

Test Medium for the Growth of *Nitrosomonas europaea*

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Received 10 September 1984/Accepted 31 January 1985

A mineral medium for studying the growth of *Nitrosomonas europaea* was developed and examined. The medium was defined in terms of chemical speciation by using chemical equilibrium computer models. The medium significantly increased the metabolic activity of the organisms compared with previously developed media, yielding a specific growth rate as high as 3.0 day^{-1} (generation time, 5.5 h). The specific growth rate was enhanced by increasing the inoculum and was linearly correlated with the inoculum-to-total-culture volume ratio on a semilog scale. A reproducible growth rate for *N. europaea* was obtained with this medium under controlled experimental conditions.

One of the most important features to be considered in the study of bacterial ecology is the choice of a proper medium. Ideally, studies should be carried out in a chemically defined growth medium to overcome difficulties in interpreting results which result when a complex or nonchemically defined medium, such as river water or wastewater, is used. Since the genus *Nitrosomonas* was first described by Winogradsky in the latter part of the nineteenth century, a number of media have been developed and used for studying the nitrifying bacteria (1, 8, 10-12, 14-18, 20, 24, 25, 27-30, 32, 33). However, there has been confusion and contradiction about the effects of various stimulating and inhibiting factors on the growth of the nitrifying bacteria. One of the problems which often obscure the interpretation of results is the lack of a standard test medium. To obtain maximum metabolic activity, enriched media may be recommended. On the other hand, minimal media may be necessary to minimize complex effects induced by medium constituents. As a result, a point of compromise must be found in developing a standard medium.

The purpose of this research was to develop a simple medium which would support high metabolic activity for *Nitrosomonas europaea*. In addition, an attempt was made to define the medium in terms of chemical speciation by using equilibrium computer models. The medium can be used for toxicity screening tests of various compounds. It is especially useful for investigating ionic and complexing effects. The use of computer-assisted chemical analysis for the medium also makes it possible to gain insight into the nutritional requirements of and the effects of toxic compounds on *N. europaea*.

MATERIALS AND METHODS

The growth medium for *N. europaea* was prepared as follows. A known volume of deionized, distilled water was sterilized by autoclaving and allowed to cool to room temperature. The medium was completed by aseptically adding nutrients in the form of concentrated stock solutions (Table 1). To prevent precipitation, the nutrient solutions, except iron, were autoclaved separately at 103 kPa (15 lb/in²) for 20 min. The iron solution was sterilized by filtration through membrane filters (0.2- μm pore size). The molar concentrations of the medium constituents are given in Table 2. Various amounts of $(\text{NH}_4)_2\text{SO}_4$ were added as a concentrated solution depending on the nature of the investigation being

carried out. The pH of the final solution was 8.5 ± 0.5 and was not adjusted further.

The original pure-culture strain of *N. europaea* ATCC 19718 was obtained from the American Type Culture Collection. Stock cultures were grown in medium containing 0.14 mM total ammonium in the dark at 25°C. Subcultures grown for 14 days were used to inoculate culture solutions. The size of the inoculum was 10 ml/liter of medium. No inoculum preparation was performed. Since the cultures were grown in a medium which was free of precipitates and which contained a low concentration of ammonium (in a reciprocating shaker operated at 80 oscillations per min), it seems likely that the organisms were uniformly dispersed in the culture medium. In addition, the amount of nitrite carried over to fresh medium was low ($<1.4 \mu\text{M}$). Mutation and subsequent variation in the nutrient requirements of the stock culture was considered unlikely (18). The culture was maintained on the proposed medium for 2 years with transfer every 14 days.

To validate the proposed medium, various combinations of nutrients were prepared in 500-ml Erlenmeyer flasks. Sterile $(\text{NH}_4)_2\text{SO}_4$ solution was then added to the medium at 3.57 mM. *N. europaea* was added to the medium at an inoculum-to-total-culture volume ratio (defined below) of 0.01. The flasks were capped with 150-ml beakers to avoid contamination but allow sufficient oxygen transfer into the medium. The initial concentrations of total ammonium and nitrite and the pH were measured immediately after mixing. Cultures were incubated in the dark at $25 \pm 0.25^\circ\text{C}$ in a reciprocating shaker at 80 oscillations per min to provide a dissolved-oxygen concentration in excess of 2.0 mg/liter (6).

