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A TOXICOLOGICAL AND COMPARATIVE STUDY
OF THE CONCENTRATION OF FREE AMINO ACIDS
IN FRESHWATER BENTHIC INVERTEBRATES

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ROBERT LOUIS GRANEY

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Major professor

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A TOXICOLOGICAL AND COMPARATIVE STUDY OF THE CONCENTRATION OF FREE AMINO ACIDS IN FRESHWATER BENTHIC INVERTEBRATES

By

Robert Louis Graney

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Abstract

A TOXICOLOGICAL AND COMPARATIVE STUDY OF THE CONCENTRATION OF FREE AMINO ACIDS IN FRESHWATER BENTHIC INVERTEBRATES

By

Robert L. Graney

The effects of pentachlorophenol (PCP) and sodium dodecyl sulfate (SDS) on the concentration of free amino acids (FAA) in the freshwater amphipod Gammarus pseudolimnaeus and bivalve Corbicula fluminea were investigated. Alterations in the FAA pool were correlated with changes in organism growth, respiration and energy status. Changes in the concentrations of FAA are discussed with respect to utility as an in situ indicator of toxicant-induced stress.

Exposure of Gammarus pseudolimnaeus to acutely toxic concentrations of PCP resulted in a significant decrease in the total FAA pool at the higher concentrations and a significant change in the FAA profile at the lesser concentration. Hyper-osmotic conditions did not alter the FAA concentrations while hypo-osmotic conditions caused a significant decrease in the total FAA pool. The observed alterations in the concentration of total FAA in stressed amphipods is most likely related to a disruption in the osmoregulatory ability of the organism.

Exposure of G. pseudolimnaeus for 45 d to sublethal concentrations of PCP caused a significant decrease in the total concentration of FAA. There was no effect on the relative concentration of individual amino acids. Whole body glycogen, protein and caloric content were significantly decreased after 15 days and lipids after 30 days exposure. Because reductions in growth and/or energy reserves can significantly

influence the fecundity of amphipods, it was concluded that alterations in the FAA pool are indicative of future adverse effects on the organism.

Significant seasonal variation was observed in the FAA concentrations of G. pseudolimnaeus. The greatest total FAA concentrations of 226.9 and 286.4 nmol/mg dry weight were observed in April and May. Free amino acid concentrations then declined through the summer months, slowly increased through the fall, reached a second smaller peak in early winter and then dropped drastically during the colder, winter months.

Acute and chronic exposure of Corbicula fluminea to SDS caused both an increase in the total concentration and a change in the relative concentration of individual amino acids. In addition, sublethal exposure decreased oxygen consumption and condition index and increased water content of C. fluminea. Alterations in FAA represent a more sensitive indicator of toxicant exposure than more traditional measures such as respiration.

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Preface

This study encompasses both a toxicological and a comparative investigation of the concentration of free amono acids (FAA) in freshwater benthic invertebrates. The majority of the research deals with toxicant-induced alterations in the FAA pool of the freshwater amphipod Gammarus pseudolimnaeus and bivalve Corbicula fluminea. Comparative aspects deal with seasonal variations in the FAA pool of G. pseudolimnaeus and the concentrations of FAA in five species of freshwater oligochaetes.

This dissertation is organized into seven chapters. Chapters 1 and 7 are the "Introduction" and "Summary and Conclusions," respectively, with the remaining chapters each representing a set of experiments. Each chapter is self-contained in that it has an Introduction, Materials and Methods and Results and Discussion section. Excluding some minor revisions to minimize redundancy among chapters, each chapter (2-6) has been submitted separately for journal publication.

The first chapter is the introduction. The purpose of this chapter is to present the "need" and "rationale" for biochemical indicators of toxicant-induced stress. It briefly discusses free amino acids; however, the majority of the discussion on the use of FAA as a sublethal indicator of stress is in the following chapters.

Chapter 2 deals with the effect of short-term toxicant (pentachlorophenol) and osmotic stress on the FAA pool of the freshwater amphipod Gammarus pseudolimnaeus. Changes in the concentration of FAA are discussed with respect to potential modes of action and application as a biochemical indicator of stress (BIS). This chapter has been

submitted for publication in The Archives of Environmental Contamination and Toxicology.

The third chapter concerns the effect of long-term exposure to PCP on the concentration of FAA and energy reserves in G. pseudolimnaeus. The relationship between FAA alterations and higher level effects such as growth are discussed. This chapter has been submitted for publication in Ecotoxicology and Environmental Safety.

Seasonal changes in the concentration of FAA in G. pseudolimnaeus are reported in Chapter 4. Potential reasons for these fluctuations are discussed as is the importance of this information when developing a BIS. This chapter has been submitted for publication in Comparative Biochemistry and Physiology.

Chapter 5 deals with changes in the FAA pool of Corbicula fluminea during acute and chronic exposures to sodium dodecyl sulfate. A comparison is made between lethal and sublethal effects and the relationship between FAA alterations and higher level effects such as respiration and condition index. This chapter has been submitted for publication in the Journal of Environmental Toxicology and Chemistry.

The sixth chapter discusses the comparative aspects of the FAA pool of five species of freshwater oligochaetes. This has been published in the Canadian Journal of Fisheries and Aquatic Sciences (Vol. 43, 1986).

The seventh chapter contains the summary and conclusions of all of the studies combined. An attempt is made to draw conclusions concerning FAA in freshwater benthic invertebrates and their potential application as a sublethal indicator of toxicant-induced stress.

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Chapter 1

Introduction

The presence of toxic chemicals in the aquatic environment has created the need to develop methods for assessing the potential affects of these contaminants on the health of individual organisms, populations and/or communities. Typically, this has entailed measuring long-term reproductive and growth effects and changes in community structure. These techniques can be expensive, time consuming, insensitive to sub-lethal effects and are often difficult to implement under field conditions. In addition, the usefulness of this type of monitoring is limited because adverse effects in the environment have already occurred. The primary purpose in establishing an in situ monitoring program is to identify problems before significant ecosystem degradation occurs. Prior to effects being manifested at the population, community or ecosystem level, toxicants exert effects at the organismal level by causing death or impairing functions. More specifically, chemicals interact with receptors such that specific and non-specific biochemical alterations occur. This being the case, measurement of specific biochemical parameters should provide the most sensitive indicator of exposure to toxicants and of potential adverse effects.

Biochemical indicators of toxicant-induced stress (BIS) can be divided into two categories: 1) general indicators, and 2) specific indicators. General biochemical indicators are not altered via inhibition of specific biochemical processes by specific chemicals, but rather are the result of a general stress response caused by toxicant-induced changes in a variety of interrelated processes. Selye (1976) described the response of an individual vertebrate organism to stressors in terms of a succession of physiological and biochemical reactions, collectively known as the "general adaptation syndrome" (GAS). The GAS is divided

into three main parts: the "alarm stage", the "resistance stage" and the "exhaustion stage." The alarm stage is characterized by the "flight or fight" response, during which the stressor elicits a response to mobilize the organisms defenses. This response is most apparent during acute exposures of relatively large intensity. The second stage of the GAS has been described as the resistance phase and offers general, long-term protection against stressors. The third and final stage of the GAS is referred to as exhaustion and occurs after long-term, continuous exposure to a stressor. In the exhaustion phase, energy stores become depleted and the organism can no longer maintain homeostasis. This phase is generally irreversible and death of the toxicant stressed organism occurs.

Based on the above discussion, a model of a general organism response to a stressor can be constructed. An undisturbed individual operates within a normal homeostatic range. Adaptation will allow an organism to adjust this range, however the ability is finite and restricted to specific processes, such as osmoregulation. The time which an organism can operate outside its normal homeostatic range will depend upon its condition (i.e., energy reserves) at stress initiation and the intensity of the stressor. General indicators are those parameters which may be indicative of changes occurring during this stress response. Examples include changes in energy reserves (Bhagyalakshmi et al., 1983; Thomas et al., 1981), adenylate energy charge (Dickson et al., 1981; Haya and Waiwood, 1983), hormonal titres (Wedemeyer, 1969; Donaldson and Dye, 1975), ionic composition (Roesijadi et al., 1976; Wedemeyer and Yasutake, 1977) and the activity of certain enzymes (Heitz et al., 1974).

The concept of a general monitor of stressor-induced responses in organisms is important because one limitation of biochemical indicators can be their specificity. Application of indicators specific for certain chemicals requires a-priori knowledge of the toxicant present. The primary application of general indicators would be when a multitude of potential toxicants may be present, such that measuring the overall health of the organism would be more advantageous than trying to pinpoint exposure to a particular toxicant or type of toxicant. A problem arises because general indicators are only useful when one can separate the effects of natural stress from toxicant-induced stress. By their nature alone, general indicators are going to be more responsive to non-toxicant types of stress and therefore, a fairly large data base will be required to differentiate between toxicant and non-toxicant stress.

Specific biochemical indicators represent parameters that are directly altered by exposure to a specific chemical. Often, these parameters are associated with the interaction of the chemical with a receptor site or are somehow associated with the chemicals' mechanism of toxicity. Specific indicators are more often used to assess an organism's exposure to a particular chemical than to assess its relative health status. Examples include inhibition of brain acetylcholinesterase activity by organophosphate compounds (Coppage, 1972) and δ aminolevulinic acid dehydratase by lead (Hodson et al., 1984).

Biochemical indicators of stress (BIS) can be used in both laboratory hazard assessment schemes and as <u>in situ</u> monitors of effects. Because there are different assumptions and requirements for both applications, it is important to distinguish between them prior to the development of any BIS.

Laboratory hazard assessment is a process used in both mammalian and aquatic toxicology to assess the potential hazards associated with the discharge, transfer or production of synthetic chemicals. In mammalian toxicology, a large array of clinical diagnostic tests exist which can be used to assess the health and pinpoint potential effects of toxicants in individual test organisms. Identification of the site of toxicity in model rat systems is useful when extrapolating to potential effects in humans. In aquatic toxicology, however, where the primary concern is protection of the structure and function of ecosystems, application of clinical indicators in laboratory hazard assessment is not as obvious. Clinical indicators which are indicative of specific organ dysfunction and are commonly used in mammalian toxicology have limited applications in aquatic laboratory hazard assessment. However, they can provide information on potential mechanisms of toxicity that can be extremely useful when attempting to develop toxicant specific indicators of exposure that can be used in field assessments. Currently, alterations in the growth and/or reproduction of fish and invertebrates are used to determine potential environmental hazards. These tests can be both expensive and time consuming. Biochemical indicators of stress have potential in laboratory tests as short-term predictors of chronic effects. If changes in general indicators, such as energy reserves, RNA/DNA ratios, adenylates or enzymes, can be correlated with long-term effects on growth and/or reproduction, then considerable time and money can be saved.

The primary application of BIS has been in the assessment of the relative "health" of organisms inhabiting potentially contaminated environments. Necessary criteria for effective <u>in situ</u> utilization of a

particular BIS include 1) the ability to separate toxicant-induced alterations from the normal background variability in the parameter of interest, 2) correlation of biochemical changes with "higher level" alterations of biological or ecological significance, and 3) a quantitative or qualitative relationship between the presence of the stressor and the response observed.

Under field conditions, organisms are exposed to a multiplicity of both chemical and physical stressors. Temporal (i.e., diurnal, seasonal) changes in salinity, turbidity, flow and temperature contribute to the variability associated with any biochemical parameter. In addition, biological processes such as molting, reproduction and food availability can have a significant influence on specific biochemical processes. It is the combination of all of the above accessory factors which determines the "normal" range of a given parameter and from which one must separate the effect of toxicant-induced stress. So, when developing an in situ BIS, one must establish a data base for the parameter of interest such that the "normal" range can be identified. Doing so will establish the statistical confidence in determining whether the parameter has deviated outside its normal range. By developing such a data base essentially identifies the "noise" level above which it is necessary to observe a toxicant related alteration.

It is extremely important that the response of biochemical indicators be related to ecologically relevant responses of individuals and/or populations (Mehrle and Mayer, 1980). Clinical measures of pathological effects are successful in mammals because a large body of correlative information exists. In aquatic organisms, there are few studies which relate changes at the biochemical level of organization to impaired

reproduction or growth. This type of information is essential for successful interpretation of changes in biochemical parameters. Measurement of a change in a specific BIS is of little use if that change is not predictive of future adverse effects on the biology and/or ecology of the organism or ecosystems.

The ideal situation for any BIS would be the existence of a quantitative dose-response relationship between the concentration of stressor and the biochemical effect observed. Given the complexity of mechanisms responsible for biochemical alterations however, this type of relationship is seldom obtained. In addition, under field conditions, it is impossible to know the actual dose to which an organism is exposed. Given this, a qualitative dose-response relationship is the greatest resolution which one can expect. In other words, biochemical alterations which occur are indicative of the presence of a stressor, but the dose cannot be predicted from the changes observed. The general lack of a quantitative dose response relationship does not limit the usefulness of BIS. If significant changes in a particular BIS occurs, one can conclude that the organism is stressed by a particular stressor and hopefully be able to predict future adverse effects. The next step in the process would be to identify the toxicant, possibly via a specific BIS or chemical analysis.

For biochemical indicators to be useful, consideration needs to be given to the choice of organism to be used in a monitoring study. This choice is dictated by a variety of factors, including 1) knowledge of the organism's physiology and biochemistry, 2) background data base, 3) size, 4) ease of sampling, 5) trophic level, and 6) importance in the ecosystem. Both fish and invertebrates have been utilized, each

possessing specific advantages and disadvantages. The aesthetic and economic importance of fish has prompted a considerable amount of research on their physiology and biochemistry; however, mobility and sensitivity to handling stress limits usefulness as biomonitors. For invertebrates, the general lack of knowledge on basic biochemistry and physiology may complicate interpretation of biochemical alterations; however, their more sedentary lifestyle and ease of sampling makes them more amenable as monitors.

Environmental regulation is a very complex science and to be effective, ecotoxicologists must make use of all of the tools available. Ultimately, the ecosystem and all of its components is the level of organization which we want to protect. Therefore, ecosystem-level monitoring will continue to be important, as will population and community level studies. Also, when examining the responses of different levels of organization to toxicants, it should be remembered that each more complex level of organization embodies information which is not included in less complex levels of organization (Giesy and Odum, 1980). That is, because of interactions and compensatory mechanisms, there are physiological properties that cannot be predicted from knowledge of reactions at the biochemical level of organization and ecological responses which cannot necessarily be predicted from knowledge of the responses of populations (Giesy and Allred, 1985). Therefore, biochemical indicators will not replace measures of toxicant effects on individuals, populations, communities or ecosystems but rather will provide another, possibly cheaper and more sensitive tool to determine when toxicant related effects occur in the field. Specifically, an understanding of biochemical responses will elucidate the modes of toxic action of

toxicants, which may lead to the development of specific indicators of toxicant exposure. In addition, this increased understanding will subsequently serve as the basis of improved predictability of toxic effects among toxicants and species and allow better extrapolation to different environmental conditions.

A large array of specific and general biochemical indicators of stress (BIS) have been developed, including: changes in enzyme activity, lysosomal stability, concentrations of adenylates, glycogen, lipids and free amino acids and the RNA/DNA ratio (Bayne et al., 1985; Giesy et al., 1986). In marine invertebrates, FAA pools have received a considerable amount of attention and have been successfully used as a general indicator of toxicant exposure (Roesijadi et al., 1976; Kasschau et al., 1980). Interest in FAA pools arose after discovery of the important role of these compounds in intracellular osmoregulation in euryhaline invertebrates (Gilles 1975; Duchateau-Bosson and Florkin, 1961). Since then, additional research has found the FAA pool of marine organisms to respond to a variety of natural and man-made perturbations (Sansome et al., 1978; Augenfeld et al., 1980). With freshwater organisms however, the situation is quite different. While information is available on amino acid metabolism in freshwater organisms (Gilles, 1979), the effect of toxicant-induced stress on the FAA pool is presently unknown.

Given the paucity of information available on the effects of stress on the FAA pool of freshwater invertebrates, a comprehensive study was initiated to determine if alterations in the FAA pool could be used as a biochemical indicator of toxicant-induced stress. This study

encompassed establishing the natural variations in the FAA pool of specific freshwater invertebrates, determining the effects of acute and chronic exposure to specific toxicants on the FAA pool and establishing a relationship between these alterations and higher level effects such as growth.

Chapter 2

The Effect of Short-Term Toxicant and Osmotic Stress on the Free Amino Acid Pool of *Gammarus pseudolimnaeus*

Introduction

This chapter discusses the effects of acute stressors on the FAA pool of the freshwater amphipod Gammarus pseudolimnaeus. In the first experiment, amphipods were exposed to pentachlorophenol for 96 h and the effects of this exposure on whole body FAA pools were determined. The second experiment investigated the influence of osmotic shock on the FAA pool of the amphipods. This experiment was conducted based on the results of the first study, in which it was hypothesized that the reduction in the FAA pool caused by PCP exposure may have been caused by a disruption in osmoregulatory ability. In a third experiment, the combined effects of osmotic shock and PCP exposure were investigated.

Materials and Methods

General Procedures

All experiments were conducted with populations of the freshwater amphipod Gammarus pseudolimnaeus, Bousfield (Crustacea: Amphipoda) collected from the field. Organisms were collected by D-frame dip net from Glass Creek, a "pollution free" second-order stream located in Barry County, Michigan. They were returned to the laboratory and prior to test initiation, acclimated for at least 96 h and for no longer than two weeks. All tests were static renewal and conducted with river water collected from Glass Creek, MI. During testing, G. pseudolimnaeus were fed Ash leaves preconditioned by soaking them in river water for 2 weeks. Amphipods were exposed to measured concentrations of pentachlorophenol (>99% pure) obtained from Fluka Chemical Company (Hauppauge, N.Y.). Pentachlorophenol (PCP) concentrations in water were measured by the method of Carr et al. (1982). Briefly, this method

entailed extracting 100 ml of acidified water with 10 ml chloroform and back extracting into 2.0 ml of 200 mM NaOH. The aqueous fraction was then measured spectrophotometrically at 320 nm.

On designated days, organisms were sampled and immediately frozen on dry ice. Whole amphipods were subsequently dried at 95 C for 24 h, weighed and extracted by homogenizing the entire organism in methanol. After centrifugation, the supernatants were decanted and stored in a freezer (-10 C) until analysis by high pressure liquid chromatography. The extract was derivitized one minute prior to injection by mixing 100 µL extract with 50 µL o-Fluoraldehyde® (O-phthaldialdehyde:OPA) (Pierce Chemical Co., Rockford, Ill.). Since the reaction of the amino acids with OPA requires the presence of a free amine group, this method does not allow quantification of proline or hydroxyproline. Separation of 19 amino acids was achieved with a 5 µm, ultrasphere® ODS C-18 column preceded by a pellicular ODS ocatadecyl (37-53 µm) guard column. A 40 min linear gradient (17-73%) of solvent A (100% methanol) to B (0.1 M sodium acetate; pH 7.0) was utilized. All solvents were filtered and degassed before use. LDS/Milton Roy constametric® pumps were interfaced with a Kratos FS-970 L.C. fluorometric detector (activation wavelength 330 nm; emission wavelength 418 nm; sensitivity 5.0; range 1.0 µAmps; time constant 0.5Sec).

Patterns of relative FAA concentrations of stressed and unstressed amphipods were compared by univariate and multivariate statistical techniques. Total FAA concentrations of different treatment groups were compared by analysis of variance (ANOVA) followed by Duncans Multiple Range Test. The patterns of relative concentrations of individual FAA were compared by profile analysis (Morrison, 1967). This technique uses

multivariate analysis of variance to compare the differences between adjacent amino acids. Significance was determined by Wilk's criterion (SAS Institute, 1982).

Pentachlorophenol Exposure

G. pseudolimnaeus were exposed for 96 h to 0.66, 1.13 or 1.68 mg/l pentachlorophenol (measured concentrations). Tests were conducted at 20 C (± 1.0), pH 8.0 and the water was continuously aerated during the test.

Osmotic Stress

Amphipods were subjected to both hypo- and hyper-osmotic conditions for 48 h. Hyposmotic conditions were obtained by adding conditioned leaves (food) to distilled water, aerating the water for 24 h and then adding the amphipods. The resulting water had a hardness of 1.9 mg/l as $CaCO_3$ (1.9×10^{-5} osmolal). Hyperosmotic conditions consisted of a 1.05% salt solution (NaCl) and were equivalent to approximately 30% saltwater or 10.5 0/00 (0.18 osmolal). Organisms were sampled at test initiation and after 48 h exposure to each osmotic condition. Test water was continuously aerated and temperature and pH were 20.0(± 1.0) C and 8.0, respectively.

Pentachlorophenol-Osmotic Stress Exposure

The combined effect of PCP and osmotic conditions on the FAA pool was investigated by exposing G. pseudolimnaeus first to pentachlorophenol and then to osmotic stress. Because organisms collected from the field were utilized and this test was conducted during the winter months, the temperature during this exposure was 10 C, considerably

lower than the other tests (20 C). Amphipods were exposed for 48 h to 0.86, 1.16, 1.51, 1.80 or 2.24 mg/l pentachlorophenol (measured concentrations), after which they were transferred and exposed for 24 h to either clean dilution water (river water) or "hyposmotic" water, similar in quality to that used in the osmotic shock experiment. Organisms were sampled and analyzed for FAA at test initiation and after 48 h of toxicant exposure. Organisms which were transferred to hyposmotic conditions after 48 h PCP exposure were sampled after 24 h of osmotic stress.

Results and Discussion

Pentachlorophenol Exposure

The 96 h LC_{50} was 1.15 mg/l (C.L. 1.04-1.36). Total amino acid concentrations of *G. pseudolimnaeus* were significantly reduced by exposure to 1.68 mg PCP/l for 48 h (Table 1). Except for the sulfo-amino acid taurine, this reduction was due to decreases in all individual amino acids. Glutamine was reduced approximately four-fold, with all the remaining amino acids being reduced by approximately half. A reduction of the total FAA concentration was also noted in individuals exposed to 1.13 mg/l PCP, however this decrease was not statistically significant ($\alpha = 0.05$). Exposure to 1.13 mg/l PCP caused both increases and decreases in concentrations of specific amino acids. These changes resulted in a qualitative profile which was significantly different from control organisms (Fig. 1). This difference was established by conducting multivariate analysis of variance (MANOVA) on the differences between adjacent amino acids. For example, the differences between alanine and leucine, leucine and glutamine, glutamine and valine, etc.

were calculated for each treatment (Fig. 1). A MANOVA was then conducted on these differences, essentially testing for parallelism, and significance was established using Wilks' criterion. In the present study, the calculated F-value was 50.28 and was found to be highly significant ($\alpha = 0.0001$). More specifically, in exposed organisms the concentrations of alanine, glutamine, isoleucine, leucine and lysine were considerably less than in organisms which were not exposed to PCP. Alternatively, concentrations of taurine and valine were greater in exposed organisms. As will be discussed below, qualitative changes such as these may represent a more sensitive indicator of stress than the changes in the total FAA concentrations observed when organisms were exposed to greater toxicant concentrations.

The PCP-induced changes in individual amino acids observed in the present study were different from results reported by other investigators. In marine invertebrates, specific amino acids have been identified as being fairly responsive to certain kinds of stress. Increases in the molar ratios of taurine to glycine has been used as a quantitative index of stress in the hard clam Mercenaria mercenaria (Jeffries, 1972). Differences in this index in clams from polluted and non-polluted environments have been demonstrated (Jeffries, 1972). Similar results have been observed for the clam Macoma inquimator (Roesijadi and Anderson, 1979) and mussel Mytilus edulis (Widdows et al., 1981) exposed to petroleum hydrocarbons. However, a decrease in the taurine/glycine ratio was observed in oil stressed bivalves (Augenfeld et al., 1980). Kasschau et al. (1980) found that glycine concentrations in sea anemones (Bunodosoma cavernata) were too variable to be an effective indicator of toxicant-induced stress. Other amino acids in marine invertebrates

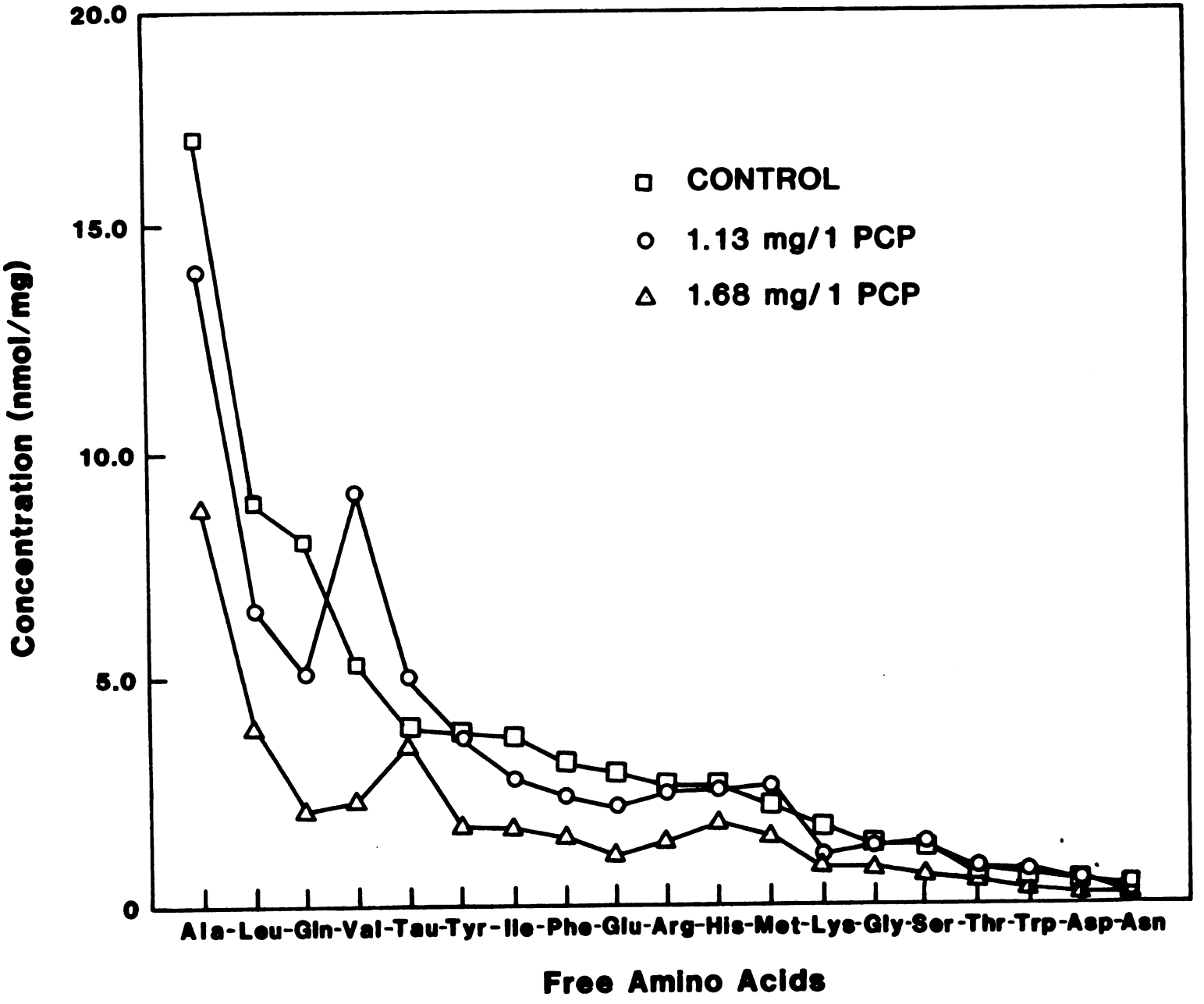
Table 1. Concentrations of individual and total amino acid (nmol/mg) of of G. pseudolimnaeus exposed to pentachlorophenol for 48 h.

	Day 0		Day 2		
Amino Acid	Control	(S.D.)[b]	Control (S.D.)	1.68 mg/l PCP (S.D.)	1.13 mg/l PCP (S.D.)
Alanine	17.21	(2.3)	16.88 (1.15)	8.80 (4.3)	14.05 (4.5)
Arginine	4.06	(0.5)	2.64 (0.69)	1.36 (0.61)	2.48 (0.69)
Asparagine	0.66	(0.07)	0.36 (0.04)	0.18 (0.76)	0.23 (0.084)
Aspartic Acid	0.79	(0.07)	0.52 (0.08)	0.22 (0.16)	0.52 (0.175)
Glutamine	8.5	(1.7)	8.05 (4.49)	2.07 (1.77)	5.14 (2.48)
Glutamic Acid	3.04	(0.26)	2.93 (0.23)	1.10 (0.86)	2.16 (0.801)
Glycine	1.83	(0.46)	1.35 (0.91)	0.80 (0.34)	1.28 (1.37)
Histidine	2.04	(0.03)	2.59 (0.48)	1.75 (0.56)	2.46 (0.615)
Isoleucine	3.59	(0.71)	3.67 (0.7)	1.66 (0.63)	2.75 (0.66)
Leucine	8.07	(1.35)	8.87 (1.67)	3.90 (1.57)	6.50 (1.56)
Lysine	2.16	(0.42)	1.72 (0.41)	0.82 (0.37)	1.09 (0.21)
Methionine	1.85	(0.24)	2.21 (0.48)	1.53 (0.75)	2.62 (1.03)
Phenylalanine	3.2	(0.46)	3.11 (0.64)	1.52 (0.52)	2.45 (0.58)
Serine	2.4	(0.74)	1.35 (0.19)	0.64 (0.30)	1.40 (0.81)
Taurine	5.75	(0.99)	3.87 (1.1)	3.49 (0.88)	5.01 (1.74)
Threonine	1.17	(0.26)	0.76 (0.07)	0.47 (0.22)	0.80 (0.36)
Tryptophan	0.74	(0.19)	0.64 (0.16)	0.31 (0.15)	0.75 (0.31)
Tyrosine	4.04	(0.78)	3.75 (0.6)	1.75 (0.65)	3.72 (1.08)
Valine	5.07	(0.79)	5.31 (0.72)	2.34 (0.86)	9.06 (0.99)
Total	76.17	(14.89)	70.58(16.30)	34.71[a](7.28)	64.46(18.41)

[a]Significantly ($\alpha = 0.05$) lower than control organisms based on Duncans Multiple Range Test.
[b]S.D. -standard deviation, n = 5.

Figure

Fig. 1. Profile of free amino acid (FAA) concentrations in *G. pseudolimnaeus* exposed to 1.13 and 1.68 mg/l PCP for 48 h. Organisms exposed to 1.13 mg/l exhibited a statistically significantly different profile ($\alpha < 0.0001$, Wilk's criterion, profile analysis).


20.0
15.0
10.0
5.0
0
Concentration (nmol/mg)
CONTROL
1.13 mg/1 PCP
1.68 mg/1 PCP
Ala-Leu-Gln-Val-Tau-Tyr-Ile-Phe-Glu-Arg-His-Met-Lys-Gly-Ser-Thr-Trp-Asp-Asn
Free Amino Acids

which have been shown to be specifically affected by toxicants include glutamate (Kasschau et al., 1980), alanine and aspartate (Riley and Mix, 1981; Powell et al., 1982; Roesijadi et al., 1976) and arginine, lysine and threonine (Augenfeld et al., 1980; Roesijadi and Anderson, 1979). Therefore, even though stress can alter specific amino acids, the inconsistency of organismal responses under different conditions and exposed to different toxicants makes interpretation of alterations extremely difficult.

