Peptidoglycan Degradation Fluorescence: Applications to Karst Groundwater Mapping

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Abstract: Natural fluorescence groundwater tracing is extremely useful in karst groundwater flow investigations, as there is no artificial dye being introduced to the system and therefore there is no limit on the geographical or chronological spacing of dye traces. However, the fluorescence properties of individual microbiological materials that would impact this tracing method have not been investigated in a pH-controlled lab setting. Through this investigation, we have found that the pH of groundwater will have an enormous impact on the fluorescence of the microbial cell wall materials found in it, and therefore an impact on the analysis of those samples. We have also found cursory evidence that the path that a groundwater flow takes through a karst system (conduit flow, flow through porous media or fractured flow, or a combination) has an impact on that water's fluorescence peak's location and intensity. Further work in this area will have to take pH into account.

Introduction

Conducting fluorescent dye traces through karst groundwater systems has yielded invaluable information about the path of flow through these difficult-to-model features. However, using artificial dye for these traces can create problems for future traces. Highly fluorescent artificial dyes commonly used for tracing can reside in a karst system for an extended period of time, limiting the turnaround between dye traces. Also, the number of fluorescent dyes that can be used for these traces is limited, restricting the number of paths that can be traced at one time in a single area. Using the natural fluorescence from the groundwater itself eliminates both of these issues. Because no dye is being introduced, natural fluorescence analysis is not limited by time or geographical proximity to other traces.

Natural fluorescence can also provide insight into the type of path followed by groundwater. Interactions between groundwater and microbiological colonies would increase the ratio of microbial organic materials to surficial organic materials in that groundwater and change the peak location of the fluorescence. Different fluorescence could indicate more interaction between the water and the microbiology, and suggest that the groundwater flow was not purely through karst conduit, but also through a porous bedrock system.

To be able to link the water's increased fluorescence with microbiological interactions, we must identify the DOM as a known compound in microbiological cells. Microbial cell wall materials make up a significant portion (~11.4%) of DOM in

terrestrial rivers (Jorgensen et al 2003). Identifying some of these common materials and testing them for fluorescence peaks would allow us to extrapolate our results to analysis of natural waters.

Present in almost all eubacteria, the common cell wall material peptidoglycan is a major contributor to DOM and appears as both the complete compound and as degradation product molecules (Van Heijenoort 2001). Gram-negative bacterial cell walls contain structures that seem to shield their peptidoglycan from breakdown by other bacteria, leaving the complete compound as DOM, while Gram-positive bacterial cell walls are almost completely peptidoglycan, which is easily broken down by other organisms and therefore can be seen as its constituent molecules (Jorgensen et al 2003). These components include N-Acetylglucosamine, N-Acetylmuramic acid and the pentapeptide chain containing D-Alanine. The fluorescence of the whole compound and the several molecules in the peptidoglycan degradation sequence can be tested.

As peptidoglycan is only common in eubacterial cell walls, the sample set was expanded to include amino sugars found commonly in all cell materials, including both phototropic and heterotrophic bacteria, as well as some methanogenic Archaea (Benner and Kaiser 2003). To include these other organisms' cell materials in our data, galactosamine and glucosamine were added. These two amino sugars are abundant in marine organic material and also occur in close conjunction with each other.

Previously, standards used for natural water fluorescence tracings in marine and estuarine settings have been derived from natural water samples themselves (Coble, 1996). These standards for fluorescence analysis unintentionally incorporate numerous environmental variables that could dramatically alter the excitation/emission spectra, such as incorporation of multiple fluorophores into one sample, differences in salinity and alterations due to proximity to marine plants (Coble, 1996). Analysis of lab-created samples of common microbial cell wall materials eliminates the unwanted variability found in the field. Therefore, lab-mixed DOM under controlled conditions (such as pH, salinity, and elimination of outside organisms) will yield more accurate, repeatable and usable results for future natural water analysis of the organic compounds.

Method:

Peptidoglycan (a common microbial cell wall material) and its constituent materials N-acetylmuramic acid, N-acetylglucosamine and D-Alanine were diluted in DI water to ppm levels, then diluted to ppb levels in four pH buffers from pH 6.5 to pH 8.0. For more complete details on the mixing of the buffers, or the dilutions, see Appendix A.

Each of the vials of diluted material was run through a Shimzu RF-5000 Spectrofluorophotometer scanning for fluorescence intensity. The scans were performed from 200nm to 800nm on both excitation and emission scales for each $\Delta\lambda$ in 5nm intervals from 10nm to 400nm.The data was then run through a Matlab program to eliminate the Raman scattering peak and to create a contour plot. The plots for each material were normalized in scale to a maximum 8.5, 15 or 38 fluorescence intensity.

