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Abstract - Summary

The production of phytotoxic air pollutants from 15 liter (Euglena gracilis) or 30 liter (Chlorella vulgaris) cultures has been studied. Effluent air from algal cultures was passed directly to plexiglas-walled plant chamber. Bean, tobacco, mustard green, cantaloupe and wheat plants all showed injury when fumigated with the algal gases for 4 hrs. Only coleus plants showed any resistance to the gases. A closed or recycled-air system is not required to produce plant injury from algal-effluent air. Typical injury symptoms for a dicotyledonous plant include: wilting, waterlogged appearance, petiole dessication and collapse, and ultimately complete leaf dessication. Younger leaves were slightly more resistsant to injury.

Using bean (Phaseolus vulgaris) plants as a bio-indicator, further studies showed that the pollutant(s) are readily trapped in acidic solutions, in 0-4C traps, or on vermiculite, $CaCO_3$ or Dri-Rite columns. The pollutant(s) are partially re-released from an acidic solution by the addition of adequate base to neutralize the solution. Maximum pollutant(s) production from Euglena cultures occurs after cell division has stopped in either photosynthetically or non-photosynthetically active cells. The levels of ethylene, ethane, hydrogen cyanide or nitrogen oxides in effluent air from Euglena or Chlorella cultures were at or below the limit of sensitivity of assay procedures used in this study. More importantly, these levels are also below the known threshold levels for these gases in producing visible injury in higher plants. Volatile amines or ammonia are discussed as possible candidates for future study.

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Introduction:

It has been necessary to create artificial environments which can be completely isolated from the external environmental stresses in order to utilize environments which are increasingly hostile to biological processes. Great success has been achieved in simulating natural environments in closed systems for either humans, higher plants or microbes. In the development of closed systems compatible to a wider range of living organisms, unforeseen problems have become evident. Korotayev _et al_. (1) and Gitel'son _et al_. (2) have reported that in a closed system with algal cultures and higher plants, there is a marked reduction in growth of the higher plants with eventual plant death. The plants appear to recover once the two systems are separated from each other which suggests the possibility of an air pollutant being introduced by the algal culture into the common air stream. Algae and higher plants are known to produce volatile by-products. The production of even the smallest amounts of toxic substances will accumulate over time in a closed system and would result in injurious effects on the most sensitive component of the closed system. Only after careful identification and evaluation of the production rates of potentially-toxic gases by algae will it be possible to recommend changes which would permit the integration of algal and higher plant production units in closed systems.

Algal Strains

The single-celled green algae used in this study included <u>Chlorella vulgaris</u> var., Berlin strain, obtained from Dr. B. Vennesland, Berlin, and <u>Euglena gracilis var bacillaris</u> obtained from Dr. J. A. Schiff, Brandeis University, Waltham, MA.

Culture Conditions

<u>Chlorella</u> was cultured photoautrophically on defined medium described by Funkhouser <u>et al</u> (3): in % w/v, 0.5% $MgSO_4$; 0.25% KH_2PO_4 0.2% NaCl; 0.2% KNO_3, 0.05% $Ca(NO_3)_2$ and micronutrients, adjusted to a pH 4.3. The medium was aerated with 5% (v/v) CO_2 supplemented air at a flow rate of 1000 cc/min. Constant culture temperature was maintained by setting the 15-liter culture flasks in an aquarium with recirculating water maintained at 22^{o}C.

Light was supplied by 6 flood lamps (30 Watts each) equally spaced around the culture flask. Light was supplied continuously and measured 145 microeinsteins/m^2/sec. at the surface of the culture flask.

<u>Euglena</u> was cultured mixotrophically on the following defined medium as described by Hutner (4): in % w/v, 0.08 % KH_2PO_4, 0.1% $MgSO_4 \cdot 7H_2O$, 0.001% $FeCl_3$, 0.0002% thiamine, 0.04ug% vitamin B_{12}, and0.04% $(NH_4)_2HPO_4$ adjusted to pH 3.5. The medium was aerated with 500cc/min of air when grown in 15-liter flasks. Cultures were maintained at 26-27C, with continuous light supplied by 8 cool white fluorescent lamps (20 Watts each) placed around the flask. Light intensity was 125 microeinstein/m^2/sec.

Chlorella cell density was monitored by the packed cell volume method and for Euglena cells by the Coulter counter method.

Chlorophyll was determined in Euglena by taking a known volume of the culture, centrifuging to collect the cells (100xg, 5 min), adding a pinch of $MgCO_3$ to neutralize the remaining culture medium, and re-suspending the cells in 80% acetone. The solution was incubated overnight, centrifuged to remove cell walls, and the solution was read at 645 and 668 nm to determine the chlorophyll content (5).

Preliminary studies showed that pinto bean (Phaseolus vulgaris cv 'Pinto') seedlings were sensitive to the effluent air from algal cultures, and were used for pollutant bioassay.

