NUTRIENT RECYCLING AND STOICHIOMETRY OF STYGOBIONTS IN THE EDWARDS AQUIFER, CENTRAL TEXAS

by

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DEDICATION

This thesis is dedicated to my dad, Gary, for encouraging me to dig in the dirt and play with earthworms and instilling in me passion for, and curiosity about, the natural world around me. Also, to Dr. Glenn Pickett, who first encouraged me to take that passion and curiosity and turn it into a career dedicated to protecting the environment.

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1. INTRODUCTION

In all ecosystems, and aquatic ecosystems in particular, metazoan animals have direct and indirect impacts on the cycling of nutrients (Vanni 2002). The assimilation and recycling of nutrients by animals can have wide-ranging effects on ecosystem processes, affecting nutrient availability, rates of primary production, and species composition (Vanni 2002; McIntyre et al. 2008). Although numerous studies have examined the role of consumer-driven nutrient cycling in lake, ocean, stream, and riverine ecosystems (e.g., Vanni et al. 2002; Sterner and Elser 2002; Sardans et al. 2012), relatively little is known about the ecology and nutrient cycling dynamics of subterranean ecosystems. Subterranean aquatic systems (i.e., groundwater-based systems) contain approximately one-third of the earth's freshwater (Ford et al. 1988), but the distribution of described obligate subterranean aquatic organisms (i.e., stygobionts) is highly regionalized with many species exhibiting restricted ranges, high levels of endemism, and therefore vulnerability to human perturbation (Culver et al. 2000). Although previous studies have examined the diversity and distribution of subterranean fauna (Culver et al. 2000; Christman and Culver 2001; Culver et al. 2003; Schneider et al. 2004; Glanville et al. 2016) and food web ecology of stygobiont communities (Pohlman et al. 1997; Graening and Brown 2003; Hutchins et al. *in press*), virtually nothing is known of the relative importance and role of stygobiont metazoans in the recycling of nutrients and the nutrient composition (elemental stoichiometry) of these organisms in aquifer ecosystems.

Groundwater ecosystems contain no *in situ* photoautotrophic primary producers, thus subterranean environments are often cited as being energy limited and resource poor, relying upon allochthonously-derived surface organic matter (OM) inputs, such as

terrestrial plant detritus, surface animal carrion, and guano from roosting bats (Simon et al. 1997; Graening and Brown 2003; Schneider et al. 2011) or *in situ* chemosynthetic bacterial production (Sarbu et al. 1996; Roach et al. 2011; Hutchins et al. *in press*) as the main sources of energy and carbon (C). In addition, nutrients, such as nitrogen (N) and phosphorus (P) can enter subterranean systems as dissolved inorganic forms (e.g., PO_4^{3-}, NO_3^-) via hydrological inputs from the surface or through the weathering of geological substrates. However, the "openness" of subterranean aquatic ecosystems to inputs of allochthonously derived OM and inorganic nutrients exists in a continuum, with some systems being relatively open (i.e., surface stream drains directly into the subsurface), while some systems are relatively "closed" to inputs of OM and nutrients. Indeed, in subterranean aquatic ecosystems with little to no direct and immediate connection to the surface, the recycling of OM and some forms of nutrients through abiotic and biotic processes may be particularly important in maintaining below ground communities (Simon and Benfield 2002; Engel et al. 2010).

In subterranean aquatic ecosystems, organisms often face conditions with low food quantity and quality (e.g., Scheider et al. 2011). It has been hypothesized that obligate subterranean organisms should exhibit a suite of adaptations and life-history traits which are consistent with living under low food (energy) availability, such as relatively low metabolic rates, slow growth rates, and increased lifespan when comparted to their surface (i.e., epigean) counterparts (Poulson 1963; Poulson and White 1969). Although these adaptations are thought to be a result of exposure to low energy conditions (the energy economy hypothesis; Poulson 1963), other potential factors, such as the high environmental stability of subterranean systems and the lack of trophic complexity may

also contribute to their presence (Kunter et al. 1999; Hüppop 2005). Some data on the metabolic function and life history of cave-dwelling organisms support this hypothesis (e.g., Barr 1968; Poulson and White 1969; Streever 1996), but data examining metabolic rates of subterranean obligate aquatic organisms in context of the energy economy hypothesis are generally lacking or equivocal (e.g., Bishop et al. 2014).

In addition to the presumptive effects of energy and food paucity, the scarcity of specific, but required, elements such as phosphorus (P) and nitrogen (N) in food items can also have impacts on shaping life history and growth rates (Sterner and Elser 2002; Elser et al. 2003). In systems in which consumers face a relatively large stoichiometric imbalance with their food, such as detritus-based and subterranean systems, there may be a strong nutrient-limited constraint on consumer growth rates and a selection for consumers that maintain relatively low tissue nutrient content (Cross et al. 2003; Elser et al. 2000; Schneider et al. 2010). It has been hypothesized that invertebrates which exhibit relatively high growth rates will have higher P demand (and subsequently higher body P content) due to the production of P-rich ribosomal RNA (rRNA) associated with rapid transcription, protein production, and overall organismal growth rates (Elser et al. 1996; Sterner and Elser 2002). In addition, the process of translation and protein synthesis may be limited by the availability of N (Hessen et al. 2007). Thus, it has been hypothesized that the P content of invertebrate organisms varies with growth rates, where more rapidly-growing species exhibit higher body P content and slower growing organisms exhibit lower body P content (i.e., the growth rate hypothesis or GRH; Elser et al. 2003). It can be further postulated that obligate subterranean organisms should exhibit relatively low body P (and possibly N) content when compared to related surface species

because of slower growth rates and lower nutrient availability in food items (e.g., Schneider et al. 2010). Most data examining the GRH and body P content has come from surface-based aquatic and terrestrial ecosystems (Elser et al. 2003; Sardans et al. 2012) and there is a comparative lack of data associated with subterranean organisms (but see Schneider et al. 2010). Indeed, the comparison of obligate subterranean organisms to related surface species represents an ideal opportunity to examine the GRH and how it may vary with life history adaptations associated with specific environmental conditions.

In this study, I examined nutrient recycling and nutrient content of a stygobiont community at a site located within the Edwards Aquifer in central Texas, USA (Fig. 1). The Edwards Aquifer is one of the world's most diverse groundwater systems for stygobionts (Culver et al. 2006; Culver and Pipan 2009; Gibson et al. 2008; Holsinger and Longley 1980; Longley 1981). In this study I had three main goals. First, I wanted to examine the abundance and biomass of stygobionts captured at a well-established site in the Edwards Aquifer that contains a relatively diverse stygobiont community. In particular, I was interested in examining the amount of temporal variation in stygobiont densities at the site and gaining information on which stygobiont species were numerically and biomass dominant in this portion of the aquifer. My second goal was to examine nutrient recycling (via measuring excretion rates) by the dominant stygobiont species at the study site. I then compared these excretion rates to closely-related surface species in the area to examine whether metabolism (excretion rates) systematically differed between stygobiont and epigean invertebrates. My third goal was to examine the elemental composition and stoichiometry of stygobionts at the study site and then compare these values to related epigean species to determine whether habitat location and

conditions (subterranean versus epigean) translated into differences in body nutrient content and ratios (specifically C, N and P).

In the first portion of the study, I hypothesized that the relative abundance and biomass of stygobiont species collected at the site should remain temporally consistent because of the presumed high degree of environmental consistency and stability associated with groundwater systems and subterranean environments. In the second portion of the study, I hypothesized that organismal excretion rates would vary with both body size and species identity, which is consistent with a variety of other studies (e.g., Vanni et al. 2002). However, I also hypothesized that the overall N and P excretion rates would systematically differ between stygobiont and epigean species, with epigean taxa exhibiting higher N and P excretion rates. In the last portion of the study, I hypothesized that the elemental composition (in particular, body P content) would differ among stygobiont species within the aquifer, but that stygobiont species would have consistently lower body P content (and potentially N content) than closely-related epigean taxa.