Results:

The fluorescence peaks for peptidoglycan (fig. 1) appear to be the sum of the compound's major components. The N-acetylmuramic acid (fig. 3) has a less intense peak than the peptidoglycan that sits higher in both excitation and emission than the N-acetylglucosamine (fig. 2) . However, the elongated peak of the N-acetylmuramic corresponds with the elongated peak region in the peptidoglycan scan and the rounder peak of the N-acetylglucosamine also appears in the peptidoglycan. The alanine peak (fig. 4) appears as a connecting region between the other two.

Figure 1

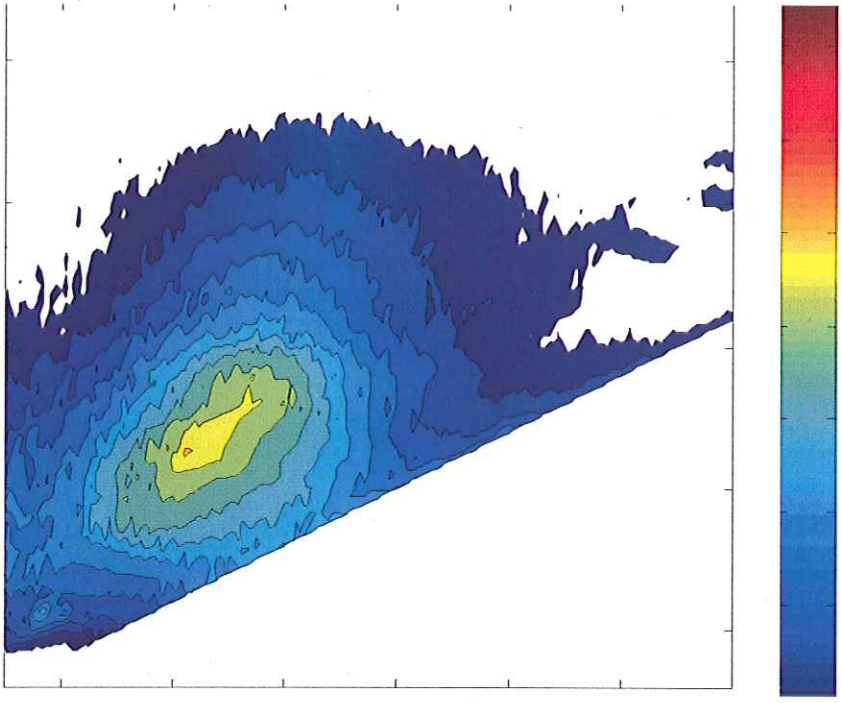


Figure 2

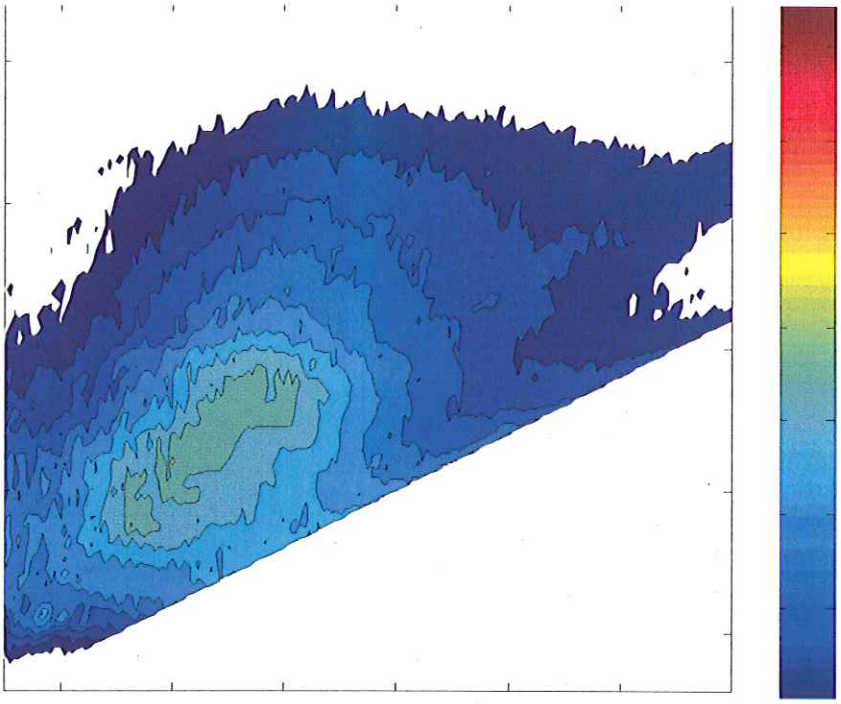


Figure 3

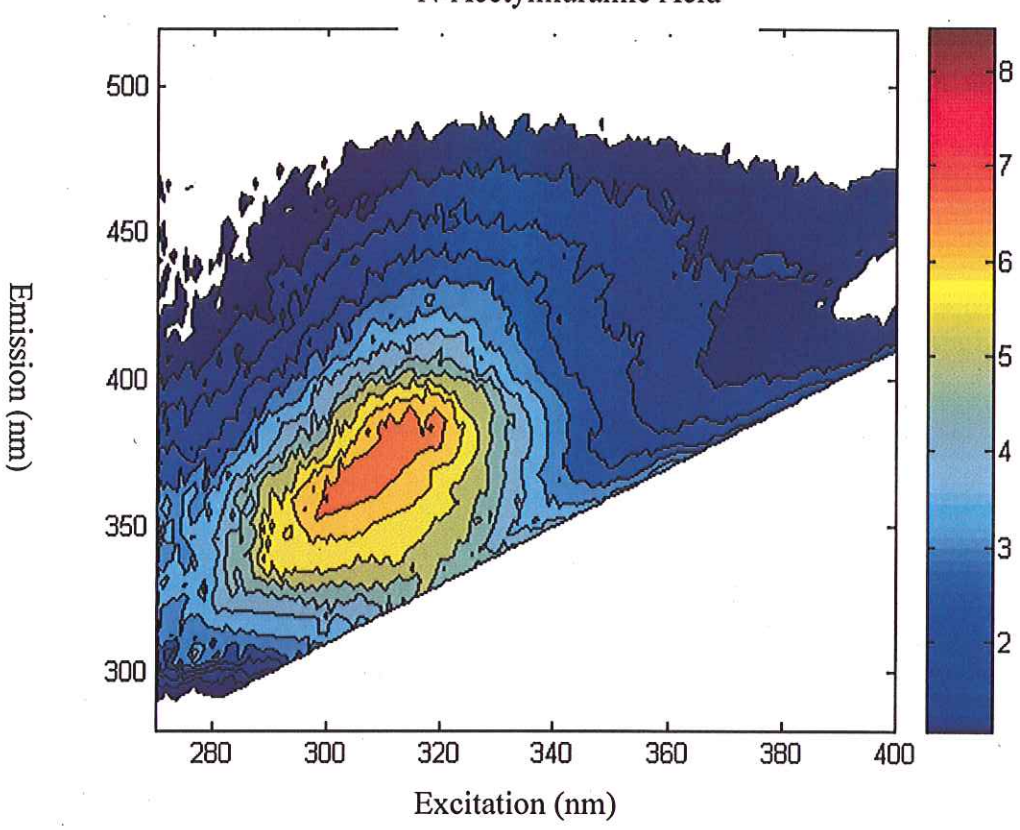


Figure 4

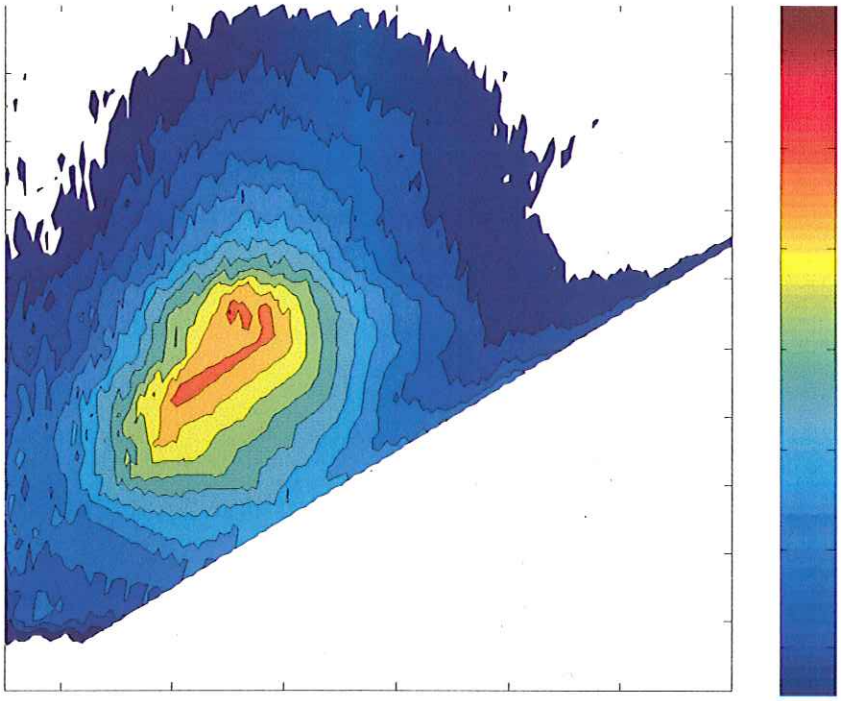
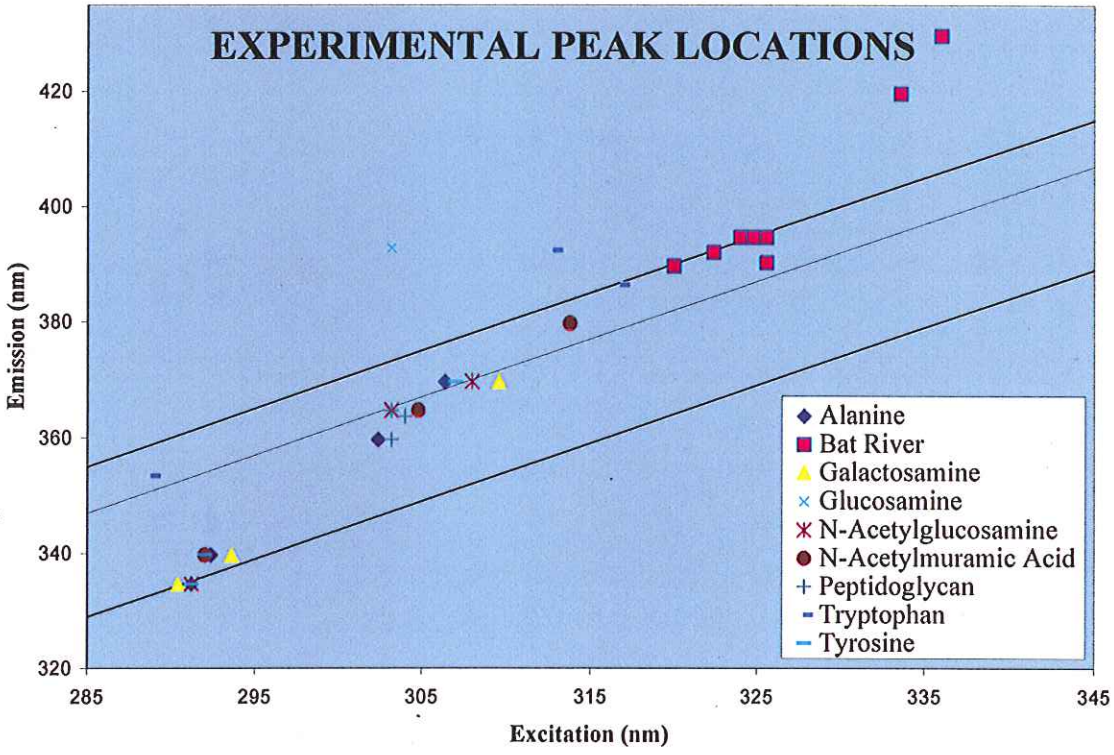