Phaseolus vulgaris 'Pinto' seeds were planted in 14-oz. styrofoam cups filled with vermiculite. Two holes were punched one inch from the base of the cup to permit drainage. Seeds were germinated at 26C. After germination had begun, at 5-6 days, the pots were transferred to a controlled-environment growth chamber until at least 14 days after planting. Chamber conditions were 12 hrs light/dark, with light temperatures at 26C and dark temperatures at 20C. Humidity was not controlled and was 30-50% relative humidity during the light period and 90-100% relative humidity during darkness. Plants were watered daily with 1/4 strength Hoagland's solution.

Fumigation chamber:

The fumigation chamber was of clear plexiglas and measuring 30 cm X 30 cm X 36 cm high. On one side wall, an inlet tube permitted fumigation from another source such as the algal culture (Figure 1). Air could exit from the chamber through a small hole on the wall opposite the inlet tube. The inlet and outlet openings were the same diameter. Generally from 2 to 4 pots could easily fit inside the chamber without crowding the foliage. White light supplied by a mixture of incandescent and fluorescent lamps provided 125 microeinsteins /m^2/sec at mid-plant height. Temperature was 26C, and there was 90-100% relative humidity since inlet gases had bubbled through algal culture medium. After plants were placed within the chamber, a plexiglas lid was clamped into place using large spring clamps. The lid was lined with a 1/2-inch band of soft styrofoam to form an airtight seal around the lid.

Ethylene and Ethane Analysis:

Ethylene and ethane were assayed using a gas chromatograph coupled to a flame ionization detector. Ambient levels were determined by sampling the effluent air and injecting 1ml directly onto a poropak column with the following conditions: 75^{o}C, hydrogen 50cc/min, air 400cc/min, nitrogen (carrier gas) 60 cc/min.

Ethylene and ethane standards were used to establish elution times and to calibrate the detector responsiveness to known quantities of gases. For ethylene the minimum level of detection was 50 ppb.

Cyanide assay

The effluent gases were passed through 50 to 250 ml of 6N NaOH in a gas-bubbling flask. The cyanide ion content was determined using the Guilbault and Krammer method (6). This method provides a linear sensitivity in nanomolar quantities of

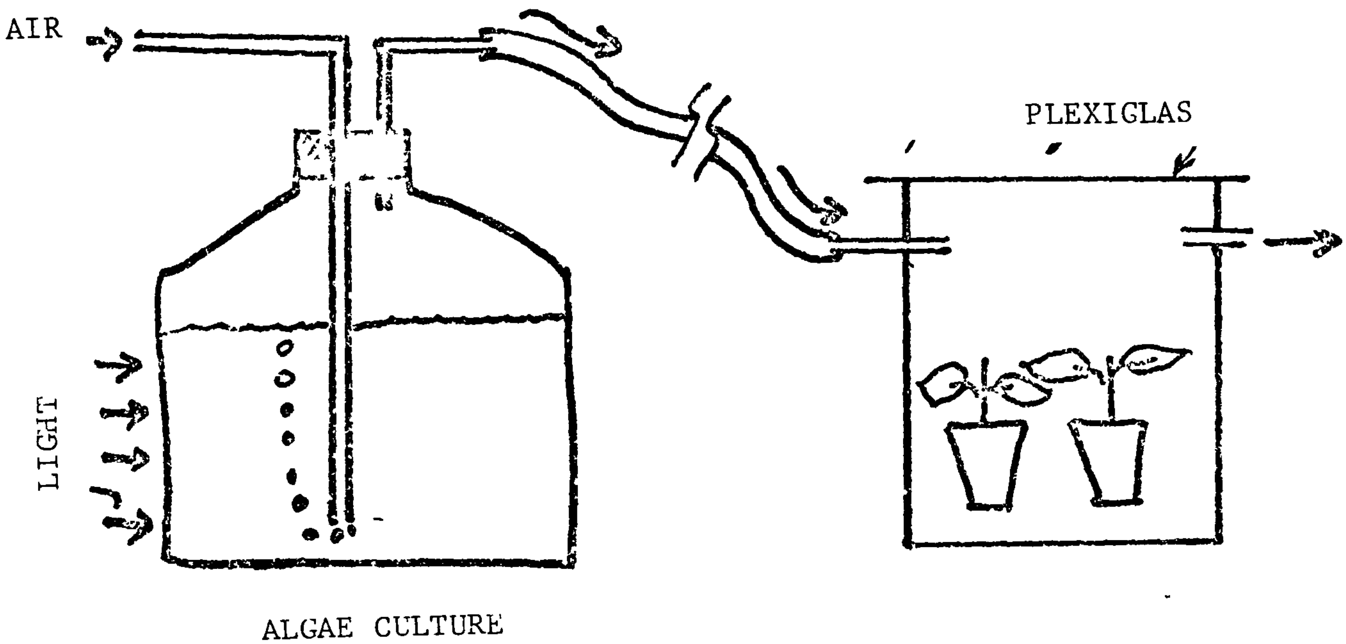

Figure 1. Fumigation system
The fumigation system used consisted of a 5 gal glass carbouy containing either Euglena or Chlorella cell culture. The air from the culture medium was passed through to a plexiglas-wall box containing plants.

cyanide. The reaction solution included: 0.5ml 0.1N NaOH, 1 ml 0.1M p-nitrobenzaldehyde in ethylene glycol monomethylether, 1 ml 0. 1M dinitrobenzene in ethyleneglycol monomethylheter, and 0.1ml of standard, blank or sample solution. Reagents were added in the sequence listed, and incubated for 30 min before reading its absorbance at 578 nm.

