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COMBO: a defined freshwater culture medium for algae and zooplankton

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Abstract

In order to conduct experiments on interactions between animals and food organisms, it is necessary to develop a medium that adequately supports the growth of both algae and zooplankton without the need to alter the medium to accommodate either the algae or the animals. We devised a freshwater medium, named COMBO, that supports excellent growth of both algae and zooplankton. Two types of algae, *Ankistrodesmus falcatus* and *Stephanodiscus hantzschii*, were reared in COMBO and their growth rates were not significantly different from those of algae grown in a reference medium (WC). One of these algae, *A. falcatus*, was then fed to a cladoceran, *Daphnia pulicaria*, which was also cultured in COMBO, and the resulting fecundities of *D. pulicaria* were compared to those of animals reared in natural surface water. We also determined whether the value of COMBO as a medium for *D. pulicaria* was affected by modifications in nitrogen or phosphorus concentration to evaluate whether the new medium will be useful in nutritional research. Lowering the N or P content of COMBO did not affect the reproductive performance of *D. pulicaria*. Other researchers have also reported excellent growth and reproduction by numerous algae and zooplankton reared in COMBO. Our results suggest that COMBO is an effective artificial, defined culture medium capable of supporting robust growth and reproduction of both freshwater algae and zooplankton.

Introduction

There are a number of defined freshwater media that are useful for growing a wide variety of algae (Stein, 1973; Guillard, 1975; Morel et al., 1975; Becker, 1994), but inadequate for growing zooplankton such as daphniids (Conklin & Provasoli, 1977; Keating, 1985; Elendt & Bias, 1990). In order to conduct experiments on interactions between animals and food organisms, it is necessary to develop a medium that adequately supports the growth of both algae and zooplankton without the need to alter the medium to accommodate either the algae or the animals.

Many defined freshwater algal media have been designed (see Stein, 1973 for early efforts), but four are in wide use: Guillard's WC (Guillard, 1975), Fraquil (Morel et al., 1975), ASM (Carmichael & Gorham, 1974), and DYIII (Lehman, 1976a). Numerous modifications of one or more of these media have been made to fit particular needs.

Keating (1985) and Elendt & Bias (1990) devised artificial media, which were derived from the earlier recipes of Murphy & Davidoff (1972) and Conklin & Provasoli (1977), to culture species of *Daphnia* under defined conditions for use in nutrition and toxicological experiments. Keating's medium, MS, supports excellent growth of daphniids, particularly *D. magna* (Keating, 1985). However, due to its high glycylglycine content, cultures must be aseptic, which is impractical for routine use. The Elendt & Bias (1990) medium, M-4, a hard-water medium, has been adopted by the Organization for Economic Cooperation and Development's (OECD) toxicology program in Europe and is especially useful for culturing *D. magna*, but M-4 does not successfully sustain algae. The Environmental Protection Agency in the United States has recommended the use of 'reconstituted water', a

Table 1. The composition of COMBO medium. A. The concentrations of all of the elements in the medium in weight and molar units. B. The composition of the primary stock solutions for the algal trace elements (ATE). C. The composition of the primary stock solutions for the animal trace elements (ANIMATE). D. The composition of the stock solution for vitamins (VIM; Guillard 1975).

A. Composition of COMBO

Compound	Stock ($g\ l^{-1}$)	Final medium ($mg\ l^{-1}$)	Final medium ($\mu mol\ l^{-1}$)		Elements ($mg\ l^{-1}$)	Elements ($\mu mol\ l^{-1}$)	Notes
Seven major stocks							
$CaCl_2\ 2H_2O$	36.76	36.76	250	Ca	10.0	250	As in WC
				Cl	17.7	500	
$MgSO_4\ 7H_2O$	36.97	36.97	150	Mg	3.91	150	As in WC
				SO_4	14.4	150	
K_2HPO_4	8.71	8.71	50	K	3.91	100	As in WC
				P	1.55	50	
$NaNO_3$	85.01	85.01	1000	Na	23.0	1000	As in WC
				N	14.0	1000	
$NaHCO_3$	12.60	12.60	150	Na	3.44	150	As in WC
				CO_3	9.00	150	
$Na_2SiO_3\ 9H_2O$	28.42	28.42	100	Na	4.6	200	As in WC
				Si	2.81	100	
H_3BO_3	24.00	24.00	388	B	4.6	426	As in WC
KCl	7.45	7.45	100	K	3.91	100	ONLY LOW P
Algal trace elements (ATE)							
$Na_2EDTA\ 2H_2O$	4.36	4.36	11.7	Na	0.54	23.4	As in WC
				EDTA	3.4	11.7	
$FeCl_3\ H_2O$	1.0	1.00	3.7	Fe	0.21	3.7	As in ASM
				Cl	0.39	11	
$MnCl_2\ 4H_2O$		0.18	0.9	Mn	0.05	0.9	As in WC
				Cl	0.06	1.8	
$CuSO_4\ 5H_2O$		0.001	0.004	Cu	0.00025	0.004	40% Lower
				SO_4	0.00038	0.004	
$ZnSO_4\ 7H_2O$		0.022	0.08	Zn	0.005	0.08	As in WC
				SO_4	0.007	0.08	
$CoCl_2\ 6H_2O$		0.012	0.05	Co	0.003	0.05	As in WC
				Cl	0.0018	0.1	
$NaMoO_4\ 2H_2O$		0.022	0.09	Mo	0.0086	0.09	As in WC
				Na	0.0042	0.18	
H_2SeO_3		0.0016	0.012	Se	0.001	0.012	As Elendt
Na_3VO_4		0.0018	0.01	V	0.0005	0.01	10% WC
				Na	0.0007	0.03	
Animal trace elements (ANIMATE)							
LiCl		0.31	7.3	Li	0.05	7.3	As Elendt
				CL	0.25	7.3	
RbCl		0.07	0.6	Rb	0.05	0.6	As Elendt
				Cl	0.03	0.6	
$SrCl_2\ 6H_2O$		0.15	0.57	Sr	0.05	0.57	As Elendt
				Cl	0.04	1.14	
NaBr		0.016	0.16	Na	0.004	0.16	As Elendt
				Br	0.0125	0.16	
KI		0.0033	0.02	K	0.0008	0.02	As Elendt
				I	0.0025	0.02	
Vitamins (VIM)							
B_{12}		0.00055	0.0004				As WC
Biotin		0.0005	0.002				As WC
Thiamin		0.1	0.3				As WC

Table 1. Continued.