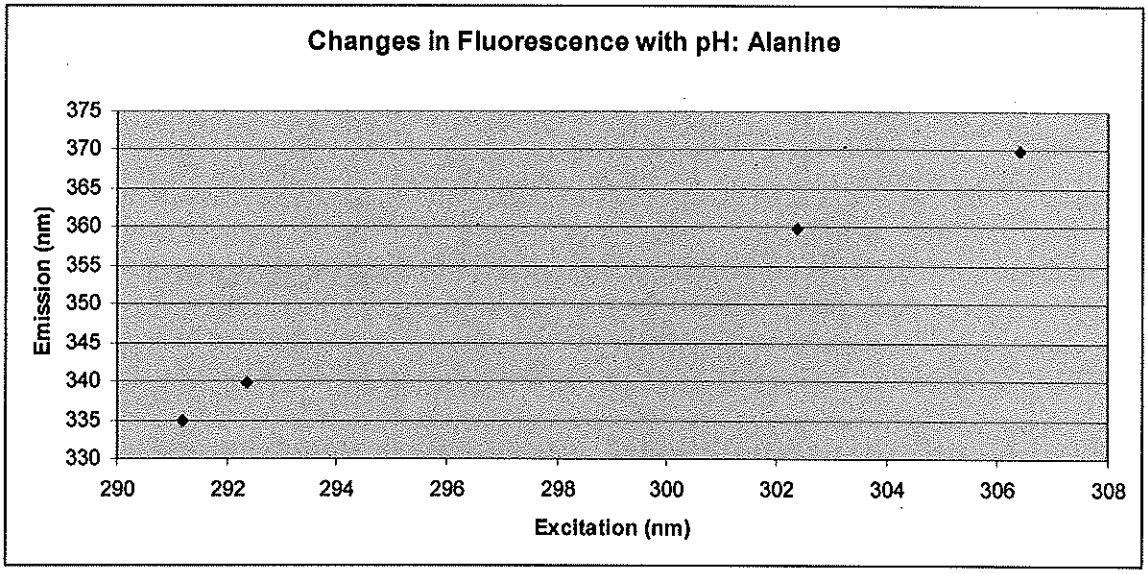


Figure 5



As shown in the "Experimental Peak Locations" graph above (fig. 5), the locations of the fluorescence peaks seem to shift up in both excitation and emission, but this is not with higher pH. Also outlined in the "Experimental Peak Locations" (above), both the test materials and the natural water samples aligned along three major $\Delta\lambda$ lines, at 44, 62 and 70.