Nitrogen oxide assay:

The NOx content was determined using the Satlzman method (7) which can detect nanomolar quantities of NOx. The effluent gases were passed through 50 to 100 ml of 1% (w/v) sulfanilamide and 0.02% (w/v) N-(naphthyl) ethylene diamine HCL in 3N HCl. Absorption was read at 540nm.

Results:

A. Symptoms on dicotyledonous plants (Figures 2-4).

After surveying the responses in 11 species of 4 genera of dicotyledonous plants, the typical esponses are as seen in *P. vulgaris*. There is an initial wilting and water-logged appearance of the leaves after 1-3 hours of fumigation with the algal-effluent air. The water-logged appearance is characterized by a deep or darker green color of the leaves with a dull appearance to the surface. Occasionally there is some chlorosis of the petioles evident at this time.

When the plants are removed from the chamber fumigation, the wilting becomes more pronounced, followed with complete dessication of the leaf blades within 24 hours. Figure 2a shows slight, moderate and severe injury several days after a

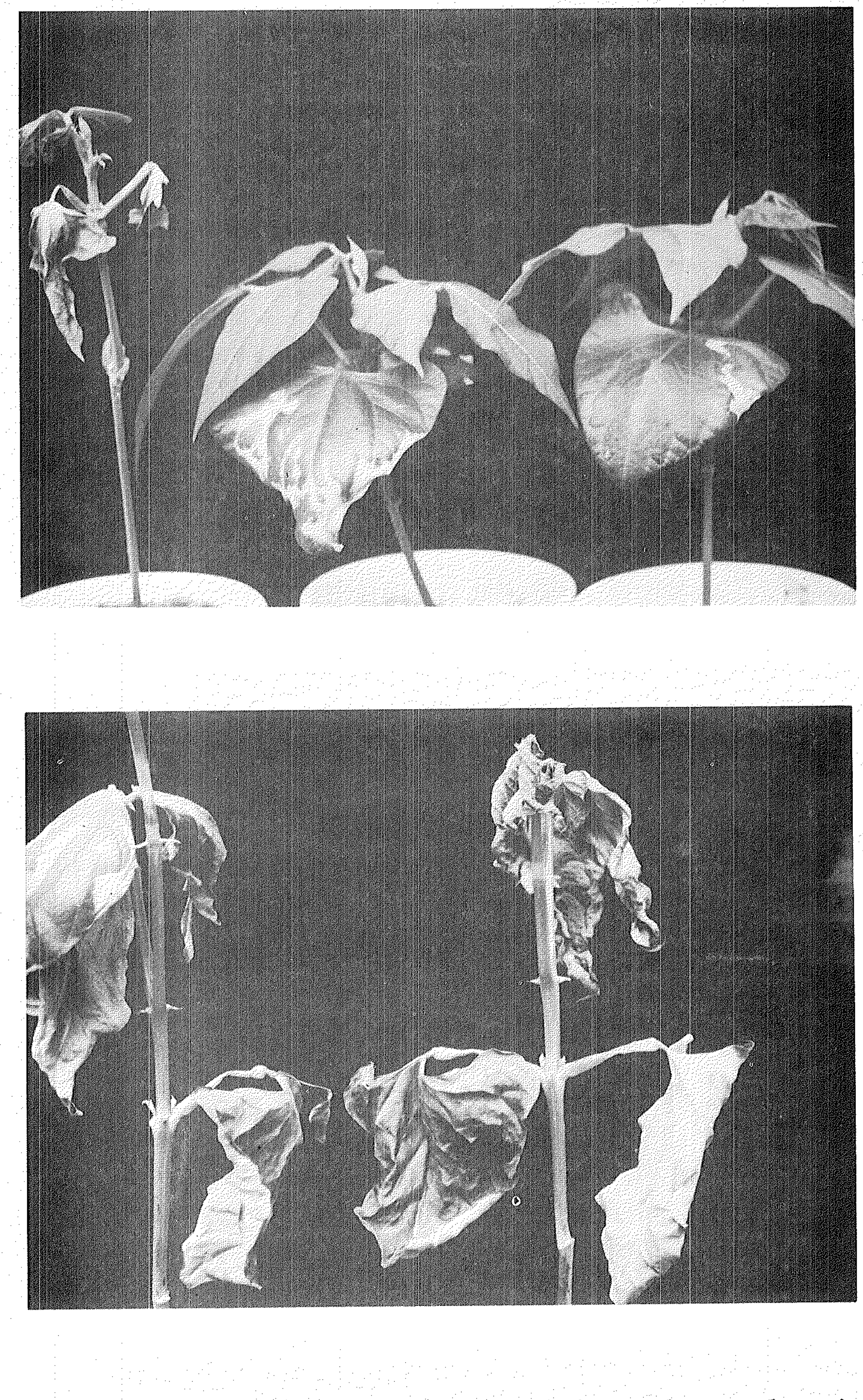

Figure 2. Injury symptoms on Phaseolus vulgaris cv 'Pinto'
a. left to right: severe, moderate and slight injury appearing 24 hrs after 4 hrs fumigation with Euglena gases;
b. severe injury is characterized by petiole collapse and extensive leaf dessication.

