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ORIGINAL PAPER

Early-earth nonprotein amino acid metabolites in modern cyanobacterial microbialites

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Abstract

Cyanobacteria are among the earth's oldest known living groups of organisms and can form layered accretions called microbialites, found in both the fossil record and existing lakes. Studies of cyanobacterial biochemical processes help to understand the evolution of life on earth. The conserved metabolism of cyanobacterial species includes the biosynthesis of unusual nonprotein amino acids such as *N*-(2-aminoethyl)glycine, hypothesized to have constituted an early form of genetic information in cells. Pavilion Lake in British Columbia, Canada, hosts a population of unique, actively growing microbialites covered in biofilms dominated by cyanobacteria. We hypothesized that the living microbial communities produce dinitrogenous nonprotein amino acids, such as *N*-(2-aminoethyl)glycine and its structural isomers β-*N*-methylamino-L-alanine, 2,4-diaminobutyric acid and β-aminomethyl-L-alanine. We analyzed samples in sediment traps collected between 2007 and 2014 in depths ranging from 11 to 46 m. *N*-(2-aminoethyl)glycine, 2,4-diaminobutyric acid and β-aminomethyl-L-alanine were found in highest concentration in the shallowest microbialite biofilms with a maximum of 22 ng/g, 33 ng/g and 0.4 ng/g, respectively. In contrast, the concentration of β-*N*-methylamino-L-alanine was highest in collections between 18 and 26 m depths and only β-*N*-methylamino-L-alanine was found in the deepest water collections. These data provide evidence indicating that the production of these nonprotein amino acids is highly conserved through the evolution of cyanobacteria and suggest that the nitrogen-rich metabolites may have had both an important role in ancient and modern cyanobacterial metabolism. Further research will determine the role of *N*-(2-aminoethyl)glycine and its isomers in early life metabolism and their current function in photosynthetic cells.

Keywords Pavilion Lake · *N*-(2-aminoethyl)glycine (AEG) · β-*N*-methylamino-L-alanine (BMAA) · Microbialites · Cyanobacteria · Microbial biofilms

Introduction

It has been hypothesized that prior to the development of DNA-based organisms about 3.5 billion years ago, the critical information for life was conserved and transmitted through RNA as the genetic material (Cech 1986; Joyce 2002). An even earlier system may have relied on polymers of *N*-(2-aminoethyl)glycine as the backbone for peptide nucleic acids for storage and transmission of genetic information in the pre-RNA era (Nielsen 1993; Banack et al. 2012). Modern cyanobacteria and some eukaryotes produce *N*-(2-aminoethyl)glycine and several isomers including β-*N*-methylamino-L-alanine, 2,4-diaminobutyric acid and β-aminomethyl-L-alanine (Cox et al. 2005; Banack et al. 2012; Réveillon et al. 2015) (Fig. 1). The metabolic function of these nonprotein amino acids has not been fully

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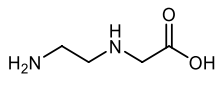
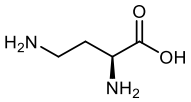
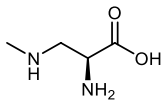


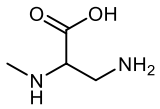
N-(2-aminoethyl)glycine



2,4-diaminobutyric acid



β-*N*-methylamino-L-alanine



β-aminomethyl-L-alanine

Chemical Formula: $C_4H_{10}N_2O_2$
Exact Mass: 118.07
Molecular Weight: 118.14
m/z: 118.07 (100.0%), 119.08 (4.3%)
Elemental Analysis: C, 40.67; H, 8.53; N, 23.71; O, 27.09

Fig. 1 Structural isomers of low molecular weight diamino nonprotein amino acids

elucidated, and it is possible that *N*-(2-aminoethyl)glycine and its isomers are remnants of ancient metabolism. We hypothesize that the study of cyanobacteria in unique ecosystems will reveal the environmental factors that influence production of *N*-(2-aminoethyl)glycine and its isomers.