As seen in "Changes in Fluorescence with pH: Alanine" (fig. 6, below), the linear-looking trend in changing fluorescence with different pH is not actually linear, as excitation and emission do not increase with increased pH. In the graph below, the progression going up in both excitation and emission wavelengths is pH 7.0, pH 8.0, pH 6.5, pH 7.5, which reflects that the fluorescence is not changing directly with the pH increasing.



The materials tested that were not directly associated with peptidoglycan degradation also showed results in the same region of the excitation-emission spectra.

Looking at the natural water samples from Bat River Cave, we cannot identify the materials in the karst groundwater samples from Bat River Cave (scans shown on schematic drawing) without enough information about the impact of pH on the fluorescence peaks of DOM. However, the peaks from various water samples were very different (see fig. 7, below), indicating that the water did interact with different parts of the karst system, and different amounts and/or types of organic material.

Comparisons with Others?

The fluorescence peaks we identified also do not correspond with the widely accepted B,T,A,M, and C regions published in Coble et al 1996. For example, the tyrptophan pH 7.5 is close, but not exactly the same as the T-region, but the other pH-buffered tryptophans are much different. Other compounds listed below are closer to the T-region than the other tryptophan scans. The fluorescence peaks' ranges used as a benchmark for most organic fluorescence analysis do not directly correspond with any of our experimental materials. Our natural water samples also do not align with published standards

Discussion:

The dramatic changes in a material's fluorescence peak with changing pH suggests that taking pH of natural water samples into account when testing for fluorescence is much more important than previously thought. Future work on pH-dependent fluorescence will yield much more information about how to identify organic materials in natural waters. The impact that pH has on a material's fluorescence also could explain the discrepancies between published fluorescence peaks (Coble et al, 1996; Biers et al, 2007). Without controlling for pH in water samples, the fluorescence peaks are not usable outside the pH at which they were mixed. Further examination of the impact of pH on fluorescence on much finer pH intervals should be able to more precisely characterize a material's fluorescent properties at a range of pH levels.

Finally, with many of the material's main fluorescence peaks lying on or very near the $\Delta\lambda$ 44, 62 and 70, scanning for their presence becomes much quicker and easier than running a full excitation-emission scan, which enables us to analyze more unique water samples and better understand karst systems.

Acknowledgements: I would like to extend my thanks to the National Science Foundation - Research Experience for Undergraduates program for funding this project. I would also like to thank John Ackerman for the use of Bat River Cave, and the local residents for their help and cooperation.

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Appendix A

Before the dilution of the organic materials could begin, pH buffers in the range of typical Minnesota groundwater had to be mixed. The two chemicals KH_2PO_4 and NaOH were mixed into a solution to serve later as components for the pH buffers needed. For the KH_2PO_4, this required a solution of 13.60912g KH_2PO_4 into 1000g DI water and for the NaOH, the solution was 4.00122g NaOH into 1000g DI water. These two solutions were then combined, based on (PAPER ON BUFFERS) guidelines to create 400g of pH buffer solution of each pH 6.5, pH7.0, pH7.5, and pH8.0. The calculated amounts needed of each material follow.

Planned pH	KH_2PO_4 (g)	NaOH (g)	DI H_2O (g)	Total (g)
6.5	200	55.6	144.4	400
7.0	200	116.4	83.6	400
7.5	200	163.6	36.4	400
8.0	200	184.4	15.6	400

The actual amounts of each material used (due to human inaccuracy) to create each buffer are listed below. After each buffer was mixed, its pH was tested with an Accumet pH/Conductivity meter calibrated just previous to measurement with a three buffer (pH4.0, pH7.0 and pH10.0) sequence. The actual pH of each buffer also listed.