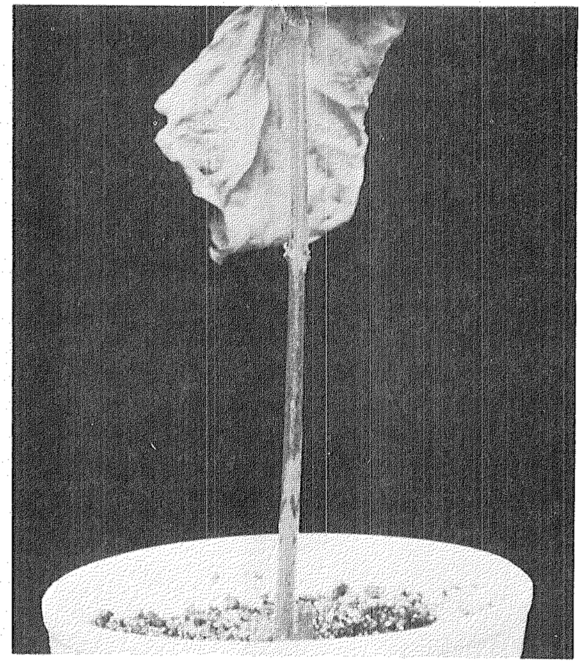

Figure 2c. Close-up of anthocyanin accumulation along main stem of _P. vulgaris_ cv 'Pinto' following fumigation with _Euglena_ gases.

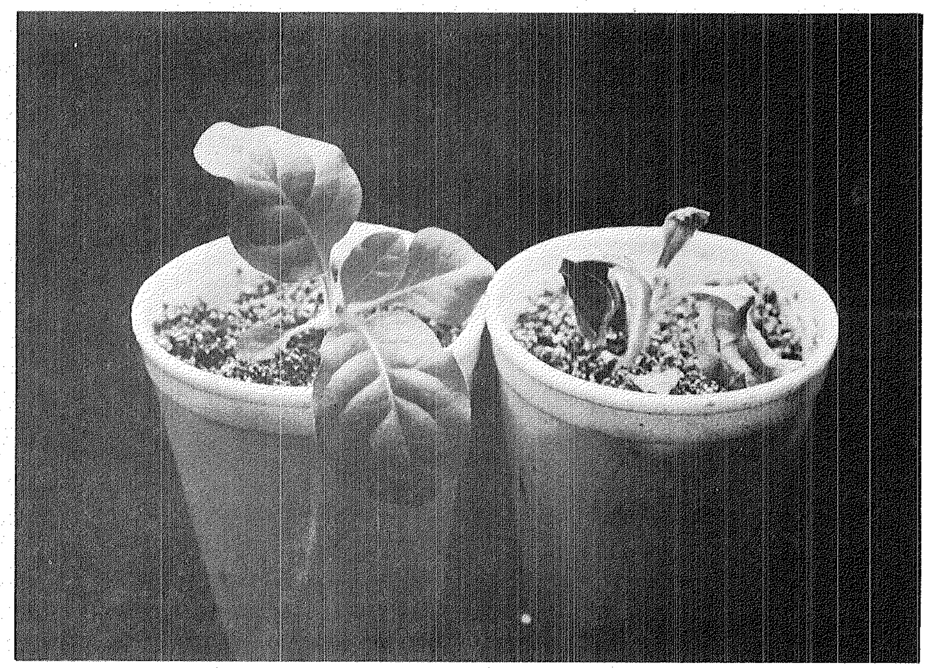

Figure 3. Injury symptoms on _Nicotiana tabacum_ following fumigation with _Euglena_ gases.

fumigation period. The leaves have large bleached areas appearing white to pale brown. Only the older leaves are sensitive to the pollutant with younger leaves showing only slight injury after fumigation. The petioles will show collapse beginning in the mid-petiole region (Figure 2b). In some cases there is anthocyanin accumulation along the main stem. (Figure 2c).

Other genera which were screened for sensitivity to the algal pollutants are listed in Table 1. Tobacco (Fig. 3), bean (Fig. 2), mustard green and cantaloupe (Fig. 4), plants all showed equivalent sensitivity to the pollutant. Only coleus plants showed any resistance. Red coleus leaves show an initial bleaching, without any subsequent leaf dessication. The red color slowly returned, but never to the normal color intensity of untreated leaves.

