EFFECT OF HYDROGEN SULFIDE IN ANOXIC MARINE SEDIMENTS ON THE ENZYMATIC DEGRADATION OF PARTICULATE PROTEINS

Gerome V. Bolalin
Xerxes dR. Cutchon

Submitted to the
Department of Biology
College of Arts and Sciences
University of the Philippines Manila
Padre Faura, Manila

In partial fulfillment of the requirements
for the degree of
Bachelor of Science in Biology
March 2004

Department of Biology
College of Arts and Sciences
University of the Philippines Manila
Padre Faura, Manila

Announcement of
Undergraduate Thesis Presentation

Gerome V. Bolalin
Xerxes dR. Cutchon

Entitled

EFFECT OF HYDROGEN SULFIDE IN ANOXIC MARINE SEDIMENTS ON THE ENZYMATIC DEGRADATION OF PARTICULATE PROTEINS

For the degree of
Bachelor of Science in Biology

March 2004
Learning Resource Center, Audio-Visual Room

THESIS ADVISERS:

Sylvia A. Sustento, MAT, MSc.
Department of Biology, CAS
UP Manila

Wolfgang Reichardt, Ph D
Marine Science Institute, CS
UP Diliman

THESIS READERS:

Samuel M.Go, M.S.P.H
Department of Biology, CAS
UP Manila

Christina A. Bordallo, MSc.
Department of Biology, CAS
UP Manila

Endorsed by:

Ma. Ofelia M. Cuevas, M. S.
Chair, Thesis Committee
Department of Biology, CAS
UP Manila

Authorized by:

Elisa L. Co, Ph. D.
Chair
Department of Biology, CAS
UP Manila

Approval Sheet

This is to certify that this undergraduate thesis entitled, ***"EFFECT OF HYDROGEN SULFIDE IN ANOXIC MARINE SEDIMENTS ON THE ENZYMATIC DEGRADATION OF PARTICULATE PROTEINS"*** submitted by Gerome V. Bolalin and Xerxes dR. Cutchon, in partial fulfillment of the requirements of the degree of Bachelor of Science in Biology, was successfully defended and approved on March 8, 2004.

SYLVIA A. SUSTENTO, MAT, MSc[1]
Thesis Adviser

WOLFGANG T. REICHARDT, Ph. D[2]
Co- Adviser

SAMUEL M. GO, M.S.P.H.
Thesis Reader

CHRISTINA A. BORDALLO, MSc.
Thesis Reader

The Department of Biology endorses the acceptance of this undergraduate thesis as partial fulfillment of the requirements for the degree of Bachelor of Science in Biology.

ELISA L. CO, Ph. D
Chairman
Department of Biology

This undergraduate thesis is hereby officially accepted as partial fulfillment of the requirements for the degree of Bachelor of Science in Biology.

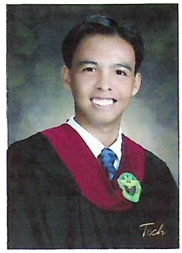
MARILOU G. NICOLAS
Dean
College of Arts and Sciences

---

[1] Department of Biology, College of Arts and Sciences, University of the Philippines Manila

[2] The Marine Science Institute, College of Science, University of the Philippines Diliman

BIOGRAPHICAL DATA



PERSONAL INFORMATION

NAME	Gerome Valladolid Bolalin
NICKNAME	Boks
BIRTHDAY	August 30, 1983
BIRTH PLACE	Baao, Camarines Sur
ADDRESS	E-12 B Camp Claudio, Tambo, Parañaque City
TELEPHONE NUMBER	512-3556
MOBILE PHONE NUMBER	(0916) – 835- 1738
EMAIL ADDRESS	geromebolalin@yahoo.com
PARENTS	Guillermo B. Bolalin
	Teresita V. Bolalin

EDUCATIONAL BACKGROUND

SCHOOLS		AWARDS
ELEMENTARY	Camp Claudio Elementary School C. Santos St., Camp Claudio Tambo, Parañaque City	1990 - 1996 Salutatorian
HIGH SCHOOL	Parañaque Science High School Dr. A. Santos Avenue, Sucat Road Parañaque City	1996 – 2000 Science Researcher of the Year
COLLEGE	University of the Philippines CAS, Padre Faura, Manila	2000 – 2004 BS Biology

ORGANIZATIONS

Lingkod E.R.
2002- 2004, member

Biology Major s Association (BIOMAS)
2003- 2004, member

Ugnayan ng Pahinungod
2001 – 2003, volunteer

UP Manila LRC
2002-2004, tutor

The information given above is true and correct.

Gerome V. Bolalin

BIOGRAPHICAL DATA

PERSONAL INFORMATION

NAME	Xerxes del Rosario Cutchon
NICKNAME	Xergs
BIRTHDAY	January 16, 1983
BIRTH PLACE	Tondo, Manila
ADDRESS	103 M. Peralta St. Kalookan City
TELEPHONE NUMBER	367-1169
MOBILE PHONE NUMBER	(0919) – 462- 2887
EMAIL ADDRESS	xerxes_cutchon@yahoo.com
PARENTS	Edgar B. Cutchon
	Carmen dR. Cutchon

EDUCATIONAL BACKGROUND

SCHOOLS		AWARDS
ELEMENTARY	La Consolacion College – Kalookan 10th Ave. A. Mabini St. Kalookan City	1990 - 1996 3rd Honorable Mention
HIGH SCHOOL	Notre Dame of Greater Manila Madre Ignacia Ave., Grace Park Kalookan City	1996 – 2000 Notre Dame Medal Recipient
COLLEGE	University of the Philippines CAS, Padre Faura, Manila	2000 – 2004 BS Biology

ORGANIZATIONS

Lingkod E.R.
2003- 2004, President

Biology Major s Association (BIOMAS)
2003- 2004, member

Ugnayan ng Pahinungod
2001 – 2003, volunteer

World Bible School
2004 - ,Coordinator

The information given above is true and correct.

Xerxes dR. Cutchon

ACKNOWLEDGMENT

We would like to express our deepest gratitude to the following people who have helped us in the course of the thesis.

Prof. Sylvia Sustento, our meticulous adviser here in UP Manila. We really thank her for her constant support, pieces of advice, and encouragement in all aspects of this thesis. Thank you for tirelessly accommodating us during consultations.

Dr. Wolfgang Reichardt our good adviser in UP-MSI. We thank him for introducing us to the world of protein recycling in marine sediments. Thank you sir for all your advice – for giving us free access to his laboratory – and for conscientiously correcting our mistakes. Thank you for the chats: academic, political, social – these lighten us up.

Sir Go and Ma'am Bordallo, our thesis readers, who gave corrections in our drafts –for further improvement of this endeavor.

Dr. Lourdes McGlone, the pretty research director of UP-MSI, for providing us a wonderful and interesting research topic– everything started there.

The DAAD, for providing the reagents, materials and equipment used in this study.

Dr. Elisa Co and **Prof. Miriam de Vera** for their advice, encouragement and help in constructing an unassailable thesis proposal last semester

Prof. Laura David and her research assistant, **Ms. Maritess Magno** for lending to us the Surfer program that was used in plotting our sampling sites right on a Bolinao map.

UP BIOMAS for its library -- the home of laptops, meeting place for hearsays and gossips, and an instant canteen for everybody – for providing us a place to meet and mingle with fellow biology majors who were also working on their thesis. Thank you also to the BIOMAS president, Ms. Paulyn Gaddi, for unwearyingly bearing with us.

Mr. Cyril Agan and **Mr. Jake Agullo** for accommodating us in their room at a time when we had nowhere to stay at – for being a great company in discovering the many wonders of Bolinao.

Ate Marie, the cook in Bolinao, for the delicious meals that nourished not only the body but the soul as well

The boatmen in Bolinao – for their smooth and swift sailing – for painstakingly assisting us during the field survey.

The BS Biology BLOCK 2 students for their share of encouragement and fun which lighten and brighten our days after sleepless nights.

Our parents, brothers and sisters for all their support, for the allowance – everything we are today – we owe it to them.

Our Almighty God, for in Him we live, move and have our being. We return all glory, praise and honor to Your holy Name. Amen.

THANK YOU.

Xerxes and Gerome

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

Appendix **Page**

ABSTRACT

In Bolinao Bay, Pangasinan, there is a continuing accumulation of particulate proteins in the form of fish feeds on the coastal marine sediments because of extensive fish farming. Establishment of anoxia and production of H_2S in sediments are some of the consequences. Hence, the influence of H_2S on the enzymatic degradation of particulate proteins is the study's main concern. Initially, a comparative study of sediments was conducted in Bolinao Bay to establish a possible association between H_2S and the enzymatic degradation of particulate proteins. Sediments near and distant from active fish cages were collected and characterized in terms of H_2S presence, proteolytic activity, macroscopic appearance, protein content and redox potential. Proteolytic activity of sediments was determined in an enzyme assay using dye-labeled scleroprotein as the enzyme substrate. This procedure measured largely the endoenzymatic degradation of solid protein into large dissolved molecules. The enzyme assay was conducted in two ways, one using the sediments directly and another using an enzyme culture fluid derived from an enriched culture of proteolytic bacteria, which were isolated from an oxic sediment sample. The data suggest that protein content had a strong negative correlation with redox potential; redox potential had a strong negative correlation with H_2S presence; and H_2S presence had a moderately negative correlation with proteolytic activity. Addition of H_2S to the enzyme culture fluid showed a direct inhibition on bacterial proteases. Proteoytic activity in anoxic sediments was significantly lower than the proteolytic activity in oxic sediments. However, the former was not significantly different from the proteolytic activity in anoxic sediments with H_2S. These results indicate that though hydrogen sulfide directly inhibited bacterial proteases, total proteolytic activity was not inhibited when proteolytic enzymes were contained in sediments. Further, removal of H_2S from natural sediments did not eliminate the inhibition of proteolytic activity.

INTRODUCTION

Background of the Study

The commercial raising of fish in coastal areas termed 'mariculture' multiplied fish output in the Philippines. The large demand for freshwater bangus (*Chanos chanos*) urged the locals in Bolinao Pangasinan to adopt mariculture. In February 2002, however, a massive fish kill devastated the mariculture industry in Bolinao. This event was widely attributed to eutrophication led by intensive cage cultivation of fish.