TABLE 1. Nutrient stock solutions

Nutrient	Concn		Amt (ml) used per liter of medium
	g/liter	mM	
Phosphate buffer ^a		500	10
Carbonate buffer ^b		600	10
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	1.84	12.5	10
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	250	1,000	2
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.1	0.36	1

^a 68.0 g of KH_2PO_4 and 87.1 g of K_2HPO_4 were dissolved separately in 1 liter of deionized, distilled water; the two solutions were mixed together in the proper amounts to give a pH of 8.2.

^b 50.4 g of NaHCO_3 and 63.6 g of Na_2CO_3 were dissolved separately in 1 liter of deionized, distilled water; the two solutions were mixed together in the proper amounts to give pH of 8.2.

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TABLE 2. Composition of medium

Constituent	Concn (mol/liter)
Metals	
K ⁺	9.65×10^{-3}
Na ⁺	6.06×10^{-3}
Ca ²⁺	1.25×10^{-4}
Mg ²⁺	2.00×10^{-3}
Fe ²⁺	3.60×10^{-7}
Ligands	
CO ₃ ²⁻	6.00×10^{-3}
PO ₄ ³⁻	5.00×10^{-3}
Cl ⁻	2.50×10^{-4}
SO ₄ ^{2-a}	1.99×10^{-3} – 5.56×10^{-3}
NH ₄ ^{++b}	7.14×10^{-5} – 9.4×10^{-3}

^a The SO₄²⁻ concentration varied with NH₄⁺ concentration because NH₄⁺ was added as (NH₄)₂SO₄.

^b The range of total ammonium (NH₃ plus NH₄⁺) concentrations tested is shown.

The flasks were removed from the shaker every 12 to 24 h, depending on the activity of the organisms, and a small sample was removed from each flask with sterilized pipettes for measurement of the nitrite concentration. The effects of total ammonium and inoculum concentrations were investigated by the procedure described above, except that a series of media containing different total ammonium concentrations and different volumes of inoculum were prepared.

During the present study, the amount of nitrite produced was used as a measure of growth. This method was validated by Engel and Alexander (8), who presented nitrite concentration which corresponded with viable cell counts of *N. europaea* (see Fig. 1, inset). Loveless and Painter (18) stated that the greater sensitivity, simplicity, and accuracy of the nitrite determination make it a better choice than the estimation of cell carbon, mass, or number. They also stated that nitrite production is a true measure of growth for *Nitrosomonas* spp. at least up to 35.7 mM NO₂⁻. Values for the specific growth rate were obtained by measuring the slope of the straight-line portion of each semilog plot (log NO₂⁻ concentration versus time) and converting these values to the natural logarithm. The analytical procedures followed the recommended standard methods (2). Nitrite nitrogen was determined by the diazotization method with *N*-(1-naphthyl)ethylenediamine dihydrochloride. Ammonia nitrogen was determined by the Nesslerization method.

RESULTS AND DISCUSSION

The preliminary medium for *N. europaea* was prepared by the directions furnished by the American Type Culture Collection. This medium was then modified by trial and error. The final medium contains five major metals (Ca²⁺, Mg²⁺, Fe²⁺, K⁺, and Na⁺) and six inorganic ligands (CO₃²⁻, SO₄²⁻, Cl⁻, PO₄³⁻, NH₄⁺, and OH⁻). These medium constituents may exist as free ions, ion pairs, colloids, or precipitates as a result of hydrolysis, hydrolytic polymerization, and oxidation-reduction reactions. These species may be subject to adsorption to particulate matter and subsequent sedimentation. They complex with the inorganic ligands present in the medium. Therefore, defining the medium with respect to chemical speciation is important to understanding the nature of the medium and the nutritional requirements of *N. europaea*. Equilibrium calculations were performed with modified versions of the COMICS (23) and MINTEQ (9) computer models. COMICS is a relatively simple model which accounts for only a dissolved phase,

whereas MINTEQ is one of the most sophisticated computer models designed to calculate geochemical equilibria accounting for both liquid and solid phases. Table 3 lists the equilibria and equilibrium constants considered in COMICS. The thermodynamic data for MINTEQ are incorporated into its data base (9). Log activities of chemical species, calculated at a total ammonium concentration of 3.57 mM at a constant pH of 8.5, are presented in Table 4. Adsorption to a solid phase was not considered in the computations because it is likely that the chemical species exist in the medium as ionic or aqueous forms and that the ammonia ligands kept the metals from adsorbing strongly to the