B. Symptoms in monocotyledonous plants:

Wheat plants were the only monocotyledon to be examined in this project. The symptoms were not as dramatic as seen with the dicotyledonous plants. During a fumigation period which would cause severe wilting in broad-leaved plants, only leaf tip chlorosis in wheat plants was observed (Figure 5). The yellowing leaf tips would progressively increase in area during fumigation and would show complete leaf dessication within 24 hours after fumigation . The dessication would appear even in regions which did now show chlorosis or yellowing during the fumigation period.

Table 1. Common crops tesed for sensitivity to *Euglena* culture gases

Sensitive:	
Beans	*Phaseolus vulgaris* cv 'Pinto'
Mustard greens	*Brassica juncea* cv 'Fordhook Fancy'
	" " cv 'Florida Broadleaf'
	" " cv 'Tendergreen'
	" " cv 'Southern Giant Curled'
Tobacco	*Nicotiana tabacum* cv 'Bel-B'
	" " cv 'Bel-W_3'
Cantaloupe	*Cucumis melo var reticulatus* cv 'Hale's Best Jumbo'
	" " " " cv 'Iroquois'
Wheat	*Triticum aestivum var aestivum* cv 'Chinese Spring'
Resistant:	
Coleus	Coleus hybrids

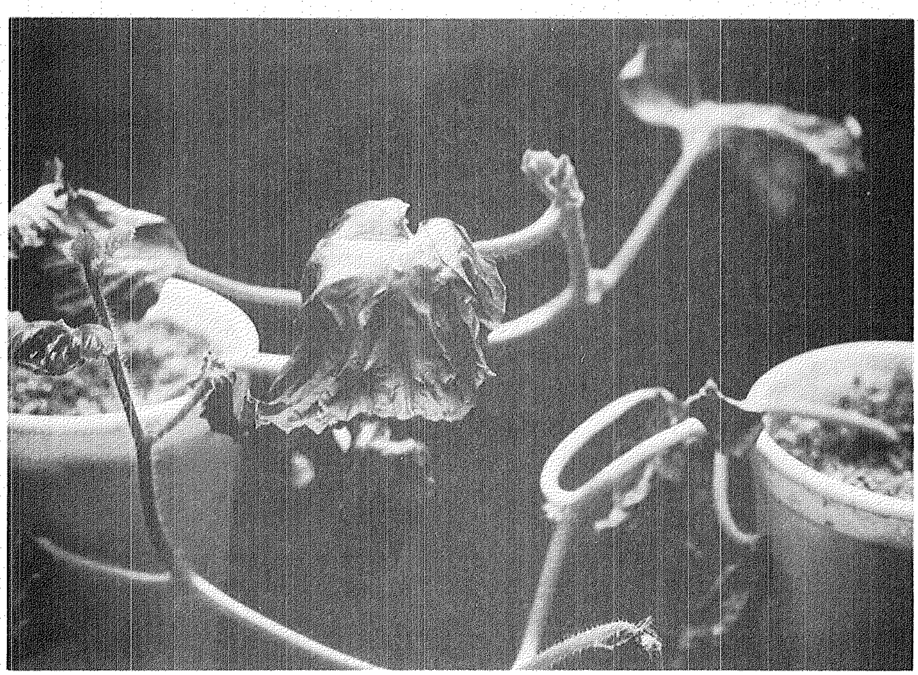

Figure 4. Injury symptoms on Cucumis melo var. reticulatus following fumigation with Euglena gases

Figure 5. Injury symptoms on Triticum aestivum following fumigation with Euglena gases.

C. Production of Pollutants

Using the bean bioassay system for monitoring the pollutant(s), we were able to determine if algal cultural conditions influenced the production of the pollutant.

The Euglena culture was grown in 15-liter flasks, and the effluent gases were monitored for toxicity at various stages during the growth phases. Bean plants were fumigated for 4 hrs (8-12 a.m.). Forty-eight hours after fumigation, injury was scored as percentage bean leaf area damaged: +, 10-30% area affected; ++, 30-70%; +++, 70-100% affected. Maximum pollutant concentrations were reached as the culture population reached non-dividing stages (Fig. 6). Some toxicity was noticed as early as the late-exponential phase of growth, which suggests that with larger culture volumes and similar gas flow volumes the effluent air should be much more toxic.

Cultures of aplastidic mutants, W_3BUL, which lack any chloroplastic DNA, also produce phytotoxic air pollutants (Table 2). Thus the production of these factors is not restricted to photosynthetic organisms.

The effluent air from Chlorella cultures did not contain as much toxic factor as found in Euglena cultures. Some toxic factor(s) was found, but required a minimum of 30 liters culture volume before even small amounts of leaf injury could be produced.