Cyanobacteria are found in virtually all terrestrial and aquatic environments, including those with extreme conditions such as high salinity or extreme temperatures, and are prominent indicators of biological activity in the fossil record for most of earth's history (Grotzinger and Knoll 1999; Paerl and Otten 2013). Fossils of microbial life on earth and layered accretion structures resulting from microbial biofilms may represent the earliest appearance of life on earth (Awramik et al. 1983; Brasier et al. 2004; Foster and Green 2011; Stal 2012). Microbialites have been defined as "living rocks" that are formed through the microbially induced lithification of calcium carbonate by bacteria and microeukaryotes such as diatoms (Dupraz et al. 2009). Microbialite history reflects complex biogeochemical processes; thus, a better understanding of microbialites and associated microbial communities can yield clues into the advent of life on earth.

Pavilion Lake, in the interior of British Columbia, Canada, hosts large, modern microbialites with unusual morphologies (Laval et al. 2000) (Fig. 2). This freshwater, ultra-oligotrophic lake offers a unique limnological setting to study *N*-(2-aminoethyl)glycine production by microbialite-associated cyanobacteria, as most other extant microbialites are found in extremely harsh environments where metazoan life forms are not viable (Lim et al. 2009). The oldest specimens in Pavilion Lake are thought to have formed approximately 11,000 years ago, after the glacial retreat of the Cordilleran Ice Sheet, and active accretion likely occurs via the microbially mediated precipitation of carbonate (Laval et al. 2000; Lim et al. 2009; Brady et al. 2010; Russell et al. 2014).

While the study of extreme ecosystems presents many difficulties for sample and data collection, we were able to access microbialite samples from Pavilion Lake collected during NASA diving expeditions between 2007 and 2014 (Fig. 2, Supplemental Tables 1 and 2). We hypothesized that the microbialites in Pavilion Lake produce *N*-(2-aminoethyl)

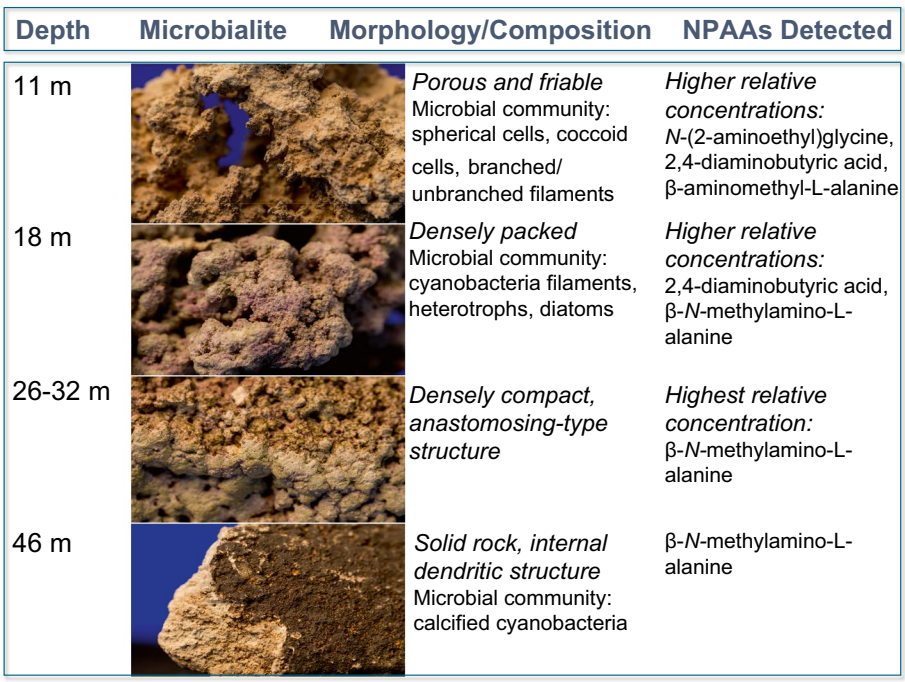


Fig. 2 Collections of microbialites from Pavilion Lake, including morphological and microbial community compositional differences at different depths. Nonprotein amino acids (NPAAs) were detected in different concentration profiles at different depths. For more detailed photographs of microbialites, see Figure S2