But mariculture may also have an impact on important organic processes in sediments. Fish farming in net cages causes the enhanced accumulation of particulate organic matter in marine sediments. This gives rise to the establishment of sediment anoxia. Eventually, anoxic conditions in the underlying marine sediment can result in the generation of ammonia and methane, and reduction of sulfate. The latter creates a real adverse condition by producing the toxic hydrogen sulfide, a compound known to inhibit the nitrification and denitrification processes (Kaspar *et al.*, 1988; as cited by Prosser *et al.* 1999). These two sediment processes are highly important in the recycling of nitrogen, which comes from the sediment detritus in marine fish farms.

However, the enzymatic hydrolysis of particulate organic material like proteins is the first rate-limiting step in the process of organic matter recycling in the sediments (Billen 1982). Bacterial extracellular enzymes hydrolyze organic polymers into oligomers and monomers before their transmembrane transport into the cell (Chrost 1989). An early

study on H_2S in oxic waters showed that the aminopeptidase activity was only partly inhibited by H_2S (Hoppe *et al* 1990). In the case of coastal marine sediments in Bolinao Bay, proteins are accumulating in the form of fish feeds or solid particulates. A study on the effect of H_2S on the enzymatic degradation of particulate proteins by bacteria is one way of assessing the potential impact of fish farming on protein recycling.

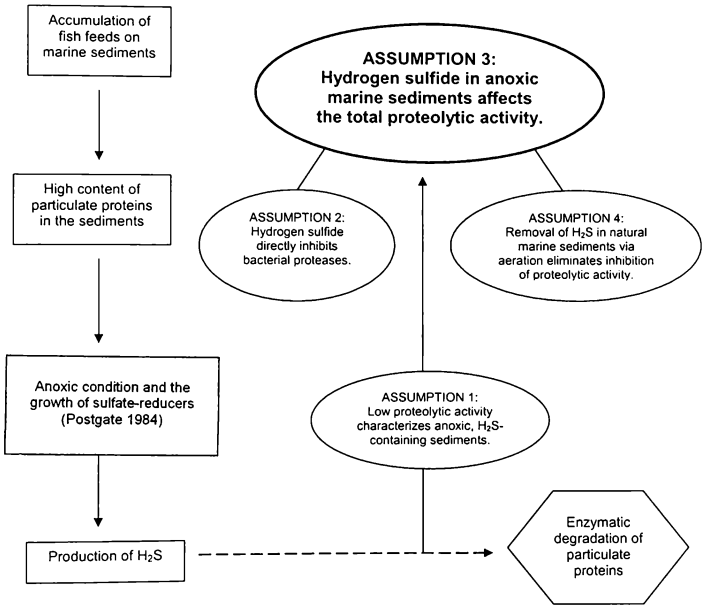
Statement of the Problem

Does H_2S in anoxic marine sediments affect the enzymatic degradation of particulate proteins?

Specific Objectives

1. To characterize marine sediments in terms of their macroscopic appearance, protein content, redox potential, H_2S presence and proteolytic activity.
2. To determine the relationships between protein content and redox potential; redox potential and H_2S presence; and H_2S presence and proteolytic activity.
3. To determine the direct effect of H_2S on bacterial proteases.
4. To determine the effect of H_2S in anoxic marine sediments on proteolytic activity.
5. To determine the effect of H_2S removal from natural sediments, via aeration, on proteolytic activity.

Theoretical Framework



When particulate proteins in the form of fish feeds accumulate on marine sediments, anoxia is established and H_2S is produced by sulfate-reducers (Postgate 1984). There is an assumption that low proteolytic activity characterizes anoxic, H_2S-containing sediments. Hence, it is hypothesized that H_2S in anoxic marine sediments affects the enzymatic degradation of particulate proteins. There are two further assumptions. First,

H_2S directly inhibits bacterial proteases. And second, removal of H_2S from natural sediments eliminates inhibition of proteolytic activity. The main assumption that H_2S in anoxic marine sediments affects proteolytic activity was tested with the following statistical hypotheses:

Ho: There is no significant difference on the total proteolytic activity between anoxic sediments with H_2S and anoxic sediments without H_2S.

Ha: There is a significant difference on the total proteoytic activity between anoxic sediments with H_2S and anoxic sediments without H_2S.

Significance of the Study

In the decomposition and mineralization of particulate proteins in marine sediments, enzymatic degradation through hydrolysis is the rate-limiting step. However, the environmental control of enzymatic hydrolysis for biopolymer recycling is an under-researched area. Among the few known factors that control microbial enzymatic degradation are substrate composition and molecular structure, temperature and pH. In anoxic environments, H_2S is the product of sulfate reduction by strict anaerobes. It is toxic to many anaerobic organisms and precipitates many metal ions. On the other hand, this compound's controlling effect on the enzymatic hydrolysis of complex biopolymers like structural proteins is not clearly explained. Hence, a study on the influence of H_2S on the enzymatic degradation of proteins would lead to a better understanding of microorganisms' main role in the recycling of complex organic matter. Further, the

Philippines being a developing country with a vast coastline is apt for aquaculture. Yet, eutrophication, which is usually attributed to unsustainable aquaculture remains to be the major cause of fish kills. High rates of protein recycling and the consequent coupled process of nitrification and denitrification are greatly important in lessening the adverse effects of eutrophication. This study is therefore, one way of assessing the impact of aquaculture on protein recycling.

Scope and Limitations

The study tested the hypothesis that H_2S in anoxic marine sediments affects the enzymatic degradation of particulate proteins. Enzymatic degradation was expressed as proteolytic activity and was directly measured by quantitative analysis of the dissolved decomposition products of a commercially available dye-labeled scleroprotein substrate (Hide powder azure (Sigma)). To establish a possible association between H_2S and enzymatic degradation, an initial field survey was conducted from September 13 to December 01, 2003 at 34 sampling sites in Bolinao Bay, Pangasinan. Selected samples collected from the survey were also used in subsequent experiments. The field study correlated only the following parameters in marine sediments: protein content, redox potential, H_2S presence and proteolytic activity. To test for the effect of H_2S on proteolytic activity, an enzyme assay was conducted in two ways, one using the sediments directly and another using an enzyme culture fluid derived from an enriched culture of proteolytic bacteria, which were isolated from an oxic sediment sample. Total

proteolyic activiy in sediments was observed under 3 conditions: oxic, anoxic, and anoxic-sulfidic. Five replicate setups were prepared for each condition. Further, an experiment was also conducted to test for the effect of H_2S removal on total proteolytic activity. Natural sediments originally containing H_2S were agitated and aerated in order to remove H_2S. This study focused only at the benthos and not on the overlying water column. Proteolytic bacterial colonies were not identified nor quantified.

REVIEW OF RELATED LITERATURE

Enzymatic Activity in Marine Sediments

Extracellular enzymatic activity is recognized as the key step in the degradation and utilization of polymeric organic matter such as proteins, polysaccharides, and nucleic acids (Gottschalk 1986). In prokaryotic bacteria, extracellular enzymes are synthesized in ribosomes and are transported through the hydrophobic layer of the cell membrane (Burns, 1978). In most cases, organic polymers must first be hydrolysed to oligomers and monomers before their transmembrane transport (Chrost 1989). Bacteria cannot consume whole particles, but will settle on their surfaces. Location of the enzymes to the cell envelope could allow for a tight coupling of hydrolysis and the uptake of the products of hydrolysis. Hollibaugh and Azam (1983) tested the hypothesis that pelagic bacteria process their potential substrates by releasing cell-surface-associated hydrolytic enzymes rather than by releasing enzymes directly into the seawater. Bacterial processing of polymeric substrates such as protein in pelagic ocean involves ectohydrolases not only on the cell surface but also in the periplasmic space; however, small substrates are hydrolytically processed only in the periplasm (Azam & Martinez 1993). In the case of bacteria in marine sediments, most of the extracellular enzymes may be bound to sediment particles or cell surfaces and not freely dissolved in the interstitial water (Meyer-Reil 1990). The activity of free extracellular enzymes in the interstitial water in the sediment is usually very low. When microbial cells excrete enzymes into the

surrounding water, the enzymes may diffuse away or undergo rapid denaturation or decomposition. Moreover, if enzymes are secreted in the vicinity of the cells or escape from them by way of diffusion, they may penetrate particle surfaces and break down the particle from within. In this case, enzymes would be more prone to degradation and a loose coupling of hydrolysis and uptake could be anticipated. In addition, enzymes can also be physically and chemically immobilized by adsorption to inorganic and organic sediment particles (Burns, 1978).

Environmental Control of Enzymatic Activity in Marine Sediments

Many factors are associated with the rate of extracellular enzymatic activity in marine sediments. If early organic matter diagenesis is largely dependent on bacterial activity (Deming & Barros 1993; as cited by Danovaro & Fabiano 1998), microbial degradation rates are dependent upon substrate composition and molecular structure (Arnosti & Repeta 1994; as cited by Danovaro & Fabiano 1998). The recycling of inorganic nitrogen compounds was found to be largely influenced by the Carbon/Nitrogen (C/N) ratio of organic matter in sediments (Kuenen & Roberton 1993; as cited by Danovaro & Fabiano 1998). Temperature is also a factor that controls the extracellular enzymatic activity (Mayer 1989). However, other factors controlling extracellular enzymatic activity especially on proteins in marine sediments remain poorly understood, despite the importance of extracellular hydrolysis to bacteria and organic matter cycling (Zehr & Ward 2002).