2. METHODS

The Edwards Aquifer is an approximately 16,000 km^2 karst aquifer with a recharge basin covering ~11,400 km^2 area over 13 counties in central and southwest Texas (Fig. 1a). A large number of well and spring sites in the Edwards Aquifer have been sampled for stygobionts and there is substantial spatial variation among sites in the individual taxa occurring at sites as well as the total number of detected taxa (e.g., species richness) (Hutchins et al. *in press*). Globally, the Edwards Aquifer is considered a stygobiont biodiversity hotspot, containing > 55 species, including diverse invertebrate assemblages and several vertebrate species (Longley 1981; Culver et al. 2003). Stygobiont assemblages were colonized by surface species at various times throughout the development of the aquifer; several arthropod groups are marine-origin (i.e., haziid and sebid amphipods, thermosbaenaceans, and cirolanid isopods) and likely colonized the aquifer sometime during the Paleocene-Eocene thermal maximum (~55 mya; Fisher and McGowan 1967; Yancey et al. 2012) or possibly earlier (Holsinger and Longley 1980). The remaining species are of freshwater origin and likely invaded the aquifer in the post-Cretaceous (Holsinger and Longley 1980). In particular, the Artesian Well (Fig. 1b) on the Texas State University campus is one of the most diverse stygobiont collection sites in aquifer (Holsinger and Longley 1980; Longley 1981; Gibson et al. 2008; Hutchins et al. *in press*). Thus, the Edwards Aquifer and the Artesian Well site in particular represents a unique opportunity to examine stygobiont diversity and nutrient excretion in an aquifer setting.

Monitoring and collection of stygobionts

In order to collect stygobionts from the aquifer, I placed a conical relatively small

aperture Nitex net (100-µm) over the outflow of the Artesian Well for 1 – 3d periods (24 – 72 h intervals) from February 13, 2013 to April 15, 2013. The net was again put over the well outflow from May 20, 2013 to May 27, 2013, from November 6, 2013 to December 19, 2013, on January 6, 2014, February 13, 2014, and April 11, 2014 to April 16, 2014. The outflow pipe from the well is contained within a locked concrete box and the net fit securely over the outflow pipe to limit the capture of any surface-associated taxa in the net. For most collection periods, the net was checked every 24-hr, and captured organisms were rinsed into a bucket with aquifer water and taken to the lab for identification using appropriate taxonomic keys and species records (Bowman 1964; Young and Longley 1976; Holsinger and Longley 1980; Lewis and Bowman 1996). Because some species have yet to be formally described, I identified some groups only to a taxonomic resolution which I was reasonably assured was correct (e.g., Copepoda, Ostracoda, Thermosbaenacea). The amount of water passing through the net during a given sampling interval was difficult to determine and varied with spring-flow and aquifer level conditions, therefore, I expressed the abundances of different species in terms of the number of individuals of each taxonomic group caught in the net over each 24-h sampling event or a 24-h density (functionally a catch-per-effort; CPE).

Estimation of nutrient excretion rates

I conducted nutrient excretion rate experiments on live and intact organisms in order to determine nutrient recycling rates of stygobionts. When I conducted nutrient excretion experiments, I only performed experiments using animals that had been collected during a 24-h sampling event so that I minimized the amount of time they were above ground and in the net. When I collected organisms for excretion experiments, I

immediately rinsed the net and placed organisms in a clean plastic bucket containing aquifer and immediately brought the organisms to a laboratory in the Freeman Aquatic Building, located adjacent to the well on the Texas State University campus. Live organisms that were obviously free of injury were immediately used for nutrient excretion experiments.

The number of nutrient excretion experiments I could conduct on a given day and the specific taxa I could use for experiments was dependent upon the composition of species I collected in the net over a given 24-h interval. A variety of species (e.g., copepods, ostracods, some amphipods) were very small-bodied, were time consuming to sort while alive, and were not likely to excrete measurable nutrients over a short-term incubation. Some species were also very fragile (e.g., *Lircelous smithii*) and collecting them in the net and handling them to conduct excretion experiments frequently damaged them. Lastly, some species were only rarely encountered, and it was difficult to collect sufficient numbers to conduct excretion experiments. Therefore, I largely focused my excretion experiment efforts on three stygobiont species that were relatively common at the Artesian well site, had larger body sizes, and were not extremely susceptible to injury. *Palaemonetes antrorum* (the Texas blind cave shrimp) is a common and widespread aquifer species considered to be a lower trophic level consumer that scrapes epilithic biofilms (Hutchins et al. *in press*). *Stygobromus flagellatus* (an amphipod) and *Cirolanides texensis* (an isopod) are also found at several other sites in the aquifer and are considered to be omnivore-predators in the aquifer food web (Hutchins et al. *in press*). I was able to conduct $n = 17$, $n = 15$, and $n = 17$ excretion experiments for *C. texensis*, *P. antrorum*, and *S. flagellatus* over the course of the study, respectively. I additionally

conducted one excretion experiment on *Texiweckeliopsis insolita*, a common, but small-bodied hadziid amphipod species.

In order to compare N excretion rates of stygobionts to surface-dwelling species, I also conducted excretion experiments on two commonly-occurring surface species that were related to two of stygobionts. I collected individuals of the surface amphipod, *Hyalella azteca,* and *Palaemonetes kadiakensis* (Mississippi grass shrimp) from the nearby upper San Marcos River. These two surface species were selected because they are related to and are within the body size range of two of the stygobiont species I examined (*S. flagellatus* and *P. antrorum*). The upper river is fed by springs from the Edwards Aquifer and has the same physicochemical conditions as the water from the Artesian Well and individuals of both species were collected from a site in the upper San Marcos River (~200 m from the Artesian Well) using kick nets and hand nets. Individuals were immediately placed into clean plastic buckets containing river water and immediately transported to the lab for excretion experiments. Over the course of the study, I conducted $n = 6$ excretion experiments for each surface species.

Once organisms were brought to the lab, individuals or groups of smaller organisms of the same species (depending on organism size) were placed into clean 200 - 500 ml high density polyethylene (HDPE) containers filled with pre-filtered (ashed Pall A/E glass fiber filter) Edwards Aquifer (stygobitic species) or river water (epigean species). Over the course of the study, an attempt was made to try to conduct experiments that would encompass the body size ranges present for each of the species. Once in containers, organisms were moved to a darkened incubator offset at aquifer and river ambient temperature (23^{o}C). Depending on the size and number of organisms in

bottles, organisms were allowed to excrete for 1-6 h and at the end of this period, organisms were removed from the incubator, organisms were removed from containers, and the water was filtered again (pre-ashed Pall A/E filter). Excretion experiment filtrate samples and initial water samples prior to incubation were analyzed for PO_4^{3-} and NH_4^+ using a Varian Cary 50 UV–Vis spectrophotometer using standard methods (Wetzel and Likens 2000). Organisms were placed in plastic weigh boats, dried for 48 h at 50°C, and mass was determined on a Mettler Toledo MX5 microbalance. The mass of one individual in incubations where multiple individuals were simultaneously excreting was determined by dividing the total mass of all individuals by the number of individuals in the sample. Mass-specific excretion rates were expressed as μg nutrient excreted/mg dry weight/hour.

Determination of body nutrient content

In order to determine the nutrient (Carbon, Nitrogen, and Phosphorous) content of organisms, individuals from the same species as above (*C. texensis*, *P. antrorum*, *S. flagellatus*, *H. azteca*, and *P. kadiakensis*) were collected from the same sites using the same methods. Organisms were euthanized by briefly placing them in a freezer and then transferring them to a drying oven set at 50°C for 48 h. Dry mass of the individual or groups of organisms was determined using the microbalance and dried animals were then ground to a fine powder with a clean mortar and pestle. Carbon and Nitrogen content of the invertebrates were determined with a CE Elantech CN Analyzer. Phosphorus content of invertebrates was determined through digestion with HCl followed by the molybdenum blue method (Wetzel and Likens 2000). Body C, N, and P content of each

species was expressed as the percent of dry mass as well as the ratios between nutrients (i.e., C:N, N:P, and C:P).