B. Primary stock solution for algal trace elements (ATE)			
Compound	Primary Stock g 100 ml^{-1}	ATE-Stock mg l^{-1}	Final Medium mg l^{-1}
$MnCl_2$ $4H_2O$	18.0	180	0.18
$CuSO_4$ $5H_2O$	0.1	1	0.001
$ZnSO_4$ $7H_2O$	2.2	22	0.022
$CoCl_2$ $6H_2O$	1.0	10	0.010
Na_2MoO_4 $2H_2O$	2.2	22	0.022
H_2SeO_3	0.16	1.6	0.0016
Na_3VO_4	0.18	1.8	0.0018

Add 1 ml l^{-1} of each Primary Stock to ca. 750 ml of LPW containing dissolved EDTA and Fe (see A. above) and bring to 1 l to make the ATE stock solution. Add 1 ml l^{-1} of ATE to final medium.

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C. Primary stock solution for animal trace elements (ANIMATE)			
Compound	Primary Stock g 100 ml^{-1}	ANIMATE-Stock mg l^{-1}	Final Medium mg l^{-1}
LiCl	31	310	0.31
RbCl	7	70	0.07
$SrCl_2$ $6H_2O$	15	150	0.15
NaBr	1.6	16	0.016
KI	0.33	3.3	0.0033

Add 1 ml l^{-1} of each Primary Stock to ca. 750 ml LPW and bring to 1 l to make the ANIMATE stock solution. Add 1 ml l^{-1} of ANIMATE to final medium.

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D. Vitamin stock solution (VIM)	
Compound	Primary stocks
Biotin	10 mg into 96 ml LPW (keep sterile and frozen)
B_{12}	10 mg into 89 ml LPW (keep sterile and frozen)

Add 1 ml of each of the two primary stocks to 100 ml LPW and add 20 mg of Thiamine HCl to make the vitamin stock solution (VIM) (keep sterile and frozen). Add 0.5 ml l^{-1} of VIM to final medium.
WC (Guillard, 1975) Elendt (Elendt & Bias, 1990) ASM (Carmichael & Gorham, 1974)

simple four salt medium developed by Marking & Dawson (1973) for fish toxicity studies. This medium is inadequate because it lacks known essential elements (Keating et al., 1989). *Daphnia* can be grown in such media with the addition of filtered lake water or Perrier water, but this defeats the purpose of having a defined medium for experimental studies.

We devised a medium, named COMBO, that supports excellent growth of both algae and zooplankton. Two types of algae were reared in COMBO and in WC (Guillard, 1975), and their growth rates were compared. One of these algae, *Ankistrodesmus falcatus*, was then fed to a cladoceran, *Daphnia pulicaria*, which was also cultured in COMBO, and the resulting fecundities of *D. pulicaria* were compared to those of animals reared in natural surface water. We also determined whether the value of COMBO as a medium for *D. pulicaria* was affected by modifications in nitrogen or phosphorus concentration to evaluate whether the new medium will be useful in nutritional research. Our results are discussed with those of other researchers who have successfully used COMBO for culturing many other species of both algae and zooplankton.

Methods

Medium composition and preparation

COMBO medium (Table 1) is prepared from working solutions, which are in turn prepared from primary stocks. Working solutions consist of the following: seven separate major element solutions, two trace element solutions (algal trace elements [ATE] and animal trace elements [ANIMATE]) and a vitamin solution (VIM). The two trace element working solutions (ATE and ANIMATE) are prepared from separate primary stocks of individual trace elements. Similarly, VIM is prepared from two separate primary vitamin stocks (Guillard, 1975). Primary stocks of the seven major elements (Table 1A) are each prepared by dissolving the prescribed amount of chemical into 1 l (final volume) of laboratory pure water (LPW: distilled and deionized). These primary stocks can be stored in polyethylene bottles at room temperature.

For culturing algae that require ammonia as a nitrogen source, an additional stock solution of NH_4Cl may be necessary, but levels above 50 μM in the final medium should be avoided (Keller et al., 1987). In addition, for experiments wherein reduced phosphorus levels are needed (e.g. low-P COMBO used in this study), a primary stock of KCl will be required (Table 1A).

Trace element stocks are stored in polyethylene bottles at 4 °C. The seven primary stocks of algal trace elements (Table 1B; not Fe or EDTA) are each prepared by dissolving the prescribed amount of each chemical into 100 ml (final vol) of LPW. The working

Table. 2. Composition of five artificial culture media: COMBO, WC (Guillard, 1975), ASM (Carmichael & Gorham, 1974), Keating MS (Keating, 1985), and Elendt M-4 (Elendt & Bias, 1990).