Hydrogen Sulfide Production in Anoxic Marine Sediments

One factor that has a great impact on coastal marine environments is fish farming. Intensive cage cultivation of fish leads to localized pollution of the adjacent seabed through accumulation of uneaten food and fecal material (Johnsen *et al.* 1993; Kaspar *et al.* 1988; as cited by Prosser *et al.* 1999). The accumulation of easily degradable organic matter can modify the sediment's characteristics and processes, leading to sediment anoxia. Under anoxic conditions some bacteria are able to use sulfate (SO_4^{2-}) as terminal electron acceptor in anaerobic respiration. When obligatory anaerobic bacteria carry out dissimilatory sulfate reduction, they are referred as to sulfate reducers, which include the traditional sulfate-reducing genera *Desulfibrio* and *Desulfotomaculum* (Postgate 1984). The reduction of sulfate results in production of hydrogen sulfide. In addition to anaerobic sulfate-reducing bacteria, some species of *Bacillus*, *Pseudomonas*, and *Saccharomyces* have been found to liberate hydrogen sulfide from sulfate, but these additional genera do not appear to play a major role in the dissimilatory reduction of sulfate. Sulfate reduction can occur over a wide range of pH, pressure, temperature, and salinity conditions. The sulfate-reducing bacteria also require a redox potential of about –200 mV for the initiation of growth. A further drop in redox potential accompanies the growth of sulfate-reducing bacteria. In sewer sediments, the rate of sulfate reduction was found to be up to 0.64 g $S^{-2}/m^2/h$ (Schmitt & Seyfield 1992). Maximum rates of sulfate reduction were found at sediment depths of between 5 and 7.5 cm. Only relatively few compounds can serve as electron donors for sulfate reduction. The most common electron

donors are pyruvate, lactate, and molecular hydrogen. Sulfate reduction is inhibited by the presence of oxygen, nitrate, or ferric ions. The rate of sulfate reduction is often carbon-limited. The addition of organic compounds to marine sediments can result in greatly accelerated rates of dissimilatory sulfate reduction.

Microbial Processes in Sediments under Organic Pollution

The accumulation of easily degradable organic matter can modify the sediment's characteristics and processes, leading to sediment anoxia and generation of hydrogen sulfide (H_2S), which are highly detrimental to farmed fish (Weston 1990; Ye *et al.*, 1991; Findlay *et al.*, 1995; Wu 1995; as cited by CSIRO Huon Estuary Study Team 2000). The output from fish farms has high nitrogen content because the major component of fish feed is protein. This may lead to eutrophication, since nitrogen is the major nutrient limiting phytoplankton growth. Bacteria release inorganic nitrogen in the form of ammonium during the process of protein decomposition called ammonification. Nitrification, the oxidation of ammonia to nitrate via nitrite, is central to the cycling of nitrogen in the environment and when coupled with denitrification, alleviates the effects of eutrophication through removal of nitrogen to the atmosphere as nitrous oxide or dinitrogen gas (Blackburn & Blackburn 1992; as cited by Prosser *et al.* 1999). Previous studies have reported that in chronic pollution, nitrification and the coupled process of denitrification are often completely suppressed (Kaspar *et al.*, 1988; as cited by Prosser *et al.* 1999). Moreover, other reports have shown that nitrification by aerobic

chemoautotrophs is sensitive to low oxygen tension, sulfur compounds, high ammonia and nitrite concentrations, and the presence of a broad range of organic compounds (Be'dard & Knowles 1989; Juliette *et al.* 1993; Keener & Arp 1994; Laanbroek *et al.*1994; as cited by Prosser *et al.* 1999).

However, the key initial process in the recycling of organic nitrogen is the enzymatic degradation of proteins by bacteria. Hoppe *et al.* (1990) investigated for the first time the effect of H_2S in oxic waters on extracellular enzymatic activity. Their investigation showed that the aminopeptidase activity in oxic waters was only partly inhibited by H_2S. In marine sediments affected by fish farming, however, most proteins are accumulating in the form of solid particulates. Hence, the use of dye-labeled substrates may be the appropriate technique in the analyses of proteolytic activity in organically polluted marine sediments. A study on the effect of H_2S on the enzymatic solubilization or degradation of particulate proteins by bacteria is one way of assessing the potential impact of fish farming on protein recycling.

MATERIALS AND METHODS

A. FIELD SURVEY

Collection of sediment samples

The study site was Bolinao Bay in Bolinao, Pangasinan (Figure 1). Bolinao is located on a cape of the western tip of Pangasinan. It is situated at the western part of Lingayen Gulf. Bolinao Bay is surrounded by the China Sea on the north and west. This study site was sampled from September 13 to December 1, 2003. Locations of the sampling sites were determined using a Global Positioning System (GPS) device. Of the total 34 sampling sites, 15 were sampled from areas near or right beside an active fish cage and 19 others were sampled relatively far from these cages. The water depth for each sampling site was also recorded. The sediments together with their overlying water were sampled to a depth of 10-12 cm by a plexiglass sediment core sampler. The sediment cores were then immediately brought back to the laboratory for macroscopic visual inspection, analysis and experiments.

Measurement of parameters in marine sediments

H_2S presence. Presence or absence of H_2S on each site was noted by virtue of smelling the characteristic H_2S odor.

Redox potential. Redox potential values are indicative of oxic and anoxic environments. Positive redox potential values suggest aerobic conditions whereas

negative redox potential values are associated with anaerobic microbial processes. Redox potential readings were determined using multi-functional pH/redox meter (Orion 1230).

Total protein content. Protein concentration was measured using a modified method by Lowry *et. al.* (1951), (Herbert *et al.*1971). Duplicates and a blank were always prepared for each sediment sample. One cm^3 of each sample in a centrifuge tube was fixed by adding 1mL NaOH. The rack containing the centrifuge tubes, which contain the fixed sample aliquots, were placed into boiling water bath to digest for exactly 5 minutes. The rack of centrifuge tubes was then immediately cooled off in a basin containing tap water. Upon cooling, 2.5mL of the Cu reagent mixture was added to each of the tubes and the rack was kept in the dark for 10 mins. After the incubation in the dark, 0.5mL of 1N Folin-Ciocalteu's phenol reagent was added rapidly. Full –blue complex was formed within 30 mins. The tubes were centrifuged for 15 mins at 3000 U/min and the absorbance of the supernatant was measured at 595nm in a spectrophotometer (Spectronic 20). Sample blanks in duplicates were made in the same way, only that distilled water was used instead of the reagents. Reagent blanks in duplicates were also made using the same reagents, this time distilled water was used instead of the sediment sample.

Proteolytic activity. Dye-release techniques measure largely endoenzymatic solubilization of biopolymers (Reichardt 1986). In this study, dye-labeled scleroprotein was made to simulate the particulate nature of proteinaceous matter settling on marine sediments. For the scleroprotein assay, duplicates and a blank were prepared for each

sample. The assay was done in centrifuge tubes. Each centrifuge tube received 1 cm^3 of sediment sample, 2 mL of 4% Triton X-100 and 2mL of 20mM Tris –HCl pH 7.56. An additional 0.5mL of 37% formaldehyde was added to the blanks. A microspoon (approx. 24 mg) of dye-labeled scleroprotein substrate (Hide powder azure (Sigma)) was then added and mixed to each of the tubes. The centrifuge tubes were mixed and incubated at 30°C for 11-17 hours. After incubation, non-blank tubes were terminated using 0.5mL of 37% formaldehyde. Color reactions were observed and the samples were centrifuged for 15 minutes at 3000 U/min. The absorbance of the supernatant was taken at 595 nm using a Spectronic 20 spectrophotometer. Proteolytic activity was calculated using the following formula:

$$\text{Proteolytic Activity Unit } (\mu g \cdot h^{-1} \cdot cm^{-3}) = (10^3 \cdot \text{absorbance} \cdot F \cdot V_a) / (V_s \cdot t)$$

where F is the substrate-specific conversion factor (4557 $\mu g \cdot l^{-1}$) V_a is the total assay volume (0.0055 L), V_s is the total volume of sediment sample used for assay (1 cm^3), and t is the incubation time (11-17 hrs.) (Reichardt 1986).

B. EXPERIMENTS

Test for the direct effect of H_2S on bacterial proteases

Enzyme culture fluid from enriched culture of proteolytic bacteria was used to determine the direct effect of H_2S on bacterial proteases. To obtain the enzyme culture

fluid, a portion of a selected natural sediment sample from an oxic site was cultured. A volume dilution (1:10) was plated on gelatin-agar (40 g gelatin, 1.33 g agar and 170 mg nutrient broth in ¾ strength aged seawater). This selective medium was prepared to allow only the growth of proteolytic marine bacteria for 2 days at 10 °C. A sample from this culture was used as an inoculum in the peptone-skim milk enrichment culture incubated for 5 days at 30 °C. The enzymes from this culture were then separated thru centrifugation for 15 minutes at 3000 U/min. The supernatant or the enzyme culture fluid (0.5 mL) was added to a centrifuge tube containing: 0.5 mL Na_2S (0, 4.2, 42 and 420 mM); 2 mL 4% Triton X-100; 2mL tris HCl buffer, 20mM, pH 7.56; and scleroprotein substrate (24 mg and 48 mg). Two fixed concentrations of the substrate were used to compute for the inhibitor constant (*Ki*) by method of Dixon plot (Segel 1968). Blanks were also prepared in the same way only that 0.5mL 37% formaldehyde was added before the addition of the scleroprotein substrate. The tubes were mixed and incubated at 30 °C for 4 hours. Incubation was terminated thru addition of 0.5 mL 37% formaldehyde. The tubes were then centrifuged for 15 minutes at 3000 U/min and the absorbance of the soluble stained hydrolyzate was determined at 595 nm. wavelength in a Spectronic 20 spectrophotometer.

Test for the effect of H_2S in anoxic marine sediments on proteolytic activity

The main experiment to determine the redox and H_2S dependence of proteolytic activity was carried out by incubating separate aliquots of a single sediment sample at 3

different redox conditions: oxic, anoxic, and anoxic-sulfidic (Batches 1 to 3). The intertidal sediment sample (Site 10; Refer to Tables 1 and 2) used in this experiment consisted of white silty sand particles. Thirty-five (35) mL aliquot of this sediment sample and 35 mL of artificial seawater were transferred to each of fifteen 80 mL containers. All the 15 setups were then divided into 3 batches. In Batch 1 (oxic control), 5 containers were continuously aerated. In Batch 2 (anoxic treatment), 5 containers were covered with a rubber stopper to maintain sediment anoxia. And in Batch 3 (anoxic-sulfidic treatment), 5 containers were supplemented with Na_2S resulting in an approximate sulfide concentration of 3.0 mM. These containers were then covered with a rubber stopper to maintain sediment anoxia. After 24 hours of incubation at 30 °C, the redox potential of the sediments was recorded. Subsamples for measurement of proteolytic activity were then taken.