TABLE 3. Equilibria considered with the COMICS model

Reaction	log K
K ⁺ + Cl ⁻ ⇌ KCl	-1.59
K ⁺ + SO ₄ ²⁻ ⇌ KSO ₄ ⁻	0.96
K ⁺ + H ⁺ + PO ₄ ³⁻ ⇌ KHPO ₄ ⁻	13.39
Na ⁺ + CO ₃ ²⁻ ⇌ NaCO ₃ ⁻	1.27
2Na ⁺ + CO ₃ ²⁻ ⇌ Na ₂ CO ₃	0.67
Na ⁺ + H ⁺ + CO ₃ ²⁻ ⇌ NaHCO ₃	10.08
Na ⁺ + SO ₄ ²⁻ ⇌ NaSO ₄ ⁻	0.72
2Na ⁺ + SO ₄ ²⁻ ⇌ Na ₂ SO ₄	1.51
Na ⁺ + H ₂ O ⇌ NaOH + H ⁺	-14.7
Na ⁺ + Cl ⁻ ⇌ NaCl	-1.60
Mg ²⁺ + PO ₄ ³⁻ ⇌ MgPO ₄ ⁻	6.59
Mg ²⁺ + H ⁺ + PO ₄ ³⁻ ⇌ MgHPO ₄	14.8
Mg ²⁺ + 2H ⁺ + PO ₄ ³⁻ ⇌ MgH ₂ PO ₄ ⁺	21.01
Mg ²⁺ + SO ₄ ²⁻ ⇌ MgSO ₄	2.36
Mg ²⁺ + H ₂ O ⇌ MgOH ⁺ + H ⁺	-11.42
Fe ²⁺ + H ⁺ + PO ₄ ³⁻ ⇌ FeHPO ₄	15.9
Fe ²⁺ + 2H ⁺ + PO ₄ ³⁻ ⇌ FeH ₂ PO ₄ ⁺	22.2
Fe ²⁺ + SO ₄ ²⁻ ⇌ FeSO ₄	2.2
Fe ²⁺ + Cl ⁻ ⇌ FeCl ⁺	0.36
Fe ²⁺ + 2Cl ⁻ ⇌ FeCl ₂	0.40
Na ⁺ + H ⁺ + PO ₄ ³⁻ ⇌ NaHPO ₄ ⁻	13.5
Ca ²⁺ + CO ₃ ²⁻ ⇌ CaCO ₃	3.2
Ca ²⁺ + H ⁺ + CO ₃ ²⁻ ⇌ CaHCO ₃ ⁺	11.6
Ca ²⁺ + PO ₄ ³⁻ ⇌ CaPO ₄ ⁻	6.5
Ca ²⁺ + H ⁺ + PO ₄ ³⁻ ⇌ CaHPO ₄	15.0
Ca ²⁺ + 2H ⁺ + PO ₄ ³⁻ ⇌ CaH ₂ PO ₄ ⁺	20.9
Ca ²⁺ + H ₂ O ⇌ CaOH ⁺ + H ⁺	12.6
Ca ²⁺ + SO ₄ ²⁻ ⇌ CaSO ₄	2.31
Mg ²⁺ + CO ₃ ²⁻ ⇌ MgCO ₃	2.18
Mg ²⁺ + H ⁺ + CO ₃ ²⁻ ⇌ MgHCO ₃ ⁺	11.33
2H ⁺ + PO ₄ ³⁻ ⇌ H ₂ PO ₄ ⁻	19.53
Fe ²⁺ + H ₂ O ⇌ FeOH ⁺ + H ⁺	-8.3
Fe ²⁺ + 2H ₂ O ⇌ Fe(OH) ₂ + 2H ⁺	-18.9
Fe ²⁺ + 3H ₂ O ⇌ Fe(OH) ₃ ⁻ + 3H ⁺	-32.0
Fe ²⁺ + 4H ₂ O ⇌ Fe(OH) ₄ ²⁻ + 4H ⁺	-46.4
Fe ²⁺ + CO ₃ ²⁻ ⇌ FeCO ₃	-5.31
Fe ²⁺ + H ⁺ + CO ₃ ²⁻ ⇌ FeHCO ₃ ⁺	13.05
Fe ²⁺ + PO ₄ ³⁻ ⇌ FePO ₄ ⁻	7.93
H ⁺ + CO ₃ ²⁻ ⇌ HCO ₃ ⁻	10.3
2H ⁺ + CO ₃ ²⁻ ⇌ H ₂ CO ₃	16.7
H ⁺ + PO ₄ ³⁻ ⇌ HPO ₄ ²⁻	12.32
3H ⁺ + PO ₄ ³⁻ ⇌ H ₃ PO ₄	21.7
H ⁺ + Cl ⁻ ⇌ HCl	-6.1
H ⁺ + SO ₄ ²⁻ ⇌ HSO ₄ ⁻	2.0
2H ⁺ + SO ₄ ²⁻ ⇌ H ₂ SO ₄	-1.0
Ca ²⁺ + NH ₃ ⇌ CaNH ₃ ²⁺	-0.2
Ca ²⁺ + 2NH ₃ ⇌ Ca(NH ₃) ₂ ²⁺	-0.8
Ca ²⁺ + 3NH ₃ ⇌ Ca(NH ₃) ₃ ²⁺	-1.6
Ca ²⁺ + 4NH ₃ ⇌ Ca(NH ₃) ₄ ²⁺	-2.7
Mg ²⁺ + NH ₃ ⇌ MgNH ₃ ²⁺	0.23
Mg ²⁺ + 2NH ₃ ⇌ Mg(NH ₃) ₂ ²⁺	-0.08
Mg ²⁺ + 3NH ₃ ⇌ Mg(NH ₃) ₃ ²⁺	-0.34
Mg ²⁺ + 4NH ₃ ⇌ Mg(NH ₃) ₄ ²⁺	-1.04
H ⁺ + NH ₃ ⇌ NH ₄ ⁺	9.26