Data analysis

In order to examine the relationships between nutrient recycling and body mass of stygobionts, I utilized ordinary least-squares (OLS) linear regression. We regressed mass-specific excretion rates and the N:P of excreted nutrients against body mass for stygobionts and epigean species; data were $\log_{10}$-transformed prior to analysis. I compared the mass-specific excretion N and P rates and the N:P of excreted nutrients for the five species using one-way ANOVA on $\log_{10}$-transformed data, followed by post-hoc comparisons using Tukey's HSD. To determine if excretion rates and ratios systematically varied between stygobiont and epigean taxa, I separated species into two groups based on origin (stygobiont *vs* epigean) and used one-way ANOVA to compare groups and included body size (individual dry mass) as a covariate in the analysis.

I compared body nutrient content and ratios across the five species using one-way ANOVA on $\log_{10}$-transformed data, followed by post-hoc comparisons using Tukey's HSD. Nutrient content was expressed as the % C, N, and P of body dry mass and body nutrient ratios (C:N, C:P, N:P) were calculated on a molar basis. Again, I separated species into two origin groups (stygobiont *vs* epigean) and used one-way ANOVA to compare nutrient content and ratios between groups.

3. RESULTS

Diversity and composition of the stygobiont community

Over the study period, individuals from 26 taxonomic groups were collected from the Artesian Well (Table 1). The stygobitic arthropod taxa at the site were particularly diverse, containing multiple orders, families and species. The most diverse group at the site was the amphipods, which was represented by 9 species across five families. Three species of isopods, each representing a different family were also present. There were two species of palaemonid shrimp, one species of dytiscid beetle, and one thermosbaenacean. The remaining arthropods were unidentified species representing Copepoda, Ostracoda, Hydracarina (Acari: Trombidiformes), and Bathynellacea. Additionally, there were three species of stygobitic gastropods (*Phreatodrobia* spp.). Individuals of an erpobdellid leech (*Mooreobdella* sp.) and a turbellarian flatworm (Kenkiidae: *Sphalloplana* sp.) also occurred in samples. Finally, the Texas blind salamander (*Eurycea rathbuni*) occasionally appeared at the site over the study period.

The mean total number of stygobionts collected in the net over a 24-h period was 314 individuals (range = 90 - 658 individuals) (Fig. 2A). Over the study period, total stygobiont density emerging from the well exhibited temporal variation, but there was no obvious overall declining or increasing trend. Three groups made up the overwhelming majority of individuals collected at the site: the Texas cave shrimp, copepods, and ostracods. Cumulatively, these three taxa accounted for 91% of the stygobionts collected over the study period. In particular, the relatively large-bodied Texas cave shrimp were

the most abundant organism collected, yielding 129 individuals per 24-h interval (range = 90 - 658 individuals).

The amphipod species *S. flagellatus*, *T. insolita, Texiweckelia texensis*, and *Seborgia relicta* commonly occurred in well samples, but these species exhibited mean 24-h densities < 7 individuals (range = 0 – 26 individuals) (Fig. 2B). A diversity of other species (*Stygobromus russelli*, *Holsingerius samacos*, *Allotexiweckelia hirsuta*, *Artesia subterranea*, and *Parabogidiella americana*) were also present in samples, but their mean 24-h densities over the study period were all <1 individual (range = 0 – 3 individuals). The isopods *C. texensis* and *Lirceolus smithii* were often found in samples, with mean 24-h densities of 2.16 (range = 0 – 6 individuals) and 4.02 individuals (range = 0 – 12 individuals) (Fig. 2C). Unidentified aquatic mites exhibited mean 24-h densities of 0.56 individuals (range = 0 – 4 individuals). A suite of other arthropod species (*Haedeoporus texanus*, *Calathaemon holthuisi*, *Tethysbaena texensis*, an unidentified Bathynellid species, and a Monocerberid isopod) had mean 24-h densities of <0.5 individuals (range = 0 – 2.94 individuals). *Phreatodrobia* snails (*P. nugax*, *P. plana*, and *P. rotunda*) exhibited mean 24-h densities of snails of 3.5 individuals (range = 0 – 8.9 individuals) (Fig. 2C). *Mooreobdella* sp. and *Sphalloplana* sp. had mean 24-h densities of 0.02 (range = 0 – 0.56) and 0.03 (range = 0 – 0.96) individuals, respectively. The Texas blind salamander had relatively low mean 24-h density over the study period (mean = 0.10 individuals, range = 0 – 1 individuals).

Nutrient excretion in stygobiont and epigean species

Across the four stygobiont species I was able to obtain with a high enough frequency or were of sufficient body size/biomass to conduct nutrient excretion trials (i.e., *C. texensis*, *P. antrorum*, *S. flagellatus*, and *T. insolita*), there was a significant decline in mass-specific NH_4^+ excretion rates with increasing body size ($F_{1, 37} = 16.17$, $P < 0.001$; Fig. 3A). When epigean species were included in the regression (*H. azteca* and *P. kadiakensis*), the overall relationship did not substantially change and was also significant ($F_{1, 49} = 72.89$, $P < 0.001$). Mass-specific PO_4^{3-} excretion rates of stygobionts also generally declined with body size, however there was a considerable amount of variation in this relationship and it was not significant ($F_{1, 49} = 2.28$, $P = 0.138$; Fig. 3B). When stygobiont and epigean species were included in the same regression, the overall fit (as indicated by R^2 value) increased substantially and the relationship was significant ($F_{1, 60} = 10.17$, $P = 0.002$). Excreted N:P did not vary with stygobiont body size ($F_{1, 28} = 0.212$, $P = 0.649$; Fig. 3C) and this relationship remained non-significant when epigean species were included in the regression ($F_{1,39} = 0.004$, $P = 0.949$).

When mass-specific NH_4^+ excretion rates of five species were compared, there was a significant difference among species ($F_{4,48} = 20.70$, $P < 0.001$; Fig. 4A). Post-hoc comparisons indicated that the stygobiont isopod *C. texensis* had a significantly lower mass-specific N excretion rates than the Texas cave shrimp, but did not significantly differ from *S. flagellatus*. There was also no significant difference in the mass-specific N excretion rates of the Texas cave shrimp and *S. flagellatus*. *H. azteca* had significantly higher mass-specific N excretion rates than all other species, whereas *P. kadiakensis* exhibited the opposite pattern, exhibiting significantly lower N excretion rates than all

other species. However, when I examined the effect of origin (epigean *vs* hypogean) on mass-specific N excretion rates using organism body size as a covariate, results indicated that origin was not a significant predictor of N excretion rates ($F_{1, 49} = 0.798$, $P = 0.376$).

When mass-specific PO_4^{3-} excretion rates were compared, there was a significant difference among species ($F_{4,49} = 6.09$, $P = 0.001$; Fig. 4B). Post-hoc comparisons indicated that *H. azteca* had a significantly higher P excretion rate than all other species. However, there was no systematic difference in PO_4^{3-} excretion rates between epigean and hypogean species when body size was used as a covariate ($F_{1, 60} = 0.159$, $P = 0.692$). Finally, there was no significant difference among species for N:P of excreted nutrients ($F_{4,38} = 1.76$, $P = 0.160$; Fig. 4C) and origin was not a significant predictor of excreted N:P when body size was used as a covariate ($F_{1, 39} = 2.33$, $P = 0.135$).

Elemental composition of stygobiont and epigean species

Nutrient content of different invertebrates, expressed as a percent dry mass, significantly differed among species for % N ($F_{4, 45} = 13.45$, $P < 0.001$; Fig. 5A) and % P ($F_{4,39} = 3.13$, $P = 0.027$; Fig. 5B), but not for % C ($F_{4,45} = 1.74$, $P = 0.160$; Fig. 5C). Post-hoc comparisons indicated that *C. texensis* had significantly lower % N and % P than most of the other species, whereas *P. kadiakensis*, *P. antrorum*, and *S. flagellatus* had higher % N content than other species. Organism origin was not a significant predictor of % N ($F_{1,45} = 1.94$, $P = 0.171$), % P ($F_{1,38} = 0.12$, $P = 0.734$), or % C ($F_{1,45} = 0.043$, $P = 0.837$).