Test for the effect of H_2S removal from natural sediments via aeration on proteolytic activity

In order to determine the influence of the removal of H_2S from natural sulfidic sediments on proteolytic activity, 3 sulfidic sediment samples (Site 11, 12, and 14) and 1 non-sulfidic sediment sample (Site 13) were reaerated. The 4 sediment samples with their overlying natural seawater were equally divided into 2 small jars. Jar 1 was aerated while Jar 2 served as a negative control. Subsamples for enzyme analyses were taken after 5 hours of aeration. Redox potential was also recorded after treatment.

C. STATISTICAL ANALYSES

Test for relationships between parameters in marine sediments

Relationships between parameters in marine sediments were tested by correlational analysis. Pearson r correlation analysis (Rao, 1998) was used for protein content versus redox, while Point Biserial correlational analysis (Downie & Heath 1983) was used for redox versus H_2S presence and H_2S presence versus proteolytic activity.

Test for significant differences between conditions in marine sediments

Significant differences in proteolytic activity between oxic, anoxic and anoxic-sulfidic conditions were tested by parametric analysis (One-way ANOVA and Tukey HSD Test) with a 0.05 level of significance (Rao, 1998).

RESULTS

A. FIELD SURVEY

Characteristics of the sediment samples

Depth. The water depth in sampling sites near active fish cages had an average value of 13.2 meters and had a range of 7.0-18.3 meters (Table 1). The sites with the minimum depth were the intertidal sampling areas (Sites10, 24, 25, 26 and 27) while the maximum value of 19.3 meters was in a site far from fish cages (Site 8).

Macroscopic appearance. Evident differences in sedimentary structure were also observed between sites (Table 1). Sediment core samples collected in areas far from active fish cages (Sites 7, 8, 13, 14, 19, 20, 21, 22, 23, 31, 32 and 33) had sediment colors of brown, dark brown and dark gray, whereas core samples taken from seagrass beds (Sites 9 and 34) and sandy beaches (Sites 10, 24, 25, 26 and 27) were much lighter in color and coarser in texture. On the other hand, sediment core samples collected in areas near fish cages (Sites 1, 2, 3, 4, 5, 6, 11, 12, 15, 16, 17, 18, 28, 29 and 30) were so flocculent and consisted mainly of decomposing fish feed and feces. Moreover, in sediment samples near active fish cages, *Beggiatoa spp.* sulfide-oxidizing bacteria formed a confluent white mat over the sediment surface, while the subsurface sediment was black. A few species of small polychaete worms were found to dominate the benthic communities in some sulfide-rich sediment samples (Sites 11 and 17).

Protein content. Sediments from 21 sampling sites were subjected to measurement of protein content (Tables 2 and 3). Protein content in sites near active fish cages had an average value of 11.56 mg/cm^3 whereas in sites far from active cages, the average value was 3.81 mg/cm^3. The minimum value of 0.27 mg/cm^3 for protein content was in a sandy beach far from fish cages (Site 10) while the highest value of 16.93 mg/cm^3 was recorded in a site near an active fish cage (Site 17).

Redox potential. Sediments from 23 sampling sites were subjected to measurement of redox potential (Tables 2 and 3). Redox potential in sites near active fish cages had an average value of –360 mV whereas in sites far from fish cages, the average value was –150 mV. The minimum value of –392 mV for redox potential was in a sampling site near an active fish cage (Site 5) while the maximum value of 109 mV was recorded in a sandy beach (Site 25).

H_2S presence. Sediments from 30 sampling sites were inspected for presence of H_2S (Tables 2 and 3). H_2S was present in all the sites near active fish cages and was also present in few sites far from fish cages (Sites 21, 22 and 23).

Proteolytic activity. Sediments from 21 sampling sites were subjected to measurement of proteolytic activity (Table 2 and 3). Proteolytic activity in sites near active fish cages had an average value of 120 $\mu g \cdot h^{-1} \cdot cm^{-3}$ whereas in sites far from fish cages, the average value was 426 $\mu g \cdot h^{-1} \cdot cm^{-3}$. Interestingly, the minimum rate of 3 $\mu g \cdot h^{-1} \cdot cm^{-3}$ for proteolytic activity was in a sampling site far from active fish cages (Site 31) while the maximum rate of 1957 $\mu g \cdot h^{-1} \cdot cm^{-3}$ was recorded in a seagrass bed (Site 34).

Relationships between parameters in marine sediments

In the scatterplot diagram (Figure 2) of protein content vs. redox potential, sediment samples with protein content values less than 5 mg/cm^3 had a narrow range of positive redox potential values (59 mV to 109 mV) whereas sediment samples with protein content values greater than or almost equal to 5 mg/cm^3 had a wide range of negative redox potential values (-245 mV to –382 mV). A correlation analyses between these two parameters showed a strong negative correlation (r_p = -0.88, Pearson r). In the boxplot diagram (Figure 3) of redox potential versus H_2S presence, sediments with H_2S tend to get limited to narrow range of negative redox potential values (-265 mV to –392 mV) whereas sediments without H_2S had a wide range of values (-310 mV to 109 mV). Similarly, there was also a strong negative correlation (r_{pb} = -0.70, Point Biserial) between redox potential and H_2S presence. In the boxplot diagram (Figure 4) of H_2S presence vs. proteolytic activity, on the other hand, proteolytic activity in sediments without H_2S had 3 times wider range of values than in sediments with H_2S. A small range of low proteolytic activity values (36 $\mu g \cdot h^{-1} \cdot cm^{-3}$ to 215 $\mu g \cdot h^{-1} \cdot cm^{-3}$) was recorded in sediments with H_2S. A correlation analysis of H_2S presence vs. proteolytic activity showed a moderate negative correlation (r_{pb} = -0.45, Point Biserial).

B. EXPERIMENTS

Direct effect of H_2S on bacterial proteases

Using 24 mg of the scleroprotein substrate, the percent (%) inhibition values of the enzyme culture fluid incubated with 4.2, 42 and 420 mM of Na_2S were 9, 22 and 88% correspondingly (Table 4). On the other hand, the percent inhibition values using 48 mg of the substrate and the same concentrations of Na_2S were 6, 16 and 68% (Table 4). A line graph drawn from these data (Figure 5) shows that the inhibition of proteolytic activity increased with increasing concentrations of Na_2S. Further, the inhibitor constant (*Ki*), which is a measure of the enzyme-inhibitor affinity, was determined by method of Dixon plot (Segel 1968). Concentration of Na_2S was plotted against the reciprocal values of proteolytic activity at 2 concentrations of the scleroprotein substrate (Figure 6). The plot shows that the *Ki* of H_2S was 20 mM.

Effect of H_2S in anoxic marine sediments on proteolytic activity

The mean proteolytic activity values in oxic, anoxic and anoxic-sulfidic sediments were 660, 560 and 512 $\mu g \cdot h^{-1} \cdot cm^{-3}$ respectively (Table 5). Proteolytic activity of the anoxic sediment without H_2S was significantly lower than the proteolytic activity of the oxic control ($p<0.05$, ANOVA-Tukey, Figure 7). However, there was no significant difference on the proteolytic activity between anoxic sediments without H_2S and anoxic sediments with H_2S ($p<0.05$, ANOVA-Tukey, Figure 7).

Effect of H_2S removal from natural sediments via aeration on proteolytic activity

The 3 sulfidic sediment samples that were not aerated had proteolytic activity values of 197, 107 and 260 $\mu g \cdot h^{-1} \cdot cm^{-3}$ whereas those sulfidic sediments that were aerated had proteolytic activity values of 81, 90 and 170 $\mu g \cdot h^{-1} \cdot cm^{-3}$ respectively (Table 6). In these 3 pairs of naturally sulfidic sediment samples, members of each pair that were reaerated for 5 hours all registered a lower proteolytic activity than their unaerated counterparts (Figure 8).

DISCUSSION

A. FIELD SURVEY

Characteristics of sediment samples

As a result of organic matter input in marine sediments, microbial sulfate reduction leads to H_2S accumulation. Presence of Beggiatoa *spp.* in sulfide-rich sediments indicates strong chemoautotrophic oxidation of H_2S into S^o, a non-toxic element at least in the sediment-water interface. *Beggiatoa spp.* are filamentous bacteria, which position themselves at the narrow oxic-anoxic interface where H_2S and oxygen coexist. Sulfur globules are deposited within their cells. In the absence of H_2S, these sulfur globules are slowly oxidized to sulfate (Jorgensen and Revsbeck 1983).

Similarly, benthic invertebrates found in sulfide-rich sediments increase the internal surfaces of sediments, thus, possibly contributing to further detoxification potential for H_2S. Enhanced survival and growth in sulfide-rich sediments by some species of polychaete worms were already observed (Tsutsumi *et al.* 2001). The bottom fauna, by building burrows, can have a marked effect on transfer rates between the sediment and the overlying water (Aller 1980). Bioturbation by polychaetes helps sustain benthic marine environments by providing for internal aeration and biofilm functions, which affect microbe-mediated biogeochemical processes in the sediments (Reise 2002).

Relationships between parameters in marine sediments

Assessment of protein content, redox potential, H_2S presence and proteolytic activity of sediment samples in Bolinao Bay, Pangasinan reveals some apparent relationships between environmental parameters. Protein content and redox have a strong negative linear relationship. Anoxic conditions characterize sediments with high concentrations of protein. The anoxic condition in sediments near active fish cages could be widely attributed to the accumulation of decomposing particulate organic protein in the form of fish feeds. In Bolinao Bay, fish farmers use organic fish feeds to maximize fish production, but only part of this high-protein feed is actually consumed by the fish. The residual feeds accumulate after sedimentation. Presence of more bio-oxidizable organic matter is expected to intensify the anoxic condition in sediments. On the other hand, a good negative correlation between redox potential and H_2S presence showed that sediments with H_2S tend to get limited to a small range of low redox potential values. This suggests a strong dependence of H_2S-producing bacteria on strictly anoxic and reducing conditions. A further drop in redox potential usually accompanies the growth of sulfate-reducing bacteria (Postgate 1984). Finally, a moderately negative correlation between H_2S presence and proteolytic activity showed that low proteolytic activity characterizes sediments with H_2S. It is assumed that H_2S has an effect on the microbial enzymatic degradation of particulate proteins.