Compared with marine organisms, relatively little work has been conducted on toxicant-induced alterations in the FAA pool of freshwater organisms. Changes in the total FAA concentration was measured in freshwater crabs exposed to Sumithion®; however, concentrations of individual amino acids were not reported (Bhagyalakshmi et al., 1983). Those authors found that the total concentration of amino acids increased during acute exposure and declined during chronic exposure. Differences in total FAA were also reported for freshwater bivalves collected from polluted and unpolluted environments (Gardner et al., 1981). The mantle tissue of organisms collected from habitats contaminated by acid and metals had a significantly greater total concentration of free amino acids. However, because these organisms were sampled from different ecosystems and reciprocal transplant controls were not utilized, the effects of accessory factors from those of the toxicants cannot be eliminated. The increase in total FAA concentrations reported by Gardner et al. (1981), is opposite to the effect observed in my study, where a significant decrease in FAAs occurred. The reason for the differences is unknown; however, given the variety of mechanisms by which the total FAA concentration can be altered, it is

not surprising that the response of the total FAA concentration varies in different organisms stressed under different conditions.

Two types of changes in the FAA pool of organisms normally occur under toxicant-induced stress. The first involves either an increase or decrease in the total FAA concentration. In the present study, acutely toxic concentrations of pentachlorophenol caused a decrease in the FAA pool. The second type of response results in alterations in specific amino acids (either increases or decreases) which may or may not cause changes in the total amino acid concentration. This effect was observed in amphipods exposed at lower PCP concentrations. The different responses of the FAA pool may reflect the presence of a variety of mechanisms responsible for amino acid alterations. These mechanisms, or the rationale for expecting changes in the FAA pool of toxicant stressed organisms, can be separated into two categories. The first category covers the effect of stress on protein metabolism and subsequent changes in the FAA pool. The second encompasses the complex mechanisms controlling osmotic regulation and the role of FAA in this process.

Protein metabolism is an extremely complex, homeostatically-regulated, process which can be altered during toxicant-induced stress and results in changes in concentrations of specific amino acids as well as the total FAA concentration. The FAA pool is the equilibrium concentration of amino acids, resulting from the interaction of a number of biochemical processes, including: 1) rates of transfer between intracellular and extracellular compartments, 2) release of amino acids *via* protein degradation and subsequent utilization in energy metabolism, 3) utilization of amino acids for protein synthesis, 4) synthesis of non-essential amino acids, 5) utilization of amino acids in the

synthesis of other important biomolecules such as purines and pyrimidines, and 6) anaplerotic reactions (Munro, 1970). Amino acid transport across membranes is an extremely complex process, controlled by a variety of interrelated processes. Toxicants can interfere directly with the energy requiring transport processes, thus affecting the intracellular pool, or they can indirectly influence FAA by altering the hormonal milieu of the organism. Hormones, such as cortisol, insulin and glucagon can directly influence amino acid uptake and distribution and are thought to be key factors controlling the metabolism of amino acids (Munro, 1970; Guidotti, 1978).

Protein degradation and subsequent utilization of the released amino acids for anaplerotic reactions and/or energy production represents another potential cause for changes in the FAA pool. For many invertebrates, amino acids may contribute significantly to the total energy budget of the organism (Gilles, 1970; Bayne, 1973; Bursell, 1966). AMP and/or ADP are covalent modifiers of many of the enzymes involved in nitrogen metabolism, such as glutamate dehydrogenase (Stryer, 1981), so that changes in the energy status of an organism can modulate the oxidation of amino acids, thus altering the relative proportions or profile of the FAA pool. Therefore, under the increased energy demand associated with toxicant-induced stress, invertebrates may degrade proteins to augment the available energy supply thus altering the FAA pool (Gould et al., 1976; Bhagyalakshmi et al., 1983; Powell et al., 1982).

Toxicant exposure may also require the repair of damaged tissue and/or the replacement of essential biomolecules. This may result in a substantial alteration in synthetic pathways such as increased protein

synthesis such that removal from the FAA pool results in an alteration in its composition.

Toxicants may also influence the FAA pool by directly or indirectly altering the osmoregulatory processes of an organism. Osmoregulation can be separated into two processes: 1) extracellular regulation, which controls the movement of water and ions between the external environment and the organisms body fluids and 2) intracellular regulation, which controls the concentration of ions and water within individual cells. In all organisms, the major objective of osmoregulation is to maintain a constant intracellular mileau such that normal metabolic processes can proceed. Marine or euryhaline organisms live under hyperosmotic conditions such that water loss and ionic influxes must be controlled. In these organisms, amino acids are extremely important intracellular osmolytes and can be adjusted during salinity fluctuations.

In freshwater organisms, however, the role of FAA in osmoregulation is less clear. Free amino acids constitute 10-20% of the active intracellular osmolytes of freshwater invertebrates (Gilles, 1979) and can play an important role in intracellular osmoregulation (Hanson and Dietz, 1976). However, freshwater crustaceans are generally considered to be efficient osmoregulators. Hyperosmotic conditions are maintained by decreased water permeability, active salt uptake and the production of hypotonic urine. Stressful conditions can interfere with osmoregulatory ability, resulting in a decrease in blood osmolality, however it is unknown whether the intracellular FAA pool concomitantly decreases to maintain isotonic conditions. Acute stress, such as handling, often results in a transitory loss of osmoregulatory ability which might result in a decrease in the entire FAA pool.

The complexity of nitrogen metabolism in aquatic invertebrates, coupled with the lack of information on the regulatory processes that control metabolism, makes it extremely difficult to discern mechanisms by which individual amino acids may be altered during toxicant-induced stress. However, when developing a general BIS, understanding the mechanism is not always essential as long as the changes observed are consistent, are quantitatively or qualitatively related to exposure, are separated from the "background noise" or variability normally associated with field conditions and are related to ecologically relevant end points.

The primary effect of acute toxicant-induced stress in the present study was a decrease in the entire FAA pool. The profile was altered at the lower concentration; however, presently it is impossible to speculate on which mechanism may be responsible for this change. With respect to the decline observed in the total pool, since all amino acids except taurine decreased, it was hypothesized that the mechanism responsible was hypoosmotic stress caused by impaired osmoregulatory ability. The breakdown in osmoregulation could be caused by changes in the hormonal status of the organism induced by the stressor or could be the result of direct damage to the gills and/or excretory organs and the subsequent loss in ability to regulate water and ion fluxes. In either situation, dilution of the hemolymph would require adjustment of the intracellular osmolality, possibly via reduction in the FAA pool. If this were the mechanism responsible in the present study, then one would also expect non-toxicant induced osmotic stress to cause a similar change in amino acid concentrations. To test this hypothesis, a study

was conducted to determine the influence of extraorganismal hyposomotic and hyperosmotic conditions on the FAA pool of G. pseudolimnaeus.

Osmotic Stress

Hyperosmotic (1.05% NaCl; 0.18 osmolal) and hyposmotic (hardness = 1.9 mg/l $CaCO_3$; 1.9×10^{-5} osmolal) conditions caused changes in the concentrations of FAA of G. pseudolimnaeus (Table 2). Under hyperosmotic conditions, there was a slight increase in the total FAA pool; however, this increase was not statistically significant ($\alpha > 0.05$). The concentrations of most free amino acids were elevated, although the majority of the increase in the total pool could be attributed to alanine, glycine, serine and taurine. The concentrations of arginine, glutamine and glutamate decreased in hyperosmotically stressed organisms. Because glutamate is extremely important in anaplerotic reactions and glutamine can be directly deaminated to glutamate, changes in concentrations of these amino acids may indicate increased flux of α keto-gluturate into both anabolic (amino acid synthesis) and catabolic (TCA cycle) pathways. The inability of G. pseudolimnaeus to significantly increase the FAA pool under conditions of hyperosmotic shock does not mean that under less severe conditions, intracellular osmoregulation via increased FAAs does not occur. Hyperosmotic regulation is generally a much slower process than hypoosmotic regulation (Bishop et al., 1981) and may require a more gradual acclimation to saline conditions than were utilized in this study.

Hyposomotic conditions resulted in a significant decrease in the total concentration of FAA (Table 2). Concentrations of most of the free amino acids decreased. Concentrations of arginine, glutamate,

Table 2. Concentrations of individual and total amino acids (nmol/mg) of G. pseudolimnaeus exposed to hyper- and hyposmotic shock for 48 h

Amino Acid	Control[a] (S.D.)	Hyperosmotic[b] (S.D.)	Hyposmotic[c] (S.D.)
Alanine	18.80 (4.4)	22.76 (1.08)	11.3 (0.92)
Arginine	7.59 (1.36)	5.14 (3.9)	2.59 (0.74)
Asparagine	0.14 (0.02)	0.31 (0.08)	N.D.
Aspartic Acid	1.02 (0.13)	1.30 (0.73)	0.53 (0.22)
Glutamine	13.16 (5.84)	9.71 (3.2)	5.20 (0.89)
Glutamic Acid	3.60 (0.10)	2.87 (0.68)	1.23 (0.22)
Glycine	1.38 (0.32)	3.63 (3.48)	0.12 (0.02)
Histidine	3.81 (0.43)	4.2 (1.1)	2.94 (1.1)
Isoleucine	3.70 (0.63)	4.34 (0.94)	2.45 (0.73)
Leucine	9.12 (1.59)	10.26 (2.5)	6.05 (2.0)
Lysine	3.06 (0.73)	2.32 (1.7)	1.10 (0.42)
Methionine	3.00 (0.37)	2.48 (0.63)	1.72 (0.54)
Phenylalanine	3.30 (0.73)	3.80 (0.98)	2.67 (1.19)
Serine	2.49 (0.81)	5.31 (6.4)	0.94 (0.21)
Taurine	4.07 (1.3)	5.95 (0.27)	7.85 (1.59)
Threonine	1.52 (0.38)	1.57 (0.88)	0.66 (0.13)
Tryptophan	0.72 (0.10)	0.96 (0.14)	0.69 (0.22)
Tyrosine	4.54 (1.09)	5.07 (0.88)	5.11 (2.8)
Valine	5.55 (0.99)	6.11 (1.27)	3.67 (1.09)
Total	90.57(21.34)	98.09(24.61)	56.89[d](14.27)

[a]S.D. - standard deviation, n = 5.
[b]1.05% NaCl; 0.18 osmolal
[c]Distilled water (1.9 mg/l as $CaCO_3$; 1.9 x 10^{-5} osmolal)
[d]Significantly ($\alpha = 0.05$) lower than control organisms based on Duncans Multiple Range Test.
N.D. - Amino acid not detectable

glutamine and glycine decreased by the largest percentage. Exceptions to this were tryptophan, which did not change at all, and taurine and tyrosine, which increased relative to control organisms. The increase in taurine, coupled with the substantial decrease in the concentration of glycine, resulted in a significant increase in the taurine/glycine ratio. Increases in this ratio have been observed in marine invertebrates subjected to a variety of stressors (Jeffries, 1972; Roesijadi and Anderson, 1979; Widdows et al. 1981). The changes in the pattern of FAA concentrations observed during hyposmotic conditions were very similar to those measured for acute PCP exposure (Tables 1 and 2). In both cases a significant decrease was observed in the total FAA concentration.

The similarity of changes in concentrations of FAA observed in organisms exposed to PCP and hyposmotic conditions indicates that similar mechanisms may be responsible for the changes in both cases. Although theoretically different mechanisms and/or stressors can cause the same alterations in the FAA pool, the wide range or variety of FAA responses reported in the literature would seem to indicate that, when consistent changes in the FAA profile occur, they are likely due to interferences with the same or similar processes. In the present study, both of the stressors, although completely different in nature, likely had the same effect on the organism; that is, a disruption in osmoregulatory ability with subsequent dilution of the hemolymph. This type of response is indicative of acutely or severely stressed individuals and does not represent a sublethal response. Therefore, decreases in the concentration of total FAA are indicative of severe stress and do not represent a very sensitive biochemical indicator. Changes in the

pattern of concentrations of individual FAA, such as those observed at the lower PCP concentrations, are more likely the type of response which will be observed in sublethally stressed organisms.

Combined PCP Exposure and Osmotic Stress

Prior to presenting the results of this experiment, a discussion of the rationale behind the study design is warranted. Many of the concepts presented below have been reviewed in an article by Stebbing (1981) that discusses the relationship between stress and homeostatic regulation in aquatic organisms. All organisms contain specific control systems, such as thermoregulation or osmoregulation, which maintain preferred conditions for a large variety of processes. Stressors, natural or man-made, can influence the ability of organisms to maintain homeostasis. Stress associated with exposure to toxicants can exert a "workload" on these homeostatic control processes. The ability of an organism to effectively resist changes in its preferred state over an extended period of time can be referred to as its "counteractive capacity." Since homeostatic control mechanisms require energy, an organism's counteractive capacity is dependent upon its initial health status. In addition, these control processes have a finite capacity, in that excessive or long-term stress can eventually force the organism to deviate from its preferred state. An organism may be able to adjust or cope with a single stressor (i.e., toxicant); however, if a second stressor is applied (i.e., osmotic), the organism may not be able to maintain homeostasis, even though under normal, unstressed conditions, the organism could have resisted the second stressor. Therefore, the ability of an organism to maintain homeostasis upon exposure to a

particular stressor is influenced by how much of its "counteractive capacity" has been utilized to cope with prior stressors.

In the present study, I hypothesized that the stress of toxicant exposure would make the organism more susceptible to osmoregulatory breakdown due to a lesser counteractive capacity, such that osmotic shock would cause changes in the FAA pool which otherwise would not have occurred. This concept could provide an approach to use biochemical measurements to assess the health of organisms collected from the field. Secondary stressors, such as osmotic shock, could be applied to organisms collected from potentially polluted sites, and their ability to resist that stressor would be indicative of prior stress and the effect of that stress on their "counteractive capacity."

A significant ($\alpha < 0.05$) decrease was observed in the total FAA concentrations (nmol/mg) of *G. pseudolimnaeus* exposed to PCP for 48 h followed by osmotic shock for 24 h (Tables 3 and 4). The 96 h-LC_{50} for the pentachlorophenol exposure was 1.37 mg/l (C.L. 1.18-1.57), considerably greater than the previous exposure. The difference is most likely due to the lower water temperature at which this test was conducted. As with the PCP exposure without subsequent hyposmotic stress, exposure to concentrations slightly greater than the 96 h LC_{50} caused a significant reduction in the total FAA concentration at 48 h. Exposure to sublethal concentrations of PCP (1.16 mg/l) had no effect on the total FAA concentration. However unlike the previous exposure, the FAA profiles of organisms exposed at the lower PCP concentration were not significantly different from control organisms. Hyposmotic conditions for 24 h did not significantly influence the total FAA pool of control organisms, although concentrations of all amino acids, except glycine, did decrease

slightly. The concentrations of FAA in G. pseudolimnaeus which had been exposed to 1.51 mg/l PCP for 48 h returned to pre-exposure values after the organisms were transferred to clean "normal" water. However, organisms which were transferred to clean hyposmotic conditions instead of clean "normal" water did not recover and had total FAA concentrations very similar to those measured after 48 h PCP exposure. FAA concentrations of G. pseudolimnaeus exposed to sublethal PCP concentrations were not significantly affected after 24 h of hypoosmotic conditions. Therefore, the stress associated with 48 h sublethal exposure to PCP did not permanently influence the ability of G. pseudolimnaeus to osmoregulate, as measured by changes in the FAA pool.

I can conclude that short-term toxicant-induced stress did not deplete the "counteractive capacity" of the organism to further resist osmotic stress. The conclusion assumes that a reduction in this capacity would be realized as a change in the FAA pool which, although theoretically possible, has not been supported by experimentation. In addition, the exposure was extremely short in this study. A longer-term toxicant-induced stress, for which the concept of counteractive capacity would be more applicable, may be more effective in reducing the organism's ability to cope with subsequent osmotic stress such that the FAA pool may be altered. The effect of osmotic stress on the organism's ability to reestablish its normal FAA pool indicates that in these organisms, toxicant stress had exhausted its capacity to maintain normal hemolymph osmotic conditions.

Before concluding this discussion, an additional point needs to be discussed concerning the initial total FAA pool of the control organisms from all three experiments. The initial mean total FAA concentrations

Table 3. Concentrations of individual and total amino acids (nmol/mg) of *G. pseudolimnaeus* exposed to pentachlorophenol for 48 h, prior to 24 h of hyposmotic stress.

	Day 0	Day 2		
Amino Acid	Control (S.D.)	Control (S.D.)[a]	1.16 mg/l (S.D.)[a]	1.51 mg/l (S.D.)[a]
Alanine	11.21 (2.49)	8.54 (2.25)	8.73 (2.36)	5.72 (1.87)
Arginine	6.86 (1.90)	6.24 (1.38)	8.41 (1.93)	3.18 (0.74)
Asparagine	0.64 (0.24)	0.52 (0.23)	0.42 (0.22)	0.13 (0.06)
Aspartic Acid	0.54 (0.26)	0.66 (0.25)	0.75 (0.50)	0.57 (0.15)
Glutamine	4.25 (0.86)	3.96 (0.72)	4.34 (1.47)	2.55 (0.38)
Glutamic Acid	2.19 (0.74)	2.33 (0.63)	2.50 (1.21)	1.97 (0.26)
Glycine	2.62 (0.58)	1.44 (0.32)	1.60 (0.62)	1.05 (0.19)
Histidine	1.53 (0.50)	1.64 (0.52)	1.43 (0.51)	0.92 (0.32)
Isoleucine	3.23 (1.01)	2.59 (0.64)	2.57 (0.95)	1.77 (0.30)
Leucine	7.45 (2.36)	6.17 (1.32)	5.75 (2.11)	3.88 (0.65)
Lysine	5.09 (1.84)	4.02 (0.90)	4.33 (1.87)	2.46 (0.52)
Methionine	1.90 (0.64)	1.77 (0.51)	1.76 (0.56)	1.33 (0.36)
Phenylalanine	2.56 (0.75)	2.24 (0.47)	2.10 (0.81)	1.31 (0.29)
Serine	1.48 (0.67)	1.48 (0.41)	1.36 (0.63)	0.89 (0.19)
Taurine	2.20 (0.64)	2.11 (0.74)	2.09 (0.93)	1.52 (0.28)
Threonine	1.12 (0.37)	1.06 (0.30)	1.15 (0.54)	0.59 (0.13)
Tryptophan	0.70 (0.24)	0.62 (0.15)	0.56 (0.36)	0.39 (0.12)
Tyrosine	4.18 (0.78)	3.43 (1.20)	3.65 (0.90)	1.98 (0.49)
Valine	3.81 (1.11)	3.36 (0.82)	3.23 (1.12)	2.27 (0.39)
Total	62.4 (16.7)	56.6 (11.45)	54.6 (17.14)	34.9[b] (7.05)

[a] - S.D. - standard deviation, n = 5
[b] - Significantly ($\alpha = 0.05$) lower than control organisms

Table 4. Concentrations of individual and total amino acids (mmol/mg) of G. pseudolimnaeus after 48 h exposure to PCP followed by 24 h hyposmotic stress. (See Table 3 for values after PCP exposure but before hyposmotic shock.)

Amino Acid	Control		1.51 mg/l		1.16 mg/l	
	Control[a]	Osm[b]	Control[c]	Osm[d]	Control[c]	Osm[d]
Alanine	8.20[e](0.66)[f]	7.05 (0.99)	8.16 (2.36)	4.95 (0.89)	8.31 (1.69)	7.66 (0.94)
Arginine	5.39 (0.26)	5.36 (2.02)	5.97 (1.22)	4.41 (1.21)	5.72 (1.63)	5.82 (1.37)
Asparagine	0.24 (0.05)	0.28 (0.15)	0.42 (0.14)	0.24 (0.07)	0.36 (0.11)	0.26 (0.07)
Aspartic Acid	0.76 (0.17)	0.86 (0.26)	0.71 (0.42)	0.51 (0.27)	0.77 (0.31)	0.85 (0.19)
Glutamine	3.49 (0.27)	4.13 (1.08)	3.62 (1.21)	2.48 (0.78)	3.91 (1.30)	3.31 (0.76)
Glutamic Acid	2.00 (0.33)	1.99 (0.43)	2.19 (1.10)	1.56 (0.36)	2.05 (0.69)	2.55 (1.01)
Glycine	2.10 (0.19)	1.50 (0.25)	1.43 (0.41)	0.88 (0.18)	1.48 (0.39)	1.79 (0.29)
Histidine	0.67 (0.33)	0.78 (0.30)	1.61 (0.48)	0.79 (0.18)	1.46 (0.61)	1.09 (0.41)
Isoleucine	2.40 (0.31)	2.68 (0.83)	2.73 (1.24)	1.72 (0.45)	2.71 (1.82)	2.33 (0.86)
Leucine	5.54 (0.56)	5.89 (1.18)	5.95 (2.77)	3.98 (0.80)	6.04 (2.11)	5.44 (1.02)
Lysine	3.83 (0.66)	3.73 (1.13)	4.48 (1.88)	2.93 (0.83)	3.95 (1.62)	3.28 (1.11)
Methionine	1.57 (0.18)	1.54 (0.30)	2.14 (0.92)	1.34 (0.29)	1.76 (0.49)	2.06 (0.22)
Phenylalanine	1.76 (0.22)	1.91 (0.32)	2.34 (1.05)	1.39 (0.28)	2.04 (0.62)	1.99 (0.27)
Serine	1.38 (0.23)	1.38 (0.36)	1.27 (0.78)	0.84 (0.31)	1.31 (0.69)	1.49 (0.52)
Taurine	1.65 (0.12)	1.83 (0.45)	1.62 (0.38)	1.34 (0.75)	1.71 (0.42)	1.88 (0.36)
Threonine	0.68 (0.22)	0.77 (0.21)	1.00 (0.42)	0.57 (0.18)	0.88 (0.39)	0.66 (0.09)
Tryptophan	0.52 (0.03)	0.56 (0.09)	0.67 (0.30)	0.39 (0.09)	0.71 (0.19)	0.92 (0.14)
Tyrosine	2.93 (0.48)	3.30 (1.10)	3.67 (0.86)	2.39 (0.63)	3.45 (0.93)	3.03 (0.92)
Valine	2.75 (0.40)	3.03 (0.50)	3.37 (1.39)	2.18 (0.46)	3.09 (0.65)	2.61 (0.74)
Total	47.8 (2.97)	48.57 (11.00)	53.3 (18.2)	34.9[g] (6.44)	51.71 (12.92)	49.02 (9.18)

[a]Control organisms subjected to no osmotic shock
[b]Control organisms hypoosmotically shocked for 24 h
[c]Exposed organisms transferred to clean water for 24 h
[d]Exposed organisms hypoosmotically shocked for 24 h
[e]Mean, n = 5
[f]Standard deviation in parentheses.
[g]Significantly ($\alpha = 0.05$) lower than all other organisms

for organisms used in experiments 1, 2 and 3 were 76.17, 90.57 and 62.4 nmol/mg, respectively. These values are considerably different, and, when developing a BIS, differences such as these need to be explained, especially when changes in the FAA, which are considered to be stress-related in one experiment, overlap with the "normal range" for control organisms in a subsequent experiment. This was the case in the present experiments, where FAA concentrations found to be significantly reduced due to osmotic stress (56.89 nmol/mg) were similar to control levels in the study of the combined effects of PCP-osmotic stress (62.4 nmol/mg). These discrepancies can be explained by comparing the time of year the organisms were collected with the seasonal variation in total FAA pools (Chapter 4). Organisms used in the combined PCP-osmotic stress experiment were collected in March when total FAA concentrations of G. pseudolimnaeus are the smallest (66.9 nmol/mg). For the other experiments, amphipods were collected in June and July, during which the total FAA concentration varied from 83-120 nmol/mg. Therefore, when conducting experiments on the effects of stress on biochemical parameters of field collected organisms, accessory factors such as seasonal variation must be considered.

Chapter 3

Effects of Long-Term Exposure to Pentachlorophenol on the Free Amino Acid Pool and Energy Reserves of *Gammarus pseudolimnaeus*

Introduction

A considerable amount of data exists on biochemical alterations in organisms during acute or lethal exposure to toxicants (Capuzzo et al., 1984; Coppage, 1972; Gould and Greig, 1983; Heitz et al., 1974; Roesijadi et al., 1976; Graney and Giesy, 1986). Although these data may be of interest in mechanistic studies and do provide insight into the type of biochemical changes which occur in stressed organisms, they are not very useful in developing BIS for laboratory hazard assessment or *in situ* monitoring of effects. One does not need to examine biochemical alterations in organisms to identify exposure to acutely toxic concentrations of chemicals. Biochemical indicators of stress are potentially useful because they are sensitive to sublethal concentrations of chemicals and may provide the earliest possible warning of future adverse effects. Therefore, when developing a BIS, it is essential to determine alterations during chronic, sublethal exposures. Although information on acute toxicity may provide the upper boundaries of dose-response relationships, it must be remembered that the "type" of effect observed in chronic, sublethal exposure can be quite different from alterations observed at greater concentrations. Examples of dose-dependant effects include toxicant alterations in respiration (Bayne et al., 1982; Weinbach and Nolan, 1956) and enzyme activity (McKim et al., 1970).

A limited amount of research has been conducted on toxicant-induced alterations in the concentrations of FAA in freshwater invertebrates (Gardner et al., 1981). In acute exposures to PCP, the total FAA concentration decreased and the relative concentrations of individual FAA changed in freshwater amphipods (*Gammarus pseudolimnaeus*) (Chapter

2). It was proposed that changes in the FAA profile, or in the relative concentrations of individual amino acids, may be a more sensitive indicator of toxicant exposure than a change in the total FAA pool. Based on those observations and the necessity of chronic exposure regimes in assessing the potential usefulness of a particular BIS, this study was initiated to determine the effect of long term sublethal toxicant exposure on FAA concentrations of freshwater amphipods. The specific objectives of the study were to 1) measure alterations in concentrations of FAA in Gammarus pseudolimnaeus exposed to sublethal concentrations of pentachlorophenol for 45 days and 2) correlate changes in the concentrations of FAA with growth, caloric content and energetic status, as measured by whole body lipid, glycogen and protein concentrations.

Materials and Methods

Freshwater amphipods, Gammarus pseudolimnaeus Bousfield (Crustacea; Amphipoda) were collected by D-framed dip net from Glass Creek, a "pollution free" second order stream located in Barry County, Michigan. Organisms were returned to the laboratory and acclimated for one week prior to test initiation. During testing, G. pseudolimnaeus was fed Ash leaves preconditioned by soaking in river water for 2 weeks. Chronic exposures were static renewal and conducted with river water collected from Glass Creek, MI. Both water and leaves were completely replaced twice a week. Amphipods were exposed to 0.77, 1.06 and 1.25 mg/l pentachlorophenol (>99% pure) (Fluka Chemical Company, Hauppauge, N.Y.) for 45 d.

Concentrations of pentachlorophenol in the water were measured by the method of Carr et al. (1982). This method uses aqueous-solvent partitioning to extract pentachlorophenol (PCP) from the water and measures the concentration spectrophotometrically at 320 nm.

On designated days, five organisms were removed from specified concentrations and immediately forzen on dry ice. Amino acid analysis was performed on control organisms from days 0, 2, 5, 9, 15, 23, 30 and 45, on organisms exposed to 0.77 mg PCP/l from days 0, 5, 15, 30 and 45, and on organisms exposed to 1.06 and 1.25 mg PCP/l from days 9 and 23, and 2, 5, and 9, respectively. Glycogen, lipid and protein concentrations of unexposed G. pseudolimnaeus and those exposed to 0.77 mg PCP/l were measured on days 0, 15, 30 and 45.

For amino acid analysis, whole amphipods were dried at 95 C for 24 h, weighed and extracted by homogenizing entire organisms in methanol. A homogenate subsample was removed and analyzed for protein by the method of Peterson (1977). The homogenate was then centrifuged at 3,000 RPM for 10 min, and a subsample of the supernatant was removed and amino acids analyzed by high pressure liquid chromatography (HPLC). The supernatant was used directly for amino acid analysis via pre-column O-phthaldialdehyde derivatization with fluorescence detection (Jones et al., 1981). A more detailed description of the procedure utilized is given in Chapter 2. Glycogen and lipids were extracted by adding an appropriate proportion of chloroform to the methanol homogenate to produce a final composition of two parts chloroform to one part methanol. This was rehomogenized, centrifuged and the supernatant removed and set aside. The pellet was dissolved in 2:1 chloroform:methanol, homogenized, centrifuged and the supernatant combined with the first. This

process was repeated a third time. The combined supernatants were brought to a constant volume, washed for 24 h with a 0.9% aqueous sodium chloride solution, and the resulting organic phase was analyzed for lipids by the phospho-vanillin method and compared to olive oil standards (Barnes and Blackstock, 1973). The pellet remaining after lipid extraction was resuspended in distilled water, vortexed for 20 sec, centrifuged and the supernatant set aside. This procedure was repeated three times, and all the supernatants were combined and subsequently split into aliquots to be used for glucose analysis or glycogen analysis.

Glucose was analyzed by the hexokinase/glucose-6-phosphate dehydrogenase method (Sigma Kit 115, Sigma Technical Bulletin No. 115, 1978). Glycogen was hydrolyzed to glucose *via* amyloglucosidase (from *Aspergillus niger*) (Sigma Chemical Co.). Conditions utilized for the digestion were outlined by Keppler and Decker (1974). The liberated glucose was measured as outlined above and glycogen concentrations were calculated as the difference in glucose concentrations before and after enzymatic hydrolysis. Oyster Type II glycogen (Sigma Chemical Co.) was used as a reference standard.

Patterns of relative FAA concentrations of PCP-exposed and unexposed amphipods were analyzed as previously discussed (Chapter 2). Growth, as measured by dry weight, concentrations of lipids, glycogen and protein and total whole organism caloric content of the different treatment groups were compared by analysis of variance (ANOVA) followed by Duncans Multiple Range Test. LC_{50} values were calculated by the probit method.

Results and Discussion

Free Amino Acids

The 30-day LC_{50} for PCP was 0.86 mg/l (95% C.L.-0.83-0.91). By test termination, 14% mortality had occurred in control organisms and 22% at the lowest PCP concentration (Table 5).

The total FAA concentrations of *G. pseudolimnaeus* were significantly reduced by chronic exposure to PCP (Tables 6, 7, 8; Fig. 2). Exposure for five days to 0.77 mg PCP/l caused a significant ($\alpha = 0.05$) decrease in the total FAA concentration (Table 7). The total FAA concentration remained significantly less than that of control values until day 45, at which time there was no longer a significant difference between PCP-exposed and unexposed organisms. This was the result of a gradual but significant decline in the FAA concentration of control amphipods (Fig. 2). Exposure to 1.06 mg PCP/l for 23 d did not significantly alter the concentration of total FAA (Table 8). However, 1.25 mg PCP/l did have a significant ($\alpha = 0.05$) effect on the total FAA concentration after exposure for five and nine days (Table 8).

Exclusing the sulfo-amino acid taurine, the reduction in total FAA concentrations due to exposure to 0.77 or 1.25 mg PCP/l can be attributed to a decrease in all individual amino acids. Essential and non-essential amino acids decreased equally, and excluding amphipods exposed to 0.77 mg/l PCP for 45 days, no change was observed in the essential/non-essential ratio (Table 9). The patterns of the relative concentrations of individual amino acids were not altered by exposure to pentachlorophenol, as determined by profile analysis and comparing the percent contribution of each individual amino acid to the total FAA concentration.

Table 5. Mortality of *G. pseudolimnaeus* exposed to pentachlorophenol for 45 days. Numbers represent the combined mortality in replicate exposures (25 organisms/replicate).

Measured Concentration (mg/l)	Mortality Day 2	5	9	15	22	30	37	45
Control	0[a]	0	0	0	0	2	3	7
0.77	0	0	0	2	6	6	7	11
1.06	1	5	23	33	41	49[b]	--	--
1.25	7	39	44	50[b]	--	--	--	--

[a] - Replicates combined

[b] - Complete mortality

Table 6. Concentration of free amino acids (nmol/mg) in Gammarus pseudolimnaeus not exposed to Pentachlorophenol (controls).