There were significant differences among species for body C:N ($F_{4,43}$ = 21.61, P < 0.001; Fig. 6A), with *C. texensis* having higher body C:N than other species. There was also a significant difference among epigean and hypogean in body C:N content ($F_{1,43}$ = 4.88, P = 0.030), with hypogean species having significantly higher body C:N than epigean species. There were no significant differences among species for body C:P ($F_{4,39}$ = 1.02, P = 0.412; Fig. 5B) or N:P ($F_{4,39}$ = 0.996, P = 0.422; Fig. 5C). In addition, there were no significant systematic differences among epigean and hypogean species for body N:P ($F_{1,39}$ = 1.75, P = 0.194) and C:P ($F_{1,39}$ = 0.406, P = 0.528).

4. DISCUSSION

Although the Edwards Aquifer is one of the most diverse aquifers in the world (Longley 1981, Gibson *et al.* 2008), I predicted that overall densities and biomasses of stygobionts at the site should be relatively low, indicating low-productivity and resource limited conditions that characterize many subterranean ecosystems. I also predicted that, due to the high degree of environmental stability, organismal abundances and composition would show relatively little temporal variation. In the present study, I detected the presence of 26 taxonomic groups at the Artesian Well site, some of which have yet to be described to the species level. Despite this relatively high taxonomic richness, the 24-h density of organisms collected from the well was overwhelmingly dominated by three taxonomic groups (ostracods, copepods, and *P. antrorum*) and there were no obvious overall temporal trends in the data, such as progressively increasing or decreasing densities of any particular taxonomic group over the study period. However, the 24-h densities of the more abundant organisms (i.e., *P. antrorum*, ostracods, copepods) were also more temporally variable that I predicted throughout the study period (89 – 658 individuals/ 24-h interval) and appeared to exhibit some fluctuation in their 24-h densities (e.g., oscillation over the study period). At this point, the circumstances influencing this variability remain unknown, but could arise from a suite of factors including fluctuation in aquifer levels, variation in flow paths leading the well site, and the timing of reproduction and/or dispersal in stygobionts within the aquifer. These overall results are generally consistent with my prediction that the composition of the stygobiont community would not be highly variable and that the overall taxonomic diversity would not substantially change over the course of the study period. However,

my results also suggest that future studies should conduct more high-resolution sampling to elucidate the temporal variation in stygobiont densities and assess the potential biotic and abiotic factors that may lead to this variability.

Previous studies have examined organismal diversity at the Artesian Well site in the Edwards Aquifer, but the focus has largely been on individual species (e.g., Bowman and Longley 1976; Botosaneanu and Iliffee 2002) or the diversity of a particular group of taxa, such as amphipods (Holsinger and Longley 1980). In the present study, we detected the presence of all of the species which have been previously identified from the site (Holsinger and Longley 1980; Lewis and Bowman 1996). Consistent with previous studies, my data suggests that the Artesian Well is a source for a particularly diverse arthropod assemblage (i.e., 20 arthropod taxa detected over the study period). In particular, we found all of the named amphipod species Holsinger and Longley (1980) identified in their earlier collections from the Artesian Well site. However, the present study found that the relative proportions of some amphipod species differed from those reported in the previous collections. Holsinger and Longley (1980) found that individuals of *T. insolita* made up ~61%, *S. flagellatus* made up 27%, and *T. texensis* made up 8.4% of all amphipods at the site; the remaining species (*S. relicta*, *A. subterannea*, *S. russelli*, *A. hirsuta*, *T. samacos*, and *P. americana*) made up $\leq$1% of total amphipods (Holsinger and Longley 1980). In the present study, *T. insolita* also comprised the highest percent of amphipods collected (~35% of total amphipods), but *S. relicta* exhibited the second highest relative abundance within the amphipod community, making up ~31% of total the amphipods collected. Of the remaining amphipods, *S. flagellatus* made up ~23% and the remaining species (*S. russelli, T. texensis, A. hirsuta,*

H. samacos, P. americana, and A. subterranea) comprised ≤ 1% of the amphipod fauna. The increased relative abundance of *S. relicta* in the current data is likely due to the use of a smaller aperture net to collect organisms. *Seborgia relicta* is small-bodied organism (~1.5 mm long) and Holsinger and Longley (1980) used a 500-μm net in their assessment of the amphipod fauna of the Artesian Well site, whereas I used a 100-μ net to collect stygobionts. Indeed, I collected and identified several other small-bodied taxa which were numerically important members of the stygofauna from the site that had not been previously noted (i.e., ostracods and copepods). Analyzing trends in time-series data can present a variety of analytical and inferential challenges (Jassby and Powell 1990; Yoccoz et al. 2001), but the results presented here emphasize the importance of taking methodological consistency into account, such as the use of similar-sized nets, when analyzing longer-term trends in the abundance and composition of communities through the comparison of contemporaneous and historical data. These results also suggest that the stygobiont faunal diversity of this site and perhaps the Edwards Aquifer may be underestimated, particularly for small-bodied taxa because many of these studies have used larger-aperture nets or have focused only on the larger-bodied species found in the aquifer (e.g., Longley 1981; Gibson et al. 2008).

Stygobiont nutrient recycling and body nutrient content

There are several important factors which influence organismal nutrient excretion rates (Vanni 2002). Organism body size has been identified as a critically important factor mediating nutrient excretion (Vanni 2002) because mass-specific nutrient excretion rates decline with increasing body size due to allometric constraints on metabolic

processes (Peters 1983; Brown et al. 2004). Environmental temperature also influences metabolic rates and potentially nutrient excretion rates because organismal metabolic rates increase with temperature (Devine and Vanni 2002; Vanni 2002; Brown et al. 2004). Other studies have shown that species- and taxon-specific characteristics are also important predictors of nutrient cycling rates (Vanni et al. 2002; Evans-White and Lamberti 2006; McIntyre *et al.* 2007). Because nutrient excretion is constrained through mass-balance processes occurring at the organismal level, excretion rates and ratio of excreted nutrients is influenced by the nutrient composition of an animal's body relative to its food resources (i.e., ecological stoichiometry) (Vanni 2002; Sterner and Elser 2002). Thus, taxonomic-specific characteristics such as morphology, growth rate, and diet/trophic level can influence nutrient recycling rates and ratios (Sterner and Elser 2002; Vanni et al. 2002; Elser et al. 2003; McIntyre et al. 2007). At the scale of communities and ecosystems, consumer species that are abundant or occupy a relatively large proportion of the community biomass can be particularly important in the supply of nutrients via recycling (Taylor et al. 2006), thus changes in the overall biomass and/or composition of animal communities can have implications for nutrient availability and the ratio at which those nutrients are supplied (Vanni et al. 2002; McIntyre et al. 2007).

In the study presented here, I found that body size was an excellent predictor for nutrient excretion rates (but not ratios) across the stygobiont species I examined. I was able to estimate mass-specific N and P excretion rates in four of the larger-bodied and more commonly encountered stygobiont species at the Artesian Well site. These species represented a substantial range in individual body sizes (0.20 – 35.0 mg DM/individual) and mass-specific N and P excretion rates declined with increasing body size across this

body size range in stygobionts. In addition, when related surface water-associated taxa were included in the data set the body size range increased (0.20 – 51.0 mg DM/individual) and the same patterns were apparent. Declining mass-specific excretion rates with increasing body size is consistent with allometric metabolic constraints (Peters 1983) and has been observed in a diversity of aquatic taxa, including benthic invertebrates (Wen and Peters 1994; Devine and Vanni; 2002; Alves et al. 2010).

In the present study, taxonomic identity was not a particularly strong predictor of nutrient recycling rates and ratios for stygobiont species. Although the three species for which I was able to obtain multiple estimates of nutrient excretion rates (*C. texensis*, *P. antrorum*, and *S. flagellatus*) differed in taxonomy and trophic ecology (Hutchins et al., *in press*), the only significant difference I observed among these species was that *C. texensis* had significantly lower N excretion rates than *P. antrorum*. These results suggest that, using nutrient excretion as a metric, there may be a relatively high degree of functional redundancy among hypogean taxa within the Edwards Aquifer. Most studies examining taxon-specific differences in excretion have been conducted on aquatic vertebrates (e.g., fish and amphibians) (Vanni et al. 2002; Torres and Vanni 2007; McIntyre et al. 2007) and studies which have examined these differences among macroinvertebrates are less common (Devine and Vanni 2002; Benstead et al. 2010; Alves et al. 2010). For freshwater invertebrates, data indicating clearly-defined taxon-specific differences in nutrient excretion rates and ratios, even among fairly disparate groups are equivocal (Devine and Vanni 2002; Spooner and Vaughn 2008; Benstead et al. 2010; Alves et al. 2010). However, it is important to note that I was only able to compare the excretion rates and ratios for three species and there are >25 species at the

Artesian Well site. Thus, when possible or practical, there should be an effort to collect additional nutrient excretion data from additional taxonomic groups within the aquifer so that researchers can more thoroughly evaluate the degree of functional redundancy within Edwards Aquifer hypogean species in terms of nutrient recycling rates and ratios.

In this study, I found that elemental composition of stygobionts varied significantly between species, with the amphipod *C. texensis* having significantly lower body nutrient content (% N and % P) and a higher body C:N than the other stygobiont species. A number of studies have found that benthic invertebrate body stoichiometry can vary significantly among taxonomic groups and with trophic position (Frost *et al.* 2003, Evans-White *et al.* 2005). My results are in contrast with other studies which have examined the elemental composition of surface isopods and found that they generally have lower body C:N than other surface-water associated invertebrates (Liess and Hillebrand 2005). Liess and Hillebrand (2005) found that the surface isopod *Asellus aquaticus* exhibited body % C, % N, and % P values of 35%, 7.7%, and 0.73%, respectively. In comparison, I found that the body nutrient composition of *C. texensis* was 37.88% (range = 33.52 – 43.25%) C, 8.08% (range = 7.34 – 9.41%) N, and 0.65% (range = 0.48 – 1.00%) P. These data indicate that, although *C. texensis* have lower nutrient content that the other stygobitic invertebrates from the Edwards Aquifer I examined, their body composition is not greatly different from data reported for other isopod species. In the present study, I did not assess the mechanisms behind the observed differences between *C. texensis* and the other two stygobiont species I examined, but differences among taxa may be related to differences among species in tissue composition and/or growth rates (Vanni et al. 2002; Elser et al. 2003).

Comparison of excretion rates and body stoichiometry of hypogean and epigean species

It has been posited that hypogean organisms have lower metabolic and growth rates than epigean species as an adaptation to the presumed low energy availability in subterranean ecosystems and some data clearly support this prediction (Barr 1968; Poulson and White 1969; Streever 1996; Hervant and Renault 2002). However, in this study, I found that mass-specific N and P excretion rates did not systematically differ between epigean and hypogean species when corrected for body size, suggesting that metabolic rates between these two groups of species does not fundamentally differ. Other studies have similarly reported lack of variation between hypogean and epigean species in terms of metabolic activity (Gannon et al. 1999; Bishop et al. 2014). Recently, Bishop et al. (2014) compared indirect measures of metabolic rates (enzymatic activity) in two of the species utilized in this study (*P. antrorum* and *P. kadiakensis*) and found that these two species did not significantly differ in measures of enzymatic activity. The lack of differences in nutrient excretion rates I observed between stygobionts and epigean species may be due to the unique conditions of the Edwards Aquifer at the Artesian Well site. Hutchins et al. (*in press*) found that the stygobiont food web at the Artesian Well site is supported by both subterranean chemolithoautotrophic production and inputs surface photosynthetic organic matter (OM) and the stygobiont community may be less susceptible to persistent energy (food resource) limitation as other portions of the aquifer that are solely reliant upon surface OM inputs (i.e., sites which are located farther west in the aquifer). To assess this hypothesis, it would be insightful to examine metabolic and/or nutrient excretion rates across a diversity of sites in the Edwards Aquifer which vary in the relative importance of chemolithoautotrophic and surface OM sources.

I predicted that stygobionts would have lower body P (and potentially N) content than related epigean species due to the hypothesized lower growth rates from living in environmental conditions associated with subterranean environments (e.g., low body P and rRNA content related to the GRH), but I did not find significant systematic differences in % P and % N body content between hypogean and epigean species. In contrast to my results, Schneider et al. (2010) compared body P content among troglophilic and troglobitic arthropods in terrestrial caves and found that troglobionts generally exhibited lower body % P than troglophilic taxa. However, I found that hypogean species exhibited higher C:N body ratios (indicative of lower N per unit C) when compared to epigean species. Higher body C:N may be reflective of lower protein content, which would be consistent with hypogean species exhibiting lower protein synthesis and growth rates (Hessen et al. 2007). In the present study I did not determine protein or rRNA content of stygobiont and epigean species and future studies should explicitly compare these groups to species to determine if any differences in body stoichiometry are related to differences in biochemical composition. Schneider et al. (2010) found that obligate cave millipedes had lower RNA/DNA ratios than related troglophilic millipedes (presumably supportive of the GRH), but that this difference was due to lower DNA content of troglophilic species and not because of lower RNA content of obligate troglobitic millipedes. In addition, Bishop et al. (2014) compared the protein content of *P. antrorum* and *P. kadiakensis* from our same field sites and determined that these species did not significantly differ. Cumulatively, results from these studies and my data indicate that there is clearly a need for further investigation into the relationships

between growth rates, elemental composition, proximate biochemical content in surface and stygobitic species.

5. CONCLUSION

To my knowledge, this is the first study to examine excretion and elemental composition of obligate subterranean aquifer species and to compare these excretion rates and elemental composition to related surface species. I found that stygobionts can recycle nutrients at rates equivalent to surface species and that the densities emerging from my study site in the Edwards Aquifer were relatively high (>300 individuals per 24 h). In addition, I found that one of the most numerically-dominant species emerging from the aquifer (*P. antrorum*) is also one of the larger-bodied organisms and thus likely to be biomass-dominant in Artesian Well portion of the Edwards Aquifer. Nutrient recycling by benthic invertebrate communities in surface ecosystems can be important nutrient sources (e.g., Devine and Vanni 2002; Hall et al. 2003) affecting the stoichiometry and production of surficial biofilms (Evans-White and Lamberti 2006; Liess and Haglund 2007). However, there is still a need to gain greater understanding of ecosystem-level influences of nutrient recycling by stygobionts in subterranean aquatic systems such as the Edwards Aquifer because we were only able to obtain excretion rate and elemental composition from several of the more common and larger-bodied species. In addition, many stygiobiont species are listed as species of concern and there is a need to relate potential changes in their biomass or abundance to ecosystem-level effects on processes such as nutrient cycling (e.g., McIntyre et al. 2007).

Table 1. Numbers of stygobiont taxa collected periodically from the Artesian Well, San Marcos, Texas (USA) between February 2013 - April 2014. Organism numbers (CPE) are reported as the mean (and range) number of organisms caught standardized for a 24-h collection period effort (catch-per-effort; CPE). Numbers in parentheses are the ranges of organisms caught per 24-h effort.