B. EXPERIMENTS

Direct effect of H_2S on bacterial proteases

Addition of H2S to the enzyme culture fluid showed a direct inhibition on bacterial proteases. Hydrogen sulfide may have formed disulfide bonds with the metal-containing proteases, which were freely exposed in the enzyme culture fluid. Conformational changes could have deactivated a big fraction of the proteases. On one hand, isolation of the proteases through centrifugation showed that not all bacterial proteases were bound to the cell surface or the periplasmic space. The data suggest that bacteria in sediments could release proteases either to get dissolved in the interstitial water or to get bound to sediment particles.

Effect of H_2S in anoxic marine sediments on proteolytic activity

Results of the controlled redox experiment (Figure 6) showed that the proteolytic activity in marine sediments was lowered by oxygen depletion or anoxia. The physiological state of microbes may have a close relation to synthesis of microbial proteases. The experiment however showed that there was no significant difference on the proteolytic activity between anoxic sediments with H_2S and anoxic sediments without H_2S. This indicates that though proteolytic enzymes were directly inhibited by H_2S, total proteolytic activity was not affected when proteolytic enzymes were contained in sediments. Most of the extracellular enzymes in sediments are bound to particles or cell surfaces. Adsorption of most of the enzymes to sediment particles may explain the

protection from H_2S inhibition. Enzymes can be physically and chemically immobilized by adsorption to inorganic and organic particles (Burns 1978). By forming complexes with humic compounds through copolymerization, entrapment or adsorption, enzymes may be more stable and more resistant to H_2S attack. Moreover, the microbial slime layer produced by bacterial mineralizers to concentrate enzymes in aquatic environments is also an important adsorption site.

Effect of H_2S removal from natural sediments via aeration on proteolytic activity

Removal of H2S from natural sediments via aeration did not eliminate the inhibition of proteolytic activity. In the 3 naturally sulfidic sediment samples used, there was long exposure of proteolytic enzymes to H_2S. Thus, H_2S may have already caused direct inhibition on a significant fraction of proteolytic enzymes. Results showed that the removal of H_2S by aeration proved to be ineffective in eliminating any inhibitory effect on proteolytic activity. This may indicate that H_2S works mainly as an irreversible inhibitor and tends to establish disulfide bonds preferentially with heavy-metal containing enzymes (e.g. metalloproteases). In the case of a pair of natural non-sulfidic sediment sample, proteolytic activity in the reaerated setup was found to be greater than the unaerated one (Figure 8). Resuspension of sediments brought about by aeration may play an important role in stimulation of proteolytic activity in non-sulfidic sediments through growth of proteolytic bacteria or rapid synthesis of proteases.

CONCLUSION

In marine sediments, protein content had a strong negative correlation with redox potential; redox potential had a strong negative correlation with H_2S presence; and H_2S presence had a moderately negative correlation with proteolytic activity. Experiments showed that hydrogen sulfide directly inhibited bacterial proteases, however, total proteolytic activity was not inhibited when proteolytic enzymes were contained in sediments. Further, removal of H_2S from natural sediments via aeration did not eliminate inhibition of proteolytic activity.

RECOMMENDATIONS

1. The complex environment of marine sediments is hard to simulate. The experimental design used in this study controls only the redox conditions of the sediments and the presence of H_2S. Designs that can optimally determine the mechanistic explanation of H_2S inhibition of enzymatic activity in sediments should be made.
2. Since the system in marine environments depends on inputs from the water column, the sinking flux of proteins into the sediments is an important parameter that should be considered.
3. The water column has a big role in changing the conditions in the sediments, which affect important microbial processes. Direct studies in the water column with corresponding situations in the sediments should be performed.
4. Future investigations should study how different parameters in sediments stabilize after long-term adaptation to reducing conditions and H_2S presence.
5. Adsorption of particulate protein solubilizing enzymes to the organic coating of sediment particles could have an effect on accurate measurement of proteolytic activity. Corrections for the inhibitory impacts of the different grain sizes of inorganic sediment particles have to be considered.

LITERATURE CITED

Aller, R. C. (1980). Relationships of tube-dwelling benthos with sediment and overlying water chemistry. In Tenore, K.R. and Coull, B. C. (eds.) Marine benthic communities. Univ. of South Carolina Press, Columbia

Arnosti, C., and D. J. Repeta. (1994) Oligosaccharide degradation by anaerobic marine bacteria: characterisation of an experimental system to study polymer degradation in sediments. *Limnol. Oceanogr.* 39:1865-1877.

Azam, F., and J. Martinez. (1993) Periplasmic aminopeptidase and alkaline phosphatase activities in a marine bacterium: implications for substrate processing in the sea. *Mar. Ecol. Prog. Ser.* 92:89-97.

Be'dard, C., and R. Knowles. (1989) Physiology, biochemistry, and specific inhibitors of CH_4, NH_4^+, and CO oxidation by methanotrophs and nitrifiers. *Microbiol. Rev.* 53:68–84.

Billen, G. (1982). Modelling the process of organic matter degradation and nutrient recycling in sedimentary system. pp. 15-52 in Nedwell, D.B. and Brown, C. M. (editors), *Sediment Microbiology*, Academic Press, London

Burns, R. G. (1978). Enzyme activity in soil: some theoretical and practical considerations. pp. 295-340 in Burns. R. G. (editor), *Soil Enzymes*, Academic Press, London

Blackburn, T. H., and N. D. Blackburn. (1992) Model of nitrification and denitrification in marine sediments. *FEMS Microbiol. Lett.*.100:517–522.

Chrost, R. (1989) Characterization and significance of β-glucosidase activity in lake water. *Limnol. Oceanogr.* 34:660-672.

CSIRO Huon Estuary Study Team. (2000) *Huon Estuary Study* "Environmental research for integrated catchment management and aquaculture." Final report to Fisheries Research and Development Corporation. Project number 96/284, June 2000. CSIRO Division of Marine Research. Marine Laboratories, Hobart.

Danovaro, R., and M. Fabiano. (1998) Enzymatic activity, bacterial distribution, and organic matter composition in sediments of the Ross Sea (Antarctica). *Appl. Environ. Microbiol.*64:3838-3845.

Danovaro, R., G. M. Luna, and E. Manini. (2002) Large fraction of dead and inactive bacteria in coastal marine sediments: comparison of protocols for determination and ecological significance. *Appl. Environ. Microbiol.* 68:3509-3513

Deming, J. W., and J. A. Barross. (1993) The early diagenesis of organic matter: bacterial activity. *In* M. H. Engel, and S. A. Macko (ed.), *Organic Geochemistry: Principles and Applications.* Plenum Press, New York, N.Y., pp. 119–144.

Downie, N. M., and R. W. Heath (1983) Basic statistical methods, 5th ed. Harper & Row, Publishers, Inc. New York

Findlay, R. H., L. Watling, and L. M. Mayer. (1995) Environmental impact of salmon net-pen culture on marine benthic communities in Maine: A case study. *Estuaries* 18:145-179.

Gottschalk, G. (1986) Bacterial metabolism. Springer-Verlag, New York, N.Y.

Herbert, D., P. J. Phipps, and R. E. Strange. (1971). Chemical analysis of microbial cells. In: Norris JR, Ribbons, DW (eds.)., Methods in Microbiology Vol 5B. Academic Press, London.

Johnsen, R. I., O. Grahl-Nielsen, and B. T. Lunestad. (1993) Environmental distribution of organic waste from a marine fish farm. *Aquaculture* 118:229–244.

Jorgensen, B. B., and N. P. Revsbeck. (1983). Colorless sulfur bacteria, *Beggiatoa* spp. and *Thiovalum* spp., in O_2 and H_2S microgradients. *Appl. Environ. Microbiol.* 45:1261-1270

Juliette, L. Y., M. R. Hyman, and D. J. Arp. (1993) Inhibition of ammonia oxidation in *Nitrosomonas europaea* by sulfur compounds: thioethers are oxidized to sulfoxides by ammonia monooxygenase. *Appl. Environ. Microbiol.* 59:3718–3727.

Kaspar, H. F., G. H. Hall, and A. J. Holland. (1988) Effects of sea cage salmon farming on sediment nitrification and dissimilatory nitrate reductions. *Aquaculture* 70:333–344.

Keener, W. K., and D. J. Arp. (1994) Transformations of aromatic compounds by *Nitrosomonas europaea*. *Appl. Environ. Microbiol.* 60:1914–1920.

Kuenen, J. G., and L. A. Roberton. (1993) Interactions among bacteria metabolizing inorganic nitrogen compounds. *In* R. Guerrero and C. Pedro`s-Alio` (ed.), *Trends in Microbial Ecology*. Spanish Society for Microbiology, Barcelona, Spain, pp. 33-36.

Laanbroek, H. J., P. L. E. Bodelier, and S. Gerards. (1994) Oxygen consumption kinetics of *Nitrosomonas europaea* and *Nitrobacter hamburgensis* in mixed continuous cultures at different oxygen concentrations. *Arch. Microbiol.* 161:156–162.

Mayer, L. M. (1989) Extracellular proteolytic enzyme activity in sediments of an intertidal mudflat. *Limnol. Oceanogr.* 34:973–981.

Meyer-Reil, L. A. (1990). Microorganisms in marine sediments: considerations concerning activity measurements. *Archiv fur Hydrobiologie Beihefte Ergebnisse Limnologie* 34: 1-6

Postgate, J. R. (1984) The sulphate-reducing bacteria. Cambridge University Press, Cambridge, England

Prosser, J. I., and A. E. McCaig. (1999) Nitrogen cycling and community structure of proteobacterial B-subgroup ammonia-oxidizing bacteria within polluted marine fish farm sediments. *Appl. and Environ. Microbiol.* 65:213-220.