Amino Acid	Day 0	2	5	9	15	23	30	45
Alanine	22.1[a] (3.2)[b]	22.6 (3.3)	22.1 (2.3)	24.5 (5.0)	18.0 (4.1)	17.0 (1.7)	14.9 (2.8)	13.6 (3.8)
Arginine	6.1 (1.5)	4.4 (2.2)	8.1 (2.3)	5.7 (2.8)	7.1 (2.9)	6.6 (1.9)	6.6 (4.0)	4.9 (2.8)
Asparagine	0.9 (0.2)	0.4 (0.1)	0.6 (0.1)	0.6 (0.2)	0.7 (0.1)	0.2 (0.1)	0.2 (0.1)	0.3 (0.1)
Aspartic Acid	0.5 (0.1)	0.8 (0.2)	0.7 (0.1)	0.5 (0.2)	0.7 (0.1)	0.8 (0.2)	0.8 (0.2)	0.5 (0.2)
Glutamine	9.0 (1.2)	9.5 (3.4)	11.1 (1.4)	8.9 (1.8)	10.0 (1.2)	6.8 (0.7)	5.5 (3.2)	4.2 (1.8)
Glutamic Acid	4.0 (0.5)	3.9 (1.0)	3.8 (0.4)	3.8 (1.3)	3.8 (0.4)	3.5 (0.7)	2.9 (0.7)	2.4 (1.0)
Glycine	2.6 (0.6)	1.8 (1.0)	2.9 (0.7)	2.2 (1.0)	2.3 (0.8)	3.8 (2.0)	2.0 (0.8)	1.3 (0.9)
Histidine	6.7 (0.4)	3.4 (0.8)	4.0 (0.6)	2.9 (1.4)	3.4 (0.5)	3.0 (0.5)	3.0 (0.3)	2.4 (0.5)
Isoleucine	5.5 (0.7)	4.9 (0.6)	4.0 (0.4)	5.0 (0.4)	5.1 (0.4)	4.1 (0.6)	3.7 (0.7)	2.9 (0.8)
Leucine	12.2 (2.1)	10.6 (1.3)	9.3 (1.0)	10.9 (1.4)	11.3 (1.3)	8.7 (1.8)	7.5 (1.7)	6.2 (1.9)
Lysine	5.3 (1.8)	3.0 (1.3)	4.9 (1.0)	4.5 (2.2)	5.6 (2.3)	4.6 (1.3)	4.1 (2.2)	3.2 (1.8)
Methionine	4.4 (0.9)	4.3 (0.4)	3.0 (0.3)	3.9 (0.4)	3.7 (0.5)	2.4 (0.5)	2.2 (0.6)	1.6 (0.6)
Phenylalanine	4.1 (0.5)	3.7 (0.6)	3.3 (0.4)	3.6 (0.4)	3.9 (0.4)	3.0 (0.6)	2.5 (0.7)	2.1 (0.6)
Serine	2.3 (0.6)	2.4 (0.9)	2.1 (0.5)	2.0 (0.7)	2.0 (0.3)	1.7 (0.4)	1.4 (0.4)	1.0 (0.5)
Taurine	5.9 (1.2)	4.9 (1.2)	4.9 (0.9)	5.1 (1.2)	3.9 (0.5)	4.3 (0.9)	4.0 (0.8)	4.4 (1.2)
Threonine	1.8 (0.3)	1.6 (0.6)	1.8 (0.2)	1.5 (0.6)	2.1 (0.5)	1.4 (0.1)	1.6 (0.5)	1.1 (0.5)
Tryptophan	1.0 (0.1)	0.9 (0.2)	0.69 (0.1)	0.7 (0.2)	0.9 (0.2)	0.6 (0.2)	0.6 (0.1)	0.4 (0.2)
Tyrosine	4.3 (0.6)	4.2 (0.6)	3.9 (0.3)	3.7 (0.5)	4.8 (0.5)	3.4 (0.5)	3.3 (0.7)	2.9 (0.5)
Valine	7.5 (0.9)	6.8 (0.7)	5.8 (0.4)	6.7 (0.6)	6.7 (0.4)	5.4 (0.9)	4.4 (0.8)	3.5 (0.9)
Total	106.6 (12.6)	94.3 (15.6)	97.3 (5.8)	96.9 (16.0)	96.2 (12.6)	81.6 (10.6)	71.7[c] (16.0)	59.3[c] (18.5)

[a] - mean amino acid concentration (nmol/mg)
[b] - standard deviation (n = 5)
[c] - significantly less than Day 0 control ($\alpha = 0.05$)

Table 7. Concentration of free amino acids in G. pseudolimnaeus exposed to 0.77 mg/l pentachlorophenol

Amino Acid	Day 5		15		30		45	
Alanine	19.2[a]	(2.0)[b]	12.5	(1.9)	10.7	(2.5)	12.2	(2.4)
Arginine	5.7	(1.1)	4.4	(2.0)	2.8	(1.4)	3.0	(1.6)
Asparagine	0.6	(0.1)	0.4	(0.2)	0.1	(0.02)	0.2	(0.1)
Aspartic Acid	0.6	(0.05)	0.4	(0.1)	0.5	(0.1)	0.4	(0.1)
Glutamine	8.3	(0.8)	6.8	(2.2)	5.3	(1.5)	5.3	(2.2)
Glutamic Acid	3.1	(0.2)	2.4	(0.7)	2.0	(0.8)	2.2	(0.5)
Glycine	1.6	(0.4)	1.0	(0.6)	0.8	(0.4)	1.0	(0.7)
Histidine	3.4	(0.5)	2.6	(0.4)	2.1	(0.8)	2.5	(0.5)
Isoleucine	3.8	(0.7)	3.1	(0.7)	3.0	(0.7)	2.4	(0.8)
Leucine	8.9	(1.4)	6.5	(2.0)	6.3	(1.0)	5.2	(1.6)
Lysine	3.5	(0.8)	3.0	(1.4)	1.8	(0.6)	1.9	(0.8)
Methionine	2.8	(0.2)	2.3	(0.5)	1.7	(0.3)	1.6	(0.6)
Phenylalanine	3.2	(0.4)	2.4	(0.6)	2.2	(0.4)	2.0	(0.6)
Serine	1.9	(0.4)	1.2	(0.4)	1.1	(0.2)	1.1	(0.4)
Taurine	4.3	(0.4)	3.8	(0.4)	3.2	(1.1)	4.0	(0.8)
Threonine	1.7	(0.4)	1.3	(0.4)	1.0	(0.4)	1.0	(0.4)
Tryptophan	0.9	(0.2)	0.5	(0.1)	0.5	(0.2)	0.4	(0.2)
Tyrosine	4.2	(0.6)	3.2	(0.6)	3.0	(0.8)	3.4	(1.2)
Valine	5.3	(1.0)	4.2	(0.8)	4.0	(0.9)	3.4	(0.9)
Total	83.4[c]	(4.9)	62.4[c]	(12.7)	52.4[c]	(11.7)	53.7	(12.8)

[a] - mean amino acid concentration (nmol/mg dry weight)
[b] - standard deviation (n = 5)
[c] - significantly ($\alpha = 0.05$) less than control organisms sampled on same day (Table 2).

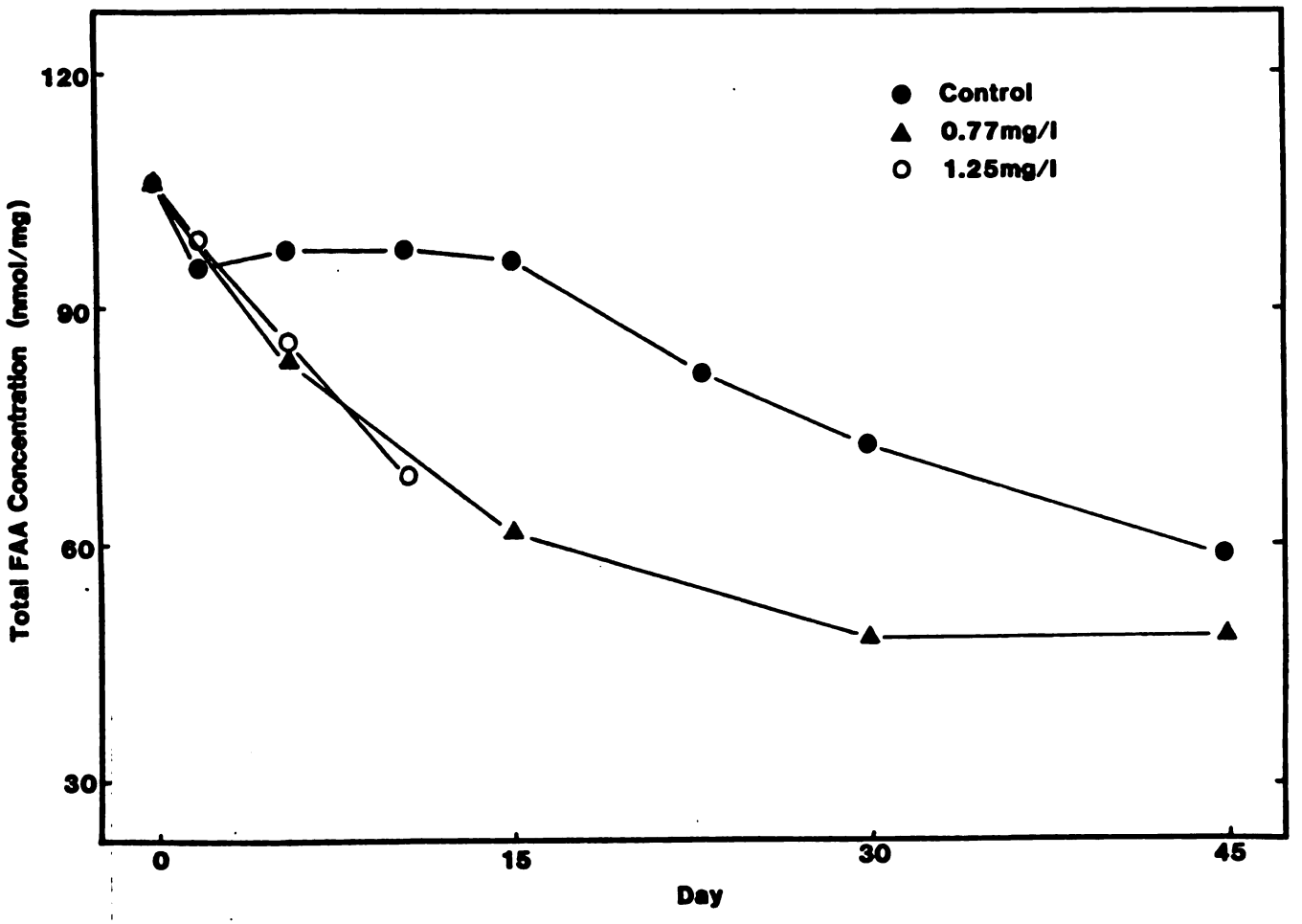
Table 8. Concentration of free amino acids in G. pseudolimnaeus exposed to 1.06 or 1.25 mg/l pentachlorophenol.

Amino Acid	1.06 mg/l		1.25 mg/l		
	Day 9	Day 23	Day 2	Day 5	Day 9
Alanine	18.9[a](5.1)[b]	22.7 (2.4)	21.6 (8.4)	20.7 (1.9)	15.3 (2.4)
Arginine	6.0 (3.6)	5.9 (2.3)	6.3 (4.1)	6.0 (1.9)	4.5 (2.5)
Asparagine	0.4 (0.2)	0.1 (0.0)	0.7 (0.4)	0.5 (0.2)	0.4 (0.1)
Aspartic Acid	0.5 (0.3)	0.6 (0.2)	0.7 (0.4)	0.6 (0.1)	0.5 (0.1)
Glutamine	6.5 (1.5)	8.6 (3.2)	8.7 (4.6)	7.5 (0.9)	6.7 (1.6)
Glutamic Acid	3.5 (1.9)	3.7 (1.0)	3.6 (2.1)	3.2 (0.6)	2.9 (0.9)
Glycine	2.5 (1.5)	2.1 (0.2)	2.8 (1.7)	2.6 (0.6)	1.8 (0.6)
Histidine	3.3 (0.7)	3.3 (0.7)	3.5 (1.0)	4.1 (0.7)	2.8 (1.3)
Isoleucine	3.6 (1.2)	4.5 (1.3)	4.8 (1.5)	3.9 (0.3)	3.5 (1.0)
Leucine	8.3 (2.7)	9.5 (2.7)	11.1 (4.5)	8.4 (0.8)	7.6 (2.5)
Lysine	4.3 (2.9)	4.8 (2.7)	4.7 (3.2)	3.6 (0.9)	2.8 (1.6)
Methionine	3.0 (1.2)	4.0 (1.5)	4.3 (1.2)	2.9 (0.2)	2.7 (0.7)
Phenylalanine	3.0 (0.9)	3.8 (1.4)	4.0 (1.5)	3.1 (0.3)	2.7 (0.9)
Serine	1.8 (0.9)	2.0 (0.5)	2.4 (1.4)	2.0 (0.3)	1.5 (0.5)
Taurine	5.1 (1.0)	5.3 (0.9)	4.9 (0.9)	5.2 (0.8)	3.2 (1.0)
Threonine	1.3 (0.7)	1.7 (0.7)	1.8 (1.0)	1.6 (0.3)	1.4 (0.4)
Tryptophan	0.6 (0.3)	0.8 (0.3)	1.0 (0.4)	0.8 (0.1)	0.6 (0.2)
Tyrosine	3.2 (1.0)	4.3 (1.5)	4.8 (1.9)	3.7 (0.4)	3.2 (0.8)
Valine	5.1 (1.3)	6.0 (1.6)	6.3 (1.9)	5.3 (0.4)	4.8 (1.3)
Total	81.4(26.5)	94.3 (21.7)	98.5 (40.6)	86.1[c] (6.2)	69.3[c] (12.3)

[a] - mean amino acid concentration (nmol/mg, dry weight)
[b] - standard deviation (n = 5)
[c] - significantly ($\alpha = 0.05$) lower than control organisms sampled on same day (Table 2)

Figure

Fig. 2. Total free amino acid concentration (nmol/mg) in *Gammarus pseudolimnaeus* exposed to 0.77 and 1.25 mg/l pentachlorophenol.


Control
0.77mg/l
1.25mg/l
Total FAA Concentration (nmol/mg)
120
90
60
30
0
15
30
45
Day

No other controlled laboratory studies investigating the effect of long-term toxicant-induced stress on free amino acid concentrations in freshwater invertebrates have been conducted. Bhagyalakshmi et al. (1983b) exposed freshwater crabs to Sumithion® for 20 d and reported a significant decline in total FAA concentration; however, concentrations of individual amino acids were not reported and the analytical procedures utilized were outdated. Gardner et al. (1981) reported significantly greater total FAA concentrations in the mantle tissue of freshwater bivalves collected from polluted ecosystems. However, this was not a controlled laboratory study and the influence of accessory factors such as diet cannot be evaluated, which makes interpretation of the results extremely difficult.

In marine invertebrates, a considerable amount of research has been conducted on the effects of natural and chemical stressors on the concentrations of free amino acids. Most of this research concerns the role of intracellular FAA in osmotic regulation (Gilles, 1979; Bishop et al., 1981). A number of studies have investigated the effects of toxicant-induced stress on FAA concentrations; however, very few have been long-term laboratory exposures in which the influence of accessory factors is controlled and the potential significance of changes in the FAA pool is assessed. Payne et al. (1983) exposed lobsters to crude oil for 14 wk and found no effect on the total FAA concentration but reported significant increases in the concentrations of the essential amino acid tryptophan and the non-essential amino acid alanine in plasma. Briggs (1979) observed a significant decrease in the concentrations of all FAA in adductor muscle of the bivalve Mytilus edulis exposed to sublethal cadmium concentrations for 16 d. Other authors

Table 9. Taurine to glycine ratio and essential to non-essential amino acid ratio for Gammarus pseudolimnaeus exposed to pentachlorophenol.

Day	Control		0.77 mg/l		1.06 mg/l		1.25 mg/l	
	T/G[a]	E/NE[b]	T/G	E/NE	T/G	E/NE	T/G	E/NE
0	2.27	1.05	2.27	1.05	2.27	1.05	2.27	1.05
2	2.70	0.86	--	--	--	--	1.75	0.94
5	1.69	0.86	2.69	0.89	--	--	2.0	0.85
9	2.32	0.88	--	--	2.04	0.91	1.83	0.93
15	1.70	1.08	3.80[c]	0.95	--	--	--	--
23	1.13	0.96	--	--	2.52[c]	0.89	--	--
30	2.00	1.02	3.78[c]	0.94	--	--	--	--
45	3.38	0.91	4.0	0.77[c]	--	--	--	--

[a] - molar ratio of taurine to glycine

[b] - ratio of essential (E) amino acids to non-essential (NE) amino acids

- essential amino acids - arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine

- non-essential amino acids - alanine, asparagine, aspartic acid, glutamine, glutamic acid, glycine, serine and taurine

[c] - significantly less than control values ($\alpha = 0.05$)

have reported changes in FAA concentrations in marine invertebrates chronically exposed in the field (Widdows et al., 1981; Carr and Neff, 1984; Jeffries, 1972); however, under field conditions, interpretation of the results is extremely difficult without corroborating laboratory data on the significance of the changes observed. Short-term laboratory exposures to toxicants at lethal and/or sublethal concentrations also have been performed with marine invertebrates (Powell et al., 1982; Riley and Mix, 1981); however, as already discussed, development of an effective BIS to be used in laboratory or field hazard assessment requires knowledge of the effect of long-term sublethal exposures on the concentration of FAA and the relationship of these changes to the overall "health" or "fitness" of the organisms.

The primary effect on marine invertebrates reported by other authors is a change in the molar ratio of taurine to glycine (Jeffries, 1972; Roesijadi and Anderson, 1979; Augenfeld et al., 1980). In general, the taurine/glycine ratio has been found to increase in marine invertebrates under toxicant stress. The increase has primarily been attributed to decreases in the concentration of glycine; although Jeffries (1972) found that increases in taurine also contributed to the greater ratio in stressed clams. In contrast, Augenfeld et al. (1980) found that a decrease in taurine concentrations resulted in a reduction of the taurine/glycine ratio in oil-stressed bivalves. In the present study, the molar ratio of taurine/glycine was significantly greater after 15 and 30 d exposure to 0.77 mg PCP/l but was not different after 45 d (Table 9). The change in the ratio was due primarily to a decrease in the glycine concentration. The taurine/glycine ratio was altered in amphipods exposed to sublethal PCP concentrations. After 9 d exposure

to 1.25 mg PCP/l, there was no significant change in the taurine/glycine ratio. Although the taurine/glycine ratio was significantly increased by sublethal PCP exposure, the ratio of taurine to any of the amino acids measured would have given similar results, because concentrations of all of the individual amino acids decreased. Changes in concentrations of individual amino acids, such as taurine and glycine, are no more sensitive indicators of stress than the total FAA pool; in fact, the total FAA pool was significantly altered earlier (5 d) than was the taurine/glycine ratio (15 d).

The mechanism responsible for toxicant-induced alterations in FAA concentrations of freshwater and marine invertebrates is unknown. Mechanisms which may be responsible for alterations in the concentrations of amino acids in G. pseudolimnaeus exposed to acutely toxic concentrations PCP were discussed in Chapter 2. Similar to the present study, a significant decrease was observed in the total concentration of FAA in amphipods exposed to acutely lethal concentrations of PCP. By comparing toxicant-induced alterations with changes in the concentration of FAA induced by osmotic stress, it was concluded that a disruption of osmoregulatory ability was responsible for the decrease in amino acids observed. Since lower concentrations of PCP did not cause a decrease in the total concentrations of FAA but rather changed the relative concentrations of individual amino acids (i.e. profile), it was hypothesized that changes in the amino acid profile may be indicative of sublethal stress whereas decreases in the total concentrations of FAA may be indicative of more severe stress. The results of the present study do not support this hypothesis however. Long-term sublethal exposure to PCP did not alter the FAA profile. However, it did cause a decrease in

the total FAA pool, an effect identical to the type observed during acute, lethal exposures. It is doubtful, however, that osmoregulatory breakdown was responsible for the FAA changes observed during chronic, sublethal stress. Crustaceans are extremely efficient hyperosmotic regulators and maintain osmotic conditions via low water permeability, excretion of dilute urine and active uptake of salts (Prosser, 1973). Generally severe stress is required to impair osmoregulation to such a degree that the osmolyte composition of the internal body fluids is altered. Other potential mechanisms which may account for the changes in amino acids observed include 1) interference with protein metabolism, 2) increased oxidation of amino acids, and 3) changes in protein synthetic rates.

The ideal situation for any BIS would be the existence of a quantitative dose-response relationship between the concentration of stressor and the biochemical effect observed. Given the complexity of mechanisms responsible for biochemical alterations however, this type of relationship is seldom obtained. Often a qualitative relationship is the greatest resolution which one can expect, especially under field conditions. In other words, the biochemical alteration which occurs is indicative of the presence of a stressor, but the dose cannot be predicted from the changes observed. In the present study, a quantitative dose-response relationship does not exist. At the lower, sublethal concentration (0.77 mg/l), the decrease in total FAA concentration is similar to the change observed at higher concentrations. However, at the 1.06 mg/l exposure level there was no affect observed. The poor relationship between dose and affect does not eliminate FAA as a potential stress indicator; although, it does limit the ability to interpret the

significance of the changes observed. If a significant decrease in the total FAA pool occurs, the conclusion that the organism was exposed to some type of stressor would be warranted. However, as with any BIS, if no change in the concentrations of FAA occurs, the conclusion that the organism is not stressed is unwarranted, since the FAA pool does not always change under toxicant-induced stress.

The decrease in the total concentration of FAA in G. pseudolimnaeus exposed to 0.77 mg PCP/l for 45 d was significant only on days 5, 15 and 30 (Tables 6, 7). After 45 d, the concentrations of FAA in unexposed organisms also had decreased such that they were no longer significantly different from exposed organisms (Fig. 2). The gradual decrease in the concentrations of FAA in unexposed organisms indicated that the exposure system utilized in this study also stressed the organisms. Laboratory-induced stress has been shown to influence the concentration of FAA in other aquatic invertebrates (Roesijadi, 1979).

The total free amino acid concentrations of Protothaca staminea, decreased when the clams were held in the laboratory for 26 d (Roesijadi, 1979). The decrease was primarily due to a decrease in the concentration of taurine. In contrast, the total concentration of FAA increased in polychaetes acclimated to laboratory conditions (Koenig et al., 1981). The increase in FAA concentration in polychaetes was attributed to several factors: 1) starvation, 2) stress of collection and transfer, 3) overcrowding and 4) lack of sufficient sediment.

In the present study, the exact nature of the stressor which decreased the concentration of FAA in control organisms is unknown. The water used was collected from the same creek as the amphipods and, thus,

should have been a suitable medium for the test. Since a static renewal system was utilized, the process of organisms transfer could have been a source of stress, although the organisms were transferred as gently as possible. The unnatural conditions of the test chamber with respect to their natural habitat may have been a primary factor. Differences in habitat characteristics such as substratum and water flow may have a direct influence on organism "health" and thus the FAA pool. Diet also has been shown to directly influence FAA concentrations of organisms (Munro, 1970; Cook et al., 1972; Riley, 1980). In Chapters 2 and 5 there were discussions of the potential influence of diet on FAA concentrations of freshwater invertebrates. In the present study, it is unknown whether the preconditioned ash leaves provided the necessary dietary requirements for G. pseudolimnaeus. It is unlikely that starvation occurred, because the organisms were observed to have full guts throughout the study. There was no differences in the essential amino acid (EAA) to non-essential amino acid (NEAA) ratio of control amphipods (Table 9). During dietary protein deficiency, the EAA/NEAA ratio has been shown to decrease in both vertebrates and invertebrates (Munro, 1970; Riley, 1980). In addition, the qualitative composition of the diet may have been inadequate, leading to an alteration in the concentrations of FAA. Most likely, a combination of all of the above factors caused the total FAA pool of the control organisms to decrease gradually.

The change in the concentration of FAA in G. pseudolimnaeus caused by laboratory conditions has important implications with respect to the application of FAA as a BIS. The affect establishes FAA as truly "general" indicators of stress in that, as the overall health of the

organism declines, the FAA concentrations is altered. Giesy et al. (1986) discussed the advantages of "general" vs. "specific" indicators of stress. General indicators would be most appropriately applied when a multitude of potential toxicants may be present, such that measuring the overall health of the organism would be more advantageous than trying to pinpoint exposure to a particular toxicant or type of toxicant. General indicators are useful only when the effects of natural stress can be separated from toxicant induced stress. By their nature alone, general indicators are going to be more responsive to non-toxicant types of stress and therefore, a large data base will be required to differentiate between toxicant and non-toxicant stress (Giesy et al., 1986).

Growth and Energy Reserves

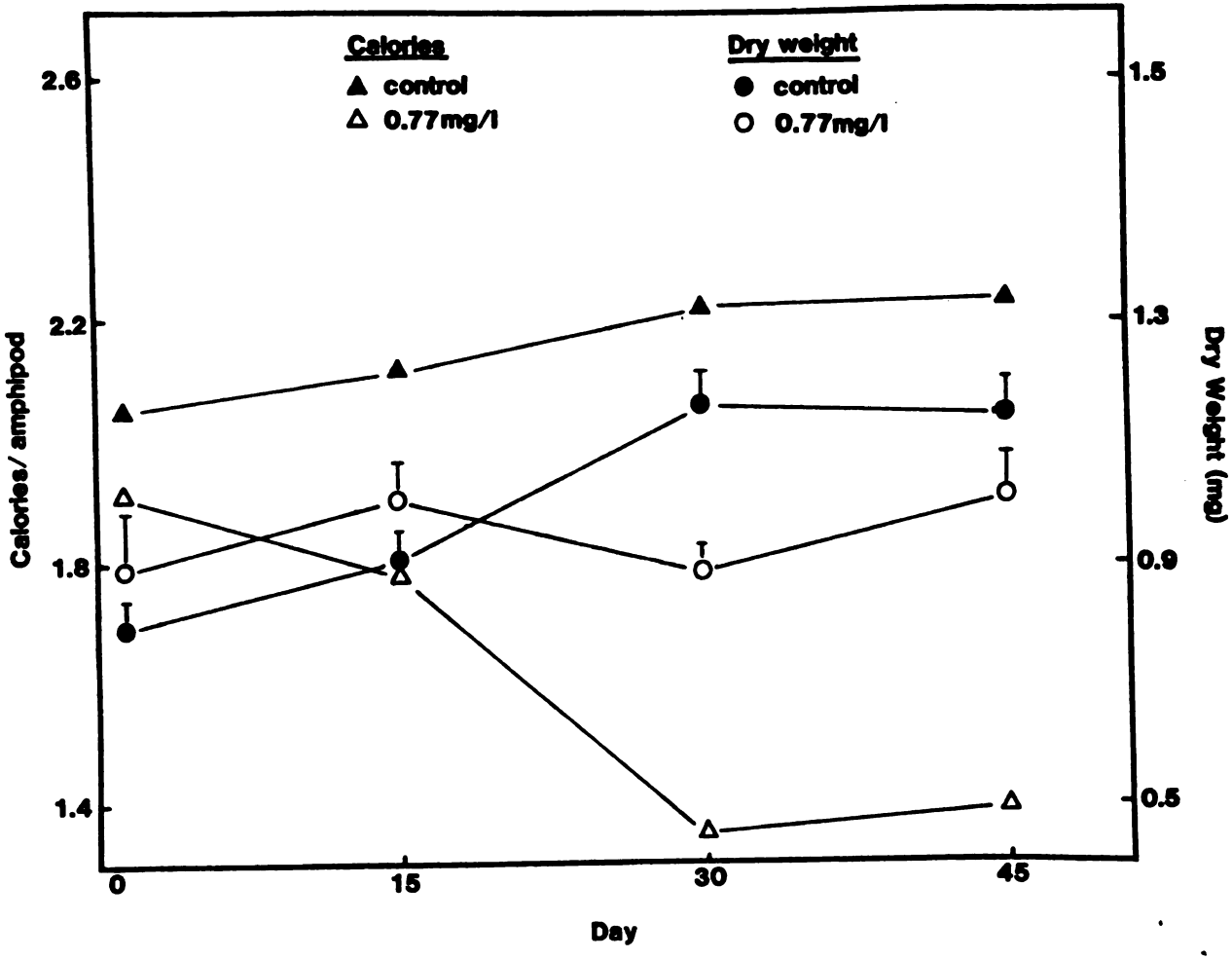
Successful application of any BIS requires establishment of a correlation between the observed biochemical alterations and detrimental effects on growth, reproduction or survival of the organism. Without such a relationship, the significance of biochemical changes is extremely difficult to interpret. Decreases in the growth of an organism can have direct effects at the population level. This may be especially true for crustaceans where fecundity is directly related to the size of the organism. In addition, an organisms energy reserves (i.e., glycogen and lipid content) can directly influence reproductive potential and ability to survive during stressful conditions, such as starvation. For these reasons, the growth and energy content of G. pseudolimnaeus were measured during PCP exposure and related to changes in the concentrations of FAA.

The dry weight of G. pseudolimnaeus was significantly less after 30 d exposure to 0.77 mg PCP/l but was not different after 45 days exposure (Fig. 3). Reduced growth is a common chronic endpoint used in both fish and invertebrate toxicity tests (Scherer, 1979; Buikema and Cairns, 1980). Tolba and Holdich (1981) found that freshwater isopods (crustaceans) collected from "polluted" streams were smaller in size and produced smaller broods than organisms collected from a "non-polluted" system. The authors felt that the energy demand associated with greater maintenance costs in isopods from pollutant stressed environments resulted in less energy being available for growth and reproduction. Calow (1979), in a paper discussing the physiological cost of reproduction, states that in organisms such as crustaceans where fecundity is positively correlated with size, a reduction in growth can reduce the future breeding potential of the organism. In the present study, size at day 30 was directly correlated with total FAA concentration on day 5. Thus, for G. pseudolimnaeus exposed to PCP, alterations in FAA concentration at early stages of exposure is indicative of reduced growth at later stages, and the reduction in growth has potential implications concerning fecundity.

The rationale for using changes in concentrations of glycogen, lipids or protein as a stress response in organisms is that energy is required to maintain homeostasis, and for an organism to resist a stressor, it must perform energy-requiring work. Chronic stress initiates compensatory physiological adjustments in energy metabolism required to maintain homeostasis and/or homeokinesis. Growth, maintenance metabolism and at certain times of the year, reproduction, demand the majority of an organisms energy expenditure. Therefore, any

Figure

Fig. 3. Caloric content and dry weight of *Gammarus pseudolimnaeus* exposed to 0.77 mg/l pentachlorophenol for 45 days.


Calories
▲ control
△ 0.77mg/l
Dry weight
● control
○ 0.77mg/l
2.6
2.2
1.8
1.4
1.5
1.3
0.9
0.5
Calories/ amphipod
Dry Weight (mg)
0
15
30
45
Day

increased maintenance requirement of an organism will result in reduced growth, and as already discussed, a possibility of reduced reproductive potential for organisms such as crustaceans, whose fecundity is directly realted to size. Thus, a comparative assessment of the energy status of an organism can be indicative of its overall condition. Toxicant- and non-toxicant-induced stress have been shown to reduce concentrations of energy storage biomolecules such as glycogen and lipids and/or alter their distribution among tissues (Thomas et al., 1981; Leatherland and Sonstegard, 1984). Because changes in energy reserves can be related to deleterious affects on organism growth and reproduction, a relationship between alterations in the concentrations of FAA, and changes in glycogen or lipid concentrations may provide insight into the potential significance of changes in the FAA pool.