Compound		COMBO	WC	ASM	Keating MS	Elendt M-4
$CaCl_2\ 2H_2O$	mg l^{-1}	36.76	36.76	29.41	38.0	293.8
	μM	250	250	200	259	2000
$MgSO_4\ 7H_2O$	mg l^{-1}	36.97	36.97	49.3	20	123.3
	μM	150	150	200	81	500
$MgCl_2\ 6H_2O$	mg l^{-1}	–	–	40.67	–	–
	μM	–	–	200	–	–
K_2HPO_4	mg l^{-1}	8.71	8.71	17.41	10	0.184
	μM	50	50	100	52	1.1
KH_2PO_4	mg l^{-1}	–	–	–	10	0.143
	μM	–	–	–	73	1.1
$NaNO_3$	mg l^{-1}	85	85	170	50	0.274
	μM	1000	1000	2000	588	0.003
$NaHCO_3$	mg l^{-1}	12.6	12.6	–	–	28.42
	μM	150	150	–	–	772
$Na_2SiO_3\ 9H_2O$	mg l^{-1}	28.42	28.42	–	10.0	10.0
	μM	100	100	–	35	35
H_3BO_3	mg l^{-1}	24.0	24.0	2.47	1.0	2.9
	μM	388	388	40	16.2	47
KCl[1]	mg l^{-1}	7.45	–	–	10	0.58
	μM	100	–	–	134	7.79
Na_2EDTA	mg l^{-1}	4.36	4.36	7.44	5.0	2.5
	μM	11.7	11.7	20.0	13.4	6.7
$FeCl_3\ 6H_2O$	mg l^{-1}	1.0	3.15	1.08	2.0	–
	μM	3.7	11.7	4.0	7.4	–
$FeSO_4\ 7H_2O$	mg l^{-1}	–	–	–	–	1.0
	μM	–	–	–	–	3.6
$MnCl_2\ 4H_2O$	mg l^{-1}	0.18	0.18	1.385	0.72	0.36
	μM	0.9	0.9	7.0	3.6	1.8
$CuSO_4\ 5H_2O$	mg l^{-1}	0.001	0.0025	0.0002	–	–
	μM	0.004	0.0098	0.00075	–	–
$CuCl_2\ 2H_2O$	mg l^{-1}	–	–	–	0.068	0.017
	μM	–	–	–	0.4	0.1
$ZnSO_4\ 7H_2O$	mg l^{-1}	0.022	0.022	0.92	–	–
	μM	0.08	0.08	3.2	–	–
$ZnCl_2$	mg l^{-1}	–	–	–	0.052	0.013
	μM	–	–	–	0.025	0.006
$CoCl_2\ 6H_2O$	mg l^{-1}	0.012	0.01	–	0.021	–
	μM	0.05	0.042	–	0.088	–
$CoSO_4\ 6H_2O$	mg l^{-1}	–	–	0.019	–	–
	μM	–	–	0.072	–	–
$NaMoO_4\ 2H_2O$	mg l^{-1}	0.022	0.0063	0.01	0.05	0.063
	μM	0.09	0.026	0.041	0.205	0.163
H_2SeO_3	mg l^{-1}	0.0016	–	0.0013	–	–
	μM	0.012	–	0.01	–	–
SeO_2	mg l^{-1}	–	–	–	0.001	–
	μM	–	–	–	0.009	–

Table. 2. Continued.

Compound		COMBO	WC	ASM	Keating MS	Elendt M-4
Na_2SeO_3	mg l^{-1}	–	–	–	–	0.0022
	μM	–	–	–	–	0.013
Na_3VO_4	mg l^{-1}	0.0018	0.018	–	–	–
	μM	0.01	0.1	–	–	–
NH_4VO_3	mg l^{-1}	–	–	–	0.0005	0.0006
	μM	–	–	–	0.004	0.005
LiCl	mg l^{-1}	0.31	–	–	0.5	0.31
	μM	7.3	–	–	11.8	7.3
RbCl	mg l^{-1}	0.07	–	–	0.14	0.07
	μM	0.6	–	–	1.2	0.6
$SrCl_2$ $6H_2O$	mg l^{-1}	0.15	–	–	0.3	0.15
	μM	0.57	–	–	1.2	0.57
NaBr	mg l^{-1}	0.016	–	–	0.064	0.016
	μM	0.16	–	–	0.62	0.16
KI	mg l^{-1}	0.0033	–	–	0.022	0.0033
	μM	0.02	–	–	0.13	0.02
B_{12}	mg l^{-1}	0.00055	0.00055	0.005	0.001	0.001
	μM	0.004	0.004	0.0036	0.007	0.007
Biotin	mg l^{-1}	0.005	0.005	0.005	0.0008	0.0008
	μM	0.002	0.002	0.002	0.0003	0.0003
Thiamine HCl	mg l^{-1}	0.1	0.1	0.0001	0.075	0.075
	μM	0.3	0.3	0.0003	0.225	0.225

1. KCl is used only for low-P cultures in COMBO.

solution of algal trace elements (ATE) is then prepared by dissolving Na_2EDTA into ca. 750 ml of LPW, adding and dissolving the $FeCI_3$ $6H_20$, adding 1 ml of each of the seven algal primary stocks, and then bringing the ATE to 1 l with LPW. The EDTA solution must be dissolved before the Fe is added, or insoluble precipitates will form. The ratio of chelator:metal in the ATE working solution is 2.4:1, which Keller et al. (1987) suggested is ideal.

Each of the five primary stocks for the animal trace elements (Table 1C) is prepared by dissolving the specified amount of chemical into 100 ml (final vol) of LPW. The working solution of animal trace elements (ANIMATE) is then prepared by adding 1 ml of each of these five primary stocks to ca. 750 ml of LPW, and then bringing the total volume to 1 l with LPW. Vitamins (Table 1D) are prepared according to Guillard (1975). Primary stocks are made of biotin (10 mg dissolved in 96 ml of LPW; keep sterile and frozen) and B_{12} (10 mg dissolved in 89 ml of LPW; keep sterile and frozen). The working vitamin solution (VIM) is made by adding 1 ml of each of biotin and B_{12} stocks to 100 ml LPW and adding 20 mg of thiamine-HCI (no primary stock of thiamine is necessary). Dispense the working VIM solution into 10 ml aliquots, autoclave, and store in a refrigerator.

