, which supports the filter. A white paper coffee filter works well as a bioassay filter. With the cap inverted, insert the mesh with the filter on top. Both mesh and filter should be cut to fit closely along the inside of the jar's lid. To filter, put a measured amount of thoroughly mixed sample water in the jar, screw the lid on tightly, and invert the bottle to allow gravity filtration. On-farm filtering may be facilitated with a commercially available hand-operated vacuum or bicycle tire pump. For field trials, a Whatman GF/C glassfiber filter (47 mm) was placed in a plastic Millipore Swinnex filter holder, and culture water was pushed through using a 50-ml plastic syringe (Knud-Hansen et al., in press)

If algal growth has been great or the culture volumes were large, thoroughly mixing the sample and filtering only 10 to 20 ml may be advisable in order to better distinguish treatment differences, and to save time on filtering. Although it does not really matter how much water is filtered, the filtrate volume *must* be identical for all treatments to permit meaningful visual comparisons. In fact, since there are only three different possible levels of algal growth in the culture bottles (see below), treatment differences may be discernable simply by comparing the relative greenness in the bottles after mixing, and filtering would not even be necessary.

Filter Interpretation for Determining Fertilization Requirements

The unaided human eye can easily separate treatment differences of algal biomass, as indicated by relative greenness on filter paper (Guttman, 1991), and posed no problem in a four-month field trial (Knud-Hansen et al., in press). Visual separation becomes ambiguous only in extremely green or turbid ponds, in which case fertilization would not be recommended at that period (discussed below). To aid in the visual comparison of filters, filters should be laid out in a format to conveniently apply Table A1.2 for interpretation. Placing filters in a straight line in the same order as the treatment heading in Table A1.2 is one option; the layout in Figure A1.1 illustrates another. Once the filters are in order, then visual color comparisons are made.

	Bioassay Response								Limiting Nutrient		Relative Input			
	Control	N	P	C	N+P	N+C	P+C	N+P+C	1°	2°	N	P	C	
1.	○	●	○	○	●	●	○	●	:	N		2	0	0
2.	○	●	○	○	●	●	○	●	:	N	P	2	1	0
3.	○	●	○	○	●	●	○	●	:	N	C	2	0	1
4.	○	●	○	○	●	●	○	●	:	N	P+C	2	1	1
5.	○	○	●	○	●	○	●	●	:	P		0	2	0
6.	○	○	●	○	●	○	●	●	:	P	N	1	2	0
7.	○	○	●	○	●	○	●	●	:	P	C	0	2	1
8.	○	○	●	○	●	○	●	●	:	P	N+C	1	2	1
9.	○	○	○	●	○	●	●	●	:	C		0	0	2
10.	○	○	○	●	○	●	●	●	:	C	N	1	0	2
11.	○	○	○	●	○	●	●	●	:	C	P	0	1	2
12.	○	○	○	●	○	●	●	●	:	C	N+P	1	1	2
13.	○	○	○	○	●	○	○	●	:	N+P		2	2	0
14.	○	○	○	○	●	○	○	●	:	N+P	C	2	2	1
15.	○	○	○	○	○	●	○	●	:	N+C		2	0	2
16.	○	○	○	○	○	●	○	●	:	N+C	P	2	1	2
17.	○	○	○	○	○	○	●	●	:	P+C		0	2	2
18.	○	○	○	○	○	○	●	●	:	P+C	N	1	2	2
19.	○	○	○	○	○	○	○	●	:	N+P+C		2	2	2
20.	●	●	●	●	●	●	●	●	:	None		0	0	0
21.	○	○	○	○	○	○	○	○	:	Other		0	0	0

Table A1.2. Guide for determining primary (1°) and secondary (2°) limiting nutrient(s) based on algal bioassay results. When visually compared to the control filter color after 2 to 3 days growth, bioassay responses are indicated by: ○ = algal response similar to control filter, ● = partial algal response, and ● = maximum algal response. Corresponding relative nitrogen (N), phosphorus (P) and/or carbon (C) fertilization input requirements are given as: 2 = full amount, 1 = half amount, and 0 = no inputs for that fertilization period. "Other" in line 21 refers to other possible limitations of algal growth such as temperature, light, and/or micronutrient availability. See text for further discussion.