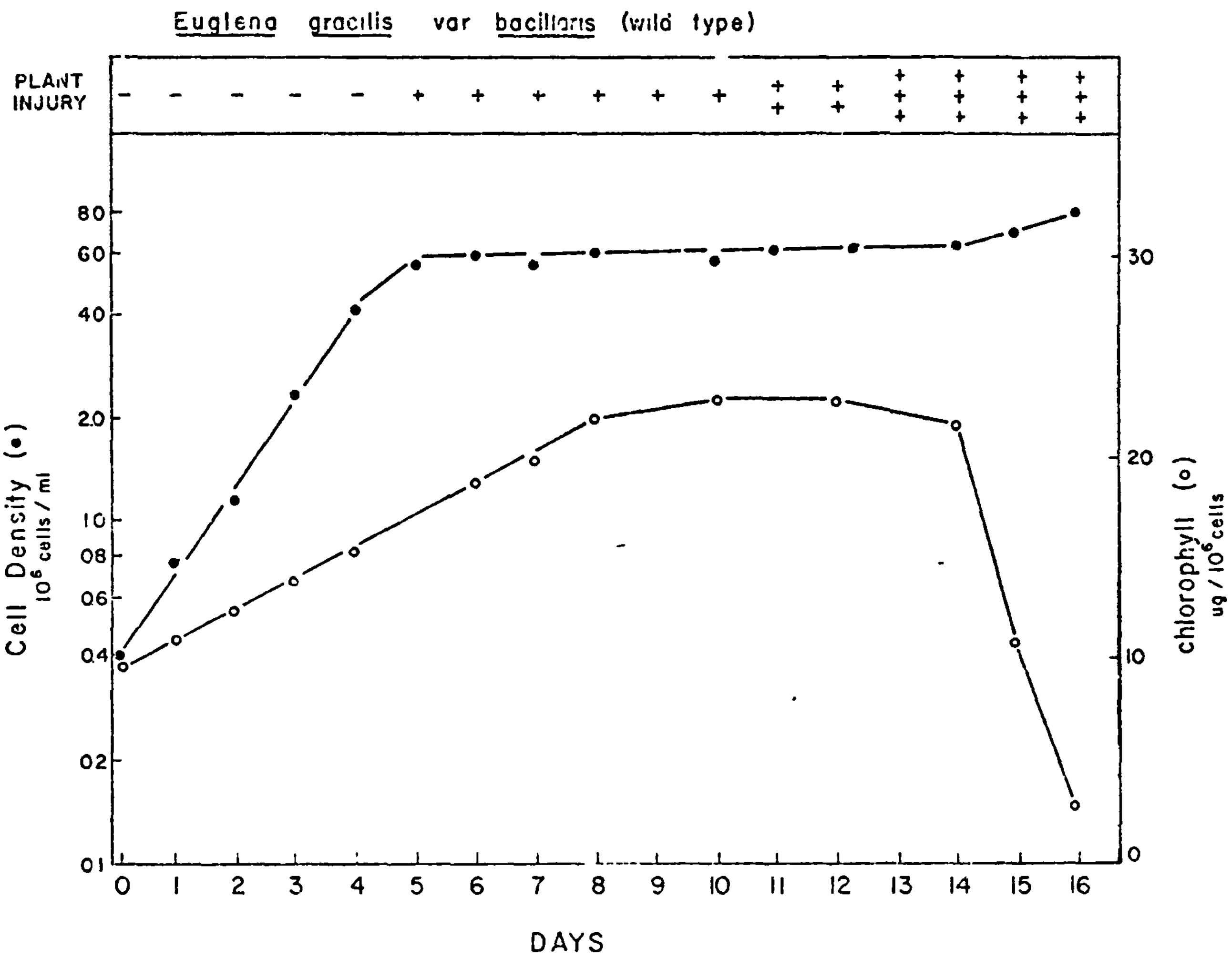

Figure 6. Pollutant production from Euglena cell culture over 16-day period. Cells were grown heterotrophically and monitored for cell density, chlorophyll content and presence of phytotoxic pollutant(s) in the effluent air. Plant injury was monitored with 15-day-old bean plants, fumigated with effluent air for 4 4 hrs and scored for injury: no injury, -; slight, +; moderate, ++; severe, +++ (see figure 2).

Table 2. Effectiveness of acid and base traps on reducing plant injury

TEST PLANT	*Euglena* STRAIN	EXPOSURE TIME (HR)	EFFLUENT TRAPS LACTIC* ACID	NaOH	PLANT INJURY
BEAN	NONE	24	absent	present	-
(*Phaseolus vulgaris*)	WILD TYPE	24	"	absent	+++
	" "	24	"	"	+++
	" "	24	"	present	+++
	" "	24	present	absent	-
	" "	48	"	"	-
	W_3BUL**	24	absent	"	+
	"	48	"	present	+++
	"	48	present	absent	-
WHEAT	NONE	48	absent	absent	-
(*Triticum aestivum*)	WILD TYPE	48	"	"	+++

ALL CULTURES WERE GROWN ON GLUTAMATE-MALATE SUPPLEMENTED MEDIUM AT pH 3.5 WITH AERATION AND LIGHT.