culture flasks. It can be seen (Table 4) that the results from COMICS and MINTEQ were in general agreement. Table 5 summarizes the percent distribution of the major components as computed by MINTEQ.

The medium was examined by varying the concentration of one constituent while holding the concentration of all other medium components constant. Once the concentration

TABLE 4. Computed medium composition

Chemical species	log activity	
	MINTEQ	COMICS
NH ₄ ⁺	-2.59	-2.61
K ⁺	-2.09	-2.12
Ca ²⁺	-4.53	-4.53
Mg ²⁺	-3.39	-3.31
Na ⁺	-2.29	-2.32
Fe ²⁺	-18.01	-8.10
Fe ³⁺	-19.04	— ^a
CO ₃ ²⁻	-4.66	-4.42
PO ₄ ³⁻	-6.55	-7.21
SO ₄ ²⁻	-2.74	-2.89
Cl ⁻	-3.67	-3.70
HPO ₄ ²⁻	-2.70	-3.39
H ₂ PO ₄ ⁻	-4.00	-4.68
NH ₃ (aq) ^b	-3.33	-3.37
NH ₄ SO ₄ ⁻	-4.22	—
MgOH ⁺	-6.67	-6.23
MgCO ₃ (aq)	-5.07	-5.55
MgHCO ₃ ⁺	-5.15	-4.90
MgSO ₄ (aq)	-3.88	-3.84
MgPO ₄ ⁻	-3.34	-3.93
MgH ₂ PO ₄ ⁺	-5.87	-6.51
MgHPO ₄ (aq)	-3.21	-4.22
CaOH ⁺	-8.63	-8.63
CaHCO ₃ ⁺	-6.34	-5.85
CaCO ₃ (aq)	-6.03	-5.75
CaSO ₄ (aq)	-4.96	-5.11
CaHPO ₄ (aq)	-4.49	-5.24
CaPO ₄ ⁻	-4.62	-5.24
CaH ₂ PO ₄ ⁺	-7.12	-7.84
NaCO ₃ ⁻	-5.68	-5.47
NaHCO ₃ (aq)	-5.37	-7.48
NaSO ₄ ⁻	-4.33	-4.49
NaHPO ₄ ⁻	-4.70	-4.53
KSO ₄ ⁻	-3.99	-4.05
KHPO ₄ ⁻	-4.50	-4.44
FeOH ⁺	-19.01	-7.90
Fe(OH) ₃ ⁻	-23.51	-14.60
FeSO ₄ (aq)	-18.50	-8.79
FeH ₂ PO ₄ ⁺	-19.30	-10.11
Fe(OH) ₂ (aq)	-21.58	-10.00
FeHPO ₄ (aq)	-17.11	-7.91
FeOH ²⁺	-12.73	—
FeHPO ₄ ⁺	-16.31	—
FeSO ₄ ⁺	-17.86	—
FeCl ²⁺	-21.23	—
FeCl ₂ ⁺	-24.26	—
FeCl ₃ (aq)	-28.93	—
Fe(OH) ₂ ⁺	-7.71	—
Fe(OH) ₃ (aq)	-7.14	—
Fe(OH) ₄ ⁻	-6.64	—
FeH ₂ PO ₄ ²⁺	-17.61	—
Fe(SO ₄) ₂ ⁻	-19.11	—
Fe ₂ (OH) ₂ ⁴⁺	-24.03	—
Fe ₃ (OH) ₄ ⁵⁺	-29.42	—
HCO ₃ ⁻	-2.83	-2.62
H ₂ CO ₃ (aq)	-4.98	-4.66
HSO ₄ ⁻	-9.26	-9.39