Planned pH	KH_2PO_4 (g)	NaOH (g)	DI H_2O (g)	Total (g)	**Actual pH**
6.5	200.00	55.61	144.42	400.03	**6.44**
7.0	200.00	116.40	83.61	400.01	**6.98**
7.5	200.00	163.67	36.33	400.00	**7.49**
8.0	200.00	184.39	15.60	400.03	**7.93**

After the initial dissolution of the organic materials to be tested for fluorescence down to ppm levels, the final dilution down to the 100ppb level was done in each of the four pH buffers. This was done for two reasons. First, using a buffer with a known pH would standardize the pH level of each diluted material as opposed to dilution in DI water, which could yield virtually any pH, due to the different material introduced. Second, using four pH buffers of levels commonly found in Minnesota groundwater could yield different levels of fluorescence for a given material in waters of different overall pH levels. Hopefully, looking at the differences in fluorescence between different pH levels of a given material will provide some insight into how these materials appear in natural water samples of varying pH.

To mimic the approximate range of concentration of our organic compounds in natural waters, the compounds needed to be diluted down to approximately 100ppb in solution. This was completed in three dilutions. First, between 0.02g and 0.03g of a given material was dissolved into approximately 8g DI water. Next, between 0.02g and 0.03g of the solution was mixed into another 8g DI water. This yielded concentrations in the ppm range. From here, differing amounts of the second solution were added to 8g of each of the four pH buffers to produce four solutions containing around 100ppb of the original

material. For example, a solution with a concentration of 7.74172ppm after the second dilution would require 0.1033362g to be mixed with 8g of the pH buffer to yield 100ppb, which would vary with the actual amounts of material and solvent in the first two dilutions. While DI water would be used for all materials in a perfect world, in reality the different organic compounds required different methods to completely dissolve them into solution. The different methods used will be discussed in conjunction with each material.

Salicylic acid dissolved readily into deionized water, so there was no need to look for alternative solvent material. Following the process outlined above, the salicylic acid was diluted to approximately 100ppb in three steps. First, the approximately 0.02g of salicylic acid was measured into an 8ml glass vial with cap. The 8g of DI water was poured into the vial onto the material while sitting on a scale. The vial was then capped and shaken to mix. Using a repeating pipette, a small amount of the solution was drawn off the top and dripped into a second glass vial replacing the first on the scale. Again, 8g DI water was added to the vial, it was capped and shaken. The equation used to find the amount of the second solution necessary for the third (into the four pH buffers) is shown below. The same method was used to pipette a small amount of solution off the top, which was then added to a third 8ml glass vial (replacing the second) on the scale. Finally, 8g of the pH buffer was added. This final step was repeated for the other three pH buffers. The salicylic acid used was Sigma Aldrich 105910 batch 00530JH.

Dilution	1		2
Salicylic Acid (g)	0.020286g	Solution 1	0.023360g
DI water (g)	7.72417g	DI water	7.92464g
Concentration (gSA/gDI)	2.62630*10^-3	Concentration	7.74172*10^-6

(Concentration 1)(Volume 1)=(Concentration 2)(Volume 2)
(concentration of second dilution)(volume needed for third)=(100*10^-9)(8g)
(7.74172*10^-6)V_1=(800*10^-9)
V_1=0.1033362g second solution into third

pH buffer	Solution 2 (g)	Buffer (g)	**Concentration**
6.5	0.10331	8.23872	**97.07783ppb**
7.0	0.10280	8.16070	**97.52213ppb**
7.5	0.10081	7.88507	**98.97778ppb**
8.0	0.10357	7.95104	**100.84340ppb**

Glucosamine hydrochloride also dissolved into DI water, so the same procedure used for the salicylic acid was used. The needed amount of solution 2 for four buffered solutions is also the same, so it will not be outlined in its entirety. The glucosamine hydrochloride used was Sigma Aldrich 64875-25G 047K0129.

Dilution	1		2

Glucosamine (g)	0.02487	Solution 1	0.04906
DI water (g)	8.28866	DI water	7.55888
Concentration (gGl/gDI)	3.00049*10^-3	Concentration	15.8786*10^-6

$(15.8786*10^{-6})V_1=(800*10^{-9})$
$V_1=0.050382$g second solution into third

pH buffer	Solution 2 (g)	Buffer (g)	**Concentration**
6.5	0.07105	7.75574	**145.463ppb**
7.0	0.05001	8.24081	**96.3605ppb**
7.5	0.05166	7.91369	**103.6543ppb**
8.0	0.06309	8.06415	**125.309ppb**

Galactosamine dissolved as easily as the salicylic acid and the glucosamine. It required no alternative methods of dilution and followed the same procedure as above.