glycine and its related isomers. We also used data collected from previous studies to form hypotheses about the effects of environmental conditions on the production of *N*-(2-aminoethyl)glycine and related isomers. The objective of this study was to incorporate new nonprotein amino acid data with existing data, such as nitrogen and light availability as well as varying microbial communities associated with microbialites present at different depths within the lake, to further understand cyanobacterial metabolism in a unique ecosystem.

Experimental

Sample collection

Pavilion Lake, British Columbia, is located in the south-central interior of British Columbia (50.51′ N; 121.44′ W) and is 5.7 km long, with a mean width of 0.8 km and a maximum depth of 65 m (Lim et al. 2009; Supplemental Figure 1). The lake is dimictic, circumneutral (median pH 8.3) and oligotrophic with phosphorus being the limiting nutrient. The current climate is mostly arid, and the lake thermally stratifies during the summer. The surface layer is typically around 20 °C in the summer, with a maximum thickness of 10 m. Lake temperature decreases rapidly below the thermocline, down to 4 °C in benthic waters (Lim et al. 2009). Carbonate microbialites with the surface-covering biofilm were collected from a variety of depths and locations via SCUBA, as described previously (Lim et al. 2009) (Fig. 2, Supplemental Table 1). In brief, diver deployed transects, consisting of a line staked to the bottom perpendicular to the shoreline from 7 to 24 m depth, were established on the east and west side of the Central Basin. SCUBA divers recovered sediment traps placed at 18 and 40 m depth along the west transect and at 30 m along the east transect. The recovered sediments were transported frozen to the collection repository at McMaster University where they were lyophilized to complete dryness. Samples were collected at: 11 m depth between April and August, 19 m depth between April and June, 26 m depth between June and July and 32–46 m depth in July. For more information about sample collection and locations, see Table S1 and Figure S1. Lyophilized subsamples were shared with UBC in October of 2015 where a small piece of each sample was ground to a fine powder and extracted.

Detection and quantification of nonprotein amino acids

Chemicals

Standard solvents and eluents included methanol (Optima LC/MS grade, Fisher Scientific, Mississauga, ON), acetonitrile (Optima LC/MS grade, Fisher), ammonium acetate (99.999%, Aldrich, Sigma, St. Louis, MO), ammonium formate (> 99.995%, Aldrich, Sigma), formic acid (98.0–100%, Sigma-Aldrich) and water (18 MΩ Direct Q3, Millipore, Mississauga, ON). Amino acids were derivatized with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) using the standard commercial kit (AccQ·Fluor Derivatization Kit, Waters Inc., Mississauga, ON).

Analytical standards

Stock standards of β-*N*-methylamino-L-alanine, 2,4-diaminobutyric acid (Sigma-Aldrich Canada Co., Oakville, ON) and *N*-(2-aminoethyl)glycine (Toronto Research Chemicals, Toronto, ON) were obtained from commercial sources. The fourth isomer, β-aminomethyl-L-alanine, was synthesized in house using previously published procedures (Bishop et al. 2018). All standards were prepared to give identical concentrations for the isomers with all initial stock solutions made at 65 µmol/mL. Three tenfold dilutions were performed, preparing concentrations of stock solutions at 0.065 µmol/mL for each isomer. A dilution series was prepared for each standard curve by combining 100 µL of each 65 nmol/mL stock solution with 700 µL of 0.1 N trichloroacetic acid (TCA) (6.5 nmol/ml of each isomer). From this, a 12-step dilution series was prepared from the higher concentration stock by taking 250 µL of the previous standard and combining it with 750 µL of 0.1 N TCA to create the next standard (1:4 dilutions). These stock standards were stored at − 20 °C for a maximum of 4 months.

Sample preparation

Each sample was prepared in triplicate beginning with approximately 50 mg of dried tissue hydrolyzed in a glass vial (4 mL). Since the samples contained carbonates, some of the samples produced substantial foam in response to the acid. Therefore, acid was added slowly in ten intervals of 50 µL of 6 N HCl with vortex mixing after each addition. Another 500 µL of acid was then added, resulting in a total volume of 1000 µL. Three of the samples reacted significantly with the acid and were hydrolyzed in larger (8 mL) vials to contain the reactions. The samples were purged with a constant nitrogen flow for 15 s before immediately capping the vials. Samples were hydrolyzed for 18–22 h at 110 °C. After hydrolysis, extracts were vortexed and filtered with centrifuge filters (Ultra Free MC PVDF, 0.22 µm, Millipore, USA) for 5 min at 13,000×*g*. An aliquot (50 µL) of each extract filtrate was transferred into a 1.5-mL Eppendorf tube and dried to complete dryness (LabConco Centrivac). Once dry, samples were derivatized by adding 80 µL of borate buffer, vortex mixing and then derivatized with 20 µL of 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AccQ-Fluor; AccQ-tag; Waters). Derivatized amino acid samples

were transferred into inserts in glass autosampler vials for injection.

Detection and quantification of nonprotein amino acids

Derivatized extracts containing nonprotein amino acids were injected in 10 µL aliquots via an Acquity I-Class Ultra-Performance Liquid Chromatography system (Waters) as previously described (Glover et al. 2015; Baker et al. 2018; Bishop et al. 2018). Amino acids were separated via reverse phase chromatography on a Phenomenex C18 column (30 × 3 mm, 2.6 µm; 100 Å; Torrance, CA) with an elution gradient of 20 mM ammonium acetate (Eluent A) and methanol (Eluent B) (90:10; 0:0–4:50, 70:30; 4:50–6:00, 25:75; 6:00–7:00; 90:10; 7:00–8:00 at a flow rate of 0.35 mL/min) at 55 °C (Baker et al. 2018) (Figure S3). The injection needle was washed between each sample with a solution of 90% acetonitrile in e-pure water.

Eluted amino acid derivatives were detected by tandem mass spectrometry (Xevo TQ-S TripleQuad, Waters) with electrospray ionization (ESI) in positive mode (ES +) as described previously (Glover et al. 2015; Baker et al. 2018). The derivatization binds a fluorescent tag to each nitrogen of the four nonprotein amino acid isomers giving a final molecular weight of 458.17 (m/z 459.18) for detection (Glover et al. 2015; Baker et al. 2018). The capillary voltage was 2.50 kV, and cone voltage was 145.00 V with a source offset of 20.0 V. The source temperature was 150 °C, and the desolvation temperature was 550 °C. The cone and desolvation gas both used nitrogen and were set to 150 L/h and 800 L/h, respectively. Argon gas was used in the collision cell, and the flow was set to 0.30 mL/min. The nebulizer gas flow was set to 7.00 bar. Data were acquired using previously optimized MRM transitions (Bishop et al. 2018) with MassLynx V4.1, and acquired data were processed using TargetLynx V4.1 (Waters) (Glover et al. 2015; Baker et al. 2018; Bishop et al. 2018).

Nonprotein amino acids were quantified by comparison to authentic standard curves prepared from the dilution series of β-aminomethyl-L-alanine, β-*N*-methylamino-L-alanine, *N*-(2-aminoethyl)glycine and 2,4-diaminobutyric acid ranging from 5 to 7.45×10^{-8} ng on column (Figure S2). Quantification and statistical analysis of nonprotein amino acids were performed using Excel™ and RStudio™. The limit of detection (LOD) determined by injection of standards was less than 0.01 pg on column, and the limit of quantification (LOQ) determined by injection of standards was less than 0.02 pg on column. The percent relative standard deviation of standards was less than 2.4% over the analysis period. Details of the Single Laboratory Validation of the analytical method has been published previously (Glover et al. 2015; Baker et al. 2018; Bishop et al. 2018).

Results and discussion

Quantification of diamino nonprotein amino acids

Distinct changes in microbialite morphology and microbial community structure exist with decreasing depth of the microbialites (Laval et al. 2000; Brady et al. 2010, 2014; Russell et al. 2014; Table S2; Fig. 2). Diamino nonprotein amino acids were found and quantified in microbialite samples ranging from depths of 11 to 46 m (Figs. 2, 3). β-*N*-methylamino-L-alanine was the only isomer identified and quantified in the samples collected in all parts of the lake, while the other isomers were found in higher concentration in the shallowest microbialites with the greatest light availability (Figs. 2, 3). *N*-(2-aminoethyl)glycine was found at a maximum concentration of 22 ng/g at a depth of 11 m and lowest concentration at 6.8 ng/g at 32 m (Fig. 3a). 2,4-diaminobutyric acid was present at the highest concentration with a maximum of 33 ng/g at 18 m depth while the 26 m samples showed a slight decrease before a more significant decrease in abundance was observed in the 32 m sample at 8.2 ng/g (Fig. 3c). While both *N*-(2-aminoethyl)glycine and β-aminomethyl-L-alanine showed an overall pattern of decreasing concentration with depth, β-aminomethyl-L-alanine was present at much lower overall concentrations (maximum 0.4 ng/g) compared to the other isomers (Fig. 3d). β-*N*-methylamino-L-alanine displayed a slightly different profile than its isomers, as it increased with depth to a maximum concentration of 5 ng/g at 26 m before decreasing in samples collected at deeper depths, to a minimum of 1.3 ng/g at 46 m (Fig. 3b). No statistical differences were found between sample depths for each isomer after ANOVA with post hoc Tukey's HSD analysis. However, statistical differences were found between 2,4-diaminobutyric acid and other isomers at different sample depths ($\alpha = 0.05$). Statistical differences between 2,4-diaminobutyric acid and isomers were found at: 11 m depth (β-*N*-methylamino-L-alanine/β-aminomethyl-L-alanine, $p < 0.01$); 18 m depth (β-*N*-methylamino-L-alanine, $p < 0.01$; β-aminomethyl-L-alanine, $p < 0.001$); 26 m depth (β-aminomethyl-L-alanine, $p < 0.05$); 32 m depth (β-*N*-methylamino-L-alanine, $p < 0.01$; β-aminomethyl-L-alanine $p < 0.001$); 46 m depth (β-*N*-methylamino-L-alanine/β-aminomethyl-L-alanine, $p < 0.01$).

Microbial metabolic responses to environmental conditions

Effect of light availability on microbial metabolism

Pavilion Lake offers an interesting opportunity to investigate metabolites produced by cyanobacteria associated

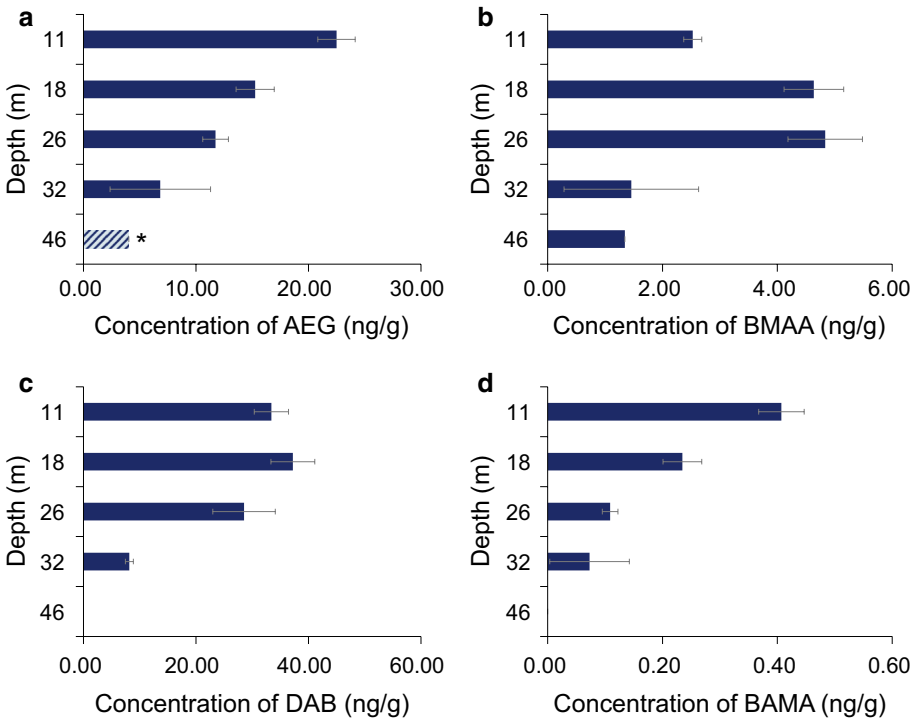


Fig. 3 Quantification of nonprotein amino acids in carbonate microbialites collected in Pavilion Lake. **a** *N*-(2-aminoethyl)glycine (AEG)*. **b** β-*N*-methylamino-L-alanine (BMAA). **c** 2,4-diaminobutyric acid (DAB). **d** 2,3-diaminobutanoic acid (β-aminomethyl-L-alanine; BAMA). For each isomer, at: 11 m depth, $n=12$; 18 m depth, $n=6$; 26 m depth, $n=6$; 32 m depth, $n=3$; 46 m depth, $n=3$.*AEG was detected in one sample at 46 m but ND in other replicates

with a modern ecosystem that is considered an analogue of ancient ecosystems present on early earth (Ansdell et al. 2011). Typically, under non-bloom conditions, which would prevail in this oligotrophic lake, light levels are greatest in surface waters and then decrease with depth. Photosynthetically active radiation falls to below 1% at depths of ~ 32 m in the summer months, and in the deeper waters, light levels fall to 0.1% of surface irradiance (Brady et al. 2009; Lim et al. 2009). Due to collection constraints, we were unable to obtain samples consistently each year and month between 2007 and 2014 for direct comparison (Table S1). However, we compared individual samples collected at 11 m depth grouped by collection month across multiple years and found that *N*-(2-aminoethyl)glycine was 3–4.5 fold higher in August (50 ng/g) than April, June or July (11–16 ng/g). β-*N*-methylamino-L-alanine was highest in April (4.2 ng/g) and August (3.2 ng/g) while 2,4-diaminobutyric acid was present in high concentrations in April (77.6 ng/g), June (35.1 ng/g) and August (20.2 ng/g), but the concentration in July was only about 0.5 ng/g. These data indicate that the detection of the diamino acids is related to the conditions of the lake and could be related to variability in temperature, light or nutrients or changes to the microbial community structure due to those factors.

Effect of nutrient availability on microbial metabolism

Previous studies have shown that the chemistry of Pavilion Lake is consistent throughout the water column, but during certain months of the year the total nitrogen: total phosphorus (TN:TP) levels decrease from surface water levels to a depth of 35 m (Lim et al. 2009). In August 2005, the TN:TP levels in the central basin fell from 55:1 at 0 m to 40:1 at 25 m depth and total nitrogen levels fell from 220 to 120 µg/L at that depth (Lim et al. 2009). As β-*N*-methylamino-L-alanine is generally found in environments with lower nitrogen levels (Downing et al. 2011), it is possible that the function of this isomer may be linked to a change in nitrogen levels in the environment during events such as a cyanobacterial bloom (Jiao et al. 2014; Pip et al. 2016; Bishop et al. 2018). Changes in TN concentrations during certain months may affect cyanobacterial metabolism, and β-*N*-methylamino-L-alanine could be produced in response to unfavorable metabolic conditions at lower depths.

Effect of microbial community structure on microbial metabolism

The concentration profiles exhibited by *N*-(2-aminoethyl) glycine and its isomers may be additionally explained by the

diversity of microbes and microbial community structure changes occurring at different depths in the lake. Previous researchers have reported that spherical, coccoid and filamentous cyanobacteria dominate the shallower microbialites while filamentous cyanobacteria, heterotrophs and eukaryotes dominate the deeper ones (Laval et al. 2000; Russell et al. 2014). The amount of microbial biomass associated with Pavilion Lake microbialites is highest in the shallow (< 20 m) microbialites and in samples collected during the summer with an increase in the relative proportion of eukaryotic microbes associated with the deeper structures (Omelon et al. 2013; Brady et al. 2014). Interestingly, eukaryotic diatoms are also known to produce β-*N*-methylamino-L-alanine, *N*-(2-aminoethyl) glycine and 2,4-diaminobutyric acid (Réveillon et al. 2016a). However, the relative amount of nonprotein amino acid produced by diatoms and other microalgae appears to vary greatly between species (Réveillon et al. 2016b). Therefore, the presence and relative concentrations of *N*-(2-aminoethyl) glycine and its isomers may be due to differences in metabolism as well as relative biomass between different species of cyanobacteria and/or eukaryotes. It is possible that the organisms adapted to lower light levels may produce more β-*N*-methylamino-L-alanine while the cyanobacteria adapted to higher light levels may produce more *N*-(2-aminoethyl)glycine, 2,4-diaminobutyric acid and β-aminomethyl-L-alanine.

A change in the microbial community with depth in the deepest microbialites was also noted in previous studies that applied molecular and biomarker techniques to investigate the microbialite biofilms (Brady et al. 2010; Russell et al. 2014). The microbial community structure changes are markedly below 26 m and the authors suggest that cyanobacteria are responsible for the shallow depth microbialites, whereas photoheterotrophs cause the formation of the deeper ones. Additionally, Omelon et al. (2013) note that a transition in growth conditions occurs at 32 m depth, where the cyanobacteria present on microbialites become overlain with calcite containing filamentous algae. To our knowledge, the dominant phototrophic communities present below 26 m, such as *Proteobacteria* and *Acidobacteria* (Russell et al. 2014), are not known to produce *N*-(2-aminoethyl)glycine or its isomers, which may explain why these diamino acids were detected in low concentration or not detected below 26 m. The results of this study provide further evidence to support the hypothesis that environmental conditions and microbial community structure affect the production of *N*-(2-aminoethyl)glycine and its isomers by cyanobacteria.

Conclusion

The identification and quantification of *N*-(2-aminoethyl)glycine and three isomers in microbialites in Pavilion Lake can offer insight into the metabolism of cyanobacteria in extreme environments and ancient formations. From the results of the current and previous studies, we hypothesize that the microbial community structure varies between microbialites at different depths and this diversity is expressed as production of different dinitrogenous nonprotein amino acids. While the exact function remains elusive, these data suggest that the metabolic pathways that produce β-*N*-methylamino-L-alanine, *N*-(2-aminoethyl)glycine, 2,4-diaminobutyric acid and β-aminomethyl-L-alanine are important enough to the viability of the organisms to be conserved over millennia. Increased knowledge about the metabolic processes involved in the formation of *N*-(2-aminoethyl)glycine may further our understanding of the evolution of the genetic material that shapes our modern world. Future studies detailing the production of nonprotein amino acids by microbes at varying depths will lead to new understandings of the metabolic mechanisms and could help us to understand the evolution of life on earth.

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Author contributions SJM, KP and PAC conceived and designed the research study. JKK and FM developed a custom synthesis method for β-aminomethyl-L-alanine and synthesized the analytical standard for method optimization. AB, GS and DSSL performed studies to characterize the microbialites in Pavilion Lake, collected, curated and shared the samples analyzed in this study. FJMT, SLB, JSM and SAB developed analytical methods and conducted the experiments. FJMT, SLB and SJM performed the statistical analysis and data interpretation. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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