Reichardt, W. T. (1973) Das reaktionspotential in okosystemen. zur problematik limnologischer enzymanalysen. *Arch. Hydrobiol., Suppl.* 42:253-272

Reichardt, W. T. (1988) Measurement of enzymatic solubilization of P.O.M. in marine sediments by using dye-release techniques. *Arch. Hydrobiol. Beih.* 31:353-363

Reise, K. 2002. Sediment mediated species interactions in coastal waters. *J. Sea Research.* 48:127-141

Schmitt, F. and C. F Seyfield (1992) Sulfate reduction in sewer sediments. Wat. Sci. Tech. 25(8):83-90

Segel, I. H. (1968) Biochemical calculations. John Wiley & Sons, Inc. Canada

Stepanaukas, R., H. Edling, and L. J. Tranvik. (1999) Differential dissolved organic nitrogen availability and bacterial aminopeptidase activity in limnic and marine waters. *Microbial Ecol.* 38:264-272

Tsutsumi, H., S. Wainright, S. Montani, M. Saga, S. Ichihara, and K. Kogure. (2001) Exploitation of a chemosynthetic food resource by the polychaete *Capitella* sp. I. *Mar. Ecol. Prog. Ser.* 216:119-127

Rao, P. V. (1998) Statistical research method in the life sciences. Brooks/Cole Pub. Inc. CA, USA

Weston, D. P. (1990) Quantitative examination of macrobenthic community changes along an organic enrichment gradient. *Mar. Ecol. Prog. Ser.* 61:233-244.

Wu, R. S. S. (1995) The environmental impact of marine fish culture: towards a sustainable future. *Marine Pollution Bulletin* 31:159-166.

Ye, L. X., D. A. Ritz, G. E. Fenton, and M. E. Lewis. (1991) Tracing the influence on sediments of solid waste from a salmonid farm using stable isotope analysis. *J. of Exp. Mar. Biol. and Ecol.* 145:161-174.

Zehr, J. P., and B. B. Ward. (2002) Nitrogen cycling in the ocean: new perspectives on processes and paradigms. *Appl. and Environ. Microbiol.* 68:1015-1024.

TABLES

Table 1: Location, site description, depth and macroscopic appearance sediment samples from Bolinao Bay (September 13 to December 01, 2003)

Site	Location (coordinates)	Site Description	Depth (m)	Macroscopic Appearance
1	16.3519° N 119.9396° E	near active cages	7.5	Black
2	16.3625° N 119.9371° E	near active cages	7.0	Brown
3	16.3716° N 119.9328° E	near active cages	14.3	Dark brown
4	16.3776° N 119.9269° E	near active cages	8.0	Black
5	16.3877° N 119.9202° E	near active cages	15.3	Black
6	16.3837° N 119.9137° E	near active cages	15.5	Dark gray
7	16.3946° N 119.9095° E	far from cages	15.5	Brown
8	16.3863° N 119.9060° E	far from cages	19.3	Brown
9	16.4121° N 119.9071° E	seagrass bed	1.0	White, carbonaceous sand; Macroalgal debris
10	16.3800° N 119.8635° E	sandy beach	intertidal	White, homogenous, silty sand; Macroalgal debris
11	16.2999° N 119.9161° E	near active cages	15.6	Black; *Beggiatoa* mats; Presence of burrowing polychaete
12	16.3117° N 119.9198° E	near active cages	14.3	Black; *Beggiatoa* mats
13	16.3518° N 119.9354° E	far from cages	4.5	Brown
14	16.3726° N 119.9318° E	far from cages	14.0	Dark brown
15	16.2999° N 119.9151° E	near active cages	12.0	Black; *Beggiatoa* mats
16	16.3055° N 119.9169° E	near active cages	13.6	Black; *Beggiatoa* mats
17	16.3107° N 119.9188° E	near active cages	16.3	Black; Presence of burrowing polychaete; *Beggiatoa* mats
18	16.3152° N 119.9242° E	near active cages	18.3	Black; *Beggiatoa* mats
19	16.3528° N 119.9344° E	far from cages	4.8	Brown; Presence of burrowing

				polychaete
20	16.3608° N 119.9352° E	far from cages	4.5	Brown
21	16.3726° N 119.9338° E	far from cages	9.0	Dark brown
22	16.3847° N 119.9147° E	far from cages	19.0	Dark brown surface (3 mm) on dark gray subsurface
23	16.3879° N 119.9264° E	far from cages	16.5	Dark gray
24	16.3810° N 119.9070° E	sandy beach	intertidal	Light brown, heterogenous, carbonaceous, coarse sand; Macroalgal debris
25	16.3810° N 119.9090° E	sandy beach	intertidal	Light brown, heterogenous, carbonaceous, coarse sand; Macroalgal debris
26	16.3810° N 119.9100° E	sandy beach	intertidal	Light brown, heterogenous, carbonaceous, coarse sand; Macroalgal debris
27	16.3800° N 119.9110° E	sandy beach	intertidal	Light brown, heterogenous, carbonaceous, coarse sand; Macroalgal debris
28	16.3009° N 119.9162° E	near active fish cages	No data	Black; *Beggiatoa* mats
29	16.3045° N 119.9159° E	near active fish cages	11.5	Black; *Beggiatoa* mats
30	16.3162° N 119.9251° E	near active fish cages	16.2	Black; *Beggiatoa* mats
31	16.3564° N 119.9368° E	far from cages	5.9	Brown
32	16.3772° N 119.9296° E	far from cages	11.7	Dark brown
33	16.3907° N 119.9076° E	far from cages	16.7	Brown
34	16.4482° N 119.9240	seagrass bed	3.2	White, carbonaceous sand; Macroalgal debris

Table 2: Protein concentration, redox potential, H_2S presence and proteolytic activity in sediment samples from Bolinao Bay

Site	Relative distance from active fish cages	Protein concentration (mg/cm^3)	Redox (mV) at 1.0 cm depth	H_2S	Proteolytic activity ($\mu g \cdot h^{-1} \cdot cm^{-3}$)
1	near	no data	-367	+	no data
2	near	no data	-312	+	no data
3	near	no data	-333	+	no data
4	near	no data	-310	+	no data
5	near	no data	-392	+	no data
6	near	no data	-393	+	no data
7	far	no data	-294	-	no data
8	far	no data	-275	-	no data
9	far	no data	-310	-	no data
10	far	0.27	+92	-	448
15	near	14.96	-370	+	158
16	near	8.75	-380	+	36
17	near	16.93	-373	+	98
18	near	16.17	-373	+	122
19	far	9.13	-245	-	161
20	far	8.95	-308	-	149
21	far	4.58	-323	+	122
22	far	15.80	-382	+	215
23	far	5.49	-265	+	90
24	far	0.52	+101	-	627
25	far	0.46	+109	-	704
26	far	0.50	+97	-	600
27	far	0.50	+59	-	555
28	near	7.36	no data	+	130
29	near	8.01	no data	+	135
30	near	8.77	no data	+	160
31	far	1.35	no data	-	3
32	far	2.60	no data	-	203
33	far	1.75	no data	-	125
34	near	1.44	no data	-	1957

Table 3: Mean values of parameters in marine sediment samples

Sites	Protein concentration (mg/cm^3)	Redox (mV) at 1.0 cm depth	H_2S	Proteolytic activity ($\mu g \cdot h^{-1} \cdot cm^{-3}$)
Near active fish cages	11.56	–360	Present in all sites	120
Far from fish cages	3.81	–150	Present in few sites	426

Table 4: Inhibition of activity of bacterial proteases after 4 hours of incubation at various Na_2S concentration. Two concentrations of the scleroprotein substrate were used.

Na_2S (mM)	Proteolytic Activity ($\mu g \cdot h^{-1} \cdot cm^{-3}$) at 24 mg substrate	Inhibition of Activity (% of the control)	Proteolytic Activity ($\mu g \cdot h^{-1} \cdot cm^{-3}$) at 24 mg substrate	Inhibition of Activity (% of the control)
0	2763	0	5098	0
4.2	2523	9	4785	6
42	2165	22	4272	16
420	342	88	1623	68

Table 5: Redox potential and proteolytic activity in sediments after 24 hours of incubation at 3 conditions: oxic, anoxic and anoxic-sulfidic.

Batch	Redox Potential (mV)	Proteolytic Activity ($\mu g \cdot h^{-1} \cdot cm^{-3}$)
Oxic		
1	+75	591
2	+77	653
3	+83	653
4	+81	761
5	+91	644
AVE	**+81**	**660**
Anoxic		
1	-35	582
2	-85	537
3	-115	546
4	-110	528
5	-98	609
AVE	**-89**	**560**
Anoxic; H_2S		
1	-290	465
2	-297	519
3	-286	510
4	-281	492
5	-304	573
AVE	**-292**	**512**

Table 6: Proteolytic activity in aerated and unaerated natural sediment samples

Site	Redox potential in unaerated samples	Proteolytic Activity in unaerated samples ($\mu g \cdot h^{-1} \cdot cm^{-3}$)	Redox potential in aerated samples	Proteolytic Activity in aerated samples ($\mu g \cdot h^{-1} \cdot cm^{-3}$)
11 sulfidic	-357	197	-113	81
12 sulfidic	-344	107	-141	90
13 non-sulfidic	-170	143	-82	152
14 sulfidic	-355	260	-28	170