*ALL TRAPPING SOLUTIONS WERE 1N CONCENTRATION.

**W_3BUL IS AN APLASTIDIC STRAIN, WITH NO DETECTABLE PLASTID DNA.

D. Trapping Solutions:

To characterize the pollutant(s) a series of traps was used to ascertain the effects of pH and temperature (Table 3). A basic solution (1 N NaOH) did not remove the toxic pollutant(s) while an acidic solution (1 N lactic acid) effectively removed the pollutant. In additon, passing the effluent air through a glass column held in an ice water bath also effectively removed the pollutant(s). Glass columns containing 200 gm of either vermiculite, $CaCO_3$ pellets, or Dririte also effectively removed the pollutant(s). Thus, the characteristics of the pollutant would be a gas which either has no charge or possibly a negative charge which can accept a proton, is water soluble, and has a vaporization point above 0-4C.

The ability to trap the solution in lactic acid or at ice water temperatures would enable an enrichment procedure which could allow further purification of these gas(es). Stability of the pollutant in trapping solutions was determined by freezing the material in lactic acid and re-releasing the pollutant in a closed chamber with the bean plants as described earlier. The frozen lactic acid solution was melted and placed in stirred beaker within the chamber. An equal volume of 1N KOH was added to the lactic acid solution and immediately the chamber lid closed. After the lid was closed, the solutions were stirred vigorously. The plants were held in this closed chamber for 4 hours and then vented. Plants were scored for any visible injury during the next two days (Table 4).

Table 3. Trapping pollutants in liquid and particulate systems.

Trapping solution or reagent	Trapping Period (days)	Bean plant injury
Water	1	+++
1N NaOH	1	+++
1N Lactic acid	2	-
4% (w/v) $Hg(CN)_2$	2	-
0-4^{o}C	1	-
$CaCO_3$ pellets	1	-
Vermiculite	1	-
Dririte	1	-

Table 4. Re-release of pollutants from trapping solutions

Trapping solution containing pollutants	Trapping period	KOH	Bean Plant Injury
1N Lactic acid	1	absent	-
		present	-
1N Lactic acid	2	absent	-
		present	++
4% $Hg(CN)_2$	2	absent	-
		present	++
0-4^{o}C water			
at 0-4^{o}C	1	absent	-
at 25^{o}C	1	absent	-

Lactic acid solutions which had been trapping algal pollutants for one day did not re-release any detectable pollutants when base was added. Only with lactic acid solutions which had been trapping for two days would produce any plant injury upon neutralizing the solution.

The $Hg(CN)_2$ solution also would release phytotoxic pollutants upon neutralization. Re-warming the 0-4 C trapped material did not produce any detectable pollutant as measured by injury to bean plants (Table 4). The ability or inability to re-release pollutants from various trapping solutions may be limited by possible further chemical reactions with the trapping solutions or by our inability to gradually re-release the pollutants over several hours as an algal culture would normally release.

Pollutant bioassays systems are notorious for having non-linear response curves (8,9), such that a high concentration presented over a very short time may give different degrees of response than if released over longer time periods. This may be caused by stomatal responses at at very high concentrations of pollutants which prevent the entry of any additional pollutant or toxic factors into the leaves. Thus, gradual release of trapped pollutant(s) may be needed to see degrees of injury resulting from the typical *Euglena* culture-bean plant system.

Since acidic solutions such as lactic acid or $Hg(CN)_2$ can effectively trap the phytotoxic factors from a *Euglena* culture, the possibility exists that the acidic culture medium itself may be a regulating factor in controlling the release of pollutants.

The culture medium for _Euglena_ is pH 3.5 and for _Chlorella_ is pH 4.3. The medium itself may be releasing some nutrient component as it increases in pH. The medium was found to increase to pH 7.0 for _Euglena_ by the time the culture is non-dividing, and to pH 6.5 - 6.9 for _Chlorella_. Using KOH to titrate 15 liters of culture medium without cells to pH 7.3 and 8.3 did not produce any visible plant injury even after 3 days of fumigation (Table 5). When _Euglena_ cell cultures which were actively producing pollutants titrated with phosphoric acid to return the culture to pH 3.5, only slight visible injury was produced on plants after 1 day of fumigation. If cultures were titrated to pH 7.3 with KOH, slight visible injury was produced on plants after 1 day of fumigation. If the culture medium were titrated to pH 8.3 with KOH, severe visible injury resulted in bean plants after 1 day of fumigation. The only component of the culture medium which may be volatile at pH 8.3 is NH_4. No visible plant injury, though, is seen when the culture medium alone is brought to pH 8.3 and its effluent air is passed to sensitive bean plants. Thus _Euglena_ cells are needed to produce some phytotoxic factor.