COMBO medium is prepared by adding the following to 750 ml LPW: a) 1 ml of each of the seven major element working solutions, b) 1 ml of ATE, c) 1 ml of ANIMATE, d) 0.5 ml of VIM. The volume is then increased to 1 l. COMBO medium should be sterilized before use and filter sterilization reduces the potential for precipitates to form. If autoclaving is used, rapid cooling in a flowing water bath is recommended. We have found the pH of COMBO to be about 8, but other researchers have found the pH to be higher (> 9) and subsequently had to titrate it with HCl (Urabe et al., 1997; R. Sterner, pers. com.) or H_2SO_4 (K. Schulz, pers. com.) to between 7–8. This acidification has not been found to alter the quality or usefulness of COMBO.

A buffer may be added to COMBO if desired. TES (N-tris[hydroxymethyl]-methyl-2aminoethane sulfonic acid) was found not to affect algal growth rates when prepared at 200 mg l^{-1}, but effects of this buffer have not been tested on zooplankton. Buffering with TES

does not support the growth of bacteria (S. S. Kilham, pers. observ.), which is important to maintain water quality, and apparently does not affect metal speciation.

Growth responses

Algae. Growth rates (day^{-1}) of two species of representative freshwater algae were compared; a green alga *Ankistrodesmus falcatus* (clone from Academy of Natural Sciences, Goulden et al., 1982) and a diatom *Stephanodiscus hantzschii* (clone from Czarnecki collection, Loras College, Dubuque IA). Growth media were WC (Guillard, 1975; with additions of B, Se and V; see Table 2), and COMBO (Table 1). Algae were cultured at 20 °C with a 14:10 hr light:dark cycle, and ca. 100 μmol m^{-2} s^{-1} of light provided by cool-white fluorescent bulbs. Cultures were maintained in 25 mm diameter glass culture tubes with Kimex caps. The *in vivo* fluorescence of well-mixed tubes was measured (Turner Designs Fluorometer) daily (Brand et al., 1981) for 16 d, and the maximum growth rate calculated as the slope of ln fluorescence units day^{-1} during the time of greatest growth rate (days 2–5 for *A. falcatus* and days 3–11 for *S. hantzschii*). There were four replicate cultures for each treatment. Statistical comparisons of algal growth in the two different culture media were performed with Student's t-tests (alpha = 0.05).

Cladocera. Daphnia pulicaria were acclimated to COMBO medium for at least two generations prior to experiments. Approximately 10–20 *D. pulicaria* were maintained in 1 l borosilicate beakers, each containing 700 ml of COMBO. Since algal cell sizes often vary over time, algal rations for *D. pulicaria* were standardized based on cell volume (Coulter Multisizer), thus maintaining consistant diet quantity (Kreeger & Langdon, 1993). Animals were fed 1.6×10^7 μm^3 ml^{-1} of *A. falcatus*, grown in COMBO, which was provided three times per week. Stock cultures of *A. falcatus* for feeding daphniids were 600 ml in 1 l borosilicate flasks, which were cultured as above, and diluted once per day to maintain a maximum growth rate.

Culture Experiment #1 with *D. pulicaria* compared the value of COMBO medium to that of natural water known to be suitable for daphniids (Goulden et al., 1982). At the start of the experiment, newly released ($\leq$ 24 h old) *D. pulicaria* were isolated from stock animal cultures and individually added to borosilicate beakers containing 60 ml of either sterile filtered Round Valley Reservoir Water (RV) (Hunterdon Co., NJ), or COMBO medium. Twenty replicates (individuals) were used for each treatment. In addition to comparing the two differing water types, experimental treatments varied in food ration of *A. falcatus* (grown in COMBO) delivered at either 5.0×10^5 μm^3 ml^{-1} d^{-1} (low ration) or 1.25×10^7 μm^3 ml^{-1} every other day (high ration). Individual animals were examined for 21 d; offspring were counted and removed daily. Standard life table methods were used to calculate the mean fecundity (total offspring per surviving female during the entire experiment), average number of broods, average brood size and average time until first brood for animals in each treatment (Goulden et al., 1982). Zooplankton survivorship and fecundity data (average fecundity, average time until first brood, average number of broods and average brood size) were compared with 2-way ANOVA (alpha = 0.05; main effects, water type, ration) using Statgraphics (Version 6.0) and Type III sum-of-squares.

Two experiments were performed to examine whether manipulation of the N or P concentration of COMBO medium affected zooplankton performance. In culture Experiment #2, *D. pulicaria* were fed *A. falcatus* at a ration of 1.57×10^7 μm^3 ml^{-1} in 100 ml beakers. Juvenile ($\leq$24 hours old) *D. pulicaria* were individually added to separate beakers each containing algal food in 60 ml of either low-N COMBO medium or standard COMBO. Both treatments consisted of 12 replicates, each in an individual beaker. The N concentration of low-N COMBO was 5 μM (0.5% of standard COMBO). The same approach was followed in culture Experiment #3 (phosphorous experiment) as in Experiment #2 (nitrogen experiment), except that the nutrient-altered COMBO contained 10% of the P in standard COMBO (5 μM).

For both Experiments #2 and #3, animals were transferred to fresh medium three times each week (Monday, Wednesday, Friday), and were fed four times per week (Monday, Wednesday, Friday and Sunday). Beakers were checked daily, for 21 d, for newly produced juveniles, and any offspring were removed and counted. For each experiment, average fecundity, average time until first brood, average number of broods and average brood size were statistically compared between media types with Student's t-tests (alpha = 0.05) using Statgraphics (Version 6.0).