^a —, Computation was not performed.

^b aq, Aqueous.

TABLE 5. Distribution of medium components

Component	Species	% Distribution
NH ₄ ⁺	NH ₄ ⁺	85.2
	NH ₃ (aq) ^a	12.9
	NH ₄ SO ₄ ⁻	1.9
K ⁺	K ⁺	98.4
	KSO ₄ ⁻	1.2
Ca ²⁺	Ca ²⁺	42.0
	CaHPO ₄ (aq)	25.7
	CaPO ₄ ⁻	22.4
	CaSO ₄ (aq)	8.7
Mg ²⁺	Mg ²⁺	35.9
	MgPO ₄ ⁻	26.2
	MgHPO ₄ (aq)	30.4
	MgSO ₄ (aq)	6.6
Na ⁺	Na ⁺	98.6
	Fe ²⁺	17.5
Fe ²⁺	FeOH ⁺	1.1
	FeHPO ₄ (aq)	77.7
	FeSO ₄ (aq)	3.1
	Cl ⁻	100.0
PO ₄ ³⁻	HPO ₄ ²⁻	72.6
	H ₂ PO ₄ ⁻	2.3
	MgPO ₄ ⁻	10.5
	MgHPO ₄ (aq)	12.2
SO ₄ ²⁻	SO ₄ ²⁻	89.8
	MgSO ₄ (aq)	3.5
	NaSO ₄ ⁻	1.4
	KSO ₄ ⁻	3.1
CO ₃ ²⁻	NH ₄ SO ₄ ⁻	1.8
	CO ₃ ²⁻	2.2
	HCO ₃ ⁻	95.8
Fe ³⁺	Fe(OH) ₂ ⁺	6.3
	Fe(OH) ₃ (aq)	20.0
	Fe(OH) ₄ ⁻	73.7

^a aq, Aqueous.

of one medium constituent was validated, the concentration of another was varied. The order in which the constituents were tested was Fe²⁺, Mg²⁺, Ca²⁺, CO₃²⁻, and PO₄³⁻. Table 6 shows the various combinations of nutrients and the specific growth rates measured. The results showed that the proposed concentrations of the medium constituents should not limit the growth of *N. europaea* and that the highest concentration (250 mM) of CaCl₂ · 2H₂O tested retarded its growth. It was observed that high levels of phosphate (>5 mM) and carbonate (>6 mM) buffers at higher pH (>8.8) caused marked precipitation in the medium. This observation is in general agreement with the computed saturation indices (9). The saturation indices for calcite and dolomite increased from -0.3 (undersaturation) at pH 8.75 to +0.1 (supersaturation) at pH 9.0 and from -0.2 at pH 8.5 to +0.6 at pH 8.75, respectively. The possibility of apatite precipitation in the proposed medium was indicated by computation, but precipitates were not observed in solution except after several days. Precipitate formation did not occur in any sample during the period when growth rates were determined. In this experiment, an extremely small number of organisms were introduced into the medium so that adsorption of metals onto bacterial cells was minimal in the early stage of growth. To summarize the experimental conditions, the pH was relatively constant, precipitation and adsorption were minimal, and chemical speciation was approximately fixed in the early stage of growth. Under these conditions, the initial medium conditions can be defined by a simple chemical equilibrium model such as COMICS. In a more complex medium, in which the sorption characteristics of