Group	Family	Species	CPE (#/24h)	CV (%)
Copepoda	--	--	90 (8 - 502)	81.26
Ostracoda	--	--	65 (14 - 280)	95.56
Amphipoda	Crangonyctidae	*Stygobromus flagellatus*	4 (0-16)	68.81
		Stygobromus russelli	0.04 (0 - 1)	423.47
	Hadziidae	*Texiweckelia texensis*	1.2 (0 - 6)	109.13
		Texiweckeliopsis insolita	6 (0 - 23)	93.94
		Holsingerius samacos	0.2 (0 - 3)	267.22
		Allotexiweckelia hirsuta	0.2 (0 - 1.4)	228.82
	Artesiidae	*Artesia subterranea*	0.2 (0 - 2)	232.96
	Sebidae	*Seborgia relicta*	5.6 (0 - 26)	91.23
	Bogidiellidae	*Parabogdiella americana*	0.3 (0 - 3)	219.29
Decapoda	Palaemondiae	*Paleomenetes antrorum*	129 (90 - 658)	61.69
		Calathaemon holthuisi	0.08 (0 - 1)	323.04
Isopoda	Cirolanidae	*Cerolanides texensis*	2.16 (0 - 6)	69.02
	Asselidae	*Lirceolus smithii*	4.02 (0 - 12)	67.65
	Microcerberidae	--	0.01 (0 - 0.3)	793.72
Insecta	Dytiscidae	*Haideoporus texanus*	0.2 (0 - 2)	262.43
Coleoptera	Elmidae	*Microcylloepus spp.*	0.02 (0-1)	653.01
		Stenelmis spp.	0.3 (0-3)	227.52
Gastropoda	Rissooidea	*Phreatodrobia nugax*	2 (0 - 7)	75.85
		Phreatodropbia plana	1.5 (0 - 5)	82.08
		Phreatodrobia rotunda	0.02 (0 - 0.7)	496.65

Table 1, Continued. Numbers of stygobiont taxa collected periodically from the Artesian Well, San Marcos, Texas (USA) between February 2013 - April 2014. Organism numbers (CPE) are reported as the mean (and range) number of organisms caught standardized for a 24-h collection period effort (catch-per-effort; CPE). Numbers in parentheses are the ranges of organisms caught per 24-h effort.

Acari: Trombidiformes	Hydrcarinadae	--	0.56 (0 - 4)	137.51
Thermosbaenacea	Monodellidae	*Tethysbaena texana*	0.3 (0 - 3)	194.29
Bathynellacea	--	--	0.1 (0 - 1.6)	259.67
Arhynchobdellida	Erpobdellidae	*Mooreobdella spp.*	0.02 (0 - 0.6)	460.77
Tricladida	Kenkiidae	*Sphalloplana spp.*	0.03 (0 - 0.96)	505.53
Vertebrata	Plethodontidae	*Eurycea rathbunii*	0.10 (0 - 1)	278.01

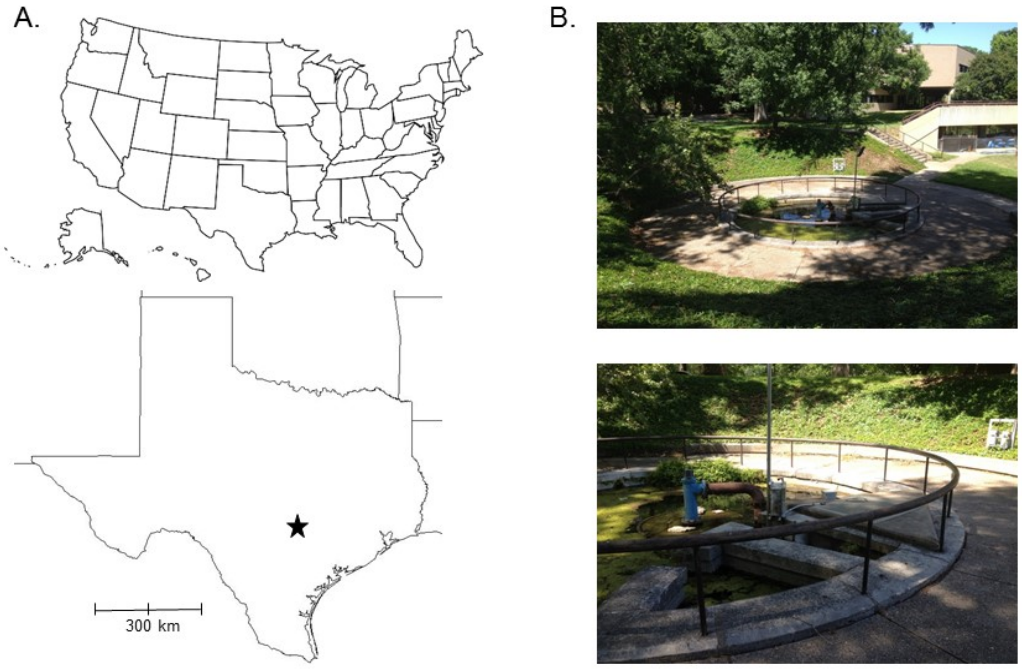

Figure 1. Location of Artesian Well on Texas State University campus. (A) Location of the Artesian Well site in central Texas, USA; (B) the Artesian Well structure located on the Texas State University campus in San Marcos, Texas.

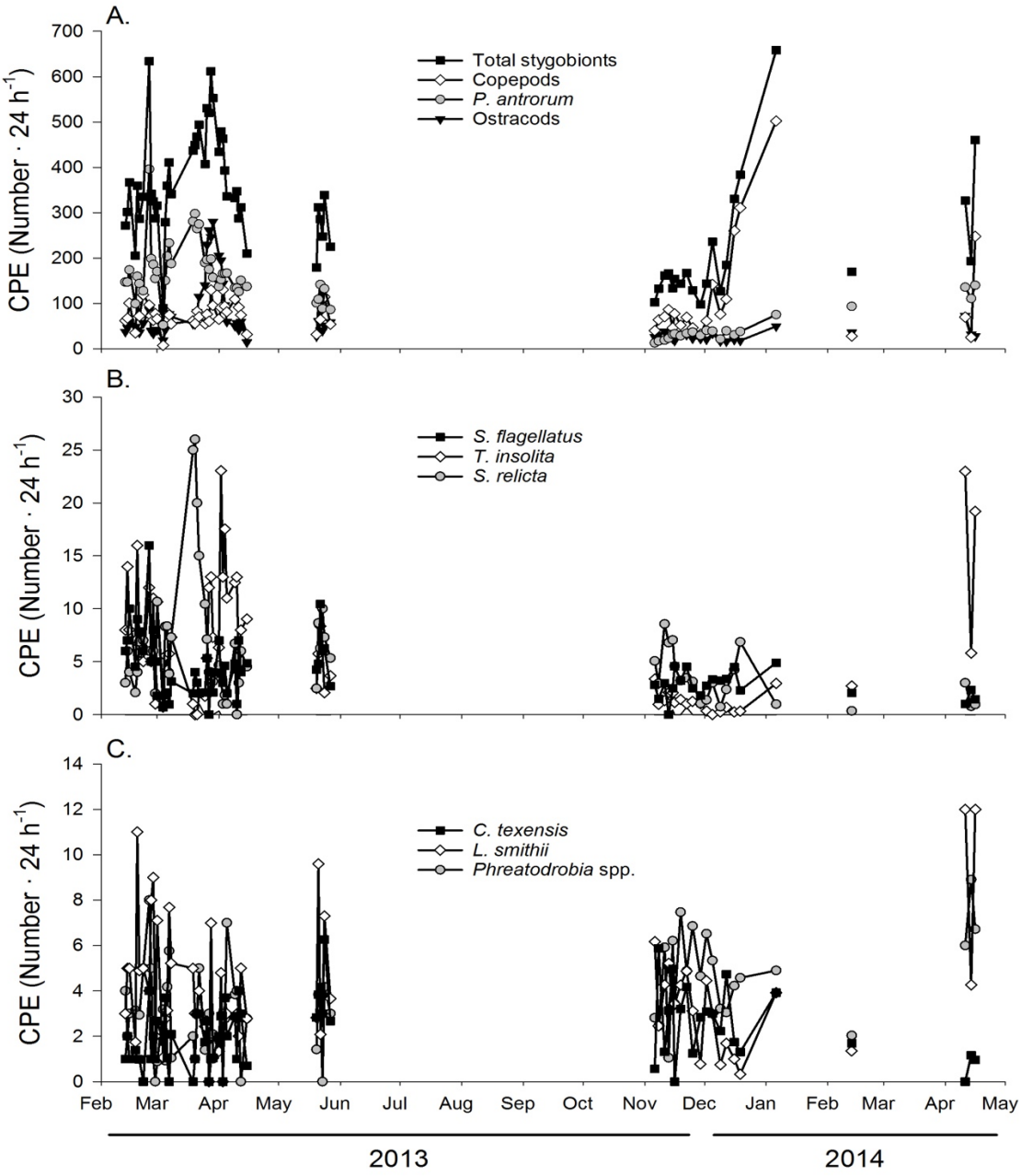

Figure 2. Time series of 24-h corrected densities of total stygobionts and several of the more common invertebrate taxa collected from the Artesian Well between February 2013 - April 2014. Catch per unit effort (CPE) is expressed as number of individuals per 24-h time period. 24-h densities of the three most commonly encountered groups of organisms to the total number of stygobionts (A), densities of the three most common amphipod species (B), and the three more common isopods and gastropods (C).