Table. 3. Comparisons of maximum growth rates of two algae, *Ankistrodesmus falcatus* and *Stephanodiscus hantzschii* grown in WC (Guillard, 1975) and COMBO. Values are means (± SD).

Species	Replicates	μ_{max} WC (d^{-1})	μ_{max} COMBO (d^{-1})	*t*-test *p*-value
A. falcatus	4	1.31 (0.03)	1.34 (0.03)	NS
S. hantzschii	4	0.39 (0.08)	0.36 (0.09)	NS

NS = not significant; $p > 0.05$.

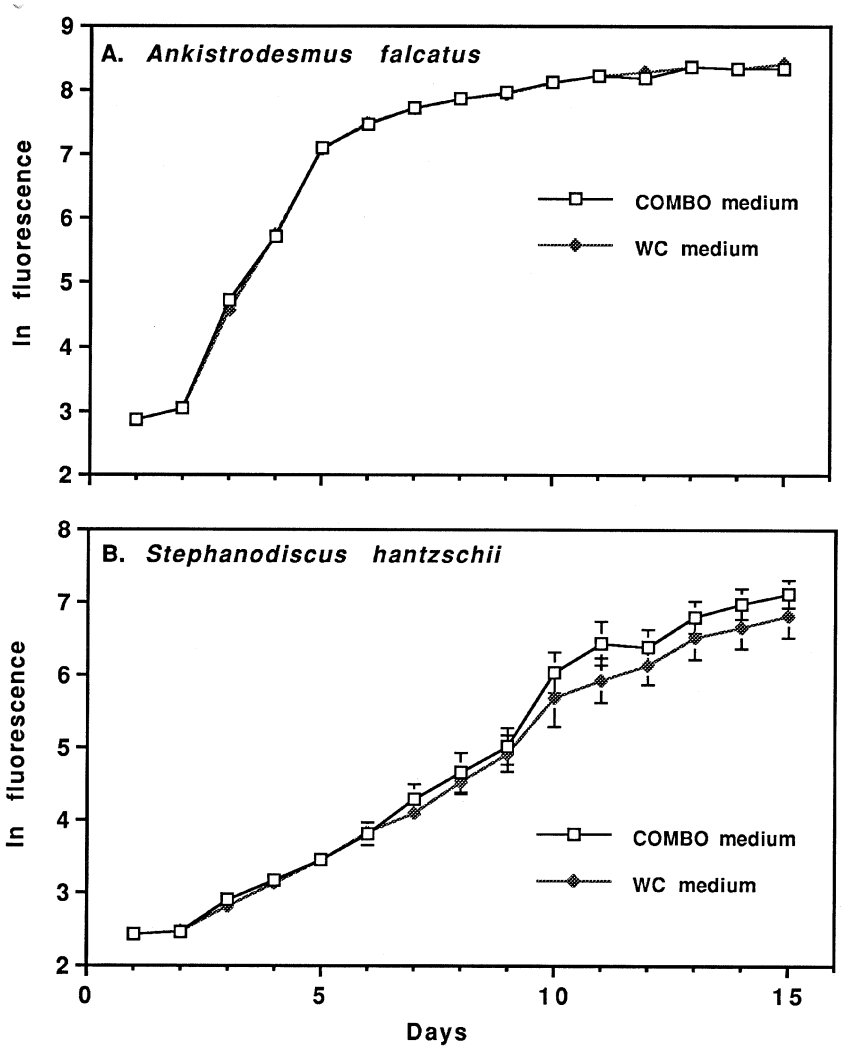

Figure 1. Growth of *Ankistrodesmus falcatus* (a) and *Stephanodiscus hantzschii* (b) in either COMBO or WC media. The means (± SD, $n = 4$) of maximum growth rates were 1.34 (0.03) in COMBO and 1.31 (0.03) in WC for *A. falcatus*, and 0.36 (0.09) in COMBO and 0.39 (0.08) in WC for *S. hantzschii*.

Table. 4. Results of life history characteristics of *D. pulicaria* reared in either COMBO or natural surface water (RV Water). Values are means (± SD). *p*-values are for 2-way ANOVA with water type and food ration size as main effects. Low ration size was significantly lower ($p < 0.001$) than the high ration size for each of the life history characteristics.

Life history characteristics	Ration size	COMBO	RV water	2-way ANOVA: water type
Average fecundity per female	Low	11.1 (2.44)	14.2 (3.21)	$p = 0.0006$
(# of neonates)	High	79.6 (23.1)	101 (13.1)	
Average time until first brood	Low	12.1 (1.73)	10.4 (0.99)	$p = 0.0001$
(days)	High	7.67 (0.72)	6.95 (0.52)	
Average number of broods	Low	4.00 (0.61)	4.60 (0.51)	$p = 0.008$
per female	High	6.73 (0.46)	6.83 (0.38)	
Average brood size	Low	2.79 (0.61)	3.07 (0.50)	$p = 0.0004$
(# of neonates)	High	11.7 (3.08)	14.8 (1.35)	