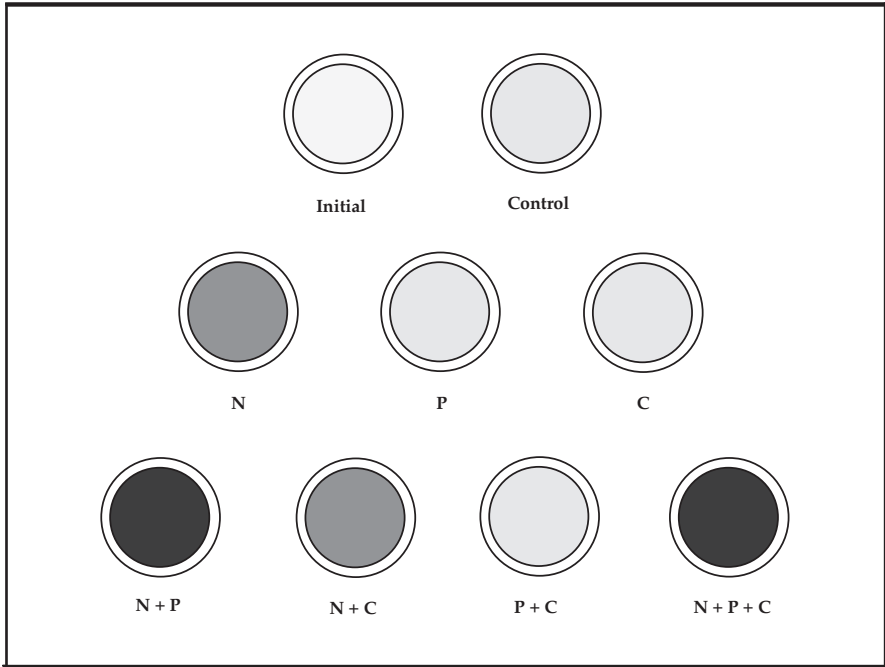
The first comparison is between the initial filter, the control filter, and the N+P+C treatment filter. If the control filter is noticeably greener than the initial filter, and looks indistinguishable when compared to the N+P+C nutrient-spiked treatment, then this result indicates that the availability of N, P, or C did not limit algal growth, and there is no need to fertilize that pond at that time (line 20, Table A1.2). If the N+P+C treatment filter is noticeably greener than the control filter, however, then there are 20 different results which are theoretically possible based on the nutrient spike combinations (Table A1.2).

The amount of greenness on the N+P+C filter represents what is considered the full response to the nutrient enrichment from spikes, and is indicated by the filled circles in Table A1.2. Other spiked samples may also show the same full response, a partial response (indicated by a half-filled circle), or a response equal to the control (i.e., no additional stimulation of algal growth, as indicated by an open circle).

Relative algal responses indicate which nutrient(s) is(are) both primarily and secondarily limiting (Table A1.2). For example, the filters illustrated in Figure A1.1 show a pond where N is primarily limiting and P is secondarily limiting. The P, C, and P+C treatments show the same response as the control, but the N+P response is greater than the N response and equal to the N+P+C response (line 2, Table A1.2). Phosphorus is secondarily limiting because it becomes limiting only after the N demand has been met; but there is not enough ambient P to satisfy algal needs once sufficient N has been added.

Table A1.2 also indicates suggested relative fertilization requirements based on algal bioassay results. If a nutrient is shown to be primarily limiting, then the pond receives the full input amount for that fertilization interval period. If a nutrient is secondarily limiting, only half the full amount is put in the pond at that time. No fertilizer is applied for a nutrient when no limitation is indicated. For example, results in Figure A1.1 indicate that the pond should be fertilized at the full rate for N, half the full rate for P, and no inputs for C for that fertilization period.

As discussed throughout this book, there is no universal recipe of "maximum" fertilization rates because of pond-specific variability. As a practical issue, however, this is not a problem. Initial maximum loading rates could be around 30 kg N ha⁻¹ wk⁻¹ and 10 kg P



Example Algal Bioassay Result

Figure A1.1. Schematic representation of results from a typical algal bioassay. Results are filter comparisons after pond water samples were spiked with nitrogen (N), phosphorus (P), and/or inorganic carbon (C). Results here show pond water with N as the primary limiting nutrient and P as the secondary limiting nutrient (see text and Table A1.2; adapted from Knud-Hansen et al., in press).

ha⁻¹ wk⁻¹, although one should check to see if ecologically applicable, predetermined fixed rates are available. Actual fertilization rates are scaled quantitatively to cover the period between algal bioassays. Nevertheless, if these rates are too high for a particular pond, then dissolved N and P will accumulate in the water and the next algal bioassay will indicate that no N or P fertilization is necessary because of the lack of nutrient limitation. Subsequent algal bioassays will reveal when a nutrient is again limiting, and should be added to the

pond to stimulate algal productivity. On the other hand, if maximum loading rates are too low for a given pond, then subsequent algal bioassays will reveal a continued demand for nutrient inputs and the farmer should fertilize accordingly.

Frequency of Algal Bioassay Testing

Algal bioassays should be conducted as frequently as reasonable and economical. Routine schedules for both algal bioassays and fertilization are certainly preferable from a farm-management perspective. If the farmer has only a few ponds, weekly analyses should be sufficient. This schedule was shown to be effective in a four-month experiment (Knud-Hansen et al., in press), as well as at the Tha Ngone farm in Lao PDR (H. Guttman, personal communication). As the farmer visually correlates algal bioassay results with pond appearance, the farmer will better understand when a pond is light-limited due to high algal productivity or high inorganic turbidity, and neither algal bioassay nor fertilization would be necessary at that time. Ultimately, the individual farmer must determine the economic trade-off between improved fertilization efficiency and the effort required to attain it.