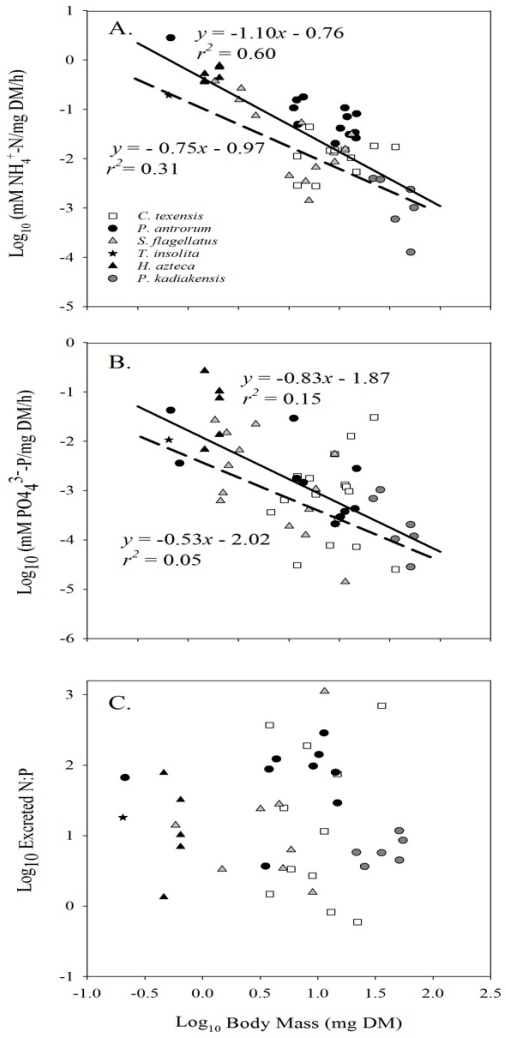

Figure 3. Relationship between mass-specific nutrient excretion rates and organism body mass for stygobiont and epigean species (mg DM). Relationships between mass-specific N excretion rate (A), mass-specific P excretion rate (B), and the N:P ratio of excreted nutrients (C) and organism body mass for stygobiont and epigean species (mg DM). Regression equations and r^2 values are shown for stygobionts only (dashed line) and stygobionts and epigean species combined (solid line). No regression lines are shown for excreted N:P (panel C) because the relationships were not significant.

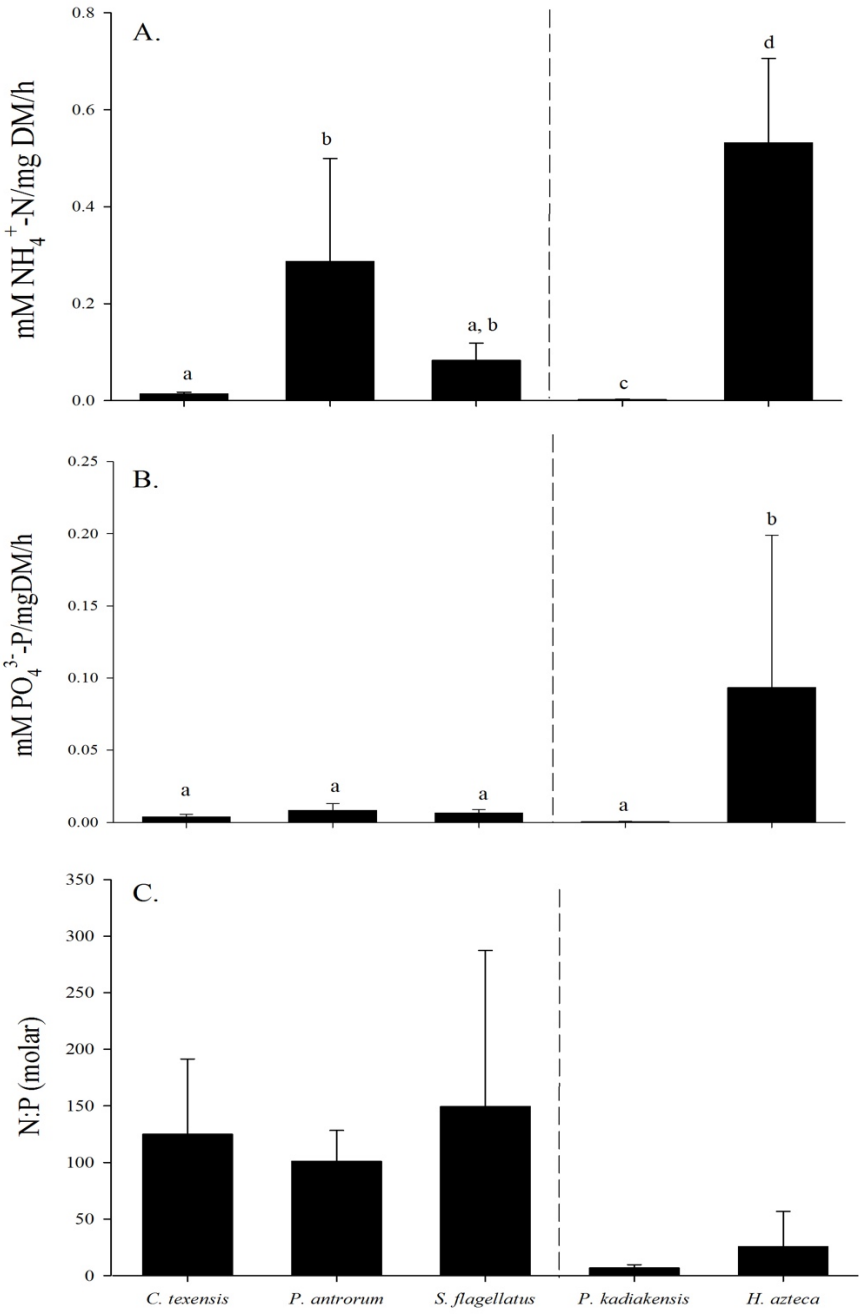

Figure 4. Mass-specific nutrient excretion rates and ratios (mean ± 1 SE) for hypogean and epigean species. Mass-specific N excretion rates (hypogean on left side of each panel; epigean on right side of each panel) (A), mass-specific P excretion rates (B), and the N:P of excretion (C). Letters at the top of each column are present if an overall significant difference among taxa was detected with one-way ANOVA and letters represent homogenous subset based upon Tukey's HSD post-hoc comparisons.

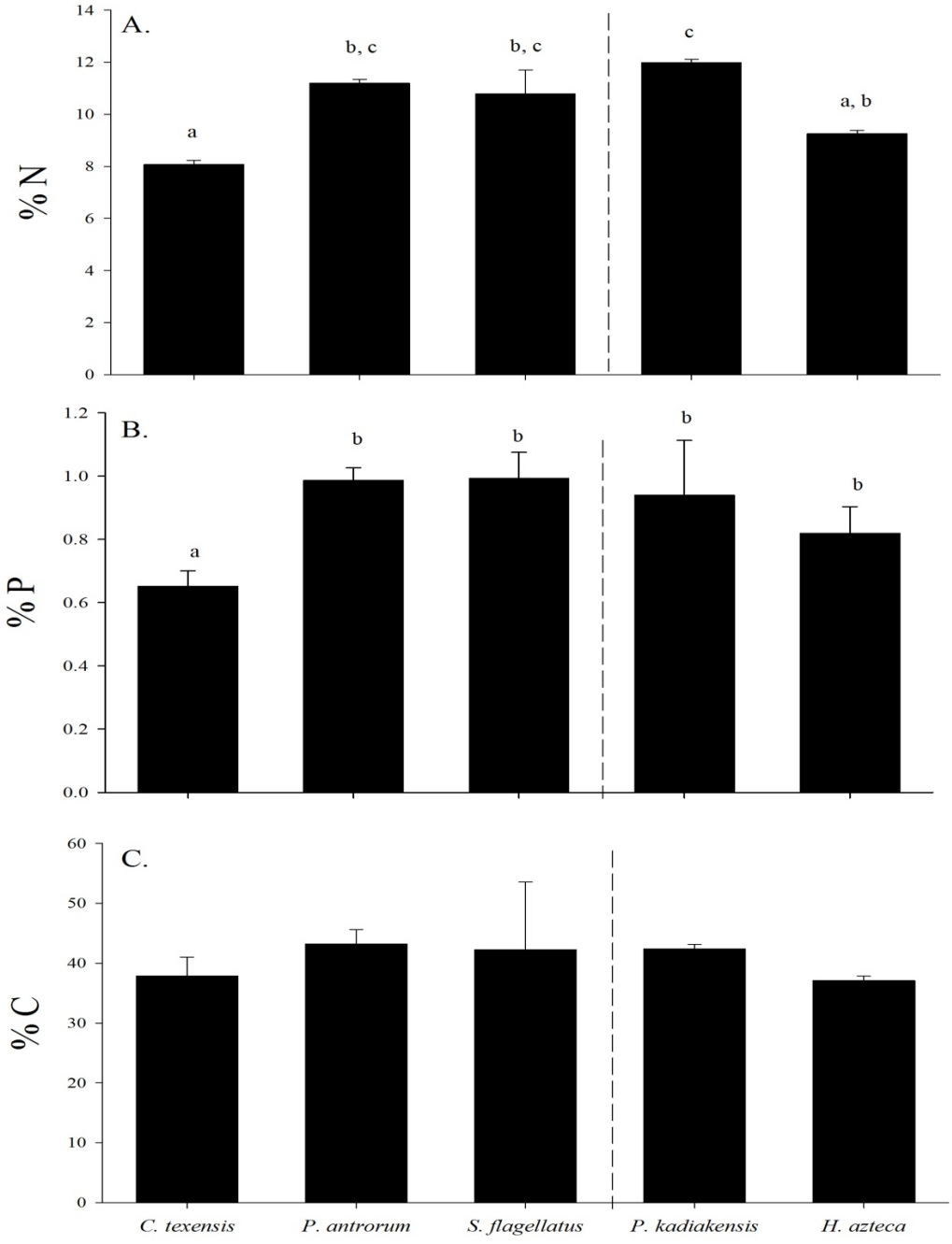

Figure 5. Percent body composition (% of dry mass) for nutrients (mean ± 1 SE) in hypogean and epigean species. Percent N (hypogean on left side of each panel; epigean on right side of each panel) (A), percent P (B), and percent C (C). Letters at the top of each column are present if an overall significant difference among taxa was detected with one-way ANOVA and letters represent homogenous subset based upon Tukey's HSD post-hoc comparisons.

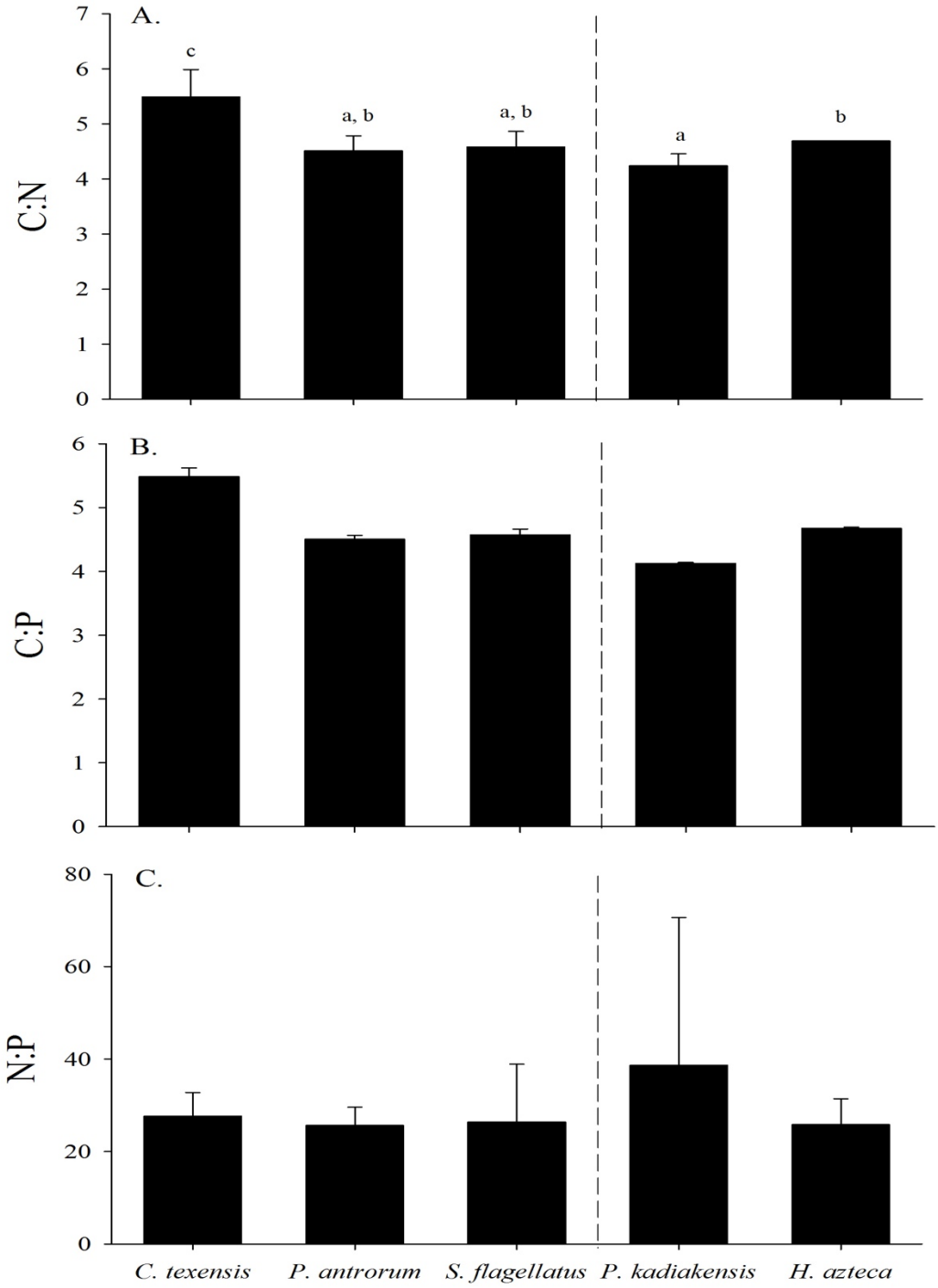

Figure 6. Elemental ratios (mean ± 1 SE) of hypogean and epigean species. Molar C:N (hypogean on left side of each panel; epigean on right side of each panel) (A), C:P (B), and N:P (C) are presented. Letters at the top of each column are present if an overall significant difference among taxa was detected with one-way ANOVA and letters represent homogenous subset based upon Tukey's HSD post-hoc comparisons.

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