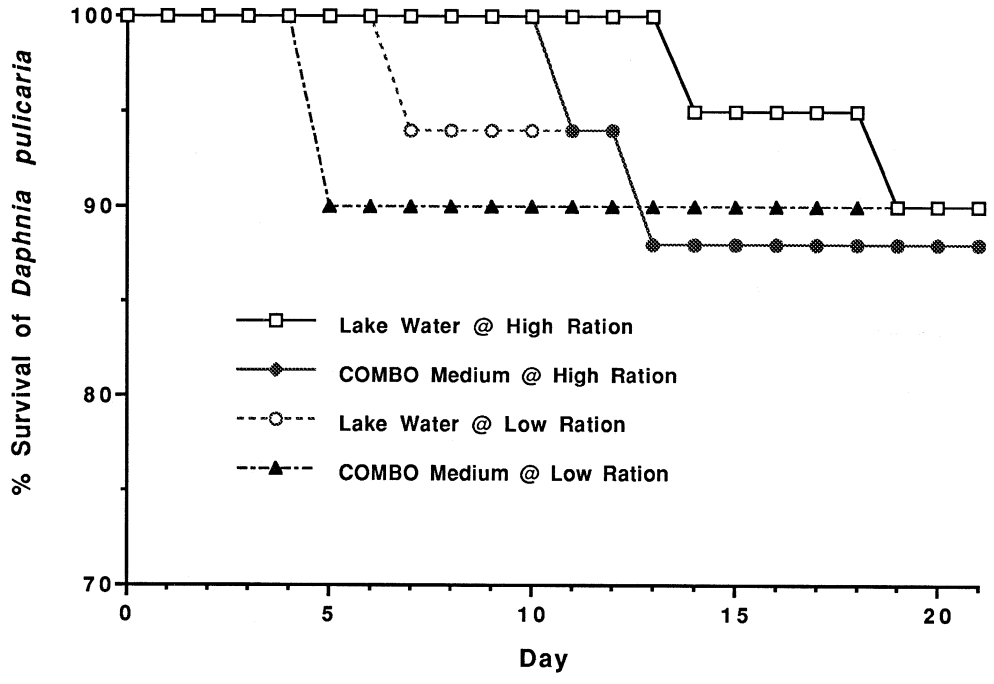

Figure 2. Survivorship of *Daphnia pulicaria* cultured for 21 d in either COMBO medium or natural lake water (RV) and fed either a high or low ration of algae.

Results

Growth of the two algal species on the two media are compared in Figure 1. Importantly, neither algal species exhibited a significant difference in growth rate between the two media (Table 3). Maximum growth rates of diatoms were more variable (± 20%) than for green algae (± 2%), however high variability in maximum growth rates of diatoms is often observed (Brand, 1981; S. S. Kilham, pers. observ.).

Survivorship of *D. pulicaria* in each of the four treatments of Experiment #1 is presented in Figure 2. One or two individuals died during the 21 d test in each treatment, but there was no significant difference ($p > 0.05$) in survivorship among treatments. Of those animals that died, individuals reared in COMBO died 2 or 3 d earlier than animals reared in RV, regardless

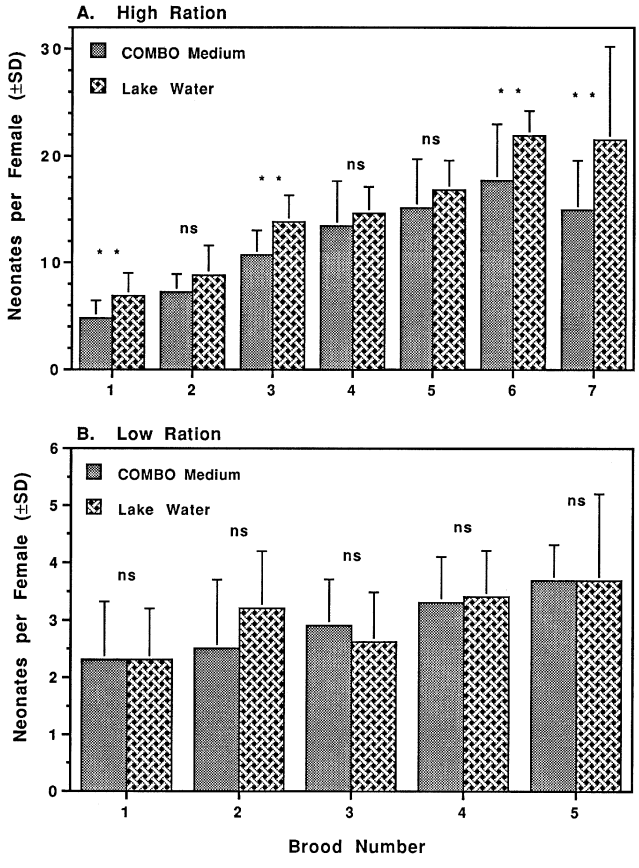

Figure 3. Average brood sizes of *Daphnia pulicaria* cultured in either COMBO medium or natural lake water (RV) and fed either a high (a) or low (b) ration of algae. Asterisks (∗∗) indicate a statistically significant difference between treatments.

of whether they were provided with a low or high food ration.

The amount of time required for *D. pulicaria* to release its first brood differed significantly between both ration size (low vs high) and medium type (COMBO vs RV) (Table 4). There were also significant differences in the number of broods during the 21 d test (Table 4). We also observed that *D. pulicaria* fed the high algal ration had significantly greater brood sizes in RV water than in COMBO (Figure 3a). Four of the seven broods produced by animals in the RV cultures were larger than broods from the animals cultured in COMBO medium; differences varied from 2 offspring per female in the first brood to 4–5 offspring per female in later broods. These differences in brood sizes did not occur when animals were fed a low ration of food (Figure 3b). The overall fecundity of *D. pulicaria* reared for 21 d on COMBO was also lower than *D. pulicaria* reared on RV water (Table 4).

The results of the nutrient manipulation experiments clearly show that lowering the N or P content of COMBO does not affect the reproductive performance of *D. pulicaria*. In Experiment #2, there was no significant difference in average fecundity between animals reared in COMBO (47.6) or in low-N COMBO (48.5). Other measures of reproductive performance such as average time until first brood (low-N = 9.1 d, COMBO = 8.8 d), average number of broods in the 21 d experiment (low-N = 4.9, COMBO = 5.0) and the average brood size (low-N = 9.9, COMBO = 9.5) also did not differ significantly among treatments. The results of Experiment #3 were similar. There was no significant difference in average fecundity between animals reared in COMBO (63) or in low-P COMBO (71). Other measures of reproductive performance such as average time until first brood (low-P = 8.2 d, COMBO = 8.3 d), average number of broods in the 21 d experiment (low-P = 5.9, COMBO = 5.3) and the

average brood size (low-P = 12, COMBO = 12) also did not differ significantly among treatments.