Dilution	1		2
Galactosamine (g)	0.01945	Solution 1	0.13692
DI water (g)	8.33362	DI water	8.12672
Concentration (gGl/gDI)	2.333919*10^-3	Concentration	39.32216^10^-6

$(39.32216*10^{-6})V_1=(800*10^{-9})$
$V_1=0.020344$g second solution into third

pH buffer	Solution 2 (g)	Buffer (g)	**Concentration**
6.5	0.02710	8.26747	**128.8944ppb**
7.0	0.02401	8.29331	**113.8418ppb**
7.5	0.01816	8.17641	**87.3355ppb**
8.0	0.02660	8.11092	**128.9582ppb**

The L-Tyrosine did not dissolve easily when mixed into DI water as outlined with the salicylic acid and the glucosamine. Several different solvents were tried, including 10%, 50% and 100% methyl alcohol (CH_3OH), as well as isopropyl alcohol and directly into the pH6.5 and pH7.0 buffer solutions. No solvent worked completely without heating and stirring, but after heating to 90°C, the tyrosine did dissolve into the DI water. Pipetting a small amount of the heated liquid off quickly (to prevent reprecipitation), the same procedure was followed as with the salicylic acid and glucosamine.

Dilution	1		2

L-Tyrosine (g)	0.02326	Solution 1	0.04432
DI water (g)	8.10595	DI water	8.04473
Concentration (gGl/gDI)	2.86949*10^-3	Concentration	15.80862*10^-6

$(15.80862*10^{-6})V_1=(800*10^{-9})$
$V_1=0.050605$g second solution into third

pH buffer	Solution 2 (g)	Buffer (g)	**Concentration**
6.5	0.06042	8.19447	**116.5612ppb**
7.0	0.06620	8.25793	**126.7304ppb**
7.5	0.05283	8.08376	**103.3145ppb**
8.0	0.05951	8.12729	**115.7546ppb**

The tryptophan also did not dissolve into 25°C DI water. It did, however, dissolve into 10% methyl alcohol (CH_3OH). The methyl alcohol solution used consisted of 30g Mallinckrodt lot 3016 KXNT methyl alcohol dissolved into DI water to yield 300.03g of total solution. The Sigma Aldrich T0254-256 tryptophan dissolved into this 10% concentration by mass solution fairly readily. The amounts used for each step of solution are listed below.

Dilution	1		2
Tryptophan (g)	0.01907	Solution 1	0.02109
10% CH_3OH (g)	7.88393	DI water	7.78220
Concentration (gGl/gDI)	2.41884*10^-3	Concentration	6.553446*10^-6

$(6.553446*10^{-6})V_1=(800*10^{-9})$
$V_1=0.122073$g second solution into third

pH buffer	Solution 2 (g)	Buffer (g)	**Concentration**
6.5	0.13144	7.86780	**109.48ppb**
7.0	0.15263	8.09790	**123.52ppb**
7.5	0.16283	8.30899	**128.43ppb**
8.0	0.13544	8.13487	**109.11ppb**

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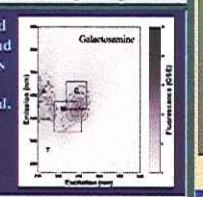
Method: Peptidoglycan (a common microbial cell wall material) and its constituent materials N-acetylmuramic acid, N-acetylglucosamine and D-Alanine were diluted in DI water to ppm levels, then diluted to ppb levels in four pH buffers from pH 6.5 to pH 8.0.

Each sample was scanned for fluorescence in a Shimazu R-5000 spectrofluorometer from 200nm to 800nm on both excitation and emission scales for each $\Delta\lambda$ in 5nm intervals from 10nm to 400nm. The data was then run through a Matlab program to eliminate the Raman scattering peak and to create a contour plot. These plots were then normalized to 8.5, 15 or 38 intensity.

Comparisons with Others?