TABLE 6. Various compositions of the medium^a and the specific growth rates measured

Constituents varied	Phosphate buffer (mM)	Carbonate buffer (mM)	CaCl ₂ · 2H ₂ O (μM)	MgSO ₄ · 7H ₂ O (mM)	FeSO ₄ · 7H ₂ O (μM)	Initial pH	μ (day ⁻¹)
Iron and calcium	20	12	12.5	3	0.18	8.4	1.9
			12.5		0.36	8.4	1.9
			12.5		0.90	8.4	2.0
			12.5		1.80	8.4	2.0
			25		0.18	8.4	2.1
			25		0.36	8.4	2.0 (2.4) ^b
			25		0.90	8.4	2.2
			25		1.80	8.4	2.0
			62.5		0.18	8.5	2.0
			62.5		0.36	8.5	2.1
			62.5		0.90	8.4	2.0
			62.5		1.80	8.5	2.0
			125		0.18	8.4	1.8
			125		0.36	8.5	2.0 (2.2)
			125		0.90	8.5	2.0
			125		1.80	8.4	2.1
Magnesium and calcium	20	12	62.5	1	0.36	8.4	2.2
			62.5	2		8.4	2.2
			62.5	3		8.4	2.4 (2.0)
			62.5	4		8.4	2.2
			125	0.5		8.5	2.1
			125	1		8.5	2.3
			125	2		8.5	2.2
			125	3		8.5	2.2 (2.0)
			125	4		8.4	2.0
			250	0.5		8.5	1.3
			250	1		8.5	1.8
			250	2		8.4	1.9
			250	3		8.4	1.8
			250	4		8.4	1.6
Phosphate and carbonate buffers	10	3	125	2	0.36	8.0	2.1
	10	6				8.3	2.3 (2.1)
	10	12				8.6	2.2
	10	18				8.7	2.2
	10	24				9.0	2.2
	20	3				8.0	2.2
	20	6				8.3	2.2
	20	12				8.4	2.3
	20	18				8.6	2.2
	20	24				8.8	2.1
	30	6				8.2	2.2
	30	12				8.4	2.3
	30	18				8.7	2.1
	30	24				8.8	2.1
Phosphate buffer	2.5	6	125	2	0.36	8.3	1.9
	5 ^c	6 ^c	125 ^c	2 ^c	0.36 ^c	8.3	2.1
	10	6	125	2	0.36	8.0	2.1

^a For each variation studied, the concentrations of the other constituents were held constant and are shown only in the first line for each section. For all experiments ($n = 42$), $\bar{x} = 2.1, \pm 0.1 \text{ day}^{-1}$ (standard deviation) for specific growth rate. Data from the media containing 250 μM CaCl₂ · 2H₂O were not used to calculate the mean or standard deviation because this high concentration retarded growth.

^b Numbers in parentheses indicate replicate experiment.

^c These concentrations are proposed for the medium.

medium components onto particulate matter and bacterial cells are important, defining the medium becomes more difficult and requires a more sophisticated model such as MINTEQ. Because sorption trends are dependent on the characteristics of the metals, ligands, and particulate matter present in the medium (4, 34), a great deal of effort may be required to obtain the necessary input data. Although there has been considerable controversy over the effect of particulate matter on the growth of *N. europaea* (7, 13), there is an advantage to using a clear rather than a suspended medium

in simplifying measurement of the chemical equilibria of the medium.