Discussion

COMBO medium was devised to provide a defined freshwater medium suitable for culturing both algae and daphniids. A number of choices were made in composing COMBO, and we detail the reasoning here.

Major elements

The major elements include the seven major ions of natural freshwater (Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}, HCO_3^{-}, SO_4^{2-}, and Cl^{-}) as well as the major nutrient elements (N, P, Si, B). Concentrations of these elements are the same as in Guillard's WC (Guillard, 1975). Differences in elemental composition from Fraquil (Morel et al., 1975) are as follows: Fraquil has only 20% of the K and Na, only 10% N, 12.5% Si and no B, but 2 times the P. The differences from ASM medium (Carmichael & Gorham 1974) are as follows: ASM has much greater Na, Cl and Mg, similar amounts of Ca, twice the K, P and N, but lacks additions of Si, B and HCO_3^{-}. Differences with DYIII (Lehman 1976a) are as follows: DYIII has double the Ca and one third more Mg, but lower Na (only 20%) and K (only 40%), and lower nutrients (but including Si and B). Typical zooplankton culture media (e.g., see Elendt & Bias 1990) have lower concentrations of major nutrients than are found in algal culture media [Note: there is a mistake in Table 2 of Elendt and Bias 1990: the nutrient ion concentrations (C in Table 2) are 100 fold concentrated, not 1000 fold concentrated as stated (Elendt, pers. com.)].

Concentrations of these major elements in COMBO medium are suitable for culturing both algae and zooplankton. An important consideration in devising COMBO was the Ca:Mg ratio. Becker (1994) reported that algae tolerate a wide range of Ca:Mg ratios; however, Lehman (1976b) determined that growth of *Dinobryon sertularia* was optimal when the molar Ca:Mg ratio was 2:1. For zooplankton, the Ca:Mg molar ratio of Keating's medium (MS) is 3.2:1 and the ratio for Elendt and Bias' medium is 4:1, both of which are somewhat higher than other media; these hard-water media were basically designed for culturing *Daphnia magna*, a species adapted for hard water habitats. As in WC and Fraquil, the molar Ca:Mg ratio of COMBO is 1.7:1.

Because COMBO has been used successfully in our lab to culture *D. magna* as well as *D. pulicaria* and *Ceriodaphnia dubia*, we have found the molar Ca:Mg ratio of COMBO is suitable for dual culturing a wide suite of both algae and zooplankton. In testing COMBO, other researchers have also reported excellent reproduction by *Daphnia pulex*, *D. pulicaria*, *D. galeata mendotae*, *D. lumholtzi* and *D. magna*, and importantly, these cultures have been maintained on COMBO for more than two years (S. Dodson pers. com.). Other cladocera successfully reared in COMBO include *Bosmina longirostris* (K. Schulz pers. com.) and *Diaphanosoma* (R. Sterner pers. com.), and in addition to cladocerans, our lab has maintained planaria and mixed rotifers in COMBO. Some researchers have suggested that COMBO can be just as effective when diluted by 50% with distilled water (S. Dodson pers. com.), however we do not recommend this dilution for hard water species such as *D. magna*.

The K concentration of COMBO (3.9 mg l^{-1}) is much lower than that in the zooplankton medium of Keating (1985) (9.8 mg l^{-1}) and slightly greater than that in the medium devised by Elendt & Bias (1990) (3.2 mg l^{-1}). We selected the lower K concentration because it is known to be suitable for zooplankton (i.e. Elendt & Bias 1990), and is not high enough to be toxic to most algae. Those wishing to grow chrysophytes, however, might consider the results of Lehman (1976b) who found that K became toxic to *Dinobryon sertularia* above 5 mg l^{-1}. We have successfully isolated and maintained the chrysophyte *Synura petersenii* in COMBO (S. J. Interlandi, pers. com.). This species had previously been successfully cultured in WC medium with half the normal trace elements and 25 μM P (Klaveness & Guillard 1975). We have also maintained numerous species of cyanobacteria, cryptophytes, green algae and diatoms in COMBO. Table 5 lists the maximum growth rates of selected algae cultured in COMBO medium and the corresponding culture conditions. Other algae include *Staurastrum* sp., *Diogenes* sp., *Selenastrum* sp., *Cryptomonas ovata* and *Cryptomonas reflexa*. Other researchers have used COMBO to grow *Chlamydomonas*, *Selenastrum* (S. Dodson, pers. com.), *Scenedesmus acutus* (R. Sterner, pers. com.) and *Chlamydomonas reinhardii* (K. Schulz, pers. com.).

COMBO has proved to be an excellent medium for nutrient manipulation experiments involving both algae and zooplankton (Kilham et al., 1997a; Kilham et al., 1997b; Urabe et al., 1997). Because P is deliv-

Table. 5. A list of algal species grown in our laboratory with culture source, culture temperature, corresponding maximum growth rate ($\mu_{\max}$), standard deviation (SD), and sample size (n). Cultures marked with an asterisk (*) are axenic.