FIGURES

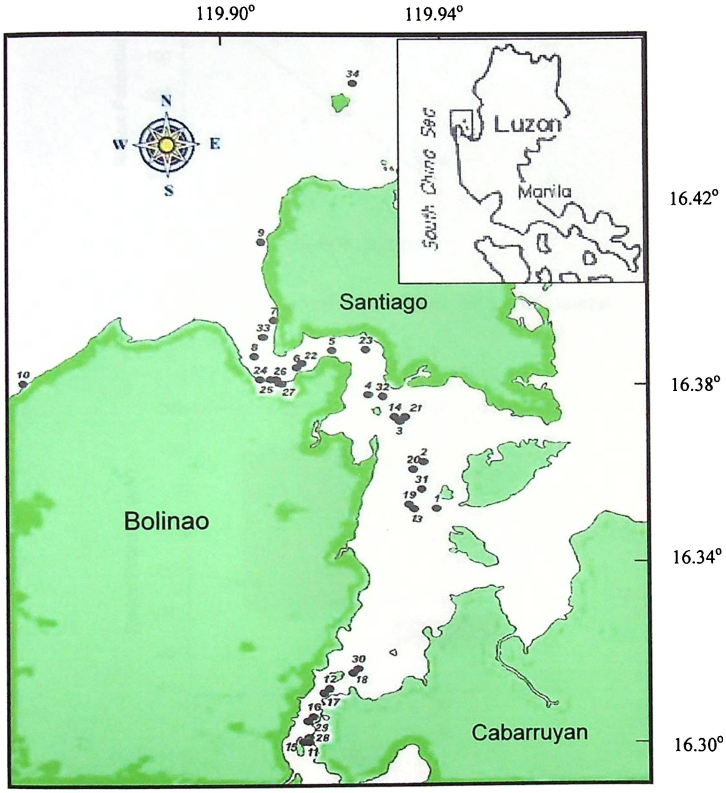


Figure 1: Sampling sites in Bolinao Bay (Philippines)

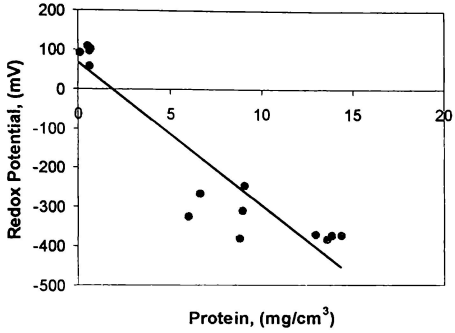


Figure 2: The relation of protein content in the sediment with its redox potential

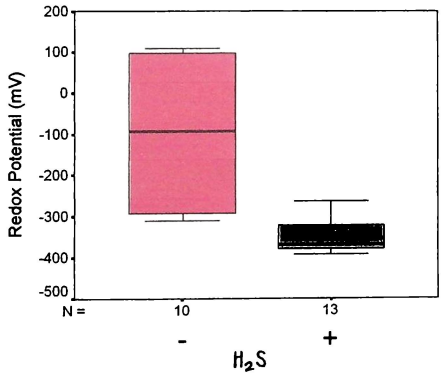


Figure 3: The relation of H_2S presence in the sediment with its redox potential

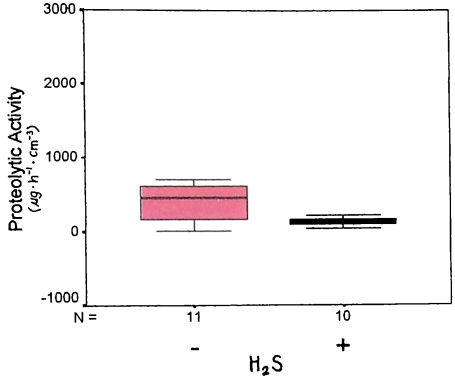


Figure 4: The relation of H_2S presence in the sediment with its proteolytic activity

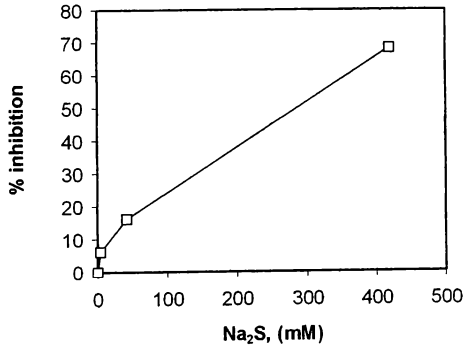


Figure 5: Inhibition of activity of bacterial proteases using 48 mg of scleroprotein substrate (expressed as % of the control) by various concentrations of Na_2S.

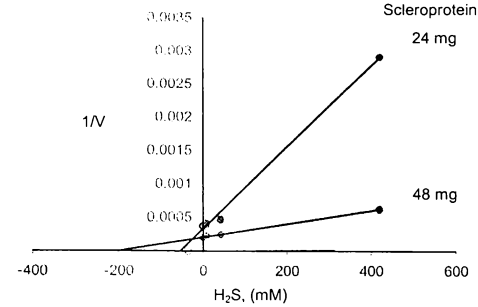


Figure 6: Dixon's plot for determination of inhibition of bacterial proteases by H_2S. Scleroprotein was used as the substrate at the indicated concentrations.

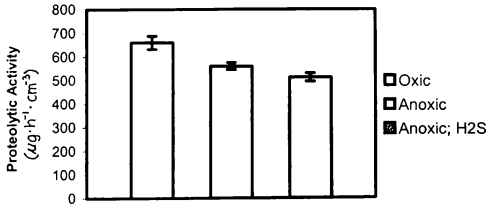


Figure 7: Proteolytic activity in sediments after 24 hours of incubation at 3 conditions: oxic, anoxic and anoxic-sulfidic.

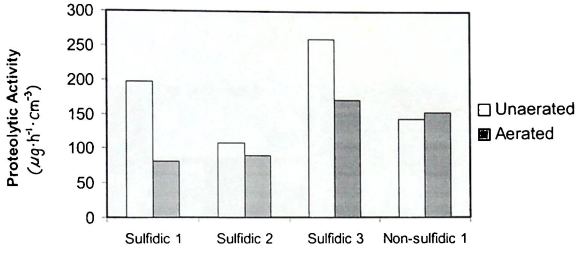


Figure 8: Proteolytic activity in aerated and unaerated natural sediments

PLATES

Plate 1: Tools and equipment used in the field (*from left to right)*: multi-functional pH/redox meter (Orion model 1230), the plexiglass sediment core sampler and field slate.

Plate 2: A sediment sample collected from an oxic site had light sediment color.

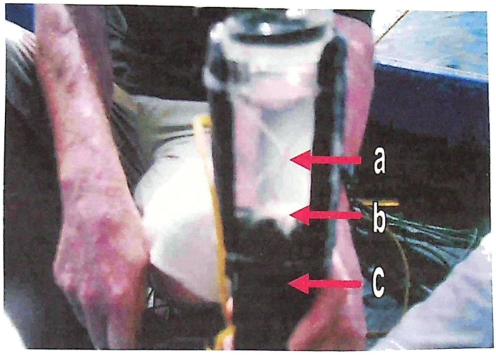


Plate 3: The core sampler shows vertical stratification of an anoxic-sulfidic site, *(a)* the water column above the sediment *(b)* mats of *Beggiatoa spp.* at the sediment and water column interface *(c)* a highly sulfidic sediment characterized by black color and strong H_2S odor.

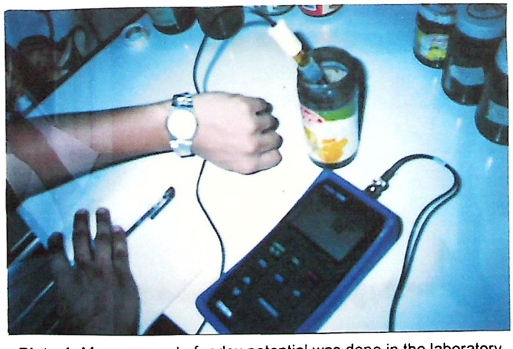


Plate 4: Measurement of redox potential was done in the laboratory.

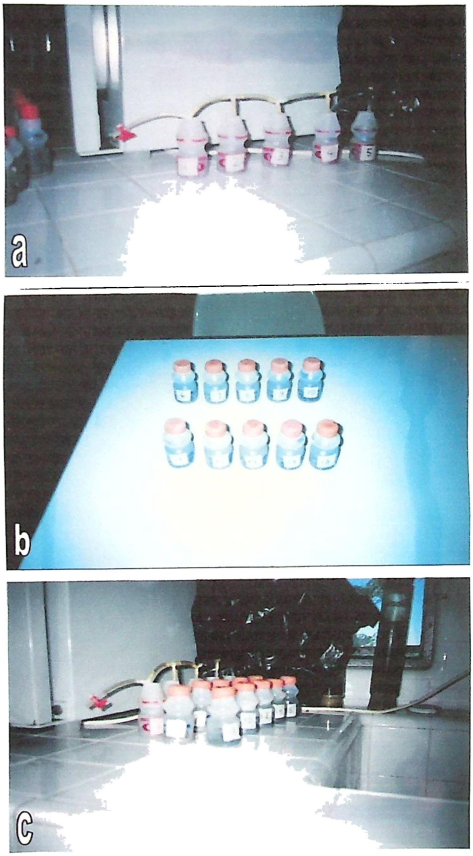


Plate 5: The experimental setup that tested for the effect of H_2S in anoxic marine sediments on total proteolytic activity. *(a)* The oxic batch was continuously aerated *(b)* The anoxic batch and as well as the anoxic-sulfidic batch were covered tightly with rubber stopper *(c)* Each batch had 5 replicates.

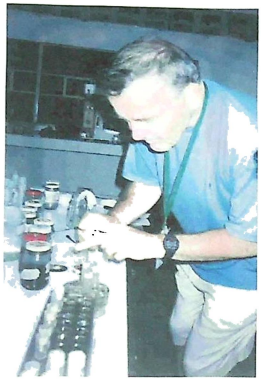
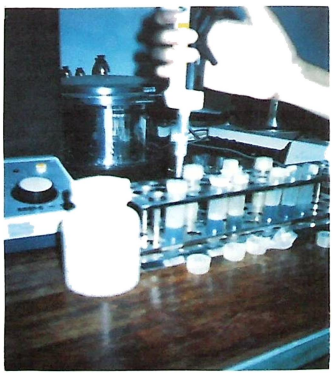


Plate 6: Addition of the dye-labeled scleroprotein substrate into the enzyme assay tubes.