Glycogen content (ug/organism) and concentration (ug/mg dry wt) of G. pseudolimnaeus was significantly reduced after 15, 30 and 45 d exposure to 0.77 mg PCP/l (Table 10). In both exposed and control amphipods, glycogen content per whole organism increased through day 30, and then began to decline. In control organisms, however, the actual concentration (ug/mg) was always greater than that of PCP-treated amphipods and compared favorably with glycogen concentrations reported for other crustaceans (Jungreis, 1968; Heath and Barnes, 1970; Hazlett et al., 1975). Other authors have reported a decrease in concentrations of glycogen in toxicant-stressed invertebrates (Bhagyalakshmi et al., 1983a; Coglianese and Neff, 1982; Srinivasa Moorthy et al., 1983; Thomas et al., 1981). In most cases, the depletion of glycogen reserves has been attributed to the increased energy demand associated with stress. Increases in glycogen reserves have also been observed during toxicant

Table 10. Glycogen, lipid and protein content (ug/organism) and concentration (ug/mg) of *G. pseudolimnaeus* exposed to 0.77 mg/l pentachlorophenol.

Day	Protein				Lipids				Glycogen			
	Control		1.0 mg/l		Control		1.0 mg.l		Control		1.0 mg/l	
0	347.0[a]	(44.7)[c]	315.0	(52.0)	66.3[a]	(10.2)	62.7	(14.3)	1.94[a]	(0.91)	2.12	(0.25)
	417.0[b]	(2.8)	369.0[d]	(9.2)	70.8[b]	(6.5)	72.9	(8.5)	1.93[b]	(0.76)	2.62	(0.95)
15	339.3	(26.0)	257.3[d]	(33.3)	74.1	(3.7)	73.4	(7.3)	7.16	(0.11)	5.31[d]	(0.59)
	326.5	(7.8)	258.0[d]	(7.0)	81.8	(8.5)	75.7	(1.9)	8.10	(0.58)	5.35[d]	(0.01)
30	339.7	(21.1)	180.4[d]	(12.7)	82.4	(4.8)	59.9[d]	(3.2)	8.37	(0.56)	5.92[d]	(0.27)
	384.0	(9.9)	194.2[d]	(1.6)	70.7	(0.8)	67.3	(4.9)	7.20	(0.16)	6.72	(0.39)
45	389.7	(25.0)	237.6[d]	(19.8)	63.7	(9.1)	42.2[e]	(1.7)	6.62	(0.55)	4.10[d]	(0.32)
	380.5	(20.5)	240.0[d]	(7.2)	59.0	(7.5)	40.8[e]	(1.1)	6.25	(0.35)	4.00[e]	(0.25)

[a] - ug/organism

[b] - ug/mg dry weight

[c] - standard deviation (n = 5)

[d] - significantly different from control ($\alpha = 0.01$)

[e] - significantly different from control ($\alpha = 0.05$)

exposure (Bhagyalakshmi et al., 1984). Changes in the concentration of glycogen can be a fairly useful indicator of toxicant stress under laboratory conditions. However, due to the influence of accessory factors, under field conditions the variability is generally great and limits the direct application of glycogen in effects monitoring.

The lipid content (ug/organism) of G. pseudolimnaeus was significantly less in organisms exposed for 30 and 45 d to 0.77 mg PCP/l than in control organisms. The lipid concentrations (ug/mg) were also significantly less than in controls after 45 d exposure (Table 10). In exposed organisms the lipid content increased through day 15 and then began to decline, while in control organisms, lipid increased through day 30 before beginning to decline. This indicates that lipids were being utilized by the stressed organisms, most likely in an effort to meet the "energy demand" caused by exposure to PCP. A decrease in percent lipid relative to whole organism dry weight was noted in amphipods exposed to fuel oil (Lee et al., 1981), and an increase in lipid catabolism was measured in oysters exposed to naphthalene (Riley and Mix, 1981). In the present study, significant changes in lipid content occured concurrent with significant reductions in amphipod growth (Day 30). Carbohydrate metabolism was significantly affected by PCP prior to lipid metabolism and may represent the more sensitive indicator of toxicant exposure in G. pseudolimnaeus.

The protein content (ug/organism) and concentration (ug/mg) of G. pseudolimnaeus exposed to 0.77 mg PCP/l for 15, 30 and 45 d were significantly less than that of control organisms (Table 10). However, on day 0, prior to any organism being exposed to toxicants, the whole body protein concentrations (but not the protein content) was significantly

greater in control organisms (Table 10). The reason for this difference is unknown. Because the organisms were randomly assigned to treatments, there should have been no difference between control and exposed organisms at test initiation.

In invertebrates, protein can represent an important energy reserve mobilized by the organism during starvation or stress (Claybrook, 1983; Florkin and Scheer, 1970; Gilles, 1970; Bayne, 1973). Protein catabolism was increased in oysters exposed to naphthalene (Riley and Mix, 1981) and in freshwater crabs exposed to sumithion® (Bhagyalakshmi, et al., 1983b). Increased proteolysis to meet the energy demands of stress were assumed by the authors to cause the observed decline in structural proteins. A similar interpretation can be made regarding the decline in structural proteins in G. pseudolimnaeus exposed to PCP.

The caloric content (cal/organism) and concentration (cal/mg) of G. pseudolimnaeus were calculated by assuming caloric values of 9.5 cal/mg for lipids, 4.3 cal/mg for glycogen and 4.1 cal/mg for proteins (Prosser and Brown, 1961; White et al., 1973) (Table 11). The caloric concentration of G. pseudolimnaeus ranged from 1.85 to 2.67 cal/mg dry weight.

The caloric contents of G. pseudolimnaeus are less than those reported for other benthic invertebrates. The freshwater amphipod Pontoporeia affinis (= hoyi) collected from different habitats had caloric concentrations ranging from 4.74 to 5.24 cal/mg, dry weight (Green, 1971). Green attributed the large caloric content to the unusually high percent lipid composition, which accounted for up to 33% of the dry weight. Similar results were reported by Gardner et al. (1985) for P. hoyi, collected from Lake Michigan. The caloric concentration in the amphipod Hyalella azetica has been reported to be 3.6

Table 11. Whole organism caloric content (cal/organism) and concentration (cal/mg) of G. pseudolimnaeus exposed to 0.77 mg PCP/l. Calories were calculated based on glycogen, lipid and protein concentrations (glycogen - 4.3 cal/mg; lipid - 9.5 cal/mg; protein - 4.1 cal/mg).

Day	Control	0.77 mg PCP/l
0	2.05[a] (0.27)[b]	1.90 (0.35)
	2.67[c] (0.28)	2.26 (1.15)
15	2.12 (0.12)	1.77[d] (0.21)
	2.50 (0.33)	1.80[d] (0.05)
30	2.21 (0.12)	1.33[d] (0.06)
	1.85 (0.09)	1.46[d] (0.11)
45	2.23 (0.18)	1.39[d] (0.09)
	2.00 (0.24)	1.40[d] (0.10)

[a] - calories/organism

[b] - standard deviation

[c] - calories/mg, dry weight

[d] - significantly less than control organisms ($\alpha = 0.05$)

cal/mg dry weight (Green, 1971) and 4.08 cal/mg ash-free dry weight (Wissing and Hasler, 1968). At this point it is unknown why the caloric concentration is so much less in G. pseudolimnaeus than other amphipods. The fact that the lipid content is less and only represents approximately 7% of the organisms dry weight can partially account for the lower caloric content. Glycogen, lipid and protein combined constituted only 50 to 60% of the dry weight of G. pseudolimnaeus, indicating that ash and chitin made up an unusually large proportion of the dry weight. This being the case, the caloric concentration may have been artificially diluted, and representing the calories on an ash-free dry weight basis may have been more representative.

The caloric content (cal/organism) and concentration (cal/mg dry weight) were significantly decreased in G. pseudolimnaeus exposed to 0.77 mg PCP/l for 15, 30 and 45 days (Table 11, Fig. 3). A reduction in the caloric content of organisms exposed to toxicants has been observed in other studies. American lobsters (Homarus americanus) acutely exposed to sublethal concentrations of petroleum hydrocarbons had a significantly lower caloric content at various stages of the molt cycle which indicates that energy available for growth was reduced by exposure to oil (Capuzzo et al., 1984). Similarly, exposure of the cichlid fish (Cichlasoma bimaculatum) to PCP resulted in more rapid use of calories during starvation (Krueger et al., 1968). Exposure of Pink Salmon to crude oil or naphthalene caused a decrease in caloric content. This was not the result of increased mobilization of energy reserves to satisfy the toxicant-induced energy requirements of the organism, as has been proposed in other studies, but rather it was the result of a toxicant-induced decrease in food consumption (Moles and Rice, 1983).

Increases in caloric content have also been reported in invertebrates exposed to toxicants. Thornton and Wilhm (1974) observed an increase in caloric content of Chironomus attenuatus exposed to phenol, which was attributed to larger bacterial populations (food) at the greater phenol concentrations.

In the present study, the observed decrease in caloric content of G. pseudolimnaeus exposed to PCP could be due to 1) increased utilization of energy reserves to "resist" the toxicant-induced stress, 2) toxicant effect on feeding behaviors or assimilative efficiency such that reduced caloric intake occurs or 3) a combination of both factors. Pentachlorophenol is a strong metabolic poison which uncouples electron transport from ADP phosphorylation (Weinbach, 1956) and which can result in increased respiratory and/or metabolic rates (Peer et al., 1983; Weinbach and Nolan, 1956). PCP may subsequently accelerate utilization of tissue energy reserves (Holmberg, et al., 1972). The decreased caloric content of G. pseudolimnaeus may result from utilization of glycogen, lipid and protein reserves. If it is assumed that the difference in caloric content of the exposed and unexposed organisms is the amount of energy required to "counteract" the toxicant induced stress, then after 15, 30 and 45 d of exposure, approximately 0.35, 0.88 and 0.84 cals/org had been utilized or mobilized. Of this, roughly 74% can be attributed to the oxidation of protein, 25% to lipid oxidation and 1% is due to the mobilization of glycogen.

The second potential explanation for the decrease in caloric content of PCP-exposed amphipods concerns the effect of the toxicant on dietary caloric intake. Because by day 45 of the test, concentrations of both lipids and glycogen decreased in amphipods which were not

exposed to PCP, the test system exerted some form of stress on the organisms. This decrease in energy reserves paralleled the decrease in the concentration of total FAA. Given change in both FAA and caloric content, diet is the most apparent accessory factor affecting controls. Glycogen and lipid reserves of invertebrates have been shown to be directly related to diet (Bayne, 1973; Barclay et al., 1983; Tessier, et al., 1983; Holland, 1978). An inadequate diet for G. pseudolimnaeus may lead to the eventual decrease in caloric content of the control organisms. In addition, dietary deficiencies may have influenced the organisms ability to "resist" the energy demand created by PCP exposure. However given the time frame and severity of the effects caused by PCP, there is no doubt that the loss of energy reserves observed was primarily the result of PCP toxicity.

Chapter 4

Seasonal Changes in the Free Amino Acid Pool of *Gammarus pseudolimnaeus*

Introduction

This chapter primarily concerns the influence of season on the FAA pool of the freshwater scud Gammarus pseudolimnaeus Bousfield (Crustacea, Amphipoda). In developing an in situ biochemical indicator of stress, seasonal variation is the most important accessory factor to be considered because many individual accessory factors such as diet, age, reproductive condition and molting stage are implicit in any seasonal fluctuations observed. Observed seasonal changes in FAA pool are discussed with respect to toxicological applications of FAA and the ecology/biology of G. pseudolimnaeus.

Materials and Methods

G. pseudolimnaeus were collected monthly from Glass Creek, a second order trout stream located in Barry County, Michigan. Organisms were collected from detrital deposition zones by D-framed dip net and frozen immediately on dry ice. Upon returning to the laboratory, organisms were transferred to liquid nitrogen for storage until extraction for amino acid analysis. Within one month after collection, whole amphipods were dried at 95 C for 24 h, weighed and FAA extracted by homogenizing the entire organism in methanol. Subsamples of the homogenate were removed, and protein was measured by the method of Peterson (1977). The homogenate was centrifuged at 3,000 RPM for 10 min, the supernatant decanted and amino acids analyzed by high pressure liquid chromatography (Jones et al., 1981). The specific technique utilized for FAA analysis is described in detail in Chapter 2.

Improper normalization of biochemical data can often bias results and lead to inappropriate conclusions (Powell et al., 1984). Normalization parameters should not vary during the study period. In the

present study, amino acids were normalized to both dry weight and protein content of whole organisms.

Patterns of relative FAA concentrations of amphipods collected in different months were compared by univariate statistical techniques. Total FAA concentrations and their percent compositions in six specimens of G. pseudolimneaus collected in different months were compared by analysis of variance (one-way ANOVA) (SAS Institute, 1982) followed by Duncans Multiple Range Test.

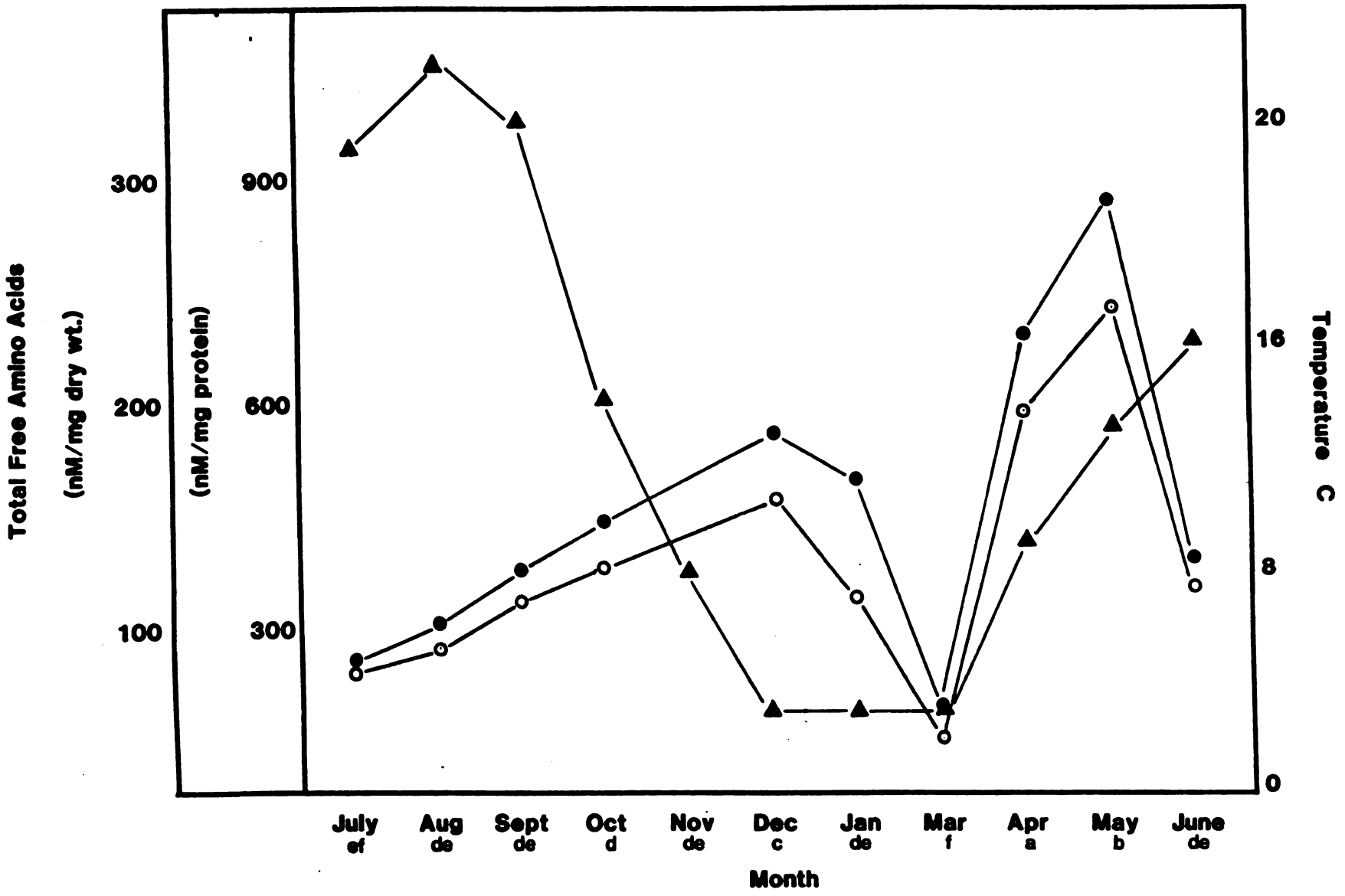
Results and Discussion

The mean total FAA concentrations of G. pseudolimnaeus varied three fold over the 12-month study period. Peak concentrations of 226.9 and 286.4 nmol/mg total FAA dry weight, were observed in April and May, and were followed by a decline through the summer months (Fig. 4; Table 12). FAA values slowly increased through the fall, had a second smaller peak in early winter and then dropped drastically during the colder, winter months. Observed patterns were identical when FAA concentrations were normalized to either total protein or dry weight. It was felt that normalization to a second parameter, such as protein, was required to ensure that the seasonal pattern observed was not an artifact caused by the disproportionate contribution of exoskeleton to the weight of the smaller organisms. Because the surface-to-volume ratio decreases as the organism grows and because the FAA pool was normalized to the weight of the entire organism, the weight of the smaller organisms likely contained a larger proportion of exoskeleton, a chitinous material devoid of FAAs. This being the case, the FAA pool of the smaller organisms collected during the summer months could be artificially diluted by the

greater exoskeleton weight. However, normalization to protein does not completely ensure that normalization bias was not present. Although inorganic constituents and chitin are the primary components of exoskeleton, protein can also be important (Dall, 1965) and, thus, may introduce the same bias as dry weight. Another important factor often overlooked when normalizing biochemical measurements is that the parameter used for normalization cannot change as a result of the treatment. In the present investigation, whole amphipod percent proteins/mg dry weight did not change significantly during the study period ($\alpha > 0.05$).

Other studies which have investigated the seasonal changes in the total FAA concentration of aquatic invertebrates have reported conflicting results. Feng et al. (1970) reported that total FAA concentrations in hemolymph of the oyster Crossostrea virginica were greatest during the winter months, opposite to the results found in the present study. Ferguson (1975) found the total FAA concentrations of gonad and digestive gland tissue of starfish were greatest in winter and least in the summer. In both of those studies, seasonal changes in the total FAA concentration were related to the reproductive cycle of the organism. Similar to the present study, Cook et al. (1972) found that the total FAA pool of adult barnacles, Balanus balanoides, decreased during the winter and reach a minimum in spring; however, the greatest FAA concentration was in the fall. Heath and Barnes (1970) found no consistent seasonal trends in the total non-protein nitrogen of the common shore crab, Carcinus maenas. Similarly, Zandee et al. (1980) measured seasonal variation in the total FAA concentration of different organs of the marine mollusk Mytilus edulis and found no seasonal pattern;

Fig. 4. Seasonal changes in the total free amino acid (FAA) concentrations of G. pseudolimnaeus. Concentrations (nmol/mg) are normalized to both whole body dry weight (O) and whole body protein (●). Also included are the seasonal changes in temperature (Δ). Organisms collected in months with the same letter place below had total FAA concentrations (nmol/mg dry weight) which were not significantly ($\alpha = 0.05$) different.


Total Free Amino Acids
(nM/mg dry wt.)
(nM/mg protein)
300
200
100
900
600
300
20
16
8
0
Temperature C
July ef
Aug de
Sept de
Oct d
Nov de
Dec c
Jan de
Mar f
Apr a
May b
June de
Month

although, concentrations of specific amino acids did change significantly. In freshwater crayfish the total FAA concentration was significantly greater in May compared with February (McWhinnie, 1972). These differences were specifically related to the molt cycle of the crayfish with the total FAA concentration increasing during the premolt period in May. Because in the majority of cases, alterations in the total FAA pool have been related to accessory factors such as molt status, reproductive condition and/or diet, it is not surprising that different species from different ecosystems exhibit different seasonal patterns.

The total FAA concentration of G. pseudolimnaeus (80-230 nmol/mg dry wt.) is similar to that reported for other freshwater invertebrates. Five different freshwater oligochaete species had total FAA concentrations which ranged from 7.8 to 50.6 nmol/mg dry weight (Chapter 6). Similarly, small concentrations of total FAA were reported for the copepod Diaptomus sp., which contained 80.9 nmol/mg (Jeffries and Alzara, 1970). The bivalves Ligumia subrostrata and Corbicula fluminea had considerably smaller total FAA concentrations of 23.6 nmol/mg (dry wt.) and 2.78 nmol/mg (wet wt.), respectively (Hanson and Dietz, 1976; Chapter 5). These low FAA concentrations are not surprising, however, because organisms with tissue in direct contact with the external media, such as clams, have adapted to freshwater by maintaining a low osmotic gradient between tissue and water. Arthropods, on the other hand, have a fairly impermeable exoskeleton which allows them to maintain a greater internal osmolyte concentration. Gardner et al. (1981) reported a total FAA concentration of 428 nmol/mg dry weight for the cladoceran Daphnia magna. Abdominal muscle of the crayfish Astacus leptodactylus had a total FAA

concentration of 545.7 and 466 nmol/mg dry weight, respectively (van Marrewijk and Ravestern, 1974; van Marrewijk and Zandee, 1975). Similarly, crayfish of the family Orconectes had total FAA concentrations of 34.6-116.3 nmol/mg wet weight in abdominal muscle (McWhinnie et al., 1972) and 42.7-50.2 nmol/mg wet weight in ventral nerve cord (Lin and Cohen, 1973). These concentrations are considerably greater than those reported here for G. pseudolimnaeus. A possible reason for this difference is that values reported for the crayfish were for specific tissues, whereas in the present study, whole organisms were analyzed. This being the case, total FAA concentrations reported for G. pseudolimnaeus may be biased toward smaller concentrations due to "dilution" of the homogenate by tissues or structures which have low concentrations of amino acids.

The concentrations of individual amino acids, normalized to dry weight and protein, are presented in Table 12. The percent compositions of each amino amino acid relative to the total FAA pool are presented in Table 13. During the entire year, alanine consistently represented the most abundant amino acid (16.2-22.4%). Arginine and leucine were the next most abundant and comprised an average of 10.2 and 10.4% of the total pool, respectively. The relative contribution of the remaining amino acids varied considerably, ranging between 1 and 10%. Both asparagine and aspartic acid were consistently less than 1%.

The pattern of relative concentrations of individual FAA in G. pseudolimnaeus is similar to that of other freshwater invertebrates. Alanine, arginine and leucine comprised approximately 40% of total FAA concentration in amphipods. Similarly, in crayfish abdominal muscle, concentrations of glycine, arginine and alanine comprised up to 73% of the total FAA concentration (van Marrewijk and Ravenstein, 1974).

Alanine and arginine also have been found to predominate in the nerve cord of the crayfish Orconectes immunis (Lin and Cohen, 1973), whole body of the freshwater cladoceran Diaptomus sp. (Jeffries and Alzara, 1970), whole body of the freshwater bivalve Ligumia substrastrata (Hanson and Dietz, 1976) and whole body of the trichopteran larvae Hydropsyche betteni (Haag and Sullivan, 1984).

In crustaceans, alanine is formed by the transamination of pyruvate with glutamate (Claybrook, 1983). The pivotal position of pyruvate with respect to glycolysis and the TCA cycle renders alanine concentrations susceptible to alterations due to these important biochemical pathways. The relatively great proportion of arginine in the total FAA pool can be attributed to the importance of arginine phosphate in crustacean muscle contraction (Onnen and Zebe, 1983). During muscular work, depletion of phosphagen stores results in an accumulation of arginine, which is subsequently converted to octopine by octopine dehydrogenase.

Glycine and taurine concentrations, which are normally relatively high in crustaceans, were relatively low in G. pseudolimnaeus. These two amino acids are considered primary osmotic effectors in the adaptation of euryhaline invertebrates to fluctuating salinites and, thus, may not be as important in freshwater crustaceans. Taurine, a sulfonic amino acid, is not found in freshwater molluscs (Awapara, 1962); however, it has been found in freshwater crustaceans (van Marrewijk and Ravestein, 1974; Lin and Cohen, 1973) and is believed to function as an emulsifying agent in gastric juices (Holwerda and Vonk, 1973). Other amino acids found to predominate in freshwater crustaceans include glutamic acid, aspartic acid, proline and histidine (Lin and Cohen, 1973; Gardner et al. 1981; Duchateau-Bosson and Florkin, 1961). None of

Table 12. Seasonal variation in the concentration of free amino acids in Gammarus pseudolimnaeus

Amino Acid	April, '84		May, '84		June, '84		July, '84		Aug., '84		Sept., '84		Oct., '84		Dec., '84		Jan., '85		March, '85	
Alanine	45.1[a]	(3.3)[b]	46.9	(2.8)	26.6	(4.9)	16.1	(4.8)	20.4	(4.9)	22.3	(2.4)	25.8	(4.1)	40.1	(9.1)	38.0	(9.8)	12.6	(1.9)
	111.2[c]	(8.4)	115.4	(7.0)	77.3	(12.5)	48.2	(14.2)	57.0	(13.1)	59.2	(5.5)	66.3	(12.0)	100.1	(23.4)	94.6	(24.8)	31.3	(4.7)
Arginine	20.9	(4.0)	39.4	(5.1)	12.6	(4.4)	6.2	(2.8)	9.9	(2.4)	12.0	(3.3)	16.2	(2.3)	21.4	(4.4)	20.4	(3.2)	5.7	(1.5)
	51.5	(9.8)	97.1	(12.6)	35.3	(7.6)	18.2	(8.1)	27.7	(6.6)	31.8	(8.2)	41.9	(6.4)	53.4	(11.2)	50.8	(7.8)	14.1	(3.7)
Asparagine	2.1	(0.6)	3.0	(0.6)	1.0	(0.3)	2.4	(3.5)	0.9	(0.3)	1.0	(0.1)	1.2	(0.5)	1.7	(0.2)	1.6	(0.1)	0.4	(0.0)
	5.3	(1.6)	7.5	(1.4)	2.8	(0.8)	6.9	(9.8)	2.5	(0.9)	2.7	(0.2)	3.0	(1.4)	4.4	(0.5)	4.0	(0.2)	1.1	(0.1)
Aspartic Acid	1.8	(1.0)	2.4	(0.5)	1.0	(0.3)	0.5	(0.2)	0.6	(0.2)	1.0	(0.2)	1.1	(0.1)	1.2	(0.5)	0.9	(0.1)	0.4	(0.0)
	4.6	(2.5)	6.0	(1.4)	2.5	(0.1)	1.5	(0.5)	1.9	(0.6)	2.6	(0.6)	2.9	(0.3)	3.0	(1.3)	2.3	(0.3)	1.1	(0.1)
Glutamine	16.6	(2.5)	21.4	(2.7)	7.5	(2.9)	7.8	(2.4)	11.1	(3.6)	9.0	(2.3)	10.8	(2.2)	10.5	(0.6)	10.2	(0.7)	5.9	(1.3)
	40.9	(6.3)	52.8	(6.6)	17.9	(7.2)	22.9	(6.5)	31.2	(10.2)	23.9	(6.0)	27.7	(6.2)	26.2	(1.7)	25.4	(1.8)	14.7	(3.1)
Glutamic Acid	3.1	(1.7)	3.4	(0.4)	1.8	(0.4)	2.1	(0.9)	3.7	(0.9)	5.1	(1.3)	6.4	(0.7)	2.2	(0.5)	2.1	(0.0)	1.3	(0.1)
	22.8	(9.1)	25.1	(3.0)	15.3	(1.4)	6.2	(2.6)	10.5	(2.5)	13.4	(3.2)	16.0	(2.1)	16.9	(4.2)	15.6	(0.5)	9.9	(1.3)
Glycine	6.3	(1.1)	8.4	(0.9)	4.3	(1.0)	1.5	(1.0)	2.0	(0.7)	5.0	(0.3)	6.4	(0.7)	12.5	(1.3)	9.0	(5.4)	4.7	(2.4)
	15.7	(2.8)	20.6	(2.3)	12.1	(2.2)	4.4	(2.9)	5.7	(2.0)	13.4	(0.7)	16.5	(1.9)	31.2	(3.0)	22.4	(13.5)	11.8	(6.0)
Histidine	9.3	(1.5)	11.9	(1.4)	3.4	(0.3)	2.5	(0.6)	3.2	(1.0)	2.5	(0.4)	2.5	(0.2)	1.6	(0.5)	1.8	(0.3)	2.2	(0.7)
	23.1	(3.9)	29.4	(3.5)	8.9	(0.9)	7.5	(1.6)	9.0	(2.7)	6.6	(1.0)	6.4	(0.6)	4.0	(1.2)	4.4	(0.8)	5.6	(1.8)
Isoleucine	12.1	(2.8)	14.7	(2.7)	6.4	(1.7)	4.6	(1.4)	4.8	(1.5)	6.2	(1.1)	6.7	(0.9)	8.9	(1.0)	8.9	(0.4)	2.0	(0.4)
	29.8	(7.0)	36.2	(6.7)	17.4	(3.0)	13.8	(4.3)	13.4	(4.2)	16.4	(2.6)	17.3	(2.5)	22.2	(2.6)	22.3	(1.2)	5.1	(1.1)
Leucine	24.6	(6.6)	32.2	(6.16)	13.7	(3.9)	10.5	(3.5)	11.2	(3.9)	14.3	(2.0)	15.7	(2.6)	19.1	(3.8)	16.9	(4.0)	4.8	(0.6)
	60.8	(16.2)	79.2	(15.1)	35.3	(4.9)	31.2	(10.5)	31.3	(10.9)	37.8	(4.8)	40.5	(7.2)	47.6	(9.5)	42.1	(9.9)	12.1	(1.6)
Lysine	9.9	(2.9)	21.6	(8.8)	9.9	(3.6)	2.2	(1.3)	4.8	(1.7)	9.0	(3.5)	13.8	(2.5)	17.8	(4.1)	7.1	(5.7)	2.7	(0.7)
	24.4	(7.2)	53.1	(21.6)	25.3	(4.7)	6.5	(3.9)	13.3	(4.7)	23.8	(9.1)	35.5	(6.6)	44.4	(10.0)	17.7	(14.2)	6.7	(1.9)
Methionine	10.9	(1.8)	9.1	(1.5)	3.7	(1.1)	3.1	(0.9)	3.5	(1.18)	5.2	(0.6)	4.6	(0.32)	5.0	(0.4)	5.1	(0.1)	1.5	(0.2)
	27.0	(4.6)	22.5	(3.7)	9.3	(1.5)	9.4	(2.8)	9.7	(3.19)	13.8	(1.5)	11.9	(0.8)	12.4	(1.1)	12.7	(0.3)	3.8	(0.4)
Phenylalanine	10.7	(2.7)	12.8	(2.5)	4.6	(1.0)	4.3	(1.4)	4.5	(1.1)	5.0	(0.3)	5.4	(0.7)	6.7	(0.3)	6.8	(0.2)	2.3	(0.4)
	26.4	(6.6)	31.6	(5.2)	12.7	(1.5)	12.7	(4.3)	12.7	(3.7)	13.3	(0.8)	13.8	(1.9)	16.9	(1.0)	16.9	(0.8)	5.8	(1.2)
Serine	6.9	(2.8)	6.6	(1.8)	2.6	(0.9)	1.3	(0.6)	1.7	(0.6)	3.2	(0.3)	3.8	(0.6)	4.1	(0.6)	3.9	(0.4)	1.8	(0.2)
	17.2	(7.1)	16.3	(4.5)	7.4	(1.7)	3.9	(1.8)	4.8	(1.8)	8.7	(0.8)	9.8	(1.5)	10.4	(1.7)	9.8	(1.0)	4.4	(0.7)
Taurine	8.0	(1.2)	11.7	(0.7)	9.8	(3.0)	3.8	(0.3)	5.3	(1.2)	7.3	(0.7)	5.9	(2.3)	6.0	(1.0)	6.0	(0.8)	7.4	(2.6)
	19.8	(3.0)	28.9	(1.9)	24.1	(8.9)	11.3	(1.3)	14.9	(3.4)	19.4	(1.7)	15.4	(6.2)	14.9	(2.5)	15.0	(2.0)	18.5	(6.5)
Threonine	4.8	(0.9)	5.4	(0.8)	2.2	(0.5)	1.7	(0.5)	1.9	(0.6)	2.2	(0.3)	2.5	(0.2)	3.8	(1.2)	3.8	(0.6)	1.5	(0.5)
	12.0	(2.4)	13.4	(1.9)	5.6	(0.7)	3.6	(1.5)	5.3	(1.6)	6.0	(1.0)	6.5	(0.7)	9.6	(3.2)	9.5	(1.7)	3.7	(1.5)
Tryptophan	3.9	(1.2)	4.0	(0.6)	1.2	(0.3)	1.1	(0.1)	1.0	(0.2)	1.5	(0.2)	1.4	(0.1)	1.9	(0.4)	1.5	(0.9)	0.9	(0.2)
	9.5	(2.9)	9.8	(1.6)	3.2	(0.5)	3.5	(1.0)	2.9	(0.5)	4.0	(0.7)	3.6	(0.3)	4.7	(1.0)	3.9	(2.3)	2.4	(0.6)
Tyrosine	14.0	(6.3)	13.8	(3.3)	6.3	(2.4)	4.9	(1.3)	5.6	(1.5)	5.6	(0.3)	7.0	(0.7)	10.8	(0.9)	10.1	(0.4)	5.1	(1.3)
	34.5	(15.5)	34.1	(8.2)	15.7	(2.8)	14.4	(3.9)	15.6	(4.2)	14.8	(1.0)	18.1	(1.9)	27.0	(2.5)	25.2	(0.9)	12.8	(3.2)
Valine	15.3	(3.5)	17.2	(2.6)	7.3	(1.7)	6.3	(1.7)	6.8	(1.8)	6.9	(1.7)	8.3	(1.2)	10.4	(6.9)	9.9	(0.8)	3.0	(0.4)
	37.7	(8.6)	42.4	(6.4)	19.7	(2.9)	18.7	(5.1)	19.1	(5.2)	18.4	(4.4)	21.3	(3.3)	25.9	(2.6)	24.6	(2.3)	7.5	(1.1)
Sum (wt.)	226.9	(31.9)	286.4	(42.9)	127.3	(10.9)	83.0	(24.1)	103.4	(28.0)	125.1	(16.8)	145.9	(17.6)	186.1	(15.4)	164.4	(13.4)	66.9	(5.83)
	(14.06)[d]		(14.98)		(24.27)		(29.04)		(27.08)		(13.43)		(12.06)		(8.28)		(8.15)		(8.71)	
Sum (Protein)	574.6	(82.5)	721.9	(100.4)	348.4	(33.5)	245.4	(68.0)	289.2	(76.9)	330.7	(39.4)	375.7	(49.5)	475.8	(44.3)	338.4	(26.4)	173.1	(15.1)

[a]average free amino acid concentration normalized to dry weight (nmol/mg) (n=6)
[b]standard deviation (n=6)
[c]average free amino acid concentrations normalized to proteins (nmol/mg protein) (n=6)
[d]coefficient of variation

Table 13. Seasonal variation in the percent composition of FAA in G. pseudolimuneus. The ratio of essential (E) to non-essential (NE) amino acids is also given. Essential and non-essential amino acids are identified.