Algal species	Source	Temp °C	μ_{max} (d^{-1})	SD	n
Aphanizomenon flos-aquae	Waynewood[1]	20	0.32[a]	0.02	8
Asterionella formosa	Yellowstone[2]	15	0.59[b]	0.02	7
Aulacoseira subarctica	Yellowstone[3]	15	0.58[b]	0.12	4
Cyclotella bodanica	Yellowstone[4]	15	0.48[b]	0.06	14
Cyclotella cryptica 1269*	Texas[5]	16	0.74[c]	0.02	4
Fragilaria crotonensis	Yellowstone[2]	15	0.67[b]	0.04	9
Microsystis aeruginosa LB2385	Texas[5]	30	0.87[d]	0.01	5
*Nitzschia frustulum**	Keating[6]	16	1.15[c]	0.04	4
Stephanodiscus minutulus	Yellowstone[4]	15	0.84[b]	0.05	7
Stephanodiscus niagarae	Yellowstone[3]	15	0.56[b]	0.06	6
Stephanodiscus yellowstonensis	Yellowstone[4]	15	0.66[b]	0.04	7

[a] (S. Datta, pers. com.); [b] (H. Roh, pers. com.); [c] (S. G. Lynn, pers. observ.); [d] (R. D. Colau, pers. com.).
[1] Isolated from Lake Waynewood, Poconos PA by S. Datta.
[2] Isolated from Lewis Lake, Yellowstone National Park WY by Theriot E. C. & S. J. Interlandi.
[3] Isolated from Jackson Lake, Yellowstone National Park WY by Therior E. C. & S. J. Interlandi.
[4] Isolated from Yellowstone Lake, Yellowstone National Park WY by Therior E. C. & S. J. Interlandi.
[5] Ordered from the Culture Collection of Algae, Department of Botany, The University of Texas at Austin, Austin TX 78713-7640, USA.
[6] Kindly provided by K. I. Keating, Department of Environmental Science, Rutgers University, New Brunswick NJ 09903, USA.

ered as K_2HPO_4, when lowering the P concentration of COMBO for use in such manipulation experiments, we recommend supplementing K concentrations with KCl (Table 1A). The added Cl is not significant. Similarly, because N is delivered mainly as $NaNO_3$, when COMBO is prepared for low N experiments, the Na levels also become lower, but as with Cl, this is also unlikely to be a problem (Becker 1994).

Trace elements

COMBO medium has two different trace element stocks. Algal trace elements (ATE) include EDTA, Fe, and seven minor elements. The animal trace elements (ANIMATE) include five additional minor elements. There can be a narrow range between deficiency and toxicity for some minor elements, and more detailed studies in this area would be desirable for freshwater algae. An appropriate trace element solution should provide sufficient concentrations to avoid limitation at high algal biomass, but not approach toxicity at low biomass. Algae can concentrate metals to a great extent (1000 fold), however the resulting high metal concentrations in algae can be detrimental to animals that eat them, so a balance must be struck between deficiency and toxicity. Morel et al. (1975) designed the low trace element mixture of Fraquil to thermodynamically reduce precipitation of various solids. Although the thermodynamic equilibrium condition causes precipitation, the rate is so low that for practical purposes it is unimportant.

EDTA levels (Table 2) are the same as in WC (11.7 μM; Fraquil = 5.0 μM; ASM and DY Ill are higher than WC; Keating is higher and Elendt and Bias is lower). A chelator:metal ratio of approximately 2.4:1 has been suggested as ideal (Keller et al., 1987). Iron concentrations are the same as in ASM, lower than WC (35%), but somewhat greater than in Fraquil, DYIII, or the media of Keating (MS) or Elendt and Bias (M-4). The concentration chosen was a compromise, because iron is a key component of metabolism, especially for cytochromes, pigment synthesis and nitrogen metabolism.

The seven minor elements required by algae (ATE) are Mn, Cu, Zn, Co, Mo, Se, and V. Suggested concen-

trations are those that Guillard (1975) recommended for the F/2 trace elements, with new additions of Se and V (Guillard & Hargraves, 1993), and a much lower Cu level. Bioassay experiments (S. S. Kilham, pers. observ.) have shown that the Cu level of WC is within a factor of 5 of concentrations that can depress growth rates of several diatom species. Hence, the Cu level in ATE is lower than in the media of Keating or of Elendt and Bias. Trace elements in Fraquil are in all cases lower than COMBO; sometimes much lower.

The five additional minor elements in ANIMATE (Table 1C) are apparently essential for animals, but have no effect on the algae (their addition neither depresses nor stimulates algal growth; Figure 1). Elendt & Bias (1990) demonstrated that Se was an essential trace element for growth of daphniids. They also showed that trace elements in their medium M-4 (similar to ANIMATE) could be increased 4 × or decreased 0.1 × with no effect on the reproduction of daphniids. Additions of Ni, Al, F and Sn to Elendt and Bias' M-4 medium did not further enhance the reproductive capacity of the animals.

Due to the varying requirements of different animals, it is unlikely that any universally acceptable culture medium can be prepared for all zooplankton. For example, our observations suggest that unaltered COMBO is not as suitable of a medium as the M-4 medium of Elendt and Bias for *D. magna*, which is best adapted for hard water. COMBO provides better growth for more soft-water animals, such as *D. pulicaria* and *C. dubia*. However, *D. magna* has been cultured successfully with minor modifications to COMBO ($3 \times CaCl$, $3 \times MgSO_4$, $10 \times NaHCO_3$), making it a suitable hard-water medium (K. Baer, pers. com.). This is an important observation because water hardness can affect the sensitivity of aquatic animals to toxicants (Rand, 1995).

COMBO supported rates of algal growth statistically comparable to those in Guillard's (1975) WC medium and supported fecundity of zooplankton close to that in natural lake water. The benefits of COMBO are that it is completely defined, readily manipulated for nutrition research, and superior to any other artificial zooplankton medium. Artificial media are essential since their composition is wholly known and can be maintained among studies, whereas the composition of natural waters is variable. Our results suggest that COMBO is an effective artificial, defined culture medium capable of supporting robust growth and reproduction of both freshwater algae and zooplankton.

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