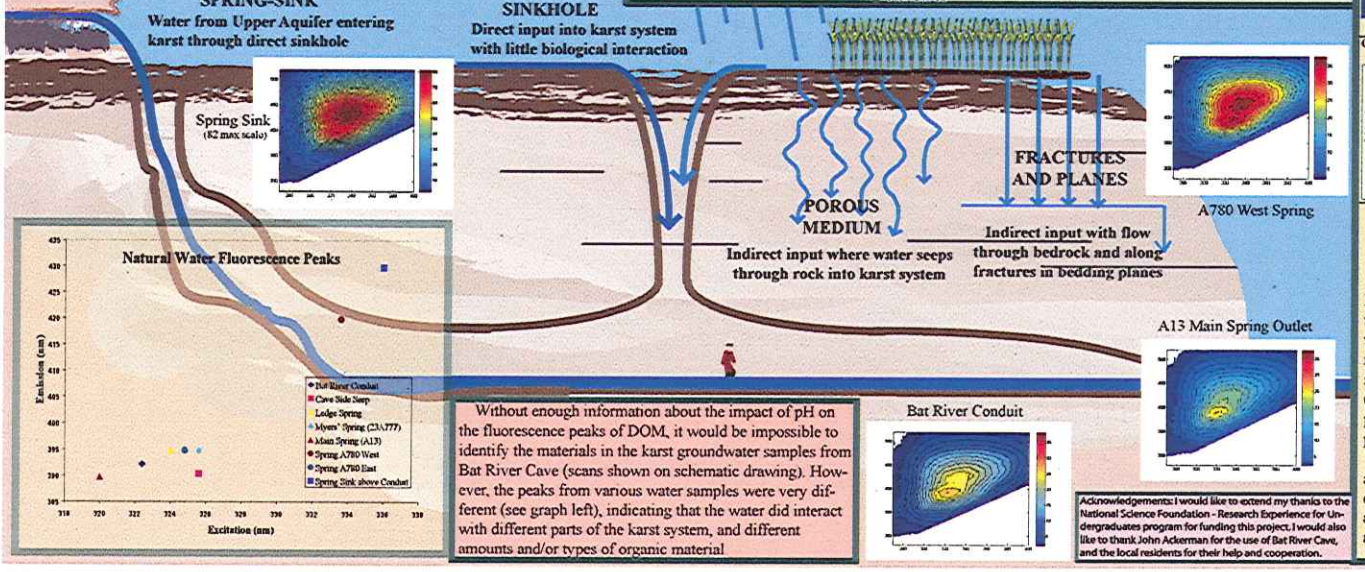
The fluorescence peaks we identified also do not correspond with the widely accepted B,T,A,M, and C regions published in Coble et al 1996. For example, the tyrptophan pH 7.5 is close, but not exactly the same as the T-region, but the other pH-buffered tryptophans are much different. Other compounds listed below are closer to the T-region than the other tryptophan scans. The fluorescence peaks' ranges used as a benchmark for most organic fluorescence analysis do not directly correspond with any of our experimental materials. Our natural water samples also do not align with published standards.



Coble et al. 1996

Peak	Ex_{max} (nm)	Em_{max} (nm)	
B	275	310	Tyrosine-like, protein-like
T	275	340	Tryptophan-like, protein-like
A	260	380–460	Humic-like
M	312	380–420	Marine humic-like
C	350	420–480	Humic-like

Our Scans	pH	Excitation	Emission
Tryptophan	7.5	288.8	353.6
Galactosamine	7	290.4	334.8
Alanine	7	291.2	334.8
N-Acetylglucosamine	7	291.2	334.8
Tyrosine	7	291.2	334.8
Tyrosine	8	291.2	334.8



Without enough information about the impact of pH on the fluorescence peaks of DOM, it would be impossible to identify the materials in the karst groundwater samples from Bat River Cave (scans shown on schematic drawing). However, the peaks from various water samples were very different (see graph left), indicating that the water did interact with different parts of the karst system, and different amounts and/or types of organic material.

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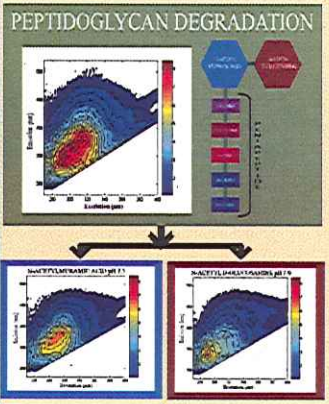
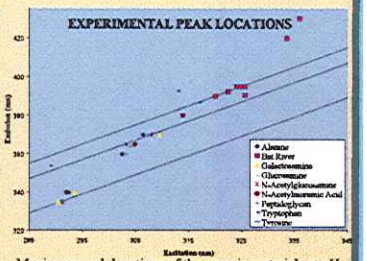
RESULTS: PEPTIDOGLYCAN DEGRADATION AND EXPERIMENTAL PEAKS

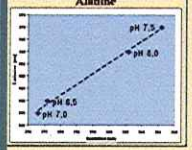




Maximum peak locations of the organic materials at pH levels between pH 6.5 and pH 8.0

Organic Material	pH	Excitation (nm)	Emission (nm)
Peptidoglycan	7.5	303.2	364.8
N-Acetylmuramic	7.5	313.8	379.8
N-Acetylglucosamine	7	291.2	334.8
Alanine	7.5	306.4	369.8
Galactosamine	7.5	309.6	369.8
Glucosamine	6.5	303.2	393

Changes in Fluorescence with pH: Alanine



Results: The fluorescence peaks for peptidoglycan appear to be the sum of the compound's major components. The N-acetylmuramic acid has a less intense peak than the peptidoglycan that sits higher in both excitation and emission than the N-acetylglucosamine. However, the elongated peak of the N-acetylmuramic corresponds with the elongated peak region in the peptidoglycan scan, and the rounder peak of the N-acetylglucosamine also appears in the peptidoglycan. The alanine peak appears as a connecting region between the other two.

As shown in the "Experimental Peak Locations" graph above, the locations of fluorescence peaