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Tolerance of flagellated protists to high sulfide and metal concentrations potentially encountered at deep-sea hydrothermal vents

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ABSTRACT: The survival rates of 3 species of deep-sea hydrothermal vent flagellates were measured after exposure to chemical conditions potentially encountered in vent environments. The survival rates, measured as viability through time, of *Caecitellus parvulus*, *Cafeteria* sp. and *Rhynchomonas nasuta* were determined and compared to shallow-water strains of the same species after exposure to increasing concentrations of sulfide or the metals Cu, Fe, Mn and Zn. Responses were variable but in all cases these flagellates showed very high tolerance to extreme conditions. *Cafeteria* spp. were remarkable in that both strains showed 100% viability after a 24 h exposure to 30 mM sulfide under anoxic conditions. By contrast, the highest naturally occurring sulfide concentrations ever measured are only 18 to 20 mM. There was little effect from metals at concentrations up to 10^{-3} M total metal, but a sharp decrease in viability occurred between 10^{-3} and 10^{-2} M total metal, due either to a rapid increase in the availability of free metal ions or colloid formation or both. This study is consistent with other previously reported studies that indicate these flagellate species are present and capable of being active members of the microbial food webs at deep-sea vents.

KEY WORDS: Flagellate · Survival · Hydrogen sulfide · Metals · Hydrothermal vent · Extreme environment

INTRODUCTION

Organisms living in extreme environments are exposed to physical and chemical conditions generally considered to be unfavorable for most other forms of life. An extreme environment may therefore be defined as one in which a restricted number of organisms can survive and grow; the majority are excluded due to unfavorable living conditions. Inhabitants of the most extreme environments found on Earth are predominantly prokaryotic organisms whose metabolic requirements often make them obligate members of specified habitats (Deming 1986, Jannasch et al. 1992, Stetter et al. 1993). However, bacterivorous protists also may be found in many of these extreme environments in close association with their bacterial prey (Small & Gross 1985, Patterson & Simpson 1996, Atkins et al. 2000). Unlike prokaryotes, which may be specialized for their particular niche, bacterivorous protists found in extreme environments may be ubiquitous species with high tolerance or adaptability to extreme conditions (Atkins et al. 2000). These organisms may be among the most tolerant or adaptable eukaryotes living on this planet, their ability to withstand or adapt to extreme environmental conditions enabling them to feed on an otherwise inaccessible food source.

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Hydrothermal vents are comprised of some of the most extreme environments on Earth. Vent fluids are hotter, more acidic and enriched with metals and dissolved gasses than the surrounding seawater (Table 1). Characterized by very steep physical and chemical gradients between vent fluids and seawater, vent fields may rival the abiotic environments characteristic of other planets. Still, life occurs at vents in abundance. The question remains as to how some organisms, particularly cosmopolitan species, are able to tolerate and survive under vent conditions. This is especially true for microorganisms like the flagellated protists that arrive in the deep sea on sinking particulate matter (Silver & Alldredge 1981, Patterson & Fenchel 1990) and have been shown to be members of deep-sea benthos (Burnett 1977, Turley et al. 1988) and deep-sea vent microenvironments (Atkins et al. 1998, 2000).

The present study determined the effects of both sulfide (H_2S, HS^- and S^{2-}) and metals, at concentrations approaching those found in end-member fluids emanating from deep-sea hydrothermal vents, on ubiquitous species of flagellates isolated from these environments. End-member vent fluid concentrations of transition metal species are enriched several orders of magnitude relative to average seawater (Seyfried & Mottl 1995), and sulfide concentrations may reach several millimolar (Table 1). Among protists, sulfide tolerance has been studied mostly in ciliates from natural environments with concentrations much lower than vent fluids (Bick & Kunze 1971, Matsuyama & Moon 1997). Other studies have measured the effect of metals at trace and somewhat higher concentrations on protistan taxa isolated from environments considered non-extreme (Brand et al. 1986, Stoecker et al. 1986, Sunda 1989, Sunda & Huntsman 1996).

To determine whether the ability to be active vent consumers exists, the survival rates of 3 species of deep-sea vent flagellates were measured after exposure to chemical conditions potentially encountered in vent environments. The flagellates used in this study were chosen on the basis of their ubiquity in very diverse and often extreme global environments, suggesting a high tolerance to a wide range of environmental conditions. The survival rates (measured as viability through time) of *Caecitellus parvulus*, *Cafeteria* sp. and *Rhynchomonas nasuta* were measured and compared to shallow-water strains of the same species after exposure to increasing concentrations of sulfide or the metals Cu, Fe, Mn and Zn.

MATERIALS AND METHODS

Isolates. Hydrothermal vent and shallow-water flagellates were collected and isolated as described by Atkins et al. (1998, 2000) (Table 2). Note that both

Table 1. Comparison among literature values for average deep-sea seawater and end-member fluids from deep-sea hydrothermal vents in the Eastern Pacific Ocean. Values shown in parenthesis for the 9° N East Pacific Rise (EPR) end-member fluids were taken during an eruption event. Also shown are total Cu, Fe, Mn and Zn concentrations measured in vent samples from which organisms were obtained. Vent strains used in the present study were obtained from the 9° N EPR hydrothermal vent sites shown below (see Table 2)

Vent	Site	Temperature (°C)	pH	H_2S (mM)	Cu (µM)	Fe (µM)	Mn (µM)	Zn (µM)
Average deep-sea seawater		**2**	**7.8**	**0**	**0.007**	**0.001**	**0**	**0.01**
Juan De Fuca	**End-member fluids**[a]	**224–285**	**3.2**	**2.9–8.1**	**2**	**18700**	**3600**	**900**
	Dante Flange Substrates (DFS)				2	5800	70	558
	Lobo Flange Subtrates (LFS)				3	7300	809	215
Guaymas Basin	**End-member fluids**[b]	**315**	**5.9**	**4.4–6.0**	**1**	**180**	**236**	**40**
	Sediment-water interface				2	3500	447	65
	Beggiatoa mat (GBB)				32	5700	2000	514
	Flange sample				10	8800	5600	521
21° N EPR	**End-member fluids**[c]	**355**	**3.4**	**7.3**	**35**	**1500**	**1000**	**106**
	Clam acres spire (CAS)				0	27	8	2
	Twin peaks chimney (TPC)				2	62	34	6
9° N EPR	**End-member fluids (eruption)**[d]	**329 (403)**	**3.5 (2.5)**	**11.3 (>65)**		**2600**	**925**	**44**
	Biovent serpulid zone (BSZ)				0	103	27	8
	Biovent riftia and mussels bed (BRM)				5	227	44	12
	East wall mussels bed (EWM)				2	30	21	2

[a] Lilley et al. (1993); Butterfield et al. (1994)
[b] Von Damm et al. (1985b); Campbell et al. (1988)
[c] Welhan & Craig (1983); Von Damm et al. (1985a); Lilley et al. (1993)
[d] Von Damm (1990); Haymon et al. (1993); Von Damm et al. (1995)

Table 2. Pure culture isolates used in this study were obtained from shallow, coastal waters (Chesapeake Bay, MD [CBR], Eel Pond, MA [EPM] and New Bedford Harbor, MA [NBH]) and the 9° N deep-sea hydrothermal vent in the Eastern Pacific Ocean. Shown are taxonomic classification, species and strain names, specific collection locations, vent sites and depths. All cultures were grown at atmospheric pressure

Classification	Species	Strain	Collection location	Vent site	Collection depth (m)
Bicosoecida, Grassé & Deflandre, 1952					
	Cafeteria sp.	VENT1	9° N vent water – H_2S reactors	9° N	2500
	Cafeteria sp.	EPM1	Eel Pond Marsh – H_2S reactors		1
	Caecitellus parvulus, Patterson et al., 1993	EWM1	East Wall Mussels Bed	9° N	2500
	Caecitellus parvulus, Patterson et al., 1993	NBH4	New Bedford Harbor, MA		3
Kinetoplastida, Honigberg, 1963					
	Rhynchomonas nasuta, Klebs, 1892	CBR1	Chesapeake Bay, MD		1
	Rhynchomonas nasuta, Klebs, 1892	BSZ1	Biovent Serpulid Zone	9° N	2500

strains of *Cafeteria* were isolated from hydrogen sulfide reactors used to culture sulfur-oxidizing bacteria from both vent and shallow-water samples (Taylor & Wirsen 1997, Taylor et al. 1999). Isolates were cultured in growth media (0.22 µM filtered, autoclaved vineyard sound seawater [VSW] [made up to 10^{-3} M with nitrilotriacetic acid {NTA} in metals experiments], 0.01% yeast extract, *Halomonas halodurens* bacteria as prey), concentrated via centrifugation and inoculated into experimental vials at a density of 1E5 cells ml^{-1}. Growth media supernatant was removed after concentrating isolates to prevent their growth during the experiments. The experimental media therefore was plain VSW (± NTA buffer) with added sulfide or metals.

Determining sulfide and metals concentration ranges used in experiments. The initial range of concentrations used in sulfide experiments was determined from literature values of end-member vent fluids shown in Table 1. The range of metal concentrations was determined by comparison between literature values and values obtained by atomic absorption spectrophotometry (AAS) of frozen, unfiltered vent samples from which flagellates were isolated (Table 1). Unfiltered samples were dried and prepared for AAS by complete digestion in hot 15% nitric acid. Acidified samples were analyzed on a Perkin Elmer AAnalyst 100 atomic absorption spectrophotometer, calibrated using standard solutions of known concentration for each metal.

Rationale for using pH range. For the trace metal experiments it was important to make sure the correct amount of metal was added to the experimental treatments. To imitate the extreme environmental situation of hydrothermal vents, very high vent-like metal concentrations are necessary that push the limits of metal solubility and are therefore hard to separate from pH effects. To achieve a 0.01M concentration requires a stock solution of at least 10-fold higher, and to solubilize such a stock solution it is necessary to decrease the pH to ~2. As a result, higher metal treatments had increased amounts of protons present. The converse of this situation would be to not acidify the stock solutions, resulting in precipitates and colloids, and thereby introducing unknown and unaccountable metal additions into the experimental treatment. Given this problem, we decided that the variation in pH that comes with acidifying our stock solutions was an unavoidable circumstance of doing metal toxicity experiments at these extremely high concentrations. Further, this pH variation was consistent with the naturally occurring pH variation between end-member fluids and seawater at hydrothermal vents (from pH 4 to 5 in end-member fluid to ca pH 8 in seawater). To account for this pH variation, an explicit effort was undertaken to model the effects of pH on both solubility and free metal concentration (Fig. 1), and the influence of pH on survival was examined.

The need to have such high concentration stock solutions necessitated their preparation at pH 2, in order to achieve complete metal dissolution. This resulted in experimental treatments that became increasingly acidic with more metal added, but the pH remained within values organisms could experience under real vent conditions. The alternatives to this effect are less attractive than the design we settled on: (1) make metal stock solutions that are neutral in pH, which would obviously be inaccurate with respect to metal concentrations upon precipitation; (2) run all the experiments at low pH, which would not be environmentally relevant; and, (3) pH adjust each of 120 experimental treatments after addition of metals, which would introduce significant variability and potential cross contamination, not to mention drastically changing the solubility of the metals.

The inclusion of elaborate speciation calculations in Fig. 1 aimed to deal with these experimental difficulties by showing in detail how metal speciation varies with pH. For similar reasons, we assume pH variation in sulfide experiments is environmentally valid as well.

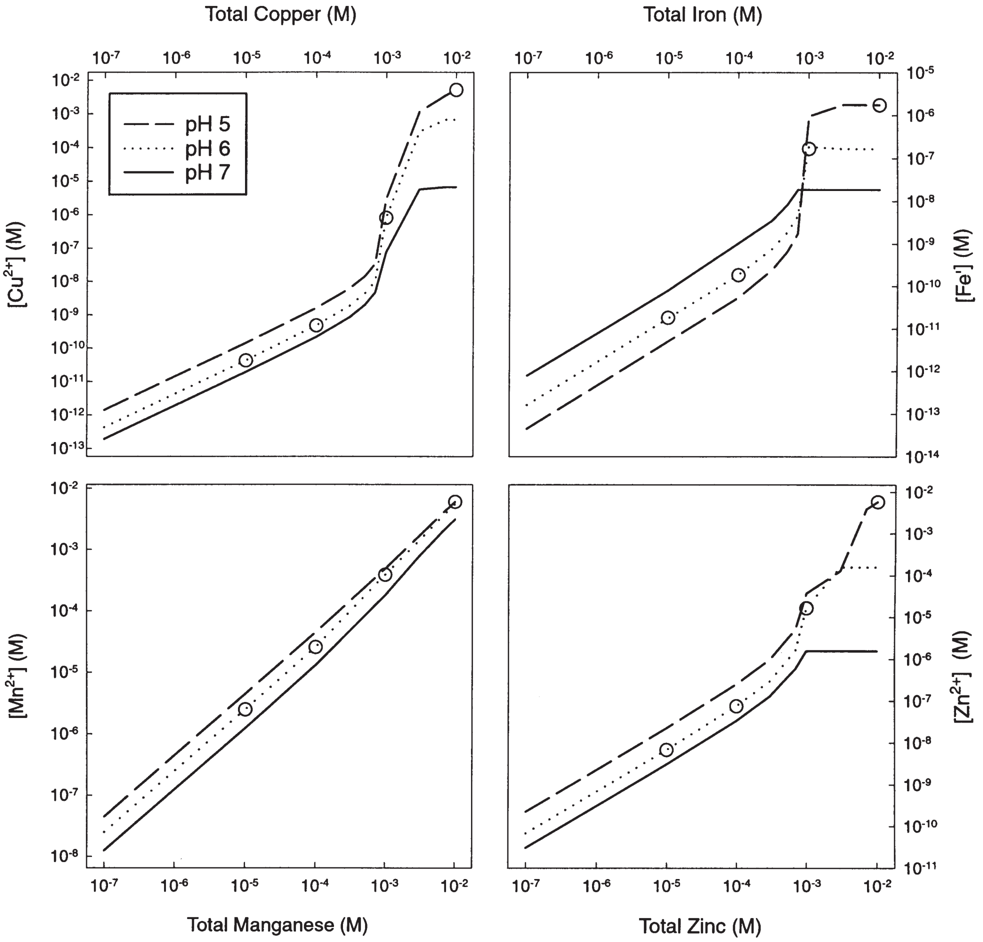

Fig. 1. Calculations of metal speciation were made using MINEQL+ (Westall et al. 1976) with stability constants verified from the NIST database (Martell & Smith 1993) and corrected for ionic strength. Typical seawater concentrations of Na^+, Cl^-, Mg^{2+},Ca^{2+}, and dissolved inorganic carbon were used in calculations to achieve correct ionic strength and free NTA concentrations. Solubility of the metals were determined relative to ferrihydrite, $Cu(OH)_{2(s)}$, $ZnCO_3\ 1H_2O_{(s)}$, and $MnCl_24H_2O_{(s)}$ solids. Highly crystalline solids were ignored due to their inability to form at the low temperatures and short time scales of this experiment. Fe′ concentrations are plotted where Fe′ is the summation of the dominant aqueous inorganic iron species ($Fe' = Fe^{3+} + FeOH^{2+} + Fe(OH)_2^+ + Fe(OH)_{3(aq)} + Fe(OH)_4^-$). Fe′ is several orders of magnitude higher than the free Fe^{3+} ion alone. Total metal concentrations were calculated from dilutions of acidified stock solutions; background concentrations of metals in vineyard sound water (VSW) were insignificant relative to the higher concentrations of metals added in the experiments. Circles show the calculated free-metal ion concentration at a given experimental total metal concentration as a function of pH.

Sulfide media. It should be noted that the term sulfide is generally used here to mean the 3 sulfide species present in these experiments: H_2S, HS^- and S^{2-}. At the pH range used in these experiments, HS^- is expected to be the dominant species. Toxicity mechanisms and effects among the 3 sulfide species are assumed to be similar. Sulfide media was prepared from anaerobic stock solutions of $Na_2S \cdot 9H_2O$ diluted to the experimental concentrations by addition to serum vials containing sterile, anoxic VSW. Sulfide concentrations in each vessel were measured at each time point using the spectrophotometric determination technique described by Cline (1969). Sulfide concentrations were relatively stable (±5%) throughout the experiments, indicating anoxic conditions were maintained. The pH was measured at each time point and ranged between 7 (lowest sulfide concentrations) and 9 (highest sulfide concentrations).

Metals media. Metal toxicity experiments were carried out in the presence of a synthetic metal ligand that buffers the free metal ion concentration (i.e. Cu^{2+}, Fe^{3+}, Mn^{2+}, Zn^{2+}) by binding the majority of the total dissolved metal and maintaining an equilibrium with the free metal ions. These free metal ion concentrations are then buffered against processes that remove metals by re-equilibration with this large reservoir of metals bound to synthetic metal ion ligands. The high concentration of synthetic buffer in these types of experimental systems results in a free ion concentration that is thought to be independent of any natural ligands present in the filtered seawater used to make the media (Brand et al. 1986). Nitrilotriacetic acid (NTA) was chosen as the synthetic metal ion ligand in expectation of a high degree of resistance to metal toxicity by these vent organisms. NTA has advantages over more commonly used metal buffers like EDTA, in that its binding constants for metals in seawater are significantly lower than that of EDTA. This allows higher free metal concentrations relative to the concentration of metal buffer (e.g. $Cu^{2+}/NTA > Cu^{2+}/CuEDTA$). Control experiments were done to test sensitivity to NTA. These experiments showed that 10^{-3} M NTA was the maximal concentration of NTA that could be used before the NTA itself became toxic (see Fig. 2). Metal stock solutions were made from salts of $CuSO_4 \cdot 5H_2O$, $FeCl_3 \cdot 6H_2O$, $MnSO_4 \cdot H_2O$ and $ZnSO_4 \cdot 7H_2O$

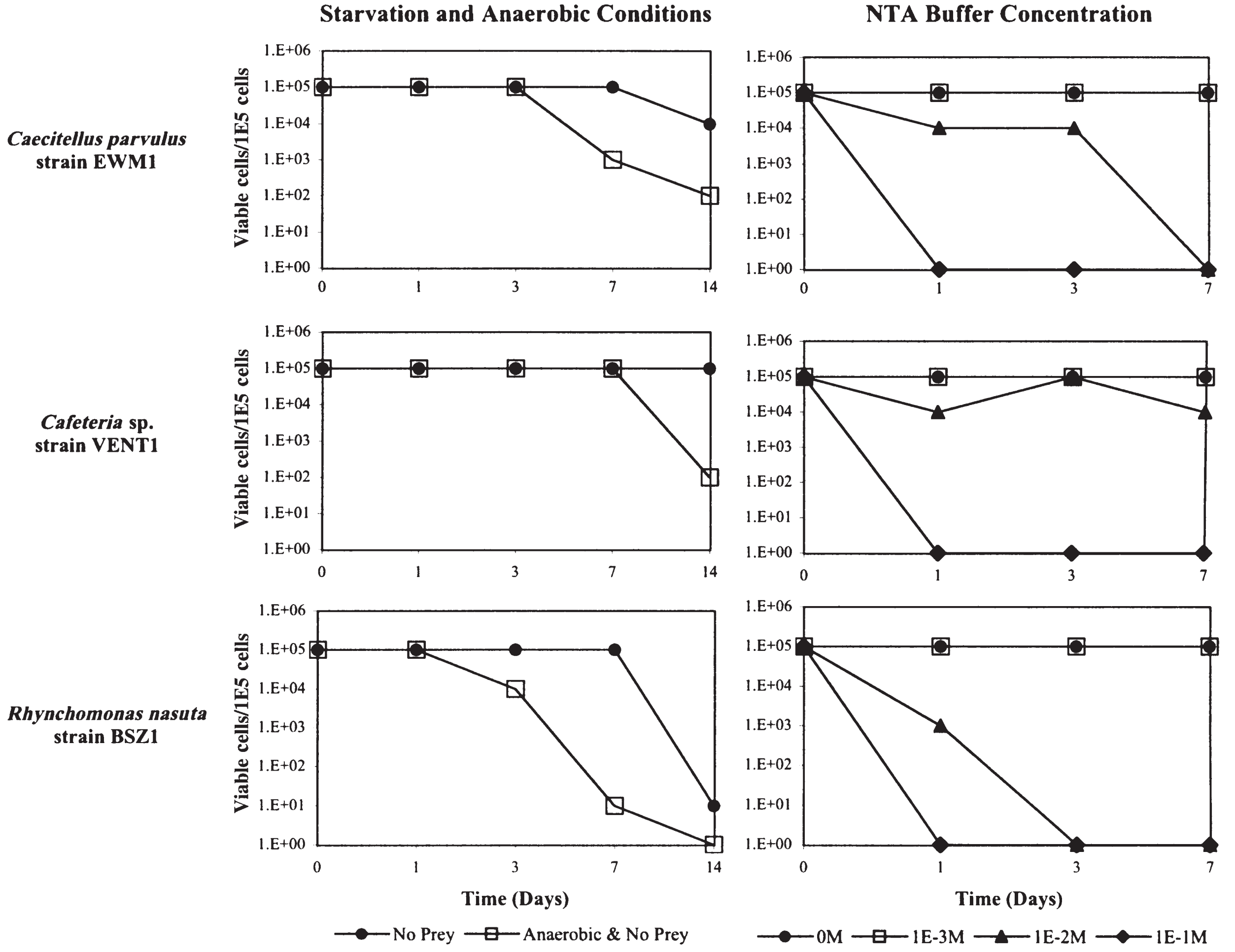

Fig. 2. Control experiments testing starvation and anaerobic conditions and NTA buffer concentrations for the 3 vent strains shown. From these results, the duration of sulfide and metals experiments and the concentration of NTA used in metals media were chosen

dissolved separately in Milli-Q water and acidified to pH 2 to prevent precipitation of solids. These metal stocks were diluted to the experimental concentrations by addition to vials containing sterile, oxic VSW buffered with 10^{-3} M NTA and were allowed to equilibrate for 24 h prior to inoculation with flagellates. The pH was measured at each time point and ranged between 5 and 7 for highest and lowest metal concentrations respectively.

Control experiments. Since these experiments measure flagellate survival as a function of exposure time, growth conditions for flagellates (i.e. bacterial prey) were not present in any of the experimental vials. Therefore, these experiments were performed under starvation conditions. Sulfide experiments were performed under the combined effect of starvation and anoxic conditions. Control experiments were done to measure the effect of these conditions through time. Flagellates were prepared as above then inoculated into vials containing sterile VSW with no prey and no prey under anoxic conditions (Fig. 2). These controls set the duration of both sulfide and metal experiments: *Rhynchomonas nasuta* was the most sensitive to anoxic/no prey conditions, with survival being affected after 24 h; therefore, this was set as the duration of sulfide experiments. All organisms showed 100% viability up to 168 h (7 d) without prey; therefore, this was set as the duration of the metals experiments.

Additional controls were done to measure the effects of both pH and NTA buffer on survival to show that exposure to sulfide or metals, not pH or buffer, was responsible for the toxicity effect measured in actual experiments. For all species, there was 100% survival after 7 d exposures to pH 5, 7 and 9 (data not shown). Concentrations of NTA began to have deleterious effects at 10^{-2} M, whereas 10^{-3} M NTA had 100% survival after 7 d (Fig. 2). As a result, 10^{-3} M NTA concentration was chosen for metal toxicity studies in order to maximize metal buffering at high concentrations of

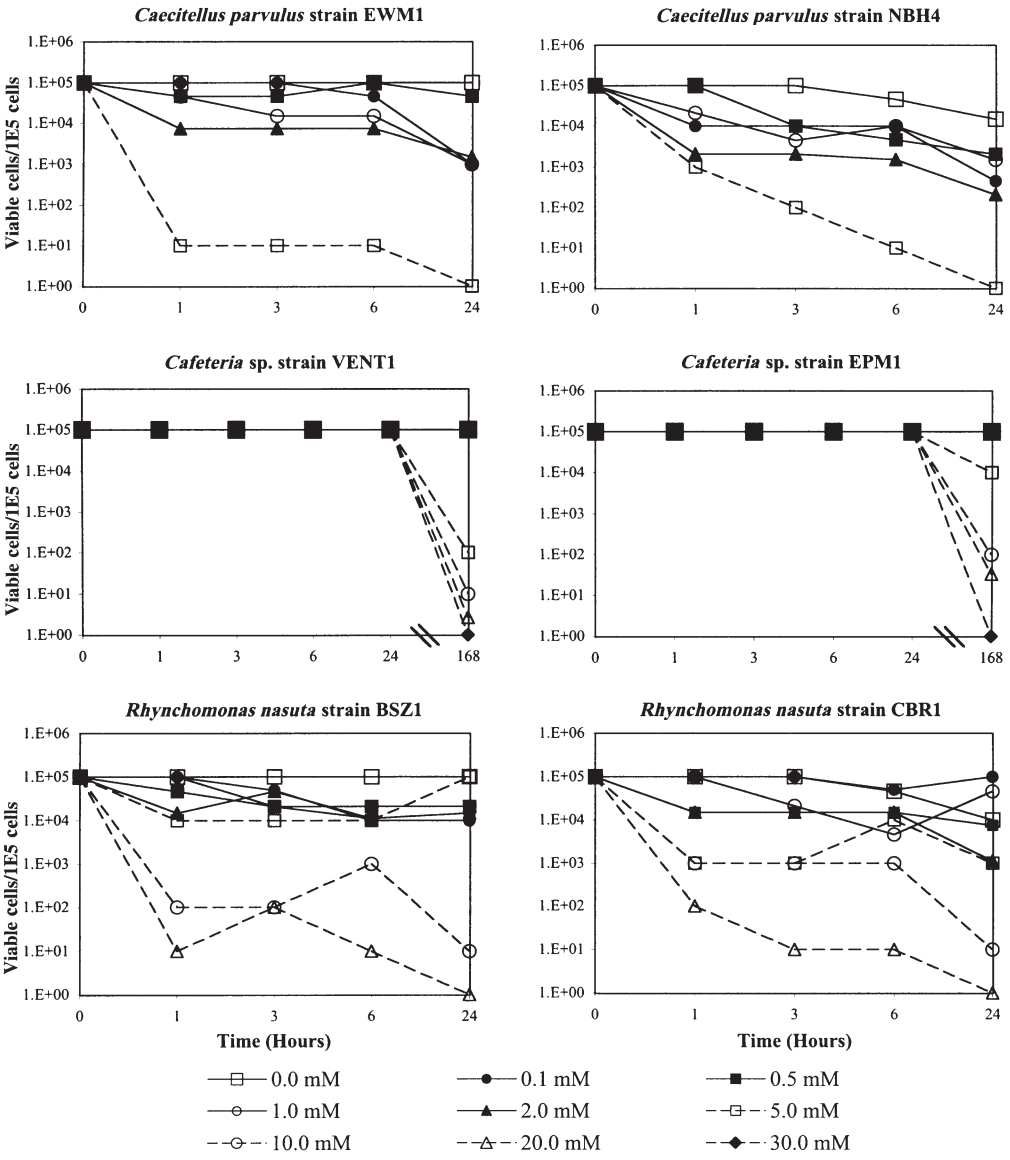

Fig. 3. Results of sulfide toxicity experiments. Deep-sea vent strains are in the left column; shallow-water strains are in the right column. All sulfide concentrations shown in the figure legend were tested on each organism; overlaying of lines occurred at lower concentrations of sulfide for *Caecitellus parvulus* strains and *Rhynchomonas nasuta* strains up to 24 h and at all concentrations of sulfide up to 24 h for *Cafeteria* sp. strains

metal. These controls proved the efficacy of the experimental design.

Measuring survival using MPN dilutions. In both sulfide and metals experiments, samples were removed at discrete time points (sulfide: 1.0, 3.0, 6.0, and 24.0 h; metals: 24, 72 and 168 h, respectively), serially diluted from 1E0 to 1E-5 ml (1/10 each subsequent dilution) and cultured in growth media up to 21 d. The cultures were then microscopically observed for positive or negative growth of flagellates. All experiments were done in triplicate and MPN tables were used to determine viable cell numbers from positive and negative growth observations.

RESULTS

Sulfide tolerance

Fig. 3 shows the results of survival experiments conducted at variable concentrations of sulfide. There was no significant difference in survival between vent and shallow-water strains of the same species. However, there was high variability among different species, including the closely related sister taxa *Cafeteria* and *Caecitellus*. Both strains of *Cafeteria* (VENT1 and EPM1) showed the greatest tolerance to high sulfide conditions, whereas *Caecitellus* strains showed the least tolerance of the 3 species tested. The dissolved sulfide concentrations used in this study are high relative to naturally occurring concentrations, which have been recorded at a global maximum of 18 to 20 mM (Brown et al. 1994, Jonas & Tuttle 1996). Anomalously high dissolved sulfide concentrations (>65 mM) have been recorded during volcanic eruptions (Haymon et al. 1993, Von Damm et al. 1995), but these are ephemeral events, occurring only on very short time scales in superheated end-member fluids and therefore are not considered as part of a stable ecosystem, extreme or otherwise.

Cafeteria strains (VENT1, vent; and EPM1, shallow) showed 100% viability at all concentrations up to 30 mM throughout the course of the experiment (24 h). In this one case, the experiment was continued to test viability after 168 h (1 wk). At 168 h, *Cafeteria* spp. showed 0.1% viability at 5 mM, 0.01% viability at 10 mM, >0.001% viability at 20 mM and no survival at 30 mM (Fig. 3).

Both strains of *Rhynchomonas nasuta* (BSZ1, vent; and CBR1, shallow) showed the second highest tolerance to high sulfide conditions. There was no significant difference in viability between 0.1 and 5 mM concentrations for either strain. Viability in this range was between 10 and 100% over the 24 h period. However, 10 mM sulfide resulted in 1.0% viability following a 6 h exposure and 0.01% viability after a 24 h exposure. Exposure to 20 mM sulfide resulted in 0.01% viability after 6 h and 0% viability after 24 h.

Both strains of *Caecitellus parvulus* (EWM1, vent; and NBH4, shallow) showed the lowest tolerance to high sulfide conditions of the 3 species tested. Viability generally was inversely proportional to sulfide concentration. Exposure to 2 mM sulfide resulted in approximately 1% viability, and exposure to 5 mM resulted in no survival in either strain after 24 h.

Comparison of literature and experimental metals concentrations

The important feature of Table 1 is to show that vent flagellates may encounter total metals (and sulfide) concentrations within the range tested in these experiments and that concentrations can reach or exceed those emitted in end-member fluids. Table 1 compares total metals concentrations from literature values in end-member fluids and AAS measurements of vent samples from the exact microhabitats (shown as acronyms in parentheses) from which vent flagellates were isolated (see Atkins et al. 2000). There is good agreement, within approximately an order of magnitude, between our measurements and literature values for Cu at all vents; for Fe at Juan de Fuca and for Zn at Juan de Fuca and Guaymas Basin. End-member fluid and sediment-water interface metals concentrations are very similar for Cu, Mn and Zn at Guaymas Basin. Generally, total metals concentrations were higher to much higher in sediments at Guaymas Basin than in end-member fluids or bottom water, suggesting accumulation and concentration of metal sulfide or other metal precipitates. Our measurements at 21° N were generally lower than literature values.

Tolerance to metal ions

Fig. 4 shows the results of the metal toxicity survival experiments. In general, there was no difference in viability between strains or between species, with the possible exception of *Caecitellus parvulus* strain EWM1. The metals exhibited differing toxicities: ferric iron had the greatest effect on survival followed by copper and zinc; manganese produced little or no effect on survival (i.e. Fe > Cu > Zn >> Mn). Of iron, copper and zinc, only the 10^{-2} M total metal concentration had an effect; lower concentrations were not significantly different and had little to no effect, in general. There seems to be a threshold response for these metals at 10^{-2} M total metal concentration that is illustrated by a sharp decrease in species viability.

Caecitellus parvulus strain EWM1 had an increased tolerance to ferric iron relative to all other strains. After a 24 h exposure to 10^{-2} M total Fe, other strains had ca 0% viability; cultures of EWM1, on the other hand, showed 10% viability after 24 h and 1% viability to the end of the experiment. Compared with the results of the shallow-water strain NBH4 and the other flagellates, these results appear anomalous rather than exceptional. However, there still was a threshold response at 10^{-2} M total metals concentrations for this strain.

Unlike in the sulfide experiment, strains of *Cafeteria* sp. did not show higher tolerance to metal exposure than the other species. This strengthens the hypothesis that *Cafeteria* sp. are especially adapted to or tolerant of sulfidic conditions and indicates that metals act on flagellates via a generalized mechanism shared at least among a wide variety of flagellate taxa.

Experimental cultures of *Rhynchomonas nasuta* strain BSZ1 were contaminated by other protozoan species midway through the metals experiments, and so the data were not used. Preliminary results prior to the contamination event suggested that there was no difference in the responses to metals between vent and shallow-water strains of this species.

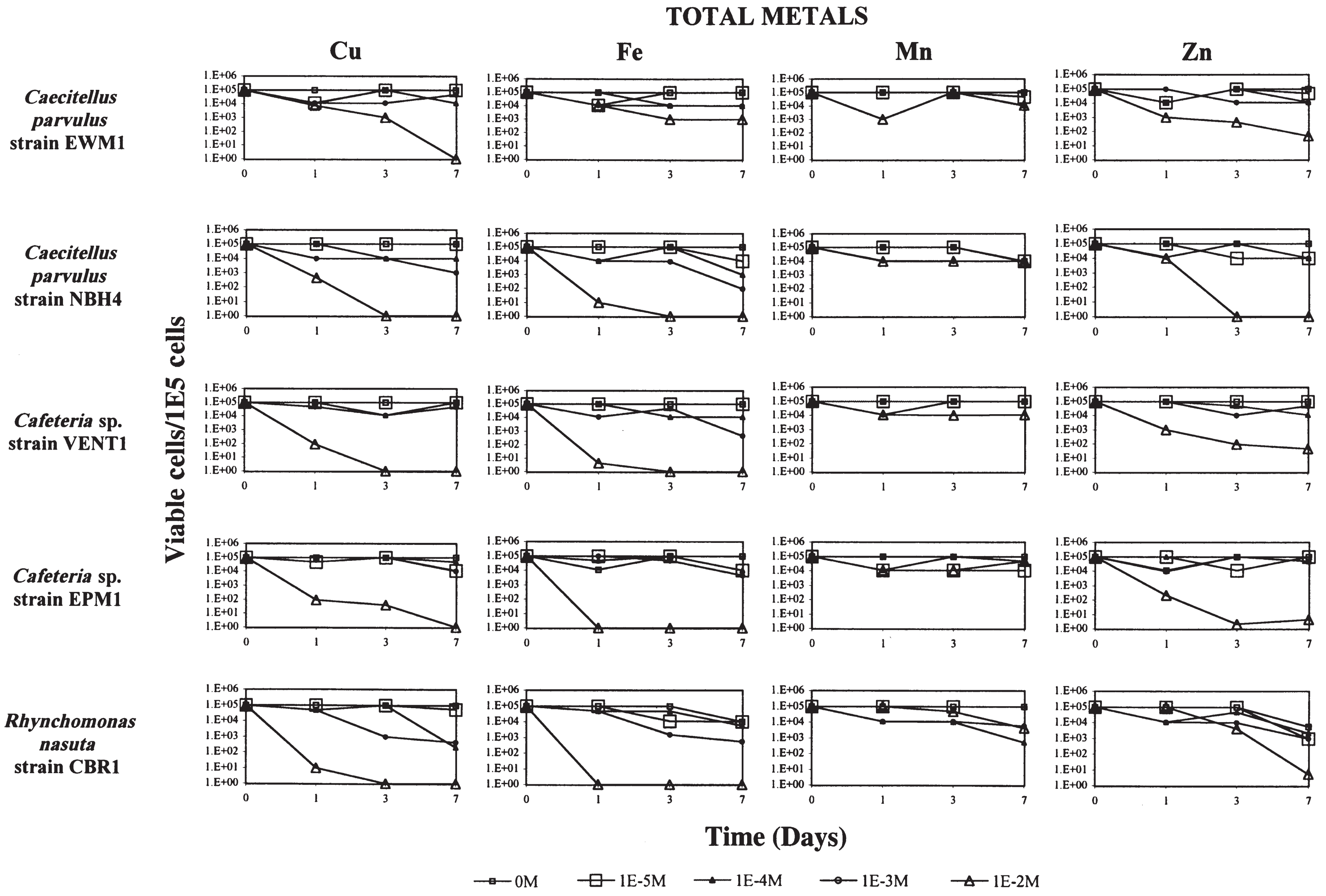

Fig. 4. Results of metal toxicity experiments. Metals concentrations represent total metals as explained in the text. All metals concentrations shown in the figure legend were tested on each organism; overlaying of lines occurred at lower concentrations of all metals. Data for *Rhynchomonas nasuta* strain BSZ1 (vent strain) not shown due to contamination by other protist species during the experiments

Calculated metal speciation under experimental conditions

Metal toxicity and bioavailability have been shown to correlate with the concentration of free metal ions rather than with the concentration of total metals in solution (Sunda 1989). Concentrations of free copper, iron, manganese, and zinc were calculated with respect to NTA buffering and solid precipitation in this experimental system and were plotted against total metal concentration (Fig. 1). The results show that free ion concentrations are linear with total metal concentration until the NTA is close to being fully titrated with metal, at which point the slope increases as added metal is no longer bound by NTA. Lower pH generally results in higher free metal ion concentration relative to higher pH (Fig. 1). Copper, iron, and zinc begin to precipitate as the free ion concentration exceeds the solubility product for the solid forms of metals included in our calculations (ferrihydrite, $Cu(OH)_{2(s)}$, $ZnCO_3\ 1H_2O_{(s)}$, Pyrocroite and $MnCl_24H_2O_{(s)}$). While the thermodynamic constants for solubility suggest a metal will precipitate, this process either may not occur or may not go to completion due to slow kinetics of solid formation. Of these 4 metals, only manganese has a redox chemistry that could result in deviations from our calculated free ion activity. Manganese (Mn) exists in seawater primarily in the Mn(II) oxidation state as Mn^{2+}. While thermodynamic predictions dictate that Mn should be oxidized to insoluble Mn(IV) oxides under typical oxic oceanic conditions, this reaction is kinetically slow on our experimental time scale. The oxidation of Mn(II) by microbial processes (Emerson et al. 1982, Moffett & Ho 1996) and the reduction of manganese oxide solids ($Mn(IV)O_{2(s)}$) by light and dissolved organic carbon (Sunda et al. 1983, Sunda & Huntsman 1988) have been shown to be important mechanisms influencing the chemistry of Mn in the surface ocean. However, other workers have observed little toxic effect of manganese on more sensitive marine organisms (Sunda & Huntsman 1998), hence any inadvertent loss of Mn^{2+} by oxidation and subsequent precipitation may not change our results significantly. Under our oxic experimental conditions, we would expect iron to remain primarily in its oxidized form as Fe(III). Anderson & Morel (1982) showed that Fe(III) uptake is controlled by the free ion model in EDTA buffered media, with slight deviations possible when those cultures are exposed to light.

DISCUSSION

The organisms used in this study were chosen because it was hypothesized that globally distributed species, especially those occurring in both extreme and non-extreme environments, must be highly-tolerant or adaptable to a wide range of environmental conditions (Atkins et al. 2000). The distribution of *Caecitellus*, *Cafeteria* and *Rhynchomonas* species is global (Fenchel & Patterson 1988, Patterson et al. 1993, Patterson & Simpson 1996, Ekelund & Patterson 1997), and they appear to be among a small group of the most commonly occurring heterotrophic flagellates on Earth (Lee & Patterson 1998). Given this, they seemed the most promising eukaryotic organisms for studies of tolerance to very extreme environmental conditions. As deep-sea hydrothermal vents are among the most extreme environments found on Earth, we chose to examine 2 parameters of importance at vents to test extreme tolerance in our flagellates: exposure to sulfide and metals.

This study of extreme tolerance in eukaryotic organisms was conducted under simplified conditions that differ from conditions found at deep-sea vents. Variables were separated to make them experimentally more tractable. Vents are much more complex systems than laboratory experiments can simulate and the interactive effects of vent temperature, pH, pressure and their complex chemistries cannot fully be explored in the more limited environment of the laboratory. Microniches inhabited by bacteria and nanoflagellates further complicate matters because physical and chemical parameters are not easily measured and replicated at this scale. These experiments began with the simplest question: how tolerant are these cosmopolitan species to individual parameters of the extreme chemistry they may encounter at deep-sea vents? The results suggest that these flagellates may be among the most tolerant eukaryotes studied. Future studies can build on this baseline study by increasing in complexity to look at the interactive effects of multiple physical and chemical parameters.

Sulfide experiments

The results from sulfide experiments truly are remarkable from both ecological and evolutionary perspectives. That *Cafeteria* species showed no negative response to 30 mM sulfide after 24 h and limited survival after 1 wk at 20 mM indicates that there is no sulfidic environment on Earth (except perhaps volcanic eruptions) where this organism cannot penetrate, for at least brief periods. *Cafeteria* spp. potentially could extend their range beyond the surficial vent field into the shallow subsurface biosphere below vents via a mechanism similar to that observed in groundwater protists (Novarino et al. 1999). It is also reasonable to assume that *Cafeteria* is capable of feeding on sulfur-oxidizing bacteria present at interfaces between H_2S and O_2 (Taylor & Wirsen 1997), by swimming into the H_2S layer metabolized by their prey. This phenomenon was observed in hydrogen sulfide reactors prepared for chemoautotrophic sulfur-oxidizers (Taylor & Wirsen pers. comm.).

Evolutionarily, *Cafeteria* may represent a unique lineage among bicosoecids and their sister taxa, which includes *Caecitellus* (Atkins et al. 2000). Unlike *Cafeteria*, *Caecitellus* was unable to tolerate sulfide at concentrations up to 5 mM. This result suggests that high sulfide tolerance is not universal among members of this taxon, but rather a trait unique to a specialized few. Further study within this group is required to test this hypothesis. The results from *Rhynchomonas* bolster the conclusion that sulfide tolerance is variable, and perhaps even specialized, among the diversity of flagellates. *Rhynchomonas* showed relatively high tolerance to sulfide, yet it is a member of the Euglenozoa at the base of the eukaryotic 18S tree, quite distant in its relationship to either *Cafeteria* or *Caecitellus* (Atkins et al. 2000).

Since there was little or no difference in tolerance between vent and shallow-water strains of each species exposed to identical sulfide conditions, these results strengthen the conclusions of Atkins et. al. (2000), who suggested that microorganisms such as these are not affected by normal barriers contributing to speciation (e.g., large-scale geographical barriers such as continents or large oceanic distances). The results presented here illustrate that there is no significant evolutionary difference between the strains with respect to sulfide tolerance and results presented by Atkins et al. (2000) show there is no difference between 18S rDNA sequences for vent and shallow strains of the same species isolated from distant geographical locations.

Metals experiments

Simulating the extreme environments of hydrothermal vent sites poses many unique challenges to the design of a metal toxicity study. Extremely high metal concentrations, and expansive proton (pH) and redox (Eh) concentration gradients within very small geographic regions are characteristic of vent environments. To tease out the toxic effects of individual metals on organism survival, we used experimental systems with oxic seawater and no sulfide. This absence of sulfide in our experimental setup is an important simplification that allows for a tractable experimental design and straightforward calculations of free metal ion concentrations. The presence of sulfide would change the metal speciation and solubility through the formation of dissolved metal sulfide species and sulfide precipitates. Given that these organisms should be responding to the free metal ion toxicity rather than the total metal concentrations or metal sulfide chemical species, these toxicity results can be related to those of a system including sulfide if metal speciation data and/or sulfide concentrations from that environment are available for free metal ion calculations.

Metal toxicity studies at these high concentrations also pose unique challenges to the metal-ion buffered media used in the experimental system. We chose NTA as a buffer because it is weak enough to allow a relatively high free metal ion concentration relative to the concentration of total metal. Control studies determined that the maximum concentration of NTA we could use without the NTA itself becoming toxic was 10^{-3} M. We included a total metal concentration in excess of the NTA for the highest metal treatment in expectation of a high degree of metal tolerance. The changes in metal speciation were calculated, including the precipitation of metals at higher total concentrations once the NTA was titrated out. Free metal concentrations also increase once NTA has been completely titrated by a metal, and it is these higher free metal concentrations resulting in precipitation of solids that were observed experimentally in the Cu and Fe systems.

The results presented here were compared with copper toxicity results from other organisms. Copper is generally considered one of the most toxic metals in marine environments: toxicity has been observed at picomolar to nanomolar concentrations of free copper. Other studies that measured protist Cu toxicity showed a 50% decrease in growth at concentrations between 10^{-10} and 10^{-13} M (Brand et al. 1986, Stoecker et al. 1986, Mann et al. 2000), 2 to 6 orders of magnitude lower than measured in this study.

Future work could incorporate sulfide chemistry into the metal toxicity studies. While hydrogen sulfide is rapidly oxidized in seawater, it is thought that it may be stabilized by complexation to metal ions (as cited in Rozan et al. 1999). Metal sulfide complexes were shown to have environmentally relevant half-lives in river waters, with Zn and Cu sulfide having 16.7 and 21.9 d half-lives respectively (Rozan et al. 1999). Extrapolation of our toxicity results to the temperature, pressure, and chemical conditions of hydrothermal vent communities is difficult without metal speciation data. To our knowledge, no studies of electrochemical speciation have been conducted in these extreme environments. However, we can speculate that given the high concentrations of sulfide at vent communities and its relatively slow degradation half-life, metal bioavailability is likely to be significantly less than the total dissolved metal concentrations.

In both cases, sulfide and metals damage organisms on a subcellular level. For unicellular organisms, the result is death of individuals and decreased viability or survival for the population. The mechanism of sulfide toxicity is disruption of the electron transport chain in mitochondria, resulting in intracellular hypoxia. Detoxification of sulfide in eukaryotes is accomplished by sulfide-binding proteins or enzymatic and non-enzymatic oxidation of sulfide compounds to nontoxic or less toxic compounds, such as thiosulfate ($S_2O_3^{2-}$) (Grieshaber & Völkel 1998). Metal ions, such as copper and iron, are thought to catalyze the conversion of hydrogen peroxide (H_2O_2) to a hydroxyl radical ($OH^\bullet$), a powerful oxidant with the ability to damage cellular components (Fridovich 1978, 1983). Alternatively, heavy metals such as Cd, Cu, Pb and Zn replace functional metals inside cells by inappropriate binding to nitrogen, oxygen and sulfur ligands in biomolecules, thereby inactivating enzymes and disrupting cellular function (Gadd & Griffiths 1978, Karlström & Levine 1991). It has been demonstrated in yeasts that there is vacuolar involvement in detoxification of Co, Mn, Ni, and Zn (Ramsay & Gadd 1997), whereas detoxification of Cu and Cd appears to be cytosolic, with metal-binding proteins and even sulfide playing a large role (e.g., metallothionein for Cu and phytochelatins for Cd) (Mehra & Winge 1991, Gadd 1993, Ahner & Morel 1995, Ahner et al. 1995, Wu et al. 1995).

An intriguing question is raised by the results in Fig. 4: Are we observing metal toxicity due to colloidal or particulate metal formation? The data in Figs 1 & 4 suggest this possibility since copper, iron, and zinc precipitation is predicted to occur in the treatments where significant toxicity is observed. At total metal concentrations between 10^{-3} and 10^{-2} M where a threshold response is observed in flagellate populations, there is a rapid increase in free metal ions due to an excess of metal over NTA concentration and the formation of metal precipitates (data points in Fig. 1). At this threshold, all flagellate populations have a survival rate effectively of zero. While toxicity due to exposure to high concentrations of free metal ions has been well documented (Gledhill et al. 1997), it is possible that exposure to particulate and colloidal metals could also result in a toxic response. Barbeau showed that *Cafeteria* and other species of protists can ingest colloidal iron at a fairly fast and efficient rate, and that colloidal iron is remineralized during passage through the cells (Barbeau et al. 1996, Barbeau 1998). Toxicity may occur due to internal poisoning via non-selective ingestion of metal colloids and other particles during feeding. This ingestion and remineralization of colloids could potentially deliver a much larger dose of toxicants to the cytosol relative to diffusion and transported uptake of dissolved metals from outside the cell. In this way, metal colloid ingestion could be faster and more lethal than cell surface uptake alone. Further study is necessary to explore this mechanism of toxicity.

A final point of inquiry remains, namely that of remediation from toxicity under conditions at deep-sea vents. Holmes et al. (1997) have shown that production of sulfide and other sulfide compounds by microorganisms may actually increase resistance to metal toxicity through the formation of intracellular metal-sulfide precipitates eliminated as waste. High external concentrations of sulfide may also reduce metal toxicity through removal of toxic metals via metal-sulfide precipitation from the surrounding environment (White & Gadd 1998), before flagellates encounter them, although metal sulfide oxidation could replace at least some of this loss through dissolution (Eberhard et al. 1995). While the precise chemistry of vent microniches remains somewhat elusive, these findings suggest that some vent-specific conditions could ameliorate toxicity of at least metals if not other compounds. Future work should examine interactive effects between sulfide and metals, as well as including physical parameters unique to vent environments.

The results of these experiments indicate that *Caecitellus parvulus*, *Cafeteria* sp. and *Rhynchomonas nasuta* have the ability to survive very high concentrations of sulfide and the metals Cu, Fe, Mn and Zn potentially encountered in deep-sea hydrothermal vent environments. The evidence presented here coupled with: (1) evidence of their presence at vents through isolation and culturing of flagellates from vent samples (Atkins et al. 2000); (2) evidence of their presence at vents through molecular analysis of uncultured vent samples (Atkins unpubl. data); and, (3) their ability to be metabolically active under deep-sea pressures (Atkins et al. 1998), shows that these flagellate species are able to tolerate extreme vent conditions and have the potential to be active members of the microbial food webs at deep-sea vents.

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