Amino Acid	April	May	June	July	August	September	October	December	January	March
						Percent				
Alanine^{NE}	19.68	16.21	22.07	19.72	19.98	17.95	17.62	20.99	22.42	18.25
Arginine^{E}	8.91	13.49	10.1	7.61	9.65	9.54	11.15	11.16	11.98	8.16
Asparagine^{NE}	0.91	1.04	0.80	2.70	0.83	0.84	0.79	0.92	1.03	0.65
Aspartic Acid^{NE}	0.78	0.83	0.73	0.60	0.65	0.79	0.78	0.62	0.54	0.65
Glutamine^{NE}	7.14	7.33	5.20	9.35	10.78	7.29	7.35	5.52	6.19	8.53
Glutamic Acid^{NE}	3.90	3.50	4.42	2.46	3.68	4.05	4.44	3.51	3.73	5.75
Glycine^{NE}	2.84	2.87	3.47	1.68	1.96	4.11	4.46	6.63	5.19	6.76
Histidine^{E}	4.17	4.09	2.60	3.11	3.14	2.04	1.74	0.87	1.13	3.22
Isoleucine^{E}	5.13	5.00	4.98	5.64	4.60	4.94	4.63	4.67	5.31	2.96
Leucine^{E}	10.43	10.94	10.11	12.71	10.71	11.43	10.75	10.02	9.99	6.98
Lysine^{E}	4.17	7.15	7.24	2.47	4.52	7.08	9.46	9.42	3.94	3.84
Methionine^{E}	4.72	3.11	2.66	3.87	3.32	4.19	3.22	2.62	3.17	2.23
Phenylalanine^{E}	4.55	4.35	3.65	5.17	4.39	4.07	3.69	3.57	3.95	3.34
Serine^{NE}	3.14	2.23	2.11	1.55	1.64	2.64	2.63	2.18	2.37	2.56
Taurine^{NE}	3.46	4.06	7.13	4.87	5.27	5.91	4.00	3.13	3.53	10.66
Threonine^{E}	2.09	1.86	1.61	1.42	1.82	1.83	1.74	2.02	2.42	2.18
Tryptophan^{E}	1.64	1.37	0.93	1.43	1.02	1.22	0.97	1.02	1.22	1.39
Tyrosine^{NE}	5.83	4.69	4.51	5.96	5.39	4.52	4.91	5.69	6.04	7.34
Valine^{E}	6.5	5.87	5.65	7.69	6.62	5.53	5.66	5.45	5.79	4.36
Total Essential	52.31	57.23	49.53	51.12	49.79	51.87	53.01	51.82	48.90	38.66
Total Non-Essential	47.68	42.76	50.44	48.89	50.18	48.10	46.98	49.19	51.04	61.35
E/NE Ratio	1.097	1.338	0.982	1.046	0.992	1.078	1.128	1.053	0.958	0.630

these amino acids predominated in G. pseudolimnaeus, in fact, aspartic acid was least abundant of all the amino acids measured.

Comparison of the relative composition of specific amino acids indicated that at certain times of the year, the profiles of relative concentration of the FAA are different. This was especially true in March, when the percent contribution of six amino acids was either significantly greater or less than all the other months (Table 13). In addition, a significant decrease in the ratio of essential to non-essential (E/NE) amino acids occurred in March. In most crustaceans, the NE amino acids dominate the FAA pool, comprising between 58-78% of the total pool (Claybrook, 1983). In G. pseudolimnaeus there was a fairly equal proportion of E and NE amino acids in all months except March, where the E and NE amino acids comprised 38.7 and 61.03% of the total concentration, respectively. There was a significant increase in the relative proportion of the NE amino acids glutamate, glycine and taurine and a decrease in the E amino acids isoleucine, leucine and valine. A change in the E/NE ratio can be indicative of a change in protein metabolism relative to the energetic status of the organism.

Because the total FAA concentration changed each month, it is difficult to determine whether the change in the E/NE ratio was caused by an absolute increase in NE amino acids (NEAA), decrease in E amino acids (EAA) or by a combination of both. July was the only month in which total FAA concentration was not significantly different from March, and thus the only month in which an appropriate comparison of the absolute values of the E and NE amino acids can be made. However, it also should be noted that July was the only other month besides March in which the profile of relative FAA concentrations seemed to be different. The

relative concentrations of the EAA and NEAA for July and March are presented in Table 14. Also in this table are the sums of the amino acids whose percent contribution to the total FAA concentration in March were significantly different from other months. For the NEAA, the concentrations in July and March were not significantly different; although, there seemed to be a slight increase when considering only isoleucine, leucine, methionine and valine. However, the concentration of EAA was significantly ($\alpha = 0.05$) less in March than in July. Therefore, the reduction in the E/NE ratio is most likely the result of a decrease in the concentration of EAA.

Essential amino acids are those that cannot be synthesized by an organism, or which cannot be synthesized at a great enough rate such that a dietary source is required to meet the nutritional requirements of the organism. In mammals, a decrease in the E/NE ratio can be indicative of a dietary protein deficiency (Munro, 1970). This decrease is a result of a depletion of EAA, with NEAA generally remaining constant. Protein catabolism, a known consequence of starvation in both vertebrates and invertebrates, may be initiated by the reduced concentrations of EAA (Munro, 1970; Claybrook 1983). Increased protein catabolism, whether initiated by energetic (Bayne, 1973) or biosynthetic requirements, can result in an alteration of the total FAA concentration as well as the pattern of individual FAA of aquatic invertebrates. Riley (1980) found that during starvation, protein degradation increased, and over time the composition of the FAA pool and that of protein hydrolysate became increasingly similar. Riley (1980) also observed a decrease in the E/NE ratio; however, he attributed the change to an increase in NEAA and felt that the EAA were conserved for protein

Table 14. Sums of the essential and non-essential amino acids (nmol/mg) in G. pseudolimnaeus collected from the field in July and March. n = 6.

	July, 1984	March, 1985
Essential Amino Acids		
Total	42.33	26.95[c]
Glu+Gly + Tau+Tyr [a]	24.68	11.51[c]
Non-Essential Amino Acids		
Total	40.67	39.95
Ile+Leu+ Met+Val [b]	12.34	18.75

[a] In March samples, the percent contribution of these amino acids to the total pool was significantly (α=0.05) higher than in other months. Glu - glutamate, Gly - glycine, Tau - taurine, Tyr - tyrosine.

[b] In March samples, the percent contribution of these amino acids to the total pool was significantly (α=0.05) lower than in other months. Ile - leucine, Leu - leucine, Met - methionine, Val - valine.

[c] Significantly lower than essential amino acids in July.

synthesis. In the present study, it was possible that the decreased E/NE ratio observed in March was the result of dietary deficiencies or starvation. March was the end of the cold season, during which temperatures were near freezing for 3-4 months (Fig. 4). Feeding activity was likely minimal during this time period, and the amphipods may have had a negative scope for growth. This would indicate that normal maintenance requirements exceed caloric intake, thus forcing the organism to mobilize energy storage reserves to maintain homeostasis. During cold winter months, when food availability is greatly reduced, marine bivalves often are in a negative scope for growth during which time they rely mostly on protein reserves to meet energetic requirements (Bayne, 1973). If this were the case in the present study, the decrease in the E/NE ratio may have been indicative of the changes in protein metabolism.

Seasonal fluctuations in the total FAA concentration or relative concentrations of individual FAA can be attributed to a variety of interrelated accessory factors. The primary factors which can affect FAA on a seasonal basis are diet, reproductive condition and molt cycle status. Other factors which also need to be considered are sex, temperature, photoperiod, developmental stage and salinity. Because all of these accessory factors are simultaneously interacting over time, it is extremely difficult to separate the effect of the individual factors. However, the influence of all of these factors are implicit in seasonal changes in the FAA pool. In addition, when developing a biochemical indicator of stress, one is not necessarily interested in the effect of any individual accessory factor on the biochemical parameter of interest but rather in the combined influence of the factors over time and

between locations. Because seasonal variation in FAA is the result of the combined influence of all the above accessory factors, season is, along with geographic variation, the primary component required to interpret the potential significance of toxicant induced changes in the FAA pool of field organisms.

The quantitative and qualitative composition of an organism's diet can directly influence its FAA pool. As discussed above, quantitative dietary deficiencies can alter protein metabolism and subsequently the relative composition of both essential and non-essential amino acids (Munro, 1970; Riley, 1980). Similarly, qualitative changes in diet can alter the composition of an organisms FAA pool. For organisms which do not feed continuously, such as humans, diet generally has little influence on the FAA pool. Immediately following protein ingestion the tissue and body fluid FAA pool may rise dramatically, however, these changes are only transitory and amino acids rapidly return to pre-ingestion concentrations, via increased protein synthesis or amino acid oxidation (Munro, 1970). Amphipods are considered continuous feeders (Marchant and Hynes, 1981), and the constant flux of FAA via protein digestion may have a more consistent influence of the FAA pool of the organisms. Given this, changes in the qualitative aspects of an organism's diet may influence the concentration of amino acid in its tissue and fluids. Amphipod growth will vary under different dietary regimes, (Barlocher and Kendrich, 1973), which indicates that there are optimal and sub-optimal dietary conditions. Whether the FAA pool is altered under these different dietary regimes is unknown. Protein metabolism will definitely be different in organisms with varying growth rates, and because the FAA pool is a highly dynamic pool of biomolecules

responsive to alterations in protein metabolism, it follows that changes in dietary quality will alter the FAA pool. The primary nutritional source for the amphipods is the microbial flora which colonization leaves (Hynes, 1972). Since this colonization is successional, in that the microbial community changes throughout the year, it is possible that the nutritional quality of the food may also change seasonally, thus affecting the organisms FAA pool.

In the present study, seasonal changes in both the quantity and quality of the amphipods diet may have contributed to the seasonal fluctuations in FAAs. In March, the end of the winter season, the total FAA concentration was at its lowest level, and the profile of relative FAA concentrations was considerably different than in the other months. Cook et al. (1972) studied seasonal changes in the FAA pool of adult barnacles Balanus balanoides. They found that starvation during summer and winter-spring months caused a significant decrease in the total FAA concentration; however, starvation during the fall-winter time period resulted in an increase in the FAA concentration. Cook et al. (1972) concluded that the effect on the FAA concentration depends upon what substrate is being mobilized to meet the energy demands of the organism. Mobilization of carbohydrates will increase the total FAA concentration while metabolism of lipid and/or protein will cause the total FAA concentration to decrease (Cook et al., 1972). Because the primary energy reserve and an organism's dietary source may shift during the year (Bayne, 1973), it is not surprising that the total FAA concentration fluctuates seasonally.

The reproductive cycle in aquatic invertebrates is an extremely complex process controlled by a variety of indogenous and exogenous cues,

including temperature, photoperiod and nutritional status. Seasonal variations in energy metabolism as reflected by changes in the relative composition of glycogen, lipid and protein storage reserves are directly related to gametogenesis (Zandee, et al., 1980; Gabbot and Bayne, 1973; Newell and Bayne, 1980). The concentrations of phosphoadenylates have been observed to vary with reproductive condition in freshwater clams (Giesy and Dickson, 1981). The time frame of gonadal maturation is unknown in G. pseudolimnaeus, as are seasonal changes in the concentrations of glycogen and lipids. However, assuming that the reproductive cycle is accompanied by changes in energy metabolism, changes in the FAA pool similar to those found in other organisms would be expected. In a variety of marine invertebrates, changes in glycine have been directly linked to reproductive conditions. Maturation of male gonads in the cirripede Balanus balanus (Barnes, 1963), the sea anemone Bunodosoma cavernata (Kasschau and McCommas, 1982; Howard and Kasschau, 1980) and the starfish Echinaster modestus (Ferguson, 1975) results in a significant accumulation of glycine in gonadal tissue. In the present study, no increase in glycine concentration was observed during the reproductively active period. However, since whole organisms were analyzed and males and females were combined, changes in the male gonadal tissue simply may have been obscured. Other studies also have shown amino acids to vary with the reproductive cycle (Zandee et al., 1980; Cook et al., 1972; Barnes et al., 1963). The changes observed in these studies, although correlated with reproductive conditions, were more directly the result of changes in energy metabolisms which are concurrent with reproductive maturation.

The periodic molting which all crustaceans undergo is an extremely complex, hormonally-controlled process essential for the growth of the organism (Skinner, 1985). Molting can be subdivided into five major stages, each of which is characterized by changes in exoskeleton hardness and by cellular and biochemical processes (Skinner, 1985). The molting frequency is greatest in early instar organisms, with the length of the intermolt period increasing with age (Sutcliffe and Carricle, 1981). During intermolt and premolt, a variety of metabolic changes occur which prepare the organism for ecdysis. Blood glucose levels increase, glycogen and lipid reserves accumulate in the midgut gland and plasma and tissue FAA concentration change (Barclay et al., 1983; Chang and O'Connor, 1983; Yamaoka and Skinner, 1976). In freshwater crayfish, tissue FAA levels increased two-three fold during premolt (McWhinnee et al., 1972). This increase may be the result of changes in protein metabolism likely initiated by hormonal changes or may simply be a response to the increased hemolymph osmolarity which normally occurs prior to ecdysis, presumably to enhance water intake (Tucker and Costlow, 1975). In the present study, it is impossible to distinguish changes resulting from difference in the stage of the molt cycle from other accessory factors. For the early instar organisms observed in July and August, during which the molt cycle interval is relatively short (5-15 days), there is a greater probability of having organisms collected at different stages of the molt cycle. This would result in greater variation in the measured FAA pool. The coefficients of variations for the total FAA pool of amphipods collected in July and August (Table 12) are 2-3 times greater than most other months. This

Chapter 5

Alterations in the oxygen consumption, condition index and concentration of free amino acids of *Corbicula fluminea* (mollusca:gastrapoda) exposed to sodium dodecyl sulfate

increased variation could be the result of measuring FAAs in organisms at different stages of their molt cycle.

The FAA pool also can change with the developmental stage of an organism (Anderson, 1984; Levenbook and Dinamarca, 1966). In larvae of the chironomid Chironomus tentans, the hemolymph FAA concentrations changed during the fourth instar (Firling, 1977). In the present study, the amphipods FAA pool increased during the initial five months of growth, from July to December. Initially it was thought that this increase could be an artifact, in that normalizations to dry weight artificially lowered the FAA concentration because of the disproportionate contribution of the exoskeleton to the dry weight. However I feel this is a real increase, because normalization to protein produced the same trend. It must be remembered however, that this increase does not necessarily indicate any inherent metabolic changes resulting in a larger FAA pool but may simply reflect the influence of accessory factors such as diet.

Seasonal fluctuations in the FAA pool do not limit the usefulness of FAAs as an in situ biochemical indicator of stress. Knowledge of seasonal trends will allow the investigator to consider these fluctuations when interpreting the significance of changes potentially caused by toxicant exposure. As long as the "normal" concentration is known for a particular time of year and/or location, one can, with a reasonable amount of certainty, determine if the changes are the result of toxicant effects. The larger the background data base with which alterations can be compared, the greater the ability to isolate toxicant-related alterations.

Introduction

An important consideration when developing an *in situ* BIS is the choice of an appropriate organism. Programs designed to monitor chemical contamination in the environment *via* measurement of residue concentrations in tissues of indigenous biota often use bivalves as the indicator organism (Goldberg et al., 1978; Phillips, 1978). Characteristics that make bivalves good indicator organisms include 1) sedentary lifestyle, 2) ease of sampling, 3) size, 4) ability to transport and maintain in the laboratory, and 5) feeding mechanism. Many of these same advantages are applicable to the use of bivalves as *in situ* monitors of biochemical effects. In freshwater systems, few bivalve mullusks have a wide enough distribution and are found in sufficient abundance to be adapted to a comprehensive BIS monitoring program. The Asiatic clam, *Corbicula fluminea*, is one organism that adequately meets all of the above criteria.

In freshwater mollusks, very little information exists on the effect of toxicant-induced stress of the FAA pool. Alterations have been observed in molluscs collected from "polluted" systems (Gardner et al., 1981); however, this study alone does not provide sufficient data to determine the potential applicability of FAA as *in situ* indicators of toxicant effects.

The objective of this study was to determine the effect of the anionic surfactant, sodium dodecyl sulfate (SDS), on the concentration of free amino acids (FAA) in the freshwater bivalve *Corbicula fluminea* and relate the sensitivity of this indicator to several other higher level stress indicators, such as condition index and respiration. The existence of such a relationship will enable interpretation of changes

in BIS with respect to potential consequences at the population and/or community level. Amino acids were measured in both adductor muscle and mantle tissue of clams exposed acutely or chronically to SDS.

Materials and Methods

General

Corbicula fluminea were collected by D-frame dip nets from the Potomac River, Virginia in April 1985, and transported in coolers to Michigan State University. Prior to test initiation, C. fluminea were held for two months in large flow-through tanks receiving dechlorinated, charcoal filtered tap water. Washed silica sand was provided as substrate and C. fluminea were fed a homogenized mixture of trout chow and frozen spinach. During this two month holding and acclimation period, less than 1 percent mortality occurred. A 12L:12D photoperiod was utilized and the temperature was 18-21°C. To ensure that the feeding regime was adequate and C. fluminea were healthy, growth in the holding tank was monitored. Twenty C. fluminea were measured and marked and after 6 weeks holding additional shell deposition and growth was observed.

Acute Exposure

A preliminary range-finding study was conducted to determine appropriate concentrations to be used in the long-term, sublethal exposure and to establish a background data base on the concentration of free amino acids in C. fluminea. Clams were exposed statically for 96 h to nominal concentrations of 5.0, 12.5, 25.0, 50.0 and 100.0 mg/l sodium dodecyl sulfate (SDS). C. fluminea were not fed during the 96 h exposure period. Amino acids were analyzed in adductor and mantle tissue

dissected from control clams sampled at test initiation and on day 4 from C. fluminea exposed to 50 mg/l for 48 h and from C. fluminea exposed to 12.5 and 25 mg/l SDS for 96 h.

Chronic Exposure

Based on the results of the acute exposure, a long term exposure was initiated to determine the effect of sublethal stress on the concentration of FAA in C. fluminea. An Ace Glass® (Ace Glass Inc., Vineland, N.J.) flow-through diluter was used to expose C. fluminea for 60 days at 20 C to measured concentrations of 0.65, 3.0, 7.8, and 23.6 mg/l SDS. Exposure tanks were 55 liter aquaria and had a turnover time of 10 h. Replicate exposure tanks, each containing 50 C. fluminea, were randomly located in a constant temperature water bath. Washed, silica sand was used as substrate and clams were fed daily a homogenized mixture of trout chow and frozen spinach. Water concentrations of SDS were measured weekly by the methylene blue method (APHA, 1976). Amino acid concentrations in adductor and mantle tissue were measured in C. fluminea sampled on the following days and at the specified concentrations: 1) day 0-control, 2) day 2-23.6 mg/l, 3) day 5-control, 7.8 and 23.6 mg/l, 4) day 10-7.8 mg/l, and 5) days 15, 30 and 60-0.65 and 3.0 mg/l. Respiration, condition indices and water content were also measured for C. fluminea exposed to 0.65, 3.0 and 7.8 mg/l SDS for 0, 5, 15, 30 and 60 days.

Analyses

Concentrations of FAA were measured in both adductor muscle and mantle tissue of C. fluminea. Dissected tissues were weighed and amino acids extracted by homogenization in methanol. A subsample of the

homogenate was taken and analyzed for protein by the method of Peterson (1977). The homogenate was then centrifuged at 3,000 RPM for 10 min and the supernatant used directly for amino acid analysis by high pressure liquid chromatography. This procedure employs pre-column O-phthaldialdehyde (OPA) derivatization, reverse-phase C-18 separation of the amino acids and fluorescence detection (Jones et al., 1981). A more detailed description of the procedure utilized is given in Chapter 2. Amino acid concentrations were normalized to both wet weight and protein.

Oxygen consumption was measured for individual clams by a method similar to that described by Britton and Morton (1982). C. fluminea were removed from their exposure chambers, placed in 100 ml screw-top jars and submerged in a constant temperature (20 C), oxygen saturated water bath. Initial O_2 determinations were made, the jars were sealed, and after 3 1/2 hours O_2 consumption was calculated as the difference between initial and final water O_2 concentrations. Oxygen concentrations were determined by micro-winkler analyses, performed within the 10 ml syringes used to sample the water.

Condition indices and water content were then calculated for C. fluminea used in the respiration study. Lengths of each C. fluminea were recorded and whole body tissues removed, weighed, dried at 90°C for 24 h and then weighed again. Condition indices were calculated by equation 1.

$$C.I. = \frac{\text{dry weight}}{\text{shell length}} \times 100 \qquad (1)$$

Percent tissue water was calculated by equation 2.

$$\% \text{ TW} = \frac{\text{wet weight - dry weight}}{\text{wet weight}} \times 100 \qquad (2)$$

Patterns of relative FAA concentrations in extracts from SDS-exposed and unexposed C. fluminea were compared by univariate and multivariate statistical techniques. Total FAA concentrations and the percent contribution of specific amino acids to the total FAA pool of different treatment groups were compared by analysis of variance (ANOVA) followed by Duncans Multiple Range Test ($\alpha = 0.05$ unless otherwise stated). The patterns of the concentration of individual FAA were compared by profile analysis (Morrison, 1967). This technique uses multivariate analysis of variance to compare the differences between adjacent amino acids. Significance was determined by Wilk's Criterion (SAS Institute, 1982). Respiration, condition indices and percent tissue water of clams from the different treatment groups were compared by analysis of variance (ANOVA) followed by Duncans Multiple Range Test. LC_{50} values were calculated by either the probit or the binomial probability method.

Results

Acute Exposure

The 96 h LC_{50} for SDS was 31.4 mg/l (C.L. 27.3-36.2).

Exposure of C. fluminea to SDS for 96 h caused a significant increase in the total FAA concentration of adductor muscle (Table 15). The concentration of total FAA in adductor muscles increased from 2.17 to 4.23 nmol/mg, wet weight after four days exposure to 25 mg/l SDS.

Table 15. The concentration of free amino acids, normalized to both protein and wet weight in mantle tissue and adductor muscle of Corbicula fluminea acutely exposed to sodium dodecyl sulfate.

	MANTLE					ADDUCTOR				
	Day 0	Day 2	Day 4			Day 0	Day 2	Day 4		
Amino Acid	Control	50 mg/l	Control	12.5	25.0	Control	50 mg/l	Control	12.5	25.0
Alanine	7.0[a] (2.3)	14.8 (3.3)	8.34(3.8)	13.2 (3.4)	17.7 (4.3)	4.64(1.4)	7.38(2.2)	6.03(0.9)	5.10(1.84)	8.48(3.76)
	0.88[b](0.1)	1.39(0.42)	0.46(0.2)	1.42(0.27)	1.28(0.21)	0.94(0.06)	1.12(0.32)	0.65(0.07)	1.12(0.48)	1.42(0.90)
Arginine	1.2 (1.0)	0.89(0.7)	1.45(0.6)	0.93(0.2)	1.61(0.8)	1.56(0.3)	0.69(0.18)	3.01(0.41)	1.64(0.58)	4.42(0.38)
	0.14(0.1)	0.08(0.05)	0.08(0.03)	0.10(0.03)	0.11(0.04)	0.32(0.05)	0.10(0.02)	0.33(0.05)	0.34(0.09)	0.70(0.22)
Asparagine	0.08(0.03)	0.05(0.02)	0.09(0.04)	0.04(0.01)	0.10(0.08)	0.03(0.01)	0.007(0.001)	0.02(0.01)	0.005(0.001)	0.03(0.01)
	0.01(0.002)	0.005(0.002)	0.005(0.002)	0.004(0.001)	0.005(0.002)	0.006(0.2)	0.001(0.0)	0.002(0.001)	0.001(0.0)	0.005(0.003)
Aspartic Acid	0.21(0.1)	0.60(0.1)	0.86(0.4)	0.60(0.4)	1.06(0.5)	0.04(0.01)	0.07(0.008)	0.09(0.06)	0.06(0.02)	0.20(0.09)
	0.02(0.01)	0.06(0.02)	0.05(0.02)	0.07(0.04)	0.08(0.03)	0.009(0.007)	0.01(0.002)	0.009(0.006)	0.013(0.005)	0.03(0.02)
Glutamine	0.77(0.5)	0.63(0.5)	2.76(1.3)	0.96(0.7)	1.87(1.2)	0.04(0.01)	0.06(0.03)	0.15(0.06)	0.06(0.03)	0.25(0.14)
	0.09(0.05)	0.06(0.03)	0.15(0.07)	0.10(0.05)	0.13(0.14)	0.01(0.004)	0.009(0.006)	0.02(0.01)	0.012(0.005)	0.04(0.02)
Glutamic Acid	4.29(1.9)	3.73(0.6)	6.98(2.1)	3.03(0.9)	7.18(1.9)	0.74(0.3)	0.46(0.05)	1.40(0.4)	0.76(0.27)	0.88(0.34)
	0.52(0.18)	0.36(0.1)	0.40(0.12)	0.33(0.09)	0.54(0.20)	0.15(0.06)	0.07(0.01)	0.15(0.04)	0.16(0.05)	0.14(0.06)
Glycine	1.24(0.4)	0.78(0.7)	0.99(0.4)	0.59(0.1)	0.87(0.2)	0.38(0.3)	0.28(0.11)	0.57(0.39)	0.36(0.28)	0.84(0.40)
	0.16(0.03)	0.07(0.05)	0.05(0.02)	0.06(0.01)	0.06(0.01)	0.09(0.06)	0.04(0.02)	0.06(0.03)	0.08(0.06)	0.14(0.09)
Histidine	0.08(0.05)	0.14(0.06)	0.13(0.05)	0.28(0.2)	0.15(0.07)	0.05(0.01)	0.03(0.02)	0.07(0.01)	0.05(0.01)	0.09(0.06)
	0.01(0.007)	0.01(0.007)	0.007(0.002)	0.03(0.02)	0.01(0.004)	0.01(0.002)	0.005(0.003)	0.008(0.001)	0.01(0.002)	0.02(0.01)
Isoleucine	0.48(0.2)	1.02(0.3)	0.86(0.4)	1.25(0.2)	1.81(0.8)	0.22(0.1)	0.33(0.08)	0.24(0.04)	0.29(0.07)	0.50(0.20)
	0.06(0.02)	0.10(0.04)	0.05(0.02)	0.14(0.03)	0.13(0.04)	0.04(0.02)	0.05(0.01)	0.027(0.004)	0.06(0.01)	0.08(0.06)

Continued

Table 15 (continued). The concentration of free amino acids, normalized to both protein and wet weight in mantle tissue and adductor muscle of Corbicula fluminea acutely exposed to sodium dodecyl sulfate.