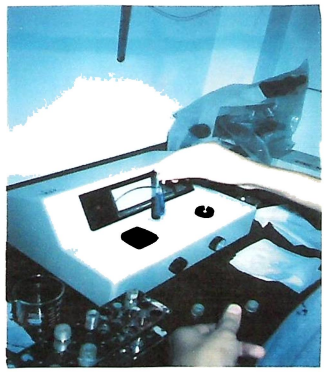


Plate 7: (*Left*) Enzyme incubation was terminated through addition of 37% formaldehyde. (*Right*) Proteolytic activity is measured by quantitative analysis of the dissolved decomposition products in a Spectronic 20 spectrophotometer.

APPENDICES

A. Computation for total proteolytic activity

Proteolytic Activity Unit ($\mu g \cdot h^{-1} \cdot cm^{-3}$) = ($10^3 \cdot$ absorbance $\cdot F \cdot V_a$) / ($V_s \cdot t$) with

F = substrate-specific conversion factor (4557 $\mu g \cdot l^{-1}$)
V_a = total assay volume (0.0055 l)
V_s = volume of sediment sample used for assay (1 cm^3)
t = incubation time (11-17 h)

Table 1: Raw data and computed proteolytic activity in Bolinao Bay sediment samples

Site	Raw data (10^{-3} · Absorbance at 595 nm)	Incubation Time (hrs)	Proteolytic Activity ($\mu g \cdot h^{-1} \cdot cm^{-3}$)
10	250	14	448
15	88	14	158
16	20	14	36
17	55	14	98
18	68	14	122
19	90	14	161
20	83	14	149
21	68	14	122
22	120	14	215
23	50	14	90
24	350	14	627
25	393	14	704
26	335	14	600
27	310	14	555
28	73	14	130
29	76	14	135
30	90	14	160
31	1.4	14	3
32	113	14	203
33	70	14	125
34	1093	14	1957

Table 2: Raw data and computed proteolytic activity in aerated and unaerated natural sediment samples

Site		Raw data (10^{-3} · Absorbance at 595 nm)	Incubation Time (hrs)	Proteolytic Activity ($\mu g \cdot h^{-1} \cdot cm^{-3}$)
11	Aerated	45	16	80.56
	Unaerated	110	16	196.93
12	Aerated	50	13.5	89.51
	Unaerated	60	13.5	107.42
13	Aerated	85	16	152.17
	Unaerated	80	16	143.22
14	Aerated	95	17	170.07
	Unaerated	145	17	259.59

Proteolytic Activity Unit ($\mu g \cdot h^{-1} \cdot cm^{-3}$) = ($10^3 \cdot$ absorbance $\cdot F \cdot V_a$) / ($V_s \cdot t$) with

F = substrate-specific conversion factor (4557 $\mu g \cdot l^{-1}$)
V_a = total assay volume (0.0055 l)
V_s = volume of sediment sample used for assay (1 cm^3)
t = incubation time (14 h)

Table 3: Raw data and computed proteolytic activity in 3 redox conditions

Batch	Raw data (10^{-3} · Absorbance at 595 nm)	Incubation Time (hrs)	Proteolytic Activity ($\mu g \cdot h^{-1} \cdot cm^{-3}$)
Oxic			
1	330	14	590.78
2	365	14	653.44
3	365	14	653.44
4	425	14	760.86
5	360	14	644.49
Anoxic			
1	325	14	581.83
2	300	14	537.08
3	305	14	546.03
4	295	14	528.12
5	340	14	608.69
Anoxic; H_2S			
1	260	14	465.47
2	290	14	519.17
3	285	14	510.22
4	275	14	492.32
5	320	14	572.88

Proteolytic Activity Unit ($\mu g \cdot h^{-1} \cdot cm^{-3}$) = ($10^3 \cdot$ absorbance $\cdot F \cdot V_a$) / ($V_s \cdot t$) with

F = substrate-specific conversion factor ($4557\ \mu g \cdot l^{-1}$)
V_a = total assay volume (0.0055 l)
V_s = volume of fluid enzyme isolate used for assay (0.5 cm^3)
t = incubation time (4 h)

Table 4: Raw data and computed proteolytic activity of bacterial proteases after 4 hours of incubation at various Na_2S concentration. Two fixed concentrations of the substrate were used.

$Na_2S \cdot 9H_2O$ (g/L)	Na_2S (mM)	Raw data (10^{-3} · Absorbance at 595 nm)	Incubation Time (hrs)	Proteolytic Activity ($\mu g \cdot h^{-1} \cdot cm^{-3}$)
24 mg of scleroprotein substrate				
0	0	485	4	2762.68
1	4.2	443	4	2523.44
10	42	380	4	2164.58
100	420	60	4	341.78
48 mg of scleroprotein substrate				
0	0	895	4	5098.14
1	4.2	840	4	4784.85
10	42	750	4	4272.19
100	420	285	4	1623.43

B. Computation for total protein content

Table 5: Absorbance values of different concentrations of lysozyme

Lysozyme Concentration (mg/L)	Raw data (10^{-3} · Absorbance at 595 nm)
0	10
16	20
80	50
400	450
2000	2350
10000	4975

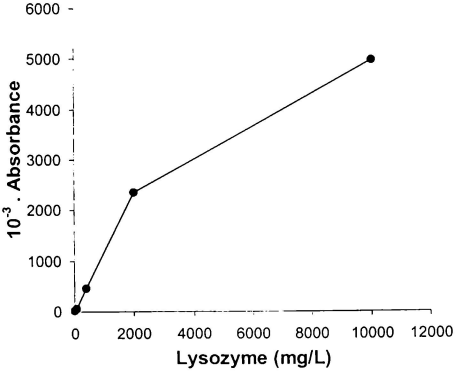


Figure 1: Absorbance values of different concentrations of lysozyme

Table 6: Raw data and computed total protein content in Bolinao Bay sediment samples using the protein standard curve in Figure 1 above.

Site	Protein Concentration (10^{-3} Absorbance at 595 nm)	Protein Concentration (mg/L)	Protein Concentration (mg/cm^3)
10	305	270	0.27
15	6632	14961	14.96
16	4582	8749	8.75
17	7282	16931	16.93
18	7032	16173	16.17
19	4707	9128	9.13
20	4647	8946	8.95
21	3207	4583	4.58
22	6907	15795	15.80
23	3507	5492	5.49
24	595	515	0.52
25	525	456	0.46
26	575	498	0.50
27	573	497	0.50
28	4125	7364	7.36
29	4337	8007	8.01
30	4590	8773	8.77
31	1579	1349	1.35
32	2552	2598	2.60
33	2272	1749	1.75
34	1685	1439	1.44

C. Statistical computations

Linear correlation (Protein content vs. Redox potential)

Data Entered

Count	Protein content (X)	Redox potential (Y)	Residual
1	0.27	+92	69.3455
2	14.96	-370	33.426
3	8.75	-380	-156.6938
4	16.93	-373	87.5655
5	16.17	-373	65.5218
6	9.13	-245	-10.672
7	8.95	-308	-78.8928
8	4.58	-323	-220.6438
9	15.80	-382	45.79
10	5.49	-265	-136.2494
11	0.52	+101	85.5967
12	0.46	+109	91.8564
13	0.50	+97	81.0166
14	0.50	+59	43.0166

Data Summary

$\sum_i X = 103.01$ $\sum_i X^2_i = 1313.7287$

$\sum_i Y = -2561$ $\sum_i Y^2_i = 1078361$

$\sum_i XY = -34964.24$

	X	Y
N	14	
Mean	7.3579	-182.9286
Variance	42.7535	46913.9176
Std.Dev.	6.5386	216.5962
Std.Err.	1.7475	57.8878

Analysis Summary

r_p	t	df
-0.8756	-6.28	12

Point Biserial Correlation Coefficient (Redox potential vs. H_2S)

Y Values Entered

(-) H_2S	(+) H_2S
-294	-367
-275	-312
-310	-333
92	-310
-245	-392
-308	-393
101	-370
109	-380
97	-373
59	-373
	-323
	-382
	-265

Summary Data

	(-) H_2S	(+) H_2S	Total
n	10	13	23
$-\Sigma Y$	-974	-4573	-5547
$-\Sigma Y^2$	456486	1627171	2083657
SS_Y	361618.4	18530.3077	745865.3043
$mean_Y$	-97.4	-351.7692	-241.1739

r_{pb}	t	df
-0.7	-4.49	21

Point Biserial Correlation Coefficient (Proteolytic activity vs. H_2S)

Y Values Entered

(-) H_2S	(+) H_2S
448	158
161	35.8
149	98.5
627	122
704	122
600	215
555	89.5
2.51	130
203	135
125	160
1957	

Summary Data

	(-) H_2S	(+) H_2S	Total
n	11	10	21
$-\Sigma Y$	5531.51	1265.8	6797.31
$-\Sigma Y^2$	5692285.300100001	180676.14	5872961.4401
SS_Y	2910685.0383	20451.176	3672798.4289
$mean_Y$	502.8645	126.58	323.6814

r_{pb}	t	df
-0.45	-2.19	19

One-Way ANOVA for 3 Independent Samples

Values Entered

Oxic (1)	Anoxic (2)	Anoxic; H_2S (3)
591	582	465
653	537	519
653	546	510
761	528	492
644	609	573

Data Summary

	Condition			
	1	2	3	Total
N	5	5	5	15
- X	3302	2802	2559	8663
-Mean	660.4	560.4	511.8	577.5333
- X^2	2195956	1574874	1316079	5086909
Variance	3828.8	1158.3	1595.7	5981.2667
Std.Dev.	61.8773	34.0338	39.9462	77.3386
Std.Err.	27.6724	15.2204	17.8645	19.9688

ANOVA Summary

Source	SS	df	MS	F	P
Treatment [between groups]	57406.53	2	28703.27	13.08	0.000967
Error	26331.2	12	2194.27		
Ss/Bl					
Total	83737.73	14			

Tukey HSD Test

HSD[.05]=79.09 M1 vs M2 P<.05 M1 vs M3 P<.05 M2 vs M3 nonsignificant	M1 = mean of Sample 1 M2 = mean of Sample 2 and so forth. --- HSD = the absolute [unsigned] difference between any two sample means required for significance at HSD [.05]