The presence of adequate concentrations of substrate and organisms is important in obtaining a high and consistent growth rate. There is now an immense body of literature on the effects of total ammonium concentration on nitrifying bacteria (26). Substrate and product inhibitions occur at high concentrations. Unionized ammonia and nitrous acid are more toxic than their ionized forms (3). The levels of substrate which have been reported to inhibit ammonia

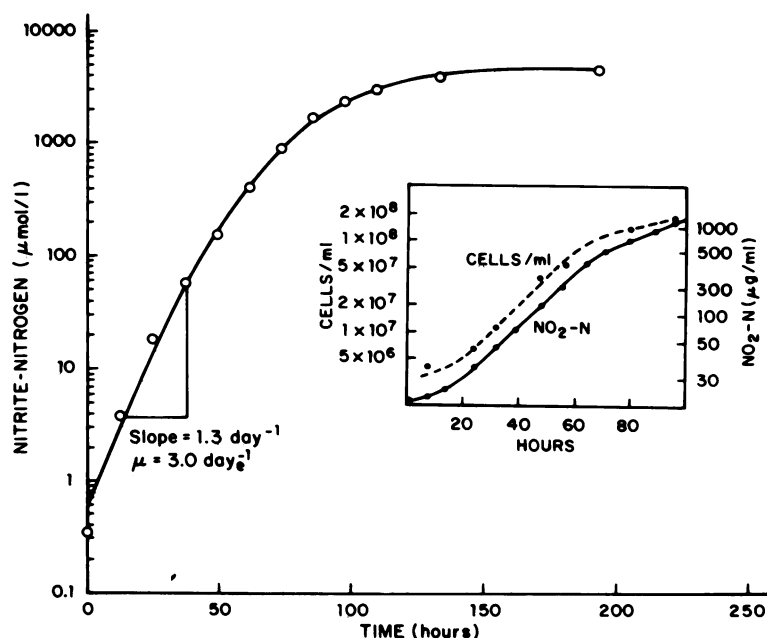


FIG. 1. Nitrite formation during growth of *N. europaea*. The medium contained 4% inoculum and 7.8 mM total ammonium. Inset: Logarithmic plot of viable cells and nitrite concentration during the growth of *N. europaea* (reproduced from reference 8).

oxidation are >75 mM total ammonium (>2.1 mM ammonia) at pH 7.7 (19), 0.71 to 10.7 mM ammonia (3), and >0.71 mM ammonia (21). The wide range of toxicities reported is primarily due to different test conditions (i.e., *Nitrosomonas* species, medium, pH, temperature). To assess the effect of total ammonium, several batch experiments were conducted in which the initial concentration of total ammonium was varied from 0 to 263 mM (40 mM ammonia). Results from several batch runs indicated that *N. europaea* could tolerate high total ammonium concentrations. Growth was not inhibited by total ammonium concentrations of up to 71 mM (10.7 mM ammonia), but a total ammonium concentration of 263 mM (40 mM ammonia) reduced the specific growth rate significantly. The results also indicated that the specific growth rate was weakly dependent on the total ammonium concentration in the range between 1.4 and 71 mM. Theoretical calculations indicate that the concentration of unionized nitrous acid in the medium should not increase to the toxic levels (16 to 200 μ M) reported by Anthonisen et al. (3) during the period when growth rates are determined if the organisms are grown at ≤ 7.1 mM total ammonium. At higher concentrations, however, unionized nitrous acid may reach toxic concentrations in the later stages of growth.

It has been shown that the number of nitrifying bacteria present in a system influences the nitrification rate (5, 26, 31, 35). In the present experiment, an extremely small number of organisms were introduced into the medium, and thus accurate determination of the number of organisms was difficult. To simplify the technique, it was defined in terms of fraction of inoculum volume (inoculum-to-total-culture volume ratio), F , where F equals the inoculum volume transferred (in milliliters) divided by the total culture volume (in milliliters).

To investigate the influence of inoculum volume on the growth of *N. europaea*, experiments were performed with total ammonium concentrations between 0 and 7.8 mM (0 to 110 mg/liter as N). Figure 1 shows a logarithmic plot of nitrite formation by *N. europaea* ($F = 0.04$) in a medium

containing 7.8 mM total ammonium. The inset is a reproduction of the plot of viable *N. europaea* cells and nitrite concentration reported by Engel and Alexander (8), which shows a good correspondence between nitrite formation and viable cell count during the growth of *N. europaea*. The effects of both total ammonium concentration and inoculum volume on the specific growth rate, μ , are shown in Fig. 2. In a control culture containing no ammonium, no growth was evident. The specific growth rate increased as the inoculum volume fraction, F , was increased. At an inoculum volume fraction of 0.04 and a total ammonium concentration of 7.8 mM, the specific growth rate reached 3.0 day^{-1} (Fig. 1). This is a considerably higher value than any previously reported for the growth of *Nitrosomonas* spp. (22). The data also indicated that the specific growth rate was less dependent on F (zero-order relationship) when F was 0.01 or higher. However, at a lower F , the specific growth rate decreased significantly, indicating a pseudo-first-order relationship. Although it appeared that the specific growth rate was enhanced by increasing the number of organisms, the fact that some of the medium from the stock culture was transferred along with the organisms suggests that such stimulation may have been caused, at least in part, by substances present in the inoculum medium. Another point of interest is that the plots of specific growth rate versus $\log F$ gave linear relationships ($r > 0.95$) with a similar slope.

To serve as a useful research tool, a successful test medium must be capable of furnishing consistent results under given experimental conditions. At a total ammonium concentration of 1.5 mM and an F of 0.01, the mean specific growth rate (standard deviation) obtained from five experiments was $2.1 \pm 0.2 \text{ day}^{-1}$. This and similar media are consistently able to yield high specific growth rates with minimal variation (Table 6). Since the use of culture media containing large quantities of insoluble constituents complicates studies of nutrition and biochemistry in *Nitrosomonas* spp. (8), the proposed clear medium can be effectively used as a test medium in studies of *N. europaea*. It is hoped that

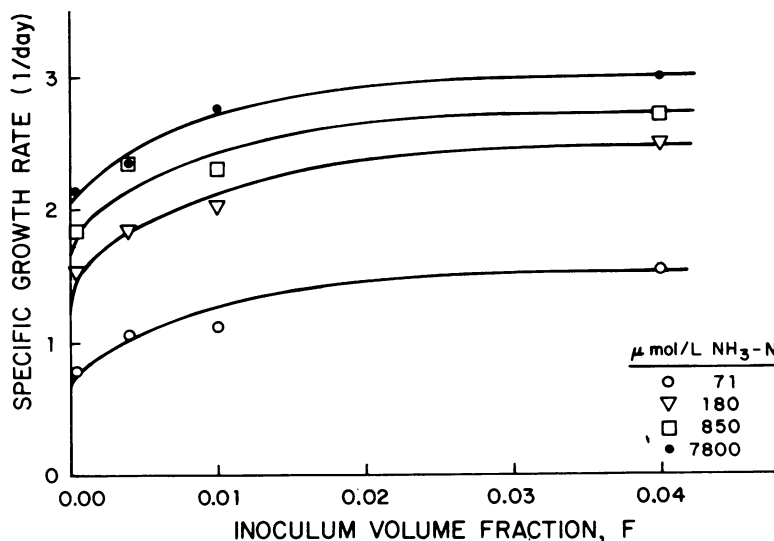


FIG. 2. Effect of inoculum volume on specific growth rate, with the amount of nitrite produced used as a measure of growth.

the medium can be used for toxicity screening tests for various compounds, especially for ionic and complexing compounds.

In conclusion, a medium for high metabolic activity and growth of *N. europaea* ATCC 19718 was developed and examined for nutritional requirements. The concentrations of the medium constituents satisfied the requirements of the organisms. The initial condition of the medium was defined in terms of chemical speciation by using two chemical equilibrium models. The medium significantly increased the activity of the bacteria compared with previously developed media, giving a specific growth rate as high as 3.0 day^{-1} (generation time, 5.5 h). The medium provided reproducible data under controlled conditions. The specific growth rate was influenced by the inoculum volume at low concentrations, but was less dependent on inoculum concentration above an inoculum-to-total-culture volume ratio (F) of 0.01. The specific growth rate was linearly correlated with the inoculum-to-total-culture volume ratio on a semilog scale within the range of F values tested.

ACKNOWLEDGMENTS

We thank James C. Lin for his assistance in computing the chemical speciations and Werner Stumm for advice on defining the medium via equilibrium calculations.

The study was sponsored by the Office of Water Research and Technology, the Iowa State Water Resources Research Institute, under grant A-0711A.

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