	MANTLE					ADDUCTOR				
	Day 0	Day 2	Day 4			Day 0	Day 2	Day 4		
Amino Acid	Control	50 mg/l	Control	12.5	25.0	Control	50 mg/l	Control	12.5	25.0
Leucine	0.99(0.4)	1.68(0.6)	1.27(0.6)	1.90(0.2)	2.64(1.1)	0.30(0.08)	0.54(0.09)	0.36(0.08)	0.48(0.12)	0.77(0.31)
	0.12(0.03)	0.16(0.07)	0.07(0.03)	0.21(0.06)	0.19(0.06)	0.06(0.01)	0.08(0.01)	0.04(0.01)	0.10(0.03)	0.13(0.08)
Lysine	0.70(0.3)	0.99(0.5)	0.99(0.2)	0.74(0.2)	1.31(0.7)	0.47(0.14)	0.72(0.12)	0.92(0.3)	0.44(0.12)	0.94(0.43)
	0.09(0.02)	0.09(0.05)	0.06(0.01)	0.08(0.02)	0.09(0.04)	0.10(0.01)	0.11(0.01)	0.10(0.02)	0.10(0.04)	0.16(0.10)
Methionine	0.42(0.1)	0.42(0.1)	0.45(0.2)	0.50(0.1)	0.67(0.3)	0.16(0.03)	0.14(0.04)	0.15(0.03)	0.14(0.03)	0.23(0.09)
	0.05(0.01)	0.04(0.02)	0.02(0.01)	0.06(0.02)	0.05(0.02)	0.03(0.005)	0.02(0.007)	0.02(0.003)	0.03(0.007)	0.04(0.02)
Phenylalanine	0.30(0.1)	0.31(0.1)	0.29(0.1)	0.40(0.1)	0.61(0.2)	0.17(0.04)	0.19(0.05)	0.16(0.03)	0.15(0.02)	0.29(0.05)
	0.04(0.01)	0.03(0.01)	0.02(0.005)	0.04(0.01)	0.04(0.01)	0.03(0.006)	0.03(0.009)	0.02(0.002)	0.03(0.004)	0.04(0.02)
Serine	0.72(0.3)	0.45(0.2)	0.75(0.3)	0.52(0.3)	0.67(0.2)	0.37(0.22)	0.19(0.07)	0.49(0.46)	0.20(0.11)	0.70(0.34)
	0.09(0.02)	0.04(0.02)	0.04(0.02)	0.05(0.01)	0.05(0.01)	0.09(0.05)	0.03(0.01)	0.05(0.03)	0.04(0.03)	0.12(0.08)
Threonine	0.19(0.1)	0.15(0.05)	0.29(0.1)	0.15(0.05)	0.27(0.1)	0.07(0.02)	0.06(0.01)	0.09(0.05)	0.05(0.02)	0.12(0.09)
	0.02(0.01)	0.02(0.01)	0.02(0.008)	0.02(0.005)	0.02(0.009)	0.02(0.01)	0.01(0.002)	0.01(0.005)	0.01(0.006)	0.02(0.01)
Tryptophan	0.13(0.06)	0.21(0.05)	0.24(0.1)	0.23(0.1)	0.29(0.03)	0.07(0.01)	0.09(0.02)	0.10(0.03)	0.11(0.01)	0.16(0.05)
	0.02(0.004)	0.02(0.01)	0.01(0.004)	0.03(0.01)	0.02(0.004)	0.01(0.003)	0.01(0.004)	0.01(0.004)	0.02(0.003)	0.02(0.003)
Tryosine	0.46(0.1)	0.53(0.2)	0.38(0.1)	0.44(0.1)	0.71(0.2)	0.16(0.04)	0.27(0.03)	0.14(0.05)	0.26(0.08)	0.45(0.06)
	0.06(0.01)	0.05(0.02)	0.02(0.01)	0.05(0.01)	0.05(0.01)	0.04(0.01)	0.04(0.005)	0.015(0.004)	0.06(0.02)	0.07(0.02)
Unknown	1.94(0.7)	1.50(0.5)	1.98(0.3)	1.09(0.4)	1.83(1.5)	3.79(1.69)	3.56(1.08)	5.87(1.23)	1.87(0.42)	5.45(3.48)
	0.24(0.05)	0.14(0.06)	0.11(0.01)	0.12(0.04)	0.13(0.09)	0.76(0.16)	0.54(0.14)	0.63(0.11)	0.41(0.12)	0.96(0.76)

Continued

Table 15 (continued). The concentration of free amino acids, normalized to both protein and wet weight in mantle tissue and adductor muscle of Corbicula fluminea acutely exposed to sodium dodecyl sulfate.

	MANTLE					ADDUCTOR				
	Day 0	Day 2	Day 4			Day 0	Day 2	Day 4		
Amino Acid	Control	50 mg/l	Control	12.5	25.0	Control	50 mg/l	Control	12.5	25.0
Valine	0.72(0.3)	1.19(0.4)	1.21(0.6)	1.36(0.2)	1.98(0.8)	0.27(0.07)	0.34(0.07)	0.25(0.08)	0.31(0.07)	0.58(0.19)
	0.09(0.03)	0.11(0.05)	0.07(0.03)	0.15(0.04)	0.14(0.04)	0.06(0.007)	0.05(0.01)	0.03(0.007)	0.07(0.02)	0.09(0.04)
Total	22.9 (9.2)	30.0 (7.9)	30.3 (10.5)	28.2 (5.5)	43.3[d](9.7)	13.6 (4.04)	15.4 (3.47)	20.1 (3.5)	12.3[f](2.9)	25.4 (9.19)
	2.78(0.55)	2.84(0.9)	1.67[e](0.51)	3.06(0.47)	3.12(0.38)	2.80(0.14)	2.34(0.47)	2.17(0.29)	2.67(0.75)	4.23[d](1.46)
E/NE[c]	0.35(0.08)	0.32(0.05)	0.34(0.06)	0.42(0.09)	0.37(0.12)	0.53(0.09)	0.38(0.09)	0.62(0.10)	0.58(0.18)	0.79(0.36)

a - nmol/mg protein (standard deviation in parentheses; n = 5)
b - nmol/mg wet weight (standard deviation in parentheses; n = 5)
c - essential amino acids (E) - arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine
non-essential amino acids (NE) - alanine, asparagine, aspartic acid, glutamine, glutamic acid, glycine, serine, tryosine
d - significantly greater than day 4 control ($\alpha = 0.05$)
e - significantly less than day 4 12.5. and 25.0 mg/l exposures and Day 0 control ($\alpha = 0.05$)
f - significantly less than day 4 control ($\alpha = 0.05$)

When normalized to protein, the concentration was also increased; however, this was not statistically significant. The increase in total FAA concentration can be attributed to significant increases in all amino acids except glutamate, glycine, lysine and threonine. However, when the adductor muscle FAA concentrations in C. fluminea exposed to 12.5 mg/l SDS for 4 days was normalized to protein content they were significantly lower than that of control C. fluminea (Table 15). This decrease was an artifact, which can be attributed to the significantly greater protein concentration in C. fluminea which were exposed to 12.5 mg/l. C. fluminea unexposed, and those exposed to 12.5 or 25 mg/l SDS had mean protein concentrations of 0.11, 0.23 or 0.14 mg protein/ mg, wet weight, respectively. It is unknown why C. fluminea exposed to 12.5 mg/l had greater protein concentrations.

The choice of an appropriate normalization parameter is often overlooked when developing BIS. Normalization parameters should be invariate during the study period. If the treatment under investigation also affects the normalization parameter, then results may be ambiguous and lead to inappropriate conclusions. Analysis of variance (ANOVA) should be performed on all normalization data to determine if significant variations occur during the experimental period. In the present study, the protein content of the adductor muscle was significantly altered by exposure to 12.5 mg/l SDS. Therefore, under these circumstances, normalization to protein was inappropriate. Whenever protein normalization was unacceptable, FAA concentrations were normalized to wet weight.

The total FAA concentration of the mantle tissue, normalized to protein, was significantly increased after 96 h exposure to 25 mg/l SDS (Table 15). The protein concentration of the mantle tissue did not vary

significantly and, thus, protein normalization was acceptable for this tissue. This increase was primarily the result of a large increase in the concentration of alanine, although isoleucine and leucine concentrations also increased significantly. Glutamine concentrations decreased and concentrations of the remaining amino acids changed little.

Exposure to 12.5 mg/l SDS did not alter the total concentration of FAA (nmol/mg protein) in mantle tissue; however, the relative concentrations of individual amino acids, or the amino acid profile, were significantly different from that of controls. This difference was established by conducting multivariate analysis of variance (MANOVA) on the differences between specific amino acids. For example, the differences between alanine and arginine, arginine and asparagine, asparagine and aspartic acid, etc., were calculated for each treatment. A MANOVA was then conducted on these differences, essentially testing for parallelism, and significance was established using Wilks' criterion. In the present study, the calculated F-value of 50.19 was found to be significant ($\alpha = 0.019$). Specific alterations included significant increases in the concentrations of alanine, isoleucine and leucine and decreases in arginine, asparagine, glutamine and glutamic acid (Table 15).

The total FAA concentration of the mantle tissue, normalized to wet weight, was significantly greater in clams exposed for four days to 12.5 and 25 mg/l SDS; however, the difference was a result of a decrease in the FAA concentration in control C. fluminea and not the result of an increase in the FAA pool of the exposed C. fluminea (Table 15). The total FAA concentrations of C. fluminea exposed to SDS were not significantly different from the initial total FAA concentrations of unexposed C. fluminea. However, after four days the total FAA concentration of

control clams was significantly less than the initial total FAA of untreated C. fluminea. It is difficult to explain the decrease in the total FAA concentration of mantle tissue of unexposed C. fluminea. Because normalization was to the wet weight of dissected tissue, it is difficult to determine if variations in the weights influenced the FAA concentrations, since the amount of tissue dissected each time may vary. However, comparison of the weights of the tissues dissected did not reveal any obvious discrepancies, so it must be assumed that the lower FAA concentration was not an artifact.

Chronic Exposure

Free Amino Acids

Exposure to 7.8 or 23.6 mg/l SDS was acutely toxic to C. fluminea (Table 16). There was no mortality of clams not exposed to SDS and exposure to 23.6 and 7.8 mg/l SDS caused complete mortality after 10 and 15 days, respectively. The 5-day LC_{50} of 16.7 mg/l SDS (C.L. 7.8-23.6), calculated for the flow-through study using measured concentrations, was considerably lower than the 4-day LD_{50} (31.4 mg/l) calculated for the preliminary static exposure. Because SDS concentrations were not measured in the static exposure and degradation of SDS likely occurred, actual concentrations of SDS may have been lower than the nominal concentrations, making the apparent LC_{50} greater.

Analysis of variance (ANOVA) on protein concentrations (mg protein/mg wet weight) indicated that significant variation occurred over the 60-day exposure period, and thus FAA concentrations were only normalized to tissue wet weight.

Table 16. Percent mortality of C. fluminea exposed to measured concentrations of sodium dodecyl sulfate.

Measured Concentration (mg/l)	Day						
	2	5	10	15	30	45	60
Control	0[a]	0	0	0	0	0	0
0.65	0	0	0	0	0	0	0
3.00	0	0	0	0	0	5	25
7.8	0	0	40	100	-	-	-
23.6	30	80	100	-	-	-	-

[a] - percent dead out of 20 clams

The concentration of FAA was significantly altered in adductor and mantle tissue of C. fluminea exposed for two days to 23.6 mg/l SDS (Tables 17 and 19; Figure 5). Only the dominant FAA of the adductor muscle and mantle tissue are presented with the concentrations of the remaining FAA being combined into a single number. Concentrations of all of the FAA increased in the adductor muscle except glutamic acid. Increases in alanine and arginine concentrations were the greatest. Similar changes in individual FAA were observed in the mantle tissue. The percent contribution of alanine to the total FAA pool increased from 20.9 to 41.4 after two days exposure to SDS (Table 19). Because essentially all of the FAA were affected by SDS exposure in a similar manner, and thus were highly intercorrelated, profile analyses could not be utilized to compare among treatments after two days of exposure to SDS. Whenever there is a high correlation among dependent variables the determinant of the correlation matrix used in the MANOVA calculations becomes zero (singular) and cannot be inverted.

The total FAA concentration of both adductor and mantle tissue continued to increase after five days exposure to 23.6 mg/l SDS (Tables 17 and 19). In adductor muscle, the pattern was the same as after two days exposure, with all the FAA increasing except glutamic acid. In mantle tissue, the profile was significantly different, as determined by Wilks' criterion ($F = 18.99$; $\alpha = 0.0003$).

Five days exposure to 7.8 mg/l SDS did not affect the total FAA concentration in adductor or mantle tissue of C. fluminea (Tables 17 and 19). Profile analysis resulted in a singular matrix for both adductor and mantle tissue. However, comparison of the percent contribution of individual amino acids to the total FAA pool, using Duncans Multiple

Table 17. The concentration (nmol/mg, wet weight) and percent composition of free amino acids in adductor muscle of Corbicula fluminea exposed to 7.8 and 23.6 mg/l sodium dodecyl sulfate for 2, 5 and 10 days.

	Day 0	Day 2	Day 5			Day 10
Amino Acid	Control	23.6 mg/l	Control	7.8	23.6 mg/l	7.8 mg/l
Alanine	0.91[a](0.23)	2.47(0.74)	0.83(0.16)	0.98(0.22)	2.68(0.95)	1.17(0.32)
	35.8[b] (3.8)	40.1 (8.17)	34.3 (2.1)	33.1 (4.0)	33.7 (8.7)	37.8 (2.9)
Arginine	0.59(0.10)	1.68(0.22)	0.69(0.22)	0.90(0.11)	1.94(0.29)	0.85(0.26)
	23.5 (3.0)	27.8 (3.65)	27.7 (2.6)	30.9 (3.9)	25.0 (3.7)	27.6 (3.3)
Glutamic	0.29(0.10)	0.21(0.06)	0.32(0.11)	0.25(0.17)	0.39(0.20)	0.15(0.08)
Acid	11.5 (2.4)	3.5 (1.02)	12.8 (2.6)	8.0 (4.5)	4.9 (2.1)	4.8 (1.6)
Lysine	0.12(0.04)	0.24(0.11)	0.18(0.05)	0.19(0.03)	0.34(0.14)	0.20(0.05)
	4.9 (1.8)	4.1 (2.1)	7.3 (1.7)	6.4 (1.2)	4.2 (1.5)	6.8 (1.1)
Glycine-	0.09(0.02)	0.21(0.05)	0.06(0.02)	0.07(0.02)	0.39(0.20)	0.08(0.02)
Threonine	3.6 (1.1)	3.4 (0.91)	2.4 (0.8)	2.4 (0.5)	5.2 (4.4)	2.5 (0.4)
Others[c]	0.52(0.11)	1.26(0.27)	0.37(0.12)	0.56(0.18)	2.06(0.38)	0.63(0.19)
	20.7 (2.2)	21.0 (2.7)	15.5 (1.9)	19.2 (2.9)	27.0 (4.6)	20.5 (3.1)
Total	2.52(0.43)	6.07*(0.76)	2.45(0.56)	2.96(0.47)	7.80*(1.11)	3.08(0.82)
E/NE[d]	0.75(0.13)	0.91(0.16)	0.82(0.08)	1.07*(0.23)	0.92(0.15)	0.99(0.11)

[a] - nmol/mg, dry weight (standard deviation in parentheses; n = 5)
[b] - percent contribution to total FAA pool (standard deviation in parentheses; n = 5)
[c] - includes asparagine, aspartic acid, glutamine, histidine, isoleucine, leucine, phenylalanine, serine, tyrosine and valine
[d] - Essential Amino Acids (E) - arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, tryptophan and valine; Non-essential Amino Acids (NE) - alanine, asparagine, aspartic acid, glutamine, glutamic acid, glycine, serine, tryosine
* - significantly different from control ($\alpha = 0.05$)

Range Test, indicated that there was no obvious alteration in the FAA pattern of the adductor muscle. In the mantle tissue, a significant increase in the percent alanine and a decrease in the percent glutamic acid and glycine-threonine was observed.

The FAA profile of the mantle tissue was significantly different after 10 days exposure to 7.8 mg/l SDS, as determined by MANOVA using Wilks criterion ($F = 12.61$; $\alpha = 0.002$). In addition, by the tenth day, the total FAA concentration of the mantle tissue had also significantly increased above values of unexposed clams (Table 19).

Long-term sublethal exposure to 0.65 or 3.00 mg/l SDS significantly altered the concentration of FAA in C. fluminea (Tables 18 and 20; Fig. 5). The total FAA concentration of the adductor muscle was not affected after 15 d exposure (Table 18). Fifteen days exposure to 3.00 mg/l SDS caused a significant increase in the total FAA concentration of mantle tissue (Table 20). No effect was observed in the FAA profile of adductor or mantle tissue. Thirty days exposure to 0.65 or 3.00 mg/l SDS caused a significant increase in the total FAA pool of C. fluminea mantle tissue; however, only the 3.0 mg/l exposure increased total FAA concentration in adductor muscle. Again, profile analysis resulted in a singular matrix, however comparison of the percent contribution of individual FAA indicates that qualitative changes did occur. These changes were most apparent in the mantle tissue at the lowest SDS concentration (0.65 mg/l SDS), where a significant increase in the percent composition of alanine and a significant decrease in the percent composition of glutamic acid was observed (Table 20). A similar decrease in glutamic acid was observed in mantle tissue when C. fluminea were

Table 18. The concentration (nmol/mg, wet weight) and percent composition of free amino acids in adductor muscle of C. fluminea exposed to 0.65 and 3.0 mg/l sodium dodecyl sulfate for 15, 30 and 60 days.

	Day 15			Day 30			Day 60		
Amino Acid	Control	0.65	3.0	Control	0.65	3.0	Control	0.65	3.0
Alanine	0.87[a](0.09)	0.96(0.12)	1.19(0.42)	0.85(0.16)	1.16(0.57)	1.74(0.59)	0.86(0.09)	1.18(0.42)	1.52(0.61)
	33.6[b] (2.3)	36.4 (1.6)	30.6 (4.1)	34.5 (1.2)	37.8 (8.8)	27.8 (4.6)	36.3 (1.2)	38.2 (3.7)	28.5 (4.0)
Arginine	0.68(0.13)	0.63(0.08)	0.98(0.20)	0.69(0.12)	0.66(0.23)	1.65(0.41)	0.60(0.08)	0.69(0.23)	1.64(0.24)
	26.4 (3.4)	24.2(2.8)	25.2 (3.7)	28.4 (2.4)	22.4 (1.4)	28.3 (9.7)	25.7 (1.1)	23.1 (4.6)	33.2 (8.2)
Glutamic Acid	0.31(0.16)	0.20(0.06)	0.28(0.08)	0.32(0.11)	0.21(0.13)	0.41(0.19)	0.28(0.08)	0.13(0.09)	0.30(0.15)
	11.9 (1.8)	7.6 (0.7)	7.2 (1.3)	12.8 (2.4)	6.6 (2.6)	6.7 (2.7)	11.5 (2.6)	4.4 (2.5)	5.1 (3.0)
Lysine	0.17(0.04)	0.17(0.05)	0.19(0.04)	0.13(0.03)	0.19(0.09)	0.32(0.07)	0.13(0.03)	0.18(0.05)	0.36(0.12)
	6.7 (0.7)	6.3 (0.9)	4.9 (2.2)	5.5 (1.5)	6.2 (1.9)	5.6 (1.9)	6.0 (1.8)	6.0 (0.8)	2.6 (0.1)
Glycine-Threonine	0.07(0.02)	0.07(0.03)	0.15(0.04)	0.06(0.03)	0.08(0.06)	0.47(0.62)	0.06(0.01)	0.14(0.08)	0.31(0.19)
	2.6 (0.5)	2.7 (0.6)	3.8 (1.6)	2.3 (0.8)	3.4 (2.4)	5.9 (3.5)	2.7 (0.7)	4.2 (1.7)	4.9 (3.2)
Others[c]	0.48(0.12)	0.60(0.10)	1.11(0.23)	0.41(0.09)	0.66(0.09)	1.79(0.83)	0.43(0.11)	0.78(0.21)	1.22(0.32)
	18.8 (3.3)	22.8 (3.0)	28.3 (5.2)	16.5 (3.1)	23.6 (5.2)	25.7 (6.4)	17.8 (4.3)	24.1 (6.2)	25.7 (4.9)
Total	2.58(0.71)	2.64(0.94)	3.91(1.09)	2.46(0.52)	2.96(1.41)	6.38*(2.84)	2.36(0.29)	3.10(1.07)	5.35*(2.28)
E/NE[d]	0.86(0.11)	0.80(0.08)	0.87(0.11)	0.82(0.09)	0.83(0.10)	1.05(0.36)	0.79(0.07)	0.85(0.11)	1.25*(0.45)

[a] - nmol/mg dry weight (standard deviation in parentheses; n = 5)
[b] - percent contribution to total FAA pool (standard deviation in parentheses; n = 5)
[c] - includes asparagine, aspartic acid, glutamine, histidine, isoleucine, leucine, phenylalanine, serine, tyrosine and valine
[d] - Essential Amino Acids (E) - arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, tryptophan and valine
[d] - Non-essential Amino Acids (NE) - alanine, asparagine, aspartic acid, glutamine, glutamic acid, glycine, serine, tryosine
* - significantly different from control ($\alpha = 0.05$)

Table 19. The concentration (nmol/mg, wet weight) and percent composition of free amino acids in mantle tissue of C. fluminea exposed to 7.8 and 23.6 mg/l sodium dodecyl sulfate for 2, 5 and 10 days.

	Day 0	Day 2	Day 5			Day 10
Amino Acid	Control	23.6 mg/l	Control	7.8	23.6	7.8
Alanine	0.59[a](0.22)	3.03(0.40)	0.97(0.37)	1.83(0.31)	4.63(0.99)	4.15(1.87)
	20.9[b] (3.4)	41.4 (2.7)	31.0 (5.6)	47.5 (3.2)	42.7 (7.3)	46.6 (4.8)
Arginine	0.16(0.02)	0.28(0.10)	0.16(0.03)	0.17(0.03)	0.44(0.31)	0.49(0.16)
	5.7 (1.2)	4.0 (1.8)	5.6 (1.3)	4.2 (0.7)	4.2 (0.6)	6.1 (3.0)
Glutamine	0.25(0.05)	0.38(0.26)	0.10(0.02)	0.18(0.07)	0.32(0.18)	0.37(0.16)
	9.5 (2.4)	5.0 (4.4)	3.6 (1.7)	5.1 (2.2)	3.1 (2.2)	4.2 (1.0)
Glutamic Acid	0.80(0.30)	0.89(0.22)	0.48(0.10)	0.37(0.14)	0.73(0.49)	0.53(0.33)
	31.1 (5.8)	12.1 (2.8)	17.7 (6.3)	10.5 (1.0)	7.0 (4.8)	5.1 (1.7)
Glycine-Threonine	0.13(0.10)	0.29(0.09)	0.20(0.14)	0.09(0.03)	0.70(0.54)	0.29(0.16)
	4.4 (1.9)	3.9 (0.9)	6.0 (2.5)	2.3 (0.4)	6.1 (4.1)	3.3. (0.8)
Valine	0.10(0.04)	0.39(0.08)	0.17(0.06)	0.18(0.06)	0.59(0.09)	0.59(0.46)
	3.5 (0.5)	5.4 (0.7)	5.6 (0.6)	4.6 (0.9)	5.4 (0.5)	6.0 (1.6)
Others[c]	0.81(0.16)	2.05(0.62)	0.97(0.12)	1.24(0.39)	3.46(1.02)	2.88(0.91)
	24.9 (2.8)	28.2 (3.3)	30.5 (5.2)	25.8 (3.9)	31.5 (4.9)	28.7 (6.2)
Total	2.84(0.85)	7.31*(0.92)	3.05(0.96)	4.06(0.71)	10.87*(1.54)	9.21*(2.94)
E/NE[d]	0.34(0.05)	0.44(0.03)	0.40(0.03)	0.41(0.09)	0.45(0.16)	0.55(0.08)

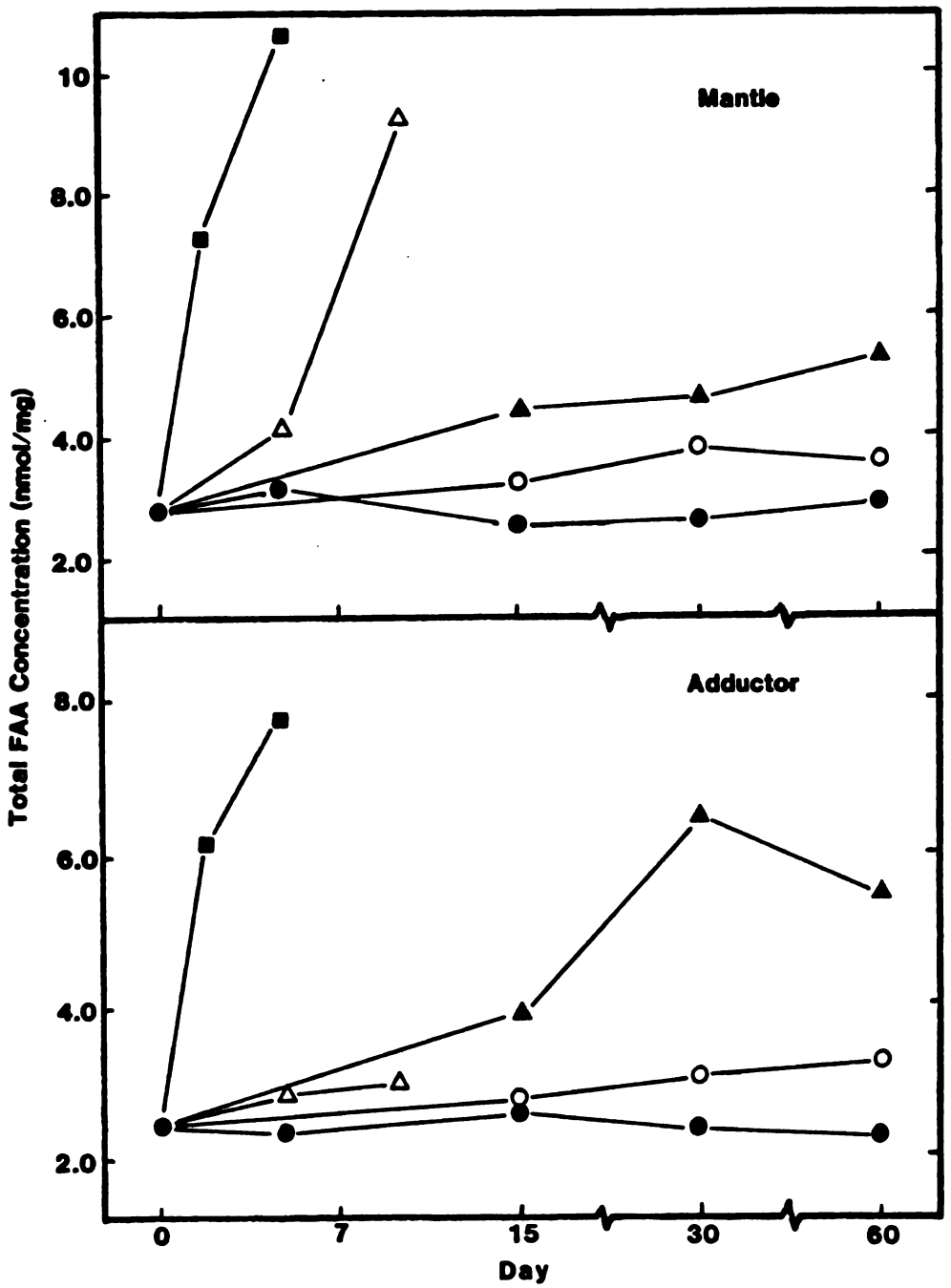
[a] - nmol/mg dry weight (standard deviation in parentheses; n = 5)
b - percent contribution to total FAA pool (standard deviation in parentheses; n = 5)
c - includes asparagine, aspartic acid, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, serine and tyrosine
[d] - essential amino acids (E) - arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, tryptophan, valine; non-essential amino acids (NE) - alanine, asparagine, aspartic acid, glutamine, glutamic acid, glycine, serine, tyrosine
* - significantly different from control ($\alpha = 0.05$)

Table 20. The concentration (nmol/mg, wet weight) and percent composition of free amino acids in mantle tissue of C. fluminea exposed to 0.65 and 3.0 mg/l sodium dodecyl sulfate for 15, 30 and 60 days.

	Day 15			Day 30			Day 60		
Amino Acid	Control	0.65	3.0	Control	0.65	3.0	Control	0.65	3.0
Alanine	0.73[a](0.22)	1.21(0.30)	1.21(0.28)	0.72(0.21)	1.75(0.56)	1.00(0.26)	0.89(0.10)	1.98(1.42)	1.80(0.65)
	29.2[b] (3.7)	37.2 (4.6)	27.3 (4.6)	27.5 (5.6)	44.2 (7.8)	21.8 (2.2)	31.2 (3.7)	48.5 (7.9)	33.9 (6.4)
Arginine	0.16(0.07)	0.17(0.07)	0.24(0.05)	0.16(0.05)	0.18(0.03)	0.20(0.07)	0.16(0.03)	0.14(0.03)	0.23(0.05)
	6.2 (0.9)	5.3 (1.1)	5.4 (0.6)	6.0 (1.4)	4.6 (0.8)	4.5 (1.2)	5.6 (0.8)	4.8 (1.6)	4.4 (0.5)
Glutamine	0.10(0.02)	0.13(0.04)	0.21(0.08)	0.14(0.04)	0.13(0.07)	0.33(0.12)	0.19(0.03)	0.07(0.06)	0.26(0.15)
	4.2 (1.0)	4.0 (1.4)	4.8 (1.1)	5.6 (1.3)	3.3 (1.3)	7.0 (1.4)	6.9 (1.6)	2.1 (0.4)	4.6 (2.2)
Glutamic Acid	0.53(0.12)	0.47(0.16)	0.91(0.18)	0.72(0.11)	0.36(0.09)	0.79(0.16)	0.48(0.11)	0.23(0.08)	0.52(0.22)
	21.3 (2.4)	14.6 (3.2)	20.5 (2.5)	28.2 (3.9)	9.4 (3.1)	17.7 (2.6)	16.8 (3.0)	8.8 (5.5)	9.7 (2.3)
Glycine-Threonine	0.14(0.05)	0.16(0.04)	0.24(0.09)	0.11(0.03)	0.15(0.10)	0.25(0.09)	0.10(0.05)	0.13(0.10)	0.30(0.18)
	5.8 (3.0)	5.0 (0.8)	5.3 (0.7)	4.3 (0.8)	4.2 (3.1)	5.4 (1.2)	3.4 (1.1)	3.7 (0.9)	6.2 (4.6)
Valine	0.14(0.03)	0.17(0.05)	0.30(0.08)	0.12(0.03)	0.19(0.04)	0.37(0.11)	0.19(0.07)	0.17(0.12)	0.44(0.12)
	5.7 (1.3)	5.3 (1.1)	6.8 (1.4)	4.8 (0.8)	4.8 (0.7)	8.1 (1.2)	6.4 (1.3)	4.9 (0.9)	8.5 (1.5)
Others[c]	0.69(0.11)	0.93(0.26)	1.33(0.19)	0.60(0.17)	1.13(0.32)	1.61(0.49)	0.87(0.18)	0.78(0.24)	1.70(0.33)
	27.6 (5.2)	28.6 (4.9)	29.9 (3.2)	23.6 (4.2)	29.5 (3.8)	35.5 (6.2)	30.0 (5.7)	27.2 (3.4)	32.7 (2.8)
Total	2.50(0.47)	3.25(0.87)	4.45*(1.32)	2.57(0.41)	3.89*(0.58)	4.55*(1.01)	2.88(0.52)	3.51(1.62)	5.25*(1.41)
E/NE[d]	0.38(0.06)	0.40(0.05)	0.43(0.07)	0.35(0.04)	0.44(0.06)	0.60(0.06)	0.47(0.07)	0.43(0.07)	0.53(0.07)

[a] - nmol/mg dry weight (standard deviation in parentheses; n = 5)
b - percent contribution to total FAA pool (standard deviation in parentheses; n = 5)
c - includes asparagine, aspartic acid, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, serine and tyrosine
d - essential amino acids (E) - arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, tryptophan, valine
d - non-essential amino acids (NE) - alanine, asparagine, aspartic acid, glutamine, glutamic acid, glycine, serine, tyrosine
* - significantly different from control ($\alpha = 0.05$)

Fig. 5. The total free amino acid (FAA) concentration (nmol/mg wet weight) in adductor muscle and mantle tissue of Corbicula fluminea exposed to sodium dodecyl sulfate.


Mantle
Adductor
Total FAA Concentration (nmol/mg)
10
8.0
6.0
4.0
2.0
8.0
6.0
4.0
2.0
0
7
15
30
60
Day

exposed to 3.0 mg/l SDS and in adductor muscle when clams were exposed to 0.65 or 3.0 mg/l SDS.

Sixty days exposure to sublethal concentrations of SDS resulted in changes identical to those observed after 30 days. The total FAA concentrations were significantly increased, profile analysis resulted in a singular matrix and the percent composition of specific FAA such as alanine and glutamic acid were significantly altered (Tables 18 and 20).

Oxygen Consumption

Since body size had a significant effect on oxygen consumption, a standard correction for body size, derived by Linear regression (equation 3), was applied before testing for treatment effects (Bayne et al., 1982).

$$Y' = YX^{-b} \qquad (3)$$

where:

Y' = mass corrected oxygen consumption, (nmol/h)

X = wet tissue weight (g)

b = weight exponent (slope of regression).

In the present experiment, the equation of the regression line was $Y = 0.589x + 0.88$ ($r^2 = 0.81$). The value of 0.589 was on the lower side of the values reported for most mollusks, although still well within the reported range (Bayne and Newell, 1983).

The weight-corrected oxygen consumption was significantly reduced in C. fluminea exposed to 3.0 or 7.8 mg/l SDS (Table 21). At 3.0 mg/l, the decrease was observed after 5, 15 and 30 days exposure, however, on

Table 21. The weight-corrected oxygen consumption (nmol/g/hr) normalized to both dry and wet weight for C. fluminea exposed to sodium dodecyl sulfate for 60 days.

Concentration (mg/l)	Normalization (weight)	Day 0	5	15	30	60
Control	Dry	11.3 (1.5)[c]	9.7 (2.21)	10.6 (1.36)	9.97 (1.57)	11.00 (1.44)
	Wet	4.07 (0.55)	3.41 (0.22)	3.70 (0.43)	3.61 (0.58)	3.78 (0.47)
0.65	Dry	-	10.96 (0.99)	11.77 (2.72)	12.3 (3.95)	11.33 (1.19)
	Wet	-	3.74 (0.19)	4.22 (0.81)	4.24 (0.87)	4.14 (0.43)
3.0	Dry	-	8.23[b] (3.46)	8.68[b] (2.39)	8.38[b] (1.74)	11.67 (2.53)
	Wet	-	2.72[a] (0.87)	3.02[b] (0.74)	2.77[a] (0.63)	3.67 (0.42)
7.8	Dry	-	5.63[a] (1.93)	-	-	-
	Wet	-	1.94[a] (0.60)	-	-	-

[a] - significantly less than control and 0.65 mg/l exposure within day ($\alpha = 0.05$)
[b] - significantly less than 0.65 mg/l exposure within day ($\alpha = 0.05$)
[c] - standard deviation (n=8)

Table 22. Condition index[a] for *Corbicula fluminea* exposed to sodium dodecyl sulfate. $\bar{X} \pm$ standard deviation in parentheses, n = 8

Measured Concentrations	Day 0	Day 5	Day 15	Day 30	Day 60
Control	0.745 (0.083)	0.790 (0.15)	0.784 (0.12)	0.770 (0.14)	0.733 (0.16)
0.65	-	0.700 (0.20)	0.794 (0.14)	0.870 (0.13)	0.827 (0.13)
3.0	-	0.690 (0.26)	0.764 (0.18)	0.610 (0.16)	0.531[b] (0.14)
7.8	-	0.770 (0.19)	-	-	-

[a] - C.I. = (Dry weight/shell length) x 100

[b] - significantly lower control and 0.65 mg/l clams within day ($\alpha = 0.05$)

Table 23. Whole body percent water of C. fluminea exposed to sodium dodecyl sulfate. $\bar{X} \pm$ standard deviation in parentheses, n = 8.

Measured Concentrations	Day 0	5	15	30	60
Control	82.2(1.6)	82.9(1.5)	83.1(1.1)	82.1(1.6)	83.5(1.3)
0.65	-	83.6(2.6)	82.1(1.6)	82.8(3.1)	81.8(1.0)
3.0	-	83.7(2.9)	83.2(1.2)	84.7(1.7)[a]	85.4(2.3)[b]
7.8	-	83.3(2.6)	-	-	-

[a] - significantly greater than control within day ($\alpha = 0.05$)
[b] - significantly greater than control and 0.65 mg/l within day ($\alpha = 0.05$)

day 60, oxygen consumption in SDS-exposed clams had increased and was no longer significantly different from controls. Oxygen consumption of control organisms did not change during the treatment period.

Condition Index and Tissue Water

A significant decrease in condition index (CI) was observed in C. fluminea exposed to 3.0 mg/l SDS for 60 days (Table 22). No change in CI was observed in control clams during the 60 day treatment period.

The percent tissue water content was significantly increased in C. fluminea exposed to 3.0 mg/l SDS for 30 and 60 days (Table 23). During the 60 day treatment period the percent water was extremely consistent in control and 0.65 mg/l SDS exposed clams.

Discussion

Species Comparisons

The concentrations of total and individual FAA in C. fluminea is similar to that reported for other freshwater bivalves. The total FAA concentration of C. fluminea foot muscle was reported to be 20.4 nmol/mg dry weight (Gainey, 1978), which when normalized to wet weight, is very similar to the values observed in the present study. Similarly, the total FAA content of Ligumia subrostrata (Say) was reported to be 23.6 nmol/mg, dry weight (Hanson and Dietz, 1976). The total FAA concentration of ventral adductor muscle of Anodonata sp. was 9.5 nmol/mg total water (Potts, 1958). Therefore, as with other freshwater molluscs, the total FAA concentration of C. fluminea is relatively small.

The relative concentration of individual FAA was also similar to those reported for other freshwater bivalves. In the present study, alanine and glutamate represented the most abundant FAA in the mantle

tissue of C. fluminea, followed by glutamine, glycine and arginine. Alanine also represented a major portion of the adductor muscle total FAA, however arginine was the next most abundant, followed by glutamate and glycine. Similar to this, Gainey (1978) found alanine to be the most abundant FAA in C. fluminea followed by glutamate, serine and aspartate. Alanine and glutamate also were found to contribute the greatest percentage to the total FAA pool in the freshwater mussels Amblema plicata (Gardner et al., 1981) and Hyridella menziesi (Bedford, 1973). In Ligumia subrostrara, arginine represented in most abundant FAA, with glutamate and alanine representing the second and fourth most abundant (Hanson and Dietz, 1976).

Acute Exposure

No other studies have been conducted on the effects of acute toxicant exposure on the concentration of FAA in freshwater mollusks, however several studies have been conducted with other freshwater aquatic invertebrates. Similar to the results of the present study, freshwater crabs acutely exposed to the organophosphate insecticide, Sumithion®, had a significantly greater total FAA concentration (Bhagyalakshmi et al., 1983). Since the authors did not measure concentrations of individual FAA, it is unknown whether the FAA profile was also altered. A significant decrease in the total FAA concentration was measured in the freshwater amphipod Gammarus pseudolimnaeus acutely and chronically exposed to pentachlorophenol, which is opposite to that observed in the present study (Chapters 2 and 3). However, similar to the present study, a significant change in the relative concentration of individual FAA was observed. Many of the FAA responsible for the profile change in

G. pseudolimnaeus, such as alanine, glutamine, isoleucine and leucine, were also responsible for the profile differences observed in C. fluminea, although the magnitude and direction of the changes were considerably different. Given this, it is likely that different mechanisms were responsible for the changes observed in C. fluminea compared with those observed in G. pseudolimnaeus.

The concentration of FAA in marine molluscs has been shown to be altered during acute toxicant exposure. The oyster Ostrea edulis exposed to naphthalene for 72 h had significantly greater gill concentrations of alanine, aspartate and glutamate (Riley and Mix, 1981). This is similar to the results of the present study in that alanine concentrations were increased, however in C. fluminea exposed to SDS, aspartic acid was not significantly altered and glutamic acid concentrations decreased. The total FAA concentrations of oysters were significantly altered when exposed to drilling effluents for two to five days (Powell et al., 1982). After two days exposure, the total FAA concentration was greater than that of control organisms. This was primarily due to elevations in cysteic acid, glutamic acid, alanine and β-alanine. Powell et al., (1982) concluded that the initial increase may be the result of self-induced anoxia, a mechanism which may have also been responsible for changes observed in the present study and which will be discussed in greater detail below. After five days exposure, however, all individual amino acids and thus the total FAA concentration had decreased in tissues of oysters exposed to drilling effluent (Powell et al., 1982). Powell et al. attributed the decrease to either the catabolism of FAA to meet a stress-generated energy demand or a general loss of FAA from the body tissues to the surrounding water.

Similar to the present study, the relative concentration of individual FAA were altered in grass shrimp (Palaemonetes pugio) exposed to polychlorinated biphinyls for 72 h (Roesijadi et al., 1976). Since the total FAA concentration was not altered, the changes were interpreted as toxicant-induced alteration in the metabolic state of the organism. As will be discussed below, this mechanism may also have been responsible for the changes observed in the pattern of FAA concentrations of C. fluminea.

There was no significant effect on the essential/non-essential FAA (E/NE) ratio of either adductor muscle or mantle tissue of clams acutely exposed to SDS (Table 15). Changes in the E/NE ratio often can be indicative of alterations in protein metabolism. In mammals, dietary protein deficiency can cause a decrease in the E/NE ratio, generally as a result of a depletion of essential amino acids (Munro, 1972). This type of change is generally observed during long-term stress and would not be expected to occur under acute conditions.

Chronic Exposure

Free Amino Acids

Exposure to 7.8 and 23.6 mg/l SDS was lethal to C. fluminea and caused alterations in the concentrations of FAA similar to those observed in the preliminary static acute exposure. Increases in the total FAA concentration were observed in both the adductor muscle and mantle tissue of clams from both exposures, although in the chronic study the affects were more dramatic and consistent. The alterations in the concentration of specific FAA were also similar in both exposures. Alanine concentrations and its percent contribution to the total FAA pool were

consistently higher in exposed organisms from both static acute and flow-through chronic experiments. In addition, glutamic acid concentrations either decreased or were not affected by SDS exposure, even though the total FAA concentrations were increasing. Thus, under acutely toxic exposure regimes, the type of alterations observed in the concentration of FAA are fairly consistent and reproducible.

The potential mechanisms responsible for changes in the concentration of FAA in chemically or physically stressed aquatic invertebrates can be separated into three categories. The first encompasses the complex mechanisms controlling osmotic regulation and the role of FAA in that process. The second category involves the effects of anoxia on the FAA pool. This mechanism can be indirectly linked to osmo-regulatory effects and primarily applies to bivalve mollusks. The third category covers the effect of stress on protein metabolism and subsequent changes in the concentration of FAA. None of these potential mechanisms are exclusive and quite often all three may be interacting to produce the observed FAA alterations. In addition, there is very little conclusive evidence in the literature establishing that any one of these mechanisms causes changes in FAA during toxicant-induced stress. Rather, these are proposed mechanisms which may be responsible for some of the FAA alterations which have been observed.

Osmoregulation maintains a constant intracellular mileau such that normal metabolic processes can proceed. Changes in the intracellular salt concentrations can inhibit enzyme activity, thus interfering with essential biochemical processes (Yancey et al., 1982; Bowlus and Somero, 1979). Many organisms have evolved mechanisms to maintain intracellular iso-osmotic conditions by regulating specific organic constituents such

as amino acids or polyhydric alcohols instead of inorganic ions. The situation is most evident in marine invertebrates, where high extracellular osmolyte concentrations exist such that intracellular organic constituents are required to maintain iso-osmotic conditions. Since freshwater organisms are generally considered to be efficient hyperosmotic regulators and are able to maintain relatively low extracellular osmolyte concentrations, relative to marine organisms, FAA are not as important in intracellular osmoregulation. However, FAA can constitute 10-20 % of the active intracellular osmolytes (Gilles, 1979) and are involved with osmoregulation in freshwater molluscs (Hanson and Dietz, 1976). Therefore, changes in the osmolyte concentrations in the extracellular fluid can potentially cause compensatory adjustments in the intracellular FAA pool.

A variety of mechanisms exist by which toxicants can alter the osmolyte composition of extracellular body fluids in freshwater molluscs. During toxicant exposure, a decrease in hemolymph osmolality via impaired osmoregulatory ability is the primary affect observed. This decreased osmolality can be caused by 1) direct damage to gill with subsequent water uptake and salt loss; 2) inhibition of gill enzyme systems responsible for ion exchange, such as Na/K^+ ATPase; 3) toxicant damage to excretory organs, such as the kidney and/or; 4) interference with endocrine control of osmoregulatory processes. Presently it is unknown whether a change in hemolymph osmolality will cause a concomitant change in the intracellular FAA concentrations. In freshwater amphipods exposed to pentachlorophenol, a significant decrease in the total FAA concentration was reported (Chapter 2). This decrease was attributed to a disruption of osmoregulatory ability. Freshwater mollusks exposed to

hyposmotic conditions have been shown to respond by decreasing the concentration of specific intracellular FAA (Gainey, 1978). However, since a significant increase in the total FAA pool was observed in the present study, a breakdown of whole organism hyperosmotic regulation was not the mechanism responsible for FAA alterations.

An increase in hemolymph osmolality may have occurred, however, and have been partially responsible for the increase in FAA. Exposure of freshwater molluscs to hyper-osmotic conditions can cause an increase in the concentration of intracellular FAA (Bedford, 1973; Gainey, 1978). Bivalve mollusks exposed to physical and/or chemical stressors, can avoid exposure by closing their shells. This avoidance response temporarily prevents exposure; however, it also creates a self-induced anoxia. The build-up of end products, such as CO_2 or lactic acid can result in acidemia, which can influence the ionic composition of the hemolymph *via* dissolution of readily available buffering substrates present in the shell. A five- to eight-fold increase in the Ca^+ concentration of hemolymph was recorded in the freshwater mussel *Ligumia subrostrata* exposed to anoxic conditions (Dietz, 1974). Such an increase in hemolymph osmolality could result in an increase in intracellular FAA concentrations (Hanson and Dietz, 1976). In the present study, an avoidance response by the clams was observed, especially exposed to 7.8 or 23.6 mg/l SDS. This may have caused osmolyte imbalances such that maintenance of iso-osmotic conditions required an increase in intracellular FAA.

Alterations in the ionic composition of hemolymph is not the only mechanism by which anoxia can influence the concentration of FAA. Many invertebrates are considered to be facultative anaerobes. The ability

to survive oxygen deprivation varies considerably among species and is primarily dependent on an organism's ability to replace oxygen with alternative electron acceptors or shift key metabolic pathways such that redox balance is maintained (DeZwaan, 1983). Substrate-level phosphorylation can be increased considerably *via* amino acid catabolism. Anaerobic degradation of aspartate results in a concomitant increase in alanine *via* specific tranasaminase reactions, with the end product succinate being produced *via* the reduction of fumurate. Thus, concentrations of aspartate, glutamate and alanine can be directly altered in faculative anaerobes exposed to anoxic conditions. Alterations in some of these FAA were observed in the present study. Alanine concentrations and percent composition were significantly increased in both adductor and mantle tissues of *C. fluminea* exposed to SDS. In addition, concentrations of glutamate, which are required for the transamination of aspartate and formation of alanine, were significantly decreased relative to other FAA. However concentrations of aspartate, which only comprised 2-3 and 0.5-1.0 percent of the total pool in mantle and adductor tissues, respectively, did not change significantly or increased slightly due to exposure to SDS. This is not consistent with the changes in aspartate observed in other faculatively anaerobic invertebrates which have been exposed to anoxic conditions (DeZwaan, 1983; Collicut, et al., 1977).

To determine if metabolic anoxia/hypoxia may have been responsible for some of the changes in FAA observed in SDS exposed organisms, two questions need to be considered. First, did exposure to SDS cause either self-induced anoxia/hypoxia *via* shell closure or toxicant-induced anoxia/hypoxia via interference with respiratory mechanisms? When

C. fluminea were exposed to 7.8 or 23.6 mg/l SDS, a decrease in siphoning activity was observed. This type of avoidance behavior could have induced anoxia and caused subsequent changes in concentrations of specific FAA. Increases in alanine concentrations in oysters exposed to drilling effluents were thought to be associated with self-induced anoxia (Powell et al., 1980). However, similar to the present study, aspartic acid concentrations did not change, and the authors concluded that the alterations in FAA were probably due to a variety of metabolic changes, only one of which was self-induced anoxia. Therefore, in the present study, valve closure may have caused oxygen deprivation and been partially responsible for the increase in alanine concentrations. However, surfactants have been shown to directly affect the adductor muscle of bivalves such that during longer exposures, valve closure is inhibited (Swedmark, et al., 1971). This being the case, it is difficult to determine whether self-induced anoxia occurred throughout the entire exposure.

The direct effect of SDS on the gills of C. fluminea may have also caused oxygen stress in the organism. During acute exposures to SDS, asphyxiation is thought to be the primary mechanism of toxicity (Abel, 1974). Extensive gill damage results in impaired oxygen exchange, thus inducing compensatory adaptive mechanisms such as anaerobic metabolism. In the present study, exposure to 3.0 or 7.8 mg/l SDS caused a significant decrease in the oxygen consumption of C. fluminea (Table 21). This being the case, FAA involved in anaerobic metabolism may be altered due to SDS-induced respiratory stress. Initially anoxia may have been caused by valve closure; whereas, after longer exposure when valve

closure was impaired, anoxia may have resulted from respiratory inhibition.

The second question concerns the presence of anaerobic pathways in C. fluminea. Although direct measurement of the capacity of these organisms to biochemically adapt to anoxic conditions has not been conducted, respiratory responses and survival of C. fluminea during anoxia indicate a limited capability to adapt to oxygen deprivation (McMahon, 1979). This would seem to indicate that C. fluminea is not a facultatively anaerobic and, thus, does not possess the anaerobic pathways discussed above. However, since this has not been established definitively, one cannot eliminate the possibility that changes observed in FAA were the result of anoxia induced FAA catabolism.

The third mechanism by which toxicant-induced stress can potentially alter the concentration of FAA involves direct and indirect effects on protein metabolism. Since proteins are composed of amino acids, changes in the complex processes controlling protein synthesis, transport and degradation can influence the concentration and/or relative composition of the FAA pool. There are a multitude of potential mechanisms by which toxicants can effect protein metabolism. However, with respect to changes in the FAA concentrations of C. fluminea exposed to SDS, only two will be discussed. The first mechanism involves the release of FAA via protein degradation and subsequent utilization in energy metabolism. The second entails the influence of SDS on enzymes involved with protein and amino acid metabolism. Other mechanisms, some of which are discussed in Chapter 2 with respect to the influence of pentachlorophenol on the concentration of FAA in the freshwater amphipod Gammarus pseudolimnaeus, include 1) alterations in amino acid membrane

transport, 2) repair of damaged tissue, 3) changes in protein synthetic rates, and 4) increased efflux to the exterior environment.

The stress associated with toxicant exposure can result in the mobilization of energy reserves, generally for the purpose of maintaining homeostasis (Giesy et al., 1986; Thomas et al., 1981; Riley and Mix, 1981). For many invertebrates, amino acids generated *via* protein degradation contribute significantly to the total energy budget of the organism (Gilles, 1970; Bayne, 1973; Bursell, 1966). Protein has been shown to be mobilized as an energy substrate in invertebrates under stressful conditions (Bayne, 1973; Riley and Mix, 1981; Bhagyalakshmi, et al., 1983). Similarly, alterations in the concentration of FAA has been attributed to proteolysis. An increase in alanine, aspartate and glutamate in oysters acutely exposed to naphthalene was attributed to accelerated protein catabolism stimulated by stress-induced energy demands (Riley and Mix, 1981). A similar explanation was proposed for the increase in alanine observed in lobsters exposed to petroleum hydrocarbons (Payne et al., 1983) and for the increase in total FAA concentrations in sea anemones collected after an oil spill (Kasschau and Howard, 1984). The decrease in the total FAA concentration in oysters exposed to drilling effluents was partially attributed to increased amino acid oxidation (Powell et al., 1982). The authors felt that the increased energy demand associated with toxicant stress created a general draw on the FAA pool.

The alterations observed in the FAA profile of *C. fluminea* exposed to SDS may be indicative of increased proteolysis. During starvation, increased protein degradation can influence the composition of the FAA pool (Munro, 1970). In the present study, the increase in the adductor

muscle E/NE ratio may be indicative of such an alteration (Table 18). In the American oyster, Crassostrea virginica, starvation increased proteolysis, and over time, the composition of the FAA pool and protein hydrolysate became increasingly similar (Riley, 1980).

Even if protein could be mobilized as an energy substrate, it is still not possible to predict the influence on the FAA pool. In the present study, the increase in the total FAA pool and the change in FAA profile may have been partially attributed to protein degradation; however, other factors were also likely to be involved.

Changes in the activity of enzymes that regulate nitrogen flow can directly influence the composition of the FAA pool. Glutamate dehydrogenase (GDH), which catalyzes the oxidative deamination of glutamate, is allosterically activated by ADP and inhibited by ATP, such that the increased energy demand of stress should activate GDH and accelerate protein degradation. Increased GDH activity in freshwater crabs exposed to Sumithion® was correlated with decreased FAA concentrations (Bhagyalakshmi, et al., 1983). Aminotransferase enzymes catalyze the reversible transfer of an amino group from glutamate to the α-keto acid of a particular amino acid. These enzymes play a pivotal role in intermediate nitrogen metabolism and alterations in their activity can result in changes in the FAA pool. Rock crabs exposed to cadmium had significantly increased heart aspartate aminotransferase (AAT) activity (Gould et al., 1976). The authors attributed this increase to compensatory adaptation by the organism in an attempt to provide additional energy to meet the demand created by toxicant stress. In addition, Kasschau et al., (1980) attributed increased concentrations of glutamate in toxicant-exposed sea anemones to changes in protein metabolism, possibly

via aminotransferase alterations. Other studies have reported alterations in transaminase activity in invertebrates under toxicant-induced stress (Chambers et al., 1978; Phelps, 1981; Blackstock, 1978).

Alteration in the structure and/or function of proteins has been proposed as a mechanism by which sublethal concentrations of surfactants exert their effect (Abel, 1974). Low concentrations of surfactants have the ability to increase or decrease the activity of enzymes (Manwell and Baker, 1967). In order for this to be a viable mechanism of surfactant toxicity, uptake of the toxicant by the animal must occur. Sodium dodecyl sulfate has been shown to be absorbed primarily through the gills and rapidly distributed throughout the body of freshwater fish (Tovell et al., 1975). Presently, it is unknown whether freshwater clams accumulate surfactants. Assuming uptake and transport of SDS occurred in C. fluminea, then interaction with enzymes involved in nitrogen metabolism may result in an alteration in the concentration of FAA. Because surfactants can influence enzyme activity in a variety of ways, it is impossible to determine the exact nature of any changes in FAA which may occur.

Oxygen Consumption

The oxygen consumption measured for C. fluminea in the present study was very similar to values reported for C. fluminea by other investigators (Britton and Morton, 1982; Habel, 1970).

Oxygen consumption in toxicant-exposed invertebrates has been shown to both increase and decrease. The magnitude and direction of the change is dependent upon the organism, type of toxicant and dose. Respiration rates have been shown to increase at low concentrations of

petroleum hydrocarbons (Gilfillan et al., 1976; Stickle et al., 1984; Stekoll, 1980) and decrease at greater concentrations (Edwards, 1978; Capuzzo et al., 1984; Stainken, 1978). Similarly, metals can cause either an increase (Johns and Miller, 1982) or decrease (Depledge, 1984) in oxygen consumption. A variety of explanations have been proposed for increased metabolic rates during toxicant exposure; although, for the most part, the exact mechanisms remain unknown. The increased energy demand and associated mobilization of stored energy reserves during toxicant stress may create a greater oxygen demand. In addition, the inhibition of specific biochemical pathways by certain toxicants may result in increased metabolic rates. For example, the uncoupling of oxidative phosphorylation by pentachlorophenol increases oxygen consumption in freshwater snails (Weinbach and Nolan, 1956). Decreases in respiration also may be caused by toxicant-induced alterations in a variety of interrelated processes. Respiration and activity are inseparably related such that toxicants which directly or indirectly suppress activity can cause a decrease in oxygen consumption (Percy, 1977; Sprague, 1971). In bivalves, avoidance of the toxicant *via* valve closure can also decrease oxygen uptake. In addition, chemicals can directly influence mechanisms involved with oxygen uptake. Narcotization of ciliary surfaces by petroleum hydrocarbons can reduce water flow over the gill surfaces, thus decreasing oxygen uptake (Galtsoff et al., 1935; Stainken, 1978). Increased mucus secretion and/or direct damage to the gill can be caused by a variety of toxicants and will interfere with oxygen uptake (Able and Skidmore, 1975; Skidmore and Tovell, 1972; Fromm, 1980). So given the variety of mechanisms possible, identifying

the cause of the changes observed in oxygen consumption can be extremely difficult.

The decrease in oxygen consumption in C. fluminea exposed to SDS was likely caused by toxicant avoidance via valve closure and/or gill damage. Reduced siphoning activity observed at the 7.8 mg/l SDS exposure may have been an avoidance response, causing decreased oxygen consumption. However at the sublethal exposure of 3.0 mg/l, an avoidance response was not observed. Surfactants are known to cause gill damage (Abel, 1974; Abel and Skidmore, 1975), and such damage may have decreased to uptake of oxygen by C. fluminea.

Condition Index

The CI, which can be used as a general indicator of the relative health of bivalves, reflects the energy stored as glycogen, lipids and protein. Since deposition of energy reserves occurs only when energy intake exceeds the organisms maintenance requirements, CI can be indicative of an organisms recent energy metabolism. Environmental factors have been shown to influence CI, including salinity (Haven, 1960), substrate (Peddicord, 1977), food (Korringa, 1952), sex (Gabbot and Bayne, 1973), and season (Zandee et al., 1980). In addition, the increased energy demand associated with toxicant-induced stress may require the mobilization of energy substrates such that CI is reduced. Decreases in CI have been reported for bivalves exposed to oil (Roesijadi and Anderson, 1979; Stekoll et al., 1980) and been correlated with reduced scope for growth (Martin et al., 1984). In the present study, the combined effect of impaired feeding and increased energy demands may have caused the mobilization of stored energy reserves, thus decreasing CI.

Tissue Water Content

The tissue water content compared favorably with values reported for other freshwater mulluscs (Bedford, 1973; Potts, 1958). Percent water varies seasonally and has been shown to be inversely related to glycogen content (Zandee et al., 1980). It is possible that water replaces the glycogen, which has been mobilized to meet the energy or reproductive requirements of the organism. This was proposed as a potential mechanism responsible for the decrease in the dry/wet weight ratio in clams chronically exposed to oil (Stekoll et al., 1980). Disruption of osmoregulatory ability is another mechanism that may alter the water content of an organism. Exposure of the marine polychaete Neanthes virens to silver caused a significant decrease in the water content (Pereira and Kanungo, 1981). This is opposite the effect observed in the present study; however, marine organisms are hypoosmotic to their environment such that osmotic imbalance would result in the loss of water, whereas hyperosmotic freshwater organisms would gain water during osmotic imbalance.

In the present study, the increase in percent water may have been caused by either a breakdown in osmoregulatory ability or the mobilization of energy reserves. Sodium dodecyl sulfate causes gill pathology and eventual loss of ionic balance (Abel, 1974). The decrease in condition index also observed indicates that mobilization of energy reserves was also occurring. So, it is likely that both of these factors were involved in the increase in percent water content.

Summary and Conclusions

The total concentrations of FAA and/or the relative FAA profile were significantly altered by sublethal exposure to SDS. This is most

likely a general stress response and not a specific alteration caused by a specific mechanism of toxicity unique to SDS. The FAA alterations observed may have been caused by self-induced anoxia stimulated by exposure to the toxicant; however, it is unlikely that this caused the changes observed at the lower SDS concentrations. The generality of the response cannot be determined until additional testing can be conducted with a wide range of toxicants. The advantages and disadvantages of a "general" vs "specific" indicator are discussed by Giesy et al. (1986).

In relation to the use of FAA as an *in situ* BIS, two additional questions need to be addressed with respect to the changes observed in FAA, oxygen consumption and CI. The first pertains to the relative sensitivity of alterations in the concentration of FAA with respect to changes in a more traditional, higher level parameter such as respiration. Comparison of the minimum effect level reveals that FAA are a more sensitive indicator of exposure to sublethal concentrations of SDS. At 0.65 mg/l SDS, no effect on respiration was observed whereas significant changes in the FAA profile were apparent. However, this raises the second question. What is the biological and/or ecological significance of changes observed in the concentration of FAA? At the 0.65 mg/l exposure, where no effects were observed on any of the other parameters investigated, it is impossible to determine whether the changes observed in the FAA profile were indicative of potential adverse effects on the organism's reproductive potential. This being the case, the fact that FAA may be sensitive to toxicant-induced stress does not automatically make them useful as a BIS. An extremely sensitive indicator is of no use if the toxicant concentration at which it responds has no adverse effect on the population, community or ecosystem. However, at the

higher, but still sublethal concentration of 3.0 mg/l SDS, alterations were observed in both oxygen consumption, CI, water content and FAA. Changes in oxygen consumption and CI can be more easily related to adverse effects on organism growth and possibly reproduction. Any effect which decreases the energy available for growth may alter reproduction potential (Bayne et al., 1978; Bayne et al., 1982; Calow, 1973). This is especially true for iteroparous species, in which fecundity can be directly related to the size of the parent (Calow, 1979). Decreased oxygen availability may impair a variety of energy (ATP) producing and homeostatic processes required to maintain growth. So, the fact the FAA change at toxicant concentrations similar to those that impair oxygen consumption and CI indicates that at these concentrations, alterations in the concentration of FAA may be indicative of future adverse effects on the biology of the organism.

Chapter 6

Free Amino Acid Pools of Five Species of Freshwater oligochaetes

Introduction

Aquatic oligochaetes are functionally important in both lentic and lotic ecosystems (Brinkhurst and Jamieson, 1971). Essential roles include sediment reworking and diagenesis (McCall and Fisher, 1980), nutrient cycling (Gardener et al., 1983) and trophic level energy transfer (Yaroskenko et al., 1980). Their ecological importance and cosmopolitan distribution has resulted in a considerable amount of research on their biology and ecology (Brinkhurst and Cook, 1980). Variations in species sensitivity to endogenous and exogenous environmental conditions such as anaerobiosis or toxic chemicals have led to the use of certain oligochaetes as "pollution indicator" species (Brinkhurst, 1980; Chapman et al., 1982). Laboratory toxicity tests, using aquatic oligochaetes, have been developed for assessing the potential hazards of environmental contaminants (Hornig, 1980; Fisher et al., 1982).

Assessment of the relative health of organisms occupying the aquatic environment is an extremely difficult task and one which has been receiving increased attention in recent years (Waldichuk, 1979). A variety of biochemical indicators of organismal level stress have been proposed as monitoring tools for assessing organism health (Livingstone, 1982; Giesy et al., 1983). In the marine environment, alterations in the FAA pool of bivalve molluscs have been successfully utilized as an indicator of toxicant exposure (Roesijadi and Anderson, 1979). The potential exists for similar applications using freshwater oligochaetes; however, extensive preliminary investigations are required to ensure adequate interpretation of any toxicant-induced alterations. Little information exists on the physiology and biochemistry of these organisms. Gross chemical composition of specific aquatic oligochaetes

(Whitten and of Tubifex tubifex (Hipp et al., 1984; Mustafa et al., 1983; Hoffman et al., 1979) have been investigated; however, no information on the free amino acid (FAA) pools of freshwater oligochaetes has been reported. Establishing such a data base is a prerequisite for developing an in situ biochemical indicator of stress based on alterations in the FAA pool. The objective of this study was to quantify the FAAs of the tubificids Limnodrilus hoffmeisteri, Tubifex tubifex, Potamothrix moldaviensis, Potamothrix vejdovskyi, and the lumbriculid Stylodrilus heringianus sampled from southeastern Lake Michigan. These organisms represent dominant oligochaete species in Lake Michigan and are extremely important in the profundal ecology of the system. The comparative aspects of the FAA pool are discussed in relation to the organisms' ecology, reproduction and distribution and potential use of FAA in oligochaete taxonomy.

Materials and Methods

Oligochaetes were obtained from sediment samples collected with successive "Ponar"® grabs on April 30, 1984, in 40 m of water in southeastern Lake Michigan (approx. 6 miles offshore of St. Joseph, MI, 42.00.81°; 86.44.11°). Sediments were returned to the lab in coolers, transferred to large holding tanks, and maintained in the dark at oxygen saturation and 10°C. Prior to selecting oligochaetes for FAA analyses, the dominant species were identified by mounting and clearing several individuals in a lactophenol solution. Fresh worms were then sorted by species, identified under a dissecting microscope, and dried at 65°C for 24 hrs. After drying, species were weighed and stored in a dessicator

until extraction. For reasons discussed below (Discussion), worms were not allowed to purge their gut contents before drying.

Amino acids were analyzed by reverse phase (C-18) high performance, liquid chromotography (HPLC) using pre-column o-phthalaldehyde (OPA) derivitization and fluorescence detection (Jones et al., 1981; Bhown et al., 1983). Two or three individual organisms were pooled for each analysis. Free amino acids were extracted from whole organisms in 500 µL of methanol in a teflon glass homogenizer. After centrifugation, supernatants were decanted and stored in a freezer (-10°C) prior to analyses. Further description of the techniques utilized are presented in Chapter 2.

Patterns of relative FAA concentrations within and among species were compared by univariate and multivariate statistical techniques. Total FAA concentrations of the different species were compared by analysis of variance (ANOVA) followed by Duncan's Multiple Range Test. Potential numerical taxonomy of the oligochaete species, based on individual FAA pools, was developed using normal and canonical discriminate analysis. Discriminant analysis is a multivariate technique used to classify objects such as oligochaete species into groups based on specified continuous variables such as amino acids (Rao, 1973). Canonical discriminate analysis is similar to principal component analysis in that given a group of objects (species) possessing specific continuous variables (amino acids), a linear combination of these variables is derived such that between-class variation is maximized (Cooley and Lohnes, 1971). Each of these techniques would have different applications. The advantage of normal discriminate analyses over canonical discriminate analyses is that once a classification data set has been

established, a test data set can be entered such that the organisms will be classified into a particular species based on their FAA pools. This technique has predictive capabilities and can be used to classify organisms with a specified probability. Canonical discriminate analysis is more of a dimension-reduction technique used to summarize or demonstrate the relative separation of objects (i.e. species). It can be used to graphically represent the difference between objects (based on quantitative variables) but has little or no predictive capabilities. All analyses were performed using the Statistical Analyses System (SAS 1982).

Results

The total FAA concentration in *P. moldaviensis* was 50.7 nmol/mg, which was significantly greater than that of the other four species (Table 24). *S. heringianus* had the second greatest total FAA concentration, which was also significantly different from all other species. The total FAA concentrations of *P. vejdovskyi*, *T. tubifex* and *L. hoffmeisteri* were not significantly different from each other.

Alanine represented between 23.1 and 41.8% of the total FAA pool and was the most abundant amino acid in all species. Leucine, valine and glutamic acid were, respectively, the next most abundant, and had similar relative concentrations in all species except *T. tubifex*. In *T. tubifex*, glutamic acid, asparagine and aspartate were the second, third and fourth most abundant FAA, respectively. In all species, each of the remaining amino acids represented between 0.5 and 5% of the total FAA pool, with their order of abundance varying from species to species.

The patterns of relative concentrations of FAA were examined by discriminate analyses based on generalized squared distances (Rao 1973) and canonical discriminate analyses based on principal component analyses and canonical correlations. In the discriminate analyses (Table 25), the "species" column lists the species to which organisms were assigned based on morphological characteristics, whereas the "classification" column lists the species grouping into which the discriminant model classified the organisms based on the concentrations of 18 FAA. In the majority of cases the correct classification based on FAA pool was 100% accurate (Table 25). The only poor classification was for one of the P. vejdovskyi organisms which had a probability of 0.27 of being classified as T. tubifex.

A perpendicular graphical representation of the first and second discriminant functions demonstrates complete separation of the five oligochaete species (Fig. 6). Means of the first canonical variable for T. tubifex, P. vejdovskyi and S. heringianus were very similar (-5.1, -5.5 and -5.2, respectively) which indicates that the first linear combination of the amino acid concentrations (which explains greater than 80% of the variation) were almost identical. However, for the second canonical variable, only P. vejdovskyi and P. moldaviensis had similar components (-0.5 and -0.6, respectively) with L. hoffmeisteri and T. tubifex being considerably smaller (-3.9 and -7.2, respectively) and S. heringianus being considerably greater (12.2). So for the first canonical variable, certain amino acids allowed separation of P. moldaviensis and L. hoffmeisteri and the second canonical variable allows separation of T. tubifex, L. hoffmeisteri and S. heringianus.

Table 24. Mean free amino acid concentrations (nmol/mg) of five oligochaete species collected from southeastern Lake Michigan.

Amino Acid	Limnodrilus hoffmeisteri	Tubifex tubifex	Potamothrix vejdovskyi	Stylodrilus heringianus	Potamothrix moldaviensis
Alanine	3.24 (0.88)[a](41.8)[b]	3.67 (0.80)(38.3)	3.39 (0.47)(28.7)	6.62 (0.05)(27.1)	11.7 (2.60)(23.1)
Leucine[c]	0.69 (0.05) (8.9)	0.70 (0.21) (7.6)	1.74 (0.13)(14.7)	3.27 (0.36)(13.4)	7.47 (2.57)(14.7)
Valine[c]	0.57 (0.04) (7.3)	0.55 (0.11) (5.8)	0.90 (0.07) (7.6)	1.75 (0.22) (7.2)	3.60 (1.20) (7.1)
Glutamic Acid	0.53 (0.20) (6.8)	1.18 (0.28)(12.4)	0.80 (0.20) (6.8)	1.56 (0.32) (6.4)	3.56 (0.59) (7.0)
Serine	0.43 (0.08) (5.5)	0.69 (0.27) (7.3)	0.62 (0.11) (5.3)	1.15 (0.11) (4.7)	2.18 (0.48) (4.3)
Tyrosine[c]	0.46 (0.09) (5.9)	0.38 (0.08) (4.0)	0.74 (0.04) (6.2)	1.24 (0.12) (5.1)	2.73 (0.57) (5.4)
Isoleucine[c]	0.38 (0.03) (4.8)	0.34 (0.09) (3.6)	0.58 (0.05) (4.9)	1.48 (0.17) (6.1)	2.50 (0.58) (4.9)
Phenylalanine[c]	0.29 (0.03) (3.8)	0.37 (0.09) (3.9)	0.62 (0.06) (5.2)	1.37 (0.14) (5.3)	2.80 (0.87) (5.5)
Glutamine	0.17 (0.06) (2.2)	0.17 (0.02) (1.7)	0.51 (0.11) (4.3)	1.18 (0.07) (4.8)	2.30 (0.61) (4.5)
Lysine[c]	0.14 (0.02) (1.3)	0.35 (0.05) (3.8)	0.28 (0.08) (2.4)	0.59 (0.09) (2.4)	2.71 (0.84) (5.4)
Methionine[c]	0.13 (0.01) (1.7)	0.22 (0.06) (2.2)	0.26 (0.04) (2.2)	0.75 (0.12) (3.1)	2.12 (0.91) (4.2)
Tryptophan	0.23 (0.08) (3.0)	0.40 (0.07) (4.5)	0.23 (0.05) (1.9)	0.51 (0.06) (2.1)	0.82 (0.17) (1.6)
Threonine[c]	0.16 (0.03) (2.1)	0.20 (0.05) (2.1)	0.35 (0.05) (3.0)	0.67 (0.08) (2.7)	1.33 (0.32) (2.6)

(continued)

Table 24. Mean free amino acid concentrations (nmol/mg) of five oligochaete species collected from southeastern Lake Michigan. (continued)

Amino Acid	Limnodrilus hoffmeisteri	Tubifex tubifex	Potamothrix vejdovskyi	Stylodrilus heringianus	Potamothrix moldaviensis
Arginine[c]	0.13 (0.05) (1.7)	0.21 (0.03) (2.3)	0.19 (0.06) (1.6)	0.21 (0.03) (0.9)	1.81 (0.68) (3.6)
Glycine	0.06 (0.02) (0.8)	(0.08) (0.02) (1.1)	0.18 (0.08) (1.5)	0.87 (0.15) (3.6)	1.17 (0.37) (2.3)
Histidine[c]	0.07 (0.03) (0.9)	0.16 (0.02) (1.7)	0.15 (0.03) (1.3)	0.39 (0.11) (1.6)	0.70 (0.19) (1.4)
Asparagine	0.08 (0.01) (1.0)	(0.15) (0.03) (0.8)	0.16 (0.03) (1.3)	0.35 (0.04) (1.4)	0.58 (0.09) (1.1)
Aspartic Acid	0.02 (0.007)(0.3)	0.08 (0.01) (0.8)	0.09 (0.03) (0.8)	0.42 (0.08) (1.7)	0.57 (0.18) (1.1)
TOTAL	7.78(A)[e]	9.90(A)[e]	11.79(A)[e]	24.38(B)[e]	50.65(C)[e]
Essential[d]	38.4	37.0	49.1	48.8	54.8

[a]Standard deviation
[b]Percent contribution to total FAA pool
[c]Essential amino acid in invertebrates
[d]Represents percent of total FAA pool which are essential amino acids
[e]Totals with the same letter are not significantly different from each other based on Duncan's Multiple Range Test ($n = 5$) ($\alpha = 0.05$).

Table 25. Classification of five oligochaete species by discriminant analysis

Species[a]	Probability of Classification of Organisms into Each Species Based on Concentrations of FAA					Classification[c]
	LH[b]	PM[b]	PV[b]	SH[b]	TT[b]	
LH	1.0000					LH
LH	1.0000					LH
LH	0.9850			0.0150		LH
LH	1.0000					LH
PM		1.0000				PM
PM		1.0000				PM
PM		1.0000				PM
PM		1.0000				PM
PV			1.0000			PV
PV			1.0000			PV
PV			1.0000			PV
PV			0.7280		0.2720	PV
SH		0.0003		0.9997		SH
SH		0.0001		0.9999		SH
SH				1.0000		SH
SH		0.0003		0.9997		SH
TT					1.0000	TT
TT					1.0000	TT
TT					1.0000	TT
TT					1.0000	TT

[a]Morphologically identified species
[b]LH - Limnodrilus hoffmeisteri; PM - Potamothrix moldaviensis; PV - Potamothrix vejdovskyi; SH - Stylodrilus heringianus; TT - Tubifex tubifex
[c]classification of organism based on free amino acid pool

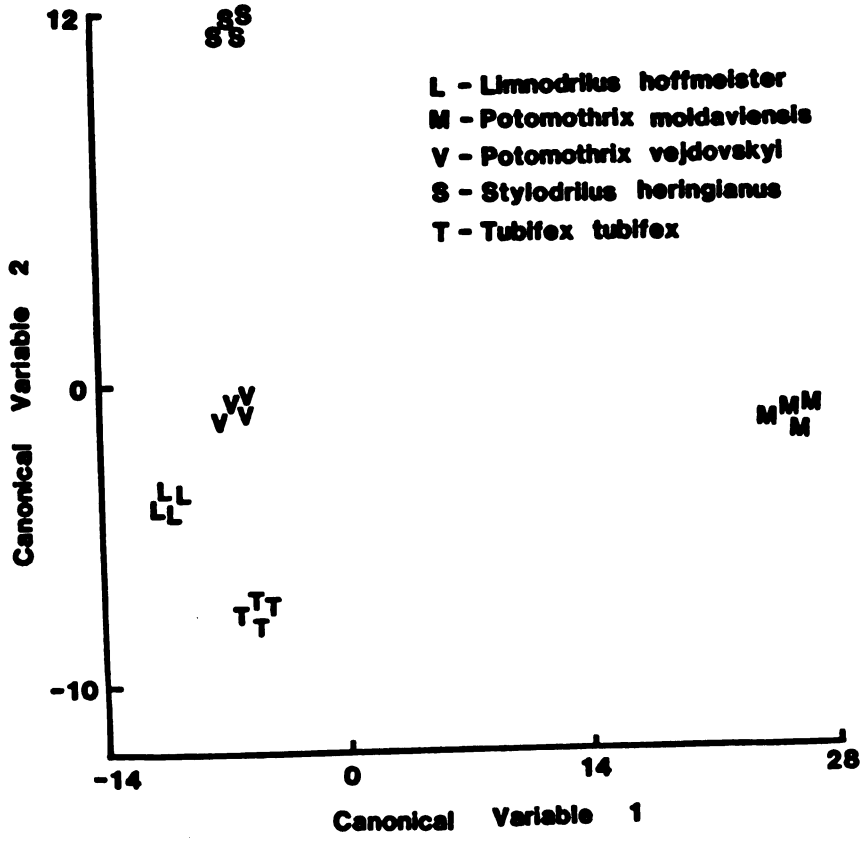
Discussion

The total FAA pool of Lake Michigan oligochaetes is small, relative to that of other freshwater invertebrates. Gardner and Miller (1981) reported a total pool of 428 nmol/mg (dry wt.) for *Daphnia magna* while the FAA pool of the copepod *Diaptomus* sp. contained 80.9 nmol/mg (Jeffries and Alzara, 1970). Van Marrewyjk and Ravestein (1974) observed total FAA concentrations of 545.7 and 330.2 nmol/mg in crayfish abdominal muscle and heptopancreas, respectively.

The relative concentrations of FAA vary among freshwater invertebrates. In crustaceans, glycine, arginine, alanine and proline are often the dominant free amino acids (van Marrewyjk and Ravestein, 1974; Claybrook, 1983). The only similarity to the Lake Michigan oligochaetes was for alanine, which was the dominant amino acid in all species (although proline was not measured). Neither arginine nor glycine represented a major proportion of the FAA pool in the Lake Michigan oligochaetes.

The sulfonic amino acid taurine was not detectable in any of the Lake Michigan oligochaetes. Taurine is found in high concentrations in marine invertebrates and is important in intracellular osmoregulation (Koenig et al., 1981). However, in freshwater invertebrates its occurrence seems to be restricted to specific phyla. Simpson et al. (1959) found no taurine in freshwater molluscan species analyzed; however, taurine has been found in freshwater crayfish (Van Marrewyjk and Ravestein, 1974; Lin and Cohen, 1973), copepods (Jeffries and Alzara, 1970) and amphipods (Chapters 2, 3 and 4). The function of taurine in freshwater invertebrates is unknown, but as with vertebrates, it may act

Figure 6. Discriminate function comparison of the concentration of 18 free amino acids from five species of freshwater oligochaetes. The first canonical variable represents the maximum between class correlation of the amino acids (continuous variables) for each species. The second canonical variable represents the next maximum multiple correlation which is uncorrelated with the first variable.


L - Limnodrilus hoffmeister
M - Potomothrix moldavienels
V - Potomothrix vejdovskyi
S - Styiodrilus heringianus
T - Tubifex tubifex
12
0
-10
-14
0
14
28
Canonical Variable 2
Canonical Variable 1

as an emulsifier of dietary fat and/or simply be an end product of sulfur amino acid metabolism (Holwerda and Vonk, 1973).

Although the essential and non-essential status of oligochaete amino acids is unknown, research conducted with crayfish indicates that requirements are very similar to those of vertebrates (van Marrewyjk and Zandee, 1975). Based on this, in P. moldaviensis, P. vejdovskyi, and S. heringianus, the essential amino acids constituted approximately 50% of the total FAA pool. Haag and Sullivan (1984) found a similar percentage of essential amino acids in whole body homogenates of two species of larval Hydropsychiadae (Trichoptera) (54%). As with this study, alanine, valine and glutamic acid were among the six most abundant amino acids measured in the Trichoptera, although the overall qualitative profile of the FAA pools were quite different. In L. hoffmeisteri and T. tubifex, the essential amino acids represent 38.3 and 33.1% of the total FAA pool, considerably less than in the other species. This difference can mainly be attributed to the high alanine content (non-essential amino acid) of these organisms.

Little is known about the specific biochemical and physiological pathways of nitrogen metabolism in freshwater annelids, therefore, it is difficult to speculate on why there are differences among such closely related Lake Michigan oligochaetes. This is especially true for P. moldaviensis and P. vejdovskyi, where a five-fold difference in the total FAA pool was caused by differences in both essential and non-essential amino-acids. The observed differences could be the result of either 1) genetically controlled differences in the metabolic pathways controlling nitrogen metabolism or 2) exogenous or environmentally induced alterations in the physiology and/or biochemistry of the

organism. An organisms free amino acid pool is the end result of the homeostatic regulation of a variety of inter-related anabolic and catabolic processes. These pathways, which include protein and amino acid synthesis and degradation, can be altered as a result of or for the purpose of homeostatic adjustment. For largely unknown reasons, each species may have different "set points" for the various amino acids around which homeostatically regulated pathways maintain the concentration of individual amino acids. Specific factors which can potentially influence the FAA pool include osmotic conditions, temperature, diet, anoxia, reproductive and molt cycles and stress Duchateau-Bosson and Florkin, 1961; Munro, 1970; Gilles, 1979). In the present study, these factors may have contributed to both the qualitative similarities and quantitative differences observed among the various species.

Dietary characteristics influence the relative composition of the FAA pool in many organisms. In mammals, immediately following protein ingestion the tissue and body fluid FAA pool rises dramatically (Munro, 1970). In many cases the FAAs that are dominant in the protein meal will increase the most in serum; however, this is not always a direct relationship and is often influenced by the uneven removal of specific amino acids by tissues for protein synthesis. For organisms which do not feed continuously, diet generally has little influence on normal composition of the FAA pool since any changes are only transitory (1-2 hrs.) and amino acids rapidly return to pre-ingestion concentrations, via increased protein synthesis and/or amino acid oxidation (Munro, 1970). However, in organisms which are generally considered to be continuous feeders (i.e. oligochaetes), the continuous digestion and absorption of proteins, oligopeptides or amino acids may have a more

consistent influence on the concentrations of FAA in tissues and fluids. Therefore, a change in an organism's diet may influence the composition of the FAA pool. In the present study all of the oligochaetes were collected from the same location and most likely had similar diets. These organisms feed by the ingestion of sediment below the surface and the subsequent deposition of fecal pellets at the sediment-water interface (McCall and Fisher, 1980). Although some studies have indicated species-specific, selective feeding by oligochaetes (Brinkhurst et al., 1972), there is no evidence in the literature to indicate that the characteristics of interspecific food resources varied such that the quantitative or qualitative composition of the protein and/or FAA consumed significantly differed among oligochaete species. Thus, the probable similarities in diet of all the species may have contributed to the qualitative similarities of the FAA pools.

It is becoming increasingly apparent that dissolved FAA in natural seawater can contribute significantly to the energetics and/or osmoregulatory ability of marine invertebrates. Direct FAA uptake from seawater occurs in bivalve larvae (Manahan, 1983), annelids (Stephens, 1964) and sea urchin larvae (Manahan et al., 1983). However, utilization of ambient dissolved FAA by freshwater invertebrates is considered to be negligible. Stephens (1964) studied twelve genera of freshwater metazoa (excluding freshwater oligochaetes) and found limited ability of any of these organisms to remove C^{14} labeled glycine from solution. Brinkhurst and Chua (1969), in studying the nutritional resources of three freshwater tubificid oligochaetes did, however, find considerable C^{14}-glycine uptake by Peloscolex multisetosus and minimal uptake by T. tubifex and L. hoffmeisteri. Although two of the species investigated in the

present study (T. tubifex and L. hoffmeisteri) have minimal capability in absorbing FAA from solution (Brinkhurst and Chua, 1969), a comparison of the dominant FAA from freshwater sediments (Brinkhurst et al., 1971) with those in the worms from the present study reveals some interesting similarities (Table 26). In the study conducted by Brinkhurst et al. (1971), dissolved FAA were measured in sediment samples from Toronto Harbor. Sediments receiving less organic input (Hanlans Point, Table 26) had smaller dissolved FAA concentrations than in Keating Channel, but the relative concentrations were very similar. The dissolved FAA concentrations of the more oligotrophic Lake Michigan sediments from which the organisms were collected in this study were not determined; however, comparison of the dominant amino acids in the oligochaetes with those in Toronto Harbor sediments reveals a great degree of concordance (Table 27). In all species, except T. tubifex, alanine and leucine were the dominant FAA in the oligochaetes and in the sediment. For alanine this may be simply coincidental because this amino acid is often a dominant amino acid in invertebrates (Claybrook, 1983) and aquatic environments (North, 1975; Vallentyne, 1957). However, leucine and valine are seldom dominant amino acids and high levels in the organisms (and the potential for high sediment levels) may be indicative of the influence of sedimentary amino acids (or diet) on the FAA pool. Likewise, based on work with other invertebrates leucine and valine are likely essential amino acids, which indicates the organisms would rely mainly on their diet to obtain them (Van Marrewyjk and Zandee, 1975). If this is the case, then the high level of leucine and valine would indicate high dietary intake, either from ingested food or via direct uptake from the surrounding medium.

Table 26. Six dominant soluble free amino acids in Toronto Harbor sediment (Brinkhurst et al. 1971) and oligochaetes collected from southern Lake Michigan.

Relative Abundance[c]	Hanlans Point[a]	Keating Channel[b]	Limnodrilus hoffmeisteri	Potamothrix moldaviensis	Potamothrix vejdovskyi	Stylodrilus heringianus	Tubifex tubifex
1	Ala[d]	Ala	Ala	Ala	Ala	Ala	Ala
2	Leu	Leu	Leu	Leu	Leu	Leu	Leu
3	Gly	Val	Val	Val	Asp	Val	Asn
4	Glu	Gly	Glu	Glu	Val	Glu	Asp
5	Ser	Glu	Tyr	Phe	Glu	Ile	Leu
6	Arg	Ser	Ser	Tyr	Tyr	Phe	Ser

a-Less eutrophic station;
b-More eutrophic station;
c-FAλ listed in order of decreasing abundance.
d-Ala-alanine; Leu-leucine; Gly-glycine; Glu-glutamic acid; Ser-serine; Arg-arginine; Val-valine; Tyr-tyrosine; Phe-phenylalanine; Asp -aspartic acid; Ile-isoleucine; Asn-asparagine

The guts of the worm were not purged prior to analyses, therefore, it is possible that the gut contents contributed to the whole body FAA pools. However, calculations based on the weight of the oligochaete gut contents, the approximate dissolved FAA of the ingested sediments (Brinkhurst et al., 1971) and the FAA pool of associated microbrial flora (DeFelice et al., 1974) indicate that the gut contents most likely contributed less than 1% to the total whole body FAA pool. Because the time and methods required for complete gut clearance would result in starvation and stress, it was decided that the small contributions of the gut contents to the total body pool would be more desirable than the potential alterations in the FAA pool induced by starvation and stress. Research with crustaceans (Torres, 1973) and molluscs (Riley, 1980) has shown the FAA pool to be significantly altered during food deprivation. Similar research on freshwater oligochaetes has not been conducted; however, energetic pathways are similar and changes in available energy sources will alter carbohydrate, lipid and protein metabolism, thus potentially affecting the FAA pool.

In the present study, variations in reproductive development may have contributed to the quantitative difference among species and/or variability within species. Based on size, all the organisms were considered to be adults, but because the reproductive status of the individual worms was unknown, each organism could have been at a different stage of reproductive development. The life history of both *S. heringianus* and *L. hoffmeisteri* can vary depending upon local conditions (Kennedy, 1966; Pickavance, 1971). In southern Lake Michigan, mature specimens of *S. heringianus* can be found all year, although the greatest density is generally found in the spring (Stimpson et al., 1975).

L. hoffmeisteri has a more defined life cycle, with the reproductively active period normally restricted to early spring and summer. In contrast to this, P. moldaviensis exhibits peak reproductive activity in late fall and P. vejdovskyi has the greatest density of individuals in the fall, although this could not be related to reproductive activity (Stimpson et al., 1975). In the present study (April 30), it is therefore possible that within and among species, individual organisms were at different stages of reproductive development. Kosiorek (1979) investigated the biochemical composition of T. tubifex at different periods of its life cycle. She found that prior to complete reproductive maturation the organism accumulated lipids and carbohydrates, while during the reproductive stage, metabolism changed considerably, resulting in reduced protein synthesis and an increase in lipid and carbohydrate metabolism. Since the concentrations of FAA's can be influenced by metabolic adjustments, changes in the reproductive status of the organism can potentially alter the FAA pool. This has been demonstrated in sea anemone (Kasschau and McCommas, 1982), starfish (Ferguson, 1975) and barnacles (Cook et al., 1972).

Differences in reproductive strategies and respiratory physiology and biochemistry has been the basis for the development of the "indicator species" concept. The trophic conditions of lakes or streams can influence the relative abundance and composition of oligochaete communities (Aston, 1973). Stylodrilus heringianus is most often associated with oligotrophic systems and is considered to be intolerant of anoxic conditions (Milbrink, 1973) while L. hoffmeisteri is a more eutrophic indicator and often dominates communities receiving organic pollution (Brinkhurst, 1980). Tubifex tubifex is considered the most resistant of

the tubificids to anoxic conditions and can be found in great numbers under extremely eutrophic conditions, although its distribution is cosmopolitan and it can also reach high densities under oligotrophic conditions (Milbrink, 1980). A considerable amount of research has been conducted on the biochemical mechanisms underlying invertebrate facultative anaerobes (Hochachka, 1975; Hipp et al., 1984). Although discussion of the complex metabolic adjustments are beyond the scope of this paper, it is interesting to note the relative distribution of alanine among the various oligochaete species. In invertebrates alanine is a major end product of anaerobic metabolism and has been shown to accumulate under anaerobic conditions in T. tubifex (Hoffman et al., 1979; Hipp et al., 1984). In the most eutrophic, anoxia-resistant species L. hoffmeisteri and T. tubifex, alanine represents 41.8 and 38.3% of the total FAA pool, whereas in the less eutrophic indicator species P. vjedovski, P. moldaviensis and S. heringianus alanine constitutes 28.7, 23.1 and 27.1 percent, respectively. Although in the present study the oxygen concentrations in the sediment were not measured when the worms were collected, given the oligotrophic condition of the collection area, the odor of the sediment and the domination of the oligochaete community by S. heringianus, one can say with reasonable certainty that the sediments were not anaerobic. This being the case, it is difficult to speculate on why the alanine levels were greater in T. tubifex and L. hoffmeisteri.

The significant quantitative and qualitative differences in the FAA pool among the various oligochaete species makes feasible the use of these variables in the area of numerical taxonomy. Biochemical taxonomy has focused mainly on the electrophoretic patterns of enzymes and found

many applications in invertebrate taxonomy (Bianchi, 1968; Jelnes et al., 1971; Milbrink and Nyman, 1973). Because direct genetic control over isozyme production and distribution is assumed, the normal problems encountered when using only morphological measures (i.e. phenotypic variability) can presumably be avoided, although in some species environmental or developmental variability can alter isozyme profiles (Bowen et al., 1969) such that geographic clines exist. Another limitation is that minor variation in technique (pH, gel composition) can result in different electrophoretic patterns, thus possibly influencing conclusions concerning species classification (Singh et al., 1976). In contrast, the analysis of FAA's is a relatively simple, straightforward procedure which requires limited subjective interpretation. Free amino acid pools have been used in invertebrate biochemical taxonomy (Micks, 1956; Micks and Gibson, 1957); however, exogenous (season) and endogenous (diet, reproductive status) factors may influence individual FAA pools, thus complicating species identification. This would most likely be true for organisms collected from different ecosystems (i.e. lentic vs. lotic), however within the same ecosystem, sufficient background data should enable one to account for this variability. The most useful application of this technique would be for the identification of sexually immature specimens, which are difficult or impossible to identify morphologically.

In this study, we were able to correctly classify each individual oligochaete to the appropriate species based on the first and second discriminant functions of the FAA profile. Although these preliminary results are encouraging, concern of seasonal and geographic intraspecific variability warrants investigation. If this variation is

excessive, then the application of this technique to oligochaete taxonomy will be limited.

Chapter 7

Summary and Conclusions

The primary objective of this study was to determine if alterations in the concentration of free amino acids (FAA) could be used as a sublethal biochemical indicator of toxicant-induced stress. Exposure of G. pseudolimnaeus to lethal and sublethal concentrations of pentachlorophenal (PCP) caused a significant change in the FAA pool. Similarly, the concentration of FAA in C. fluminea was significantly altered in both acute and chronic exposures to sodium dodecyl sulfate (SDS). However, the type of changes observed were considerably different and were most likely the result of different mechanisms causing the FAA alterations. Seasonal changes in the FAA pool were observed and must be considered when applying a BIS in situ.

The specific reasons for the different type of responses observed in the FAA pool of G. pseudolimnaeus vs. C. fluminea are unknown. The most probable explanation involves differing mechanisms of toxicity of PCP vs. SDS. In addition, the physiology and biochemical pathways of crustaceans vs. molluscs can be considerably different. These differences can result in different responses by the organisms to the same toxicant. For example, the ability of bivalves to avoid toxicants by shell closure can result in biochemical alterations completely different from those observed in crustaceans exposed to the same chemical concentration. The occurrence of such differences does not limit the usefulness or applicability of BIS, but rather emphasizes the importance of picking the appropriate organism and developing a data base and monitoring scheme based on only that organism.

At this point, the primary need is to develop a thorough understanding of one organism and its response to toxicants, not to develop a limited understanding of the responses of a variety of organisms. With

this approach, it becomes extremely important to choose the appropriate organism for an *in situ* monitoring program. The fairly limited and patchy distribution of amphipods, their small size and the difficulty of working with them in the laboratory eliminates these organisms as a useful BIS. Molluscs, on the other hand, have been routinely used as monitors for chemical contamination. *Corbicula fluminea* is widely distributed, easily collected and extremely amenable to laboratory toxicological studies.

Alterations in the concentration of FAA can result from toxicant-induced changes in a variety of biochemical pathways. In *G. pseudolimnaeus*, the decrease in FAA during acute exposure to PCP was proposed to be caused by a disruption of osmoregulatory ability. During chronic exposure, a similar decrease was observed, however it was unlikely the result of osmoregulatory breakdown, but rather the result of changes in protein metabolism. In *C. fluminea*, there was a significant increase in the total FAA pool and a change in the relative concentration of individual FAA during both lethal and sublethal exposure to SDS. This was a surprising result. Since the primary mode of action of SDS is gill damage, it was felt that this would also cause a disruption of osmoregulatory ability and a subsequent decrease in the total FAA pool. However, hypoxia also occurred and this most likely contributed to the increase observed in specific FAA. However, this does not explain all of the FAA alterations observed. Possible explanations for the other changes observed include 1) enzyme inhibition, 2) energy mobilization, and 3) general changes in protein metabolism.

Based on the results of the amphipod and bivalve exposures, changes in the FAA pool would be classified as a "general" indicator.

Alterations in the FAA pool were observed in organisms exposed to two entirely different chemicals. At this point it is unknown whether the changes observed were the result of specific mechanisms of toxicity of the toxicants or whether they were caused by a general toxicant-induced stress response. If different types of changes in the FAA pool occur as a result of exposure to different types of chemicals, then the potential exists for FAA to be a "specific" indicator. This would require extensive documentation of the effect of specific chemicals on the concentration of FAA and would require that one could separate the effect of these chemicals from this influence of natural stressors or accessory factors.

Biochemical indicators of stress must be sensitive to sublethal concentrations of chemicals. A BIS which only responds to acutely lethal concentrations of chemicals are of little use. The FAA concentrations of both amphipods and molluscs were altered during sublethal exposures. In both cases, changes in the FAA pool were more sensitive to toxicant stress than more traditional, higher level effects such as growth, caloric content and respiration. However, the fact that they are more sensitive does not necessarily make them more useful as an indicator of stress. An extremely sensitive indicator is of no use if the toxicant concentration at which it responds has no adverse effect on the population, community or ecosystem. Additional research is required to determine the physiological, biological and/or ecological implications of changes in the FAA pool.

The results of these studies indicate that measuring changes in the concentration of FAA does have potential as an _in situ_ BIS, especially in the freshwater bivalve _C. fluminea_. Changes in the FAA pool have

been related to higher level effects, such as respiration, however it is still unknown whether the alterations observed at the lower concentrations are of biological/ecological significance. Seasonal changes in the FAA pool have been shown to occur. As long as these changes are known, they should not impede ones ability to separate toxicant-induced effects from the influences of accessory factors. There does not appear to be a dose-response relationship between chemical concentrations and FAA alterations. This poor relationship does not eliminate FAA as a potential stress indicator, although it does limit one's ability to interpret the significance of the changes observed. Under field exposure conditions, it will be very difficult to develop any type of effects indicator capable of predicting previous exposure concentrations.

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