Finally, the production rates of various known biological air pollutants were measured, e.g. ethylene, ethane nitrogen oxides and cyanide (Table 6.) The air from _Chlorella_ (30 liters) or _Euglena_ (15 liters) was either sampled or concentrated from cultures which were producing toxic levels of pollutants. For ethylene and ethane only ambient levels of these gases were measured and were less than 50 ppb or less than the sensitivity of the flame ionization detection system used in this study. Cyanide levels measured less than 50 nanomoles per 48 hrs in both algae. Nitrogen oxides production measured less than 5 nanomoles per 48 hrs for both algae. For all three gases the measured levels would be less than threshold levels of any toxic effects (10).

Table 5. Culture mediums as an acid trap for pollutants.

pH (MEDIA)	*Euglena* (WILD TYPE)	FUMIGATION PERIOD (DAYS)	INJURY[3]
3.5	--	3	-
3.5[1]	+	1	+
7.3[2]	--	3	-
7.3[2]	+	1	+
8.3[2]	--	3	-
8.3[2]	+	1	+++

1 pH ADJUSTED WITH PHOSPHORIC ACID
2 pH ADJUSTED WITH POTASSIUM HYDROXIDE
3 14-DAY-OLD BEAN PLANTS EXPOSED TO EFFLUENT GASES FROM CULTURE MEDIUM

Table 6. Production rates of ethylene, ethane, cyanide and nitrogen oxides from *Euglena* and *Chlorella* cultures.

Algae:	Euglena	Chlorella
Ethylene[1]	<0.05 ppm	<0.05 ppm
Ethane[1]	<0.05 ppm	<0.05 ppm
HCN[2]	<50 nanomoles	<50 nanomoles
NO_x[2]	<5 nanomoles	<5 nanomoles

Gases were sampled during periods of pollutant production as measured by injury to bean plants. *Euglena* culture was 15 liters and *Chlorella* culture was 30 liters.

[1]: Ambient concentration in culture effluent air

[2]: Collected over a 48 hr period (1,730 liters of effluent air) in acid for the NO_x or base for the HCN traps.

Discussion:

Origin of pollutant:

The pollutant is not a component of the culture medium which is volatilized. The medium without cells when titrated to alkaline pH does not release any detectable pollutant, at least in toxic quantities (Table 5).

During the first several days following inoculation of the cell culture, there is a low production rate of phytotoxic components. This low production rate may be caused by several factors which include: low cell number, limited ability of the cells to produce the pollutant, and the ability of the acidic culture medium to trap the pollutant. The latter aspect was specifically tested and indeed if an actively pollutant-producing culture medium is adjusted to pH 3.5, no detectable pollutants are subsequently measurable by bioassay of bean plants. In conclusion, though the medium itself is not a source of the pollutant, it does play a part in controlling the actual release of the toxic factors into the effluent air line.

Chemical nature of the pollutant(s):

From our studies on trapping and regenerating the pollutant, we can conclude that it is not trapped or dissolved in (25C) water or NaOH solution, but is readily trapped in a lactic acid solution, Dri-Rite column, vermiculite, or $CaCO_3$ pellets as well as in a 0-4C trap. Several possible gaseous products of biological systems were measured, including HCN, nitrogen oxides and light hydrocarbons such as ethylene and ethane, and found to be at concentrations well below threshold levels reported for any toxic effects in biological systems (10). The ability to trap the pollutant in an acidic solution would suggest that it is a gas which can accept a proton. Amines or ammonia are possible candidates for further consideration.

The most resistant plants found included coleus hybrids. The other plants tested (Table 1) all showed similar sensitivity as found with Phaseolus vulgaris var. 'pinto.' Interestingly, the red-colored, anthocyanin-containing leaves of the coleus lose the red coloration of the leaves within 1 hour of fumigation with algal gases and recovered their coloration within several hours after removing the pollutants. There appears to be a reversible change in the anthocyanin color, strongly suggestive of pH changes within the cytoplasm. Such changes would be consis- with a volatile amine or ammonia as the major pollutant.

Some research on the production of ammonia from algae and human cabin air in closed systems has been reported (1). In the closed human cabin the ammonia level was 4 mg/m^3 measured over a 90-day period. A Chlorella culture containing bacteria releases 1.78 mg/m^3 of NH_3. The threshold for ammonia injury in higher plants is 8 mg/m^3 for 1 hour (10). Since the injury thresholds of many pollutant(s) are greatly influenced by both environmental and cultural factors (11) it would be possible that under certain conditions plants could be more sensitive to ammonia fumigation than indicated by the published values.

The present study has been restricted to examining the pollutant(s) produced in an open system, i.e. without recycling any air between plant and algal chambers. The possibility remains that additional pollutant(s) may be produced in the "closed" system with recycled air.

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16. Abstract

This report considers the production of phytotoxic air pollutants by cultures of Chlorella vulgaris and Euglena gracilis. Discussed are algal and plant culture systems, a fumigation system, and ethylene, ethane, cyanide, and nitrogen oxides assays. Bean, tobacco, mustard green, cantaloupe and wheat plants all showed injury when fumigated with algal gases for 4 hours. Only coleus plants showed any resistance to the gases. A closed or recycled-air effluent system is not required to produce plant injury from algal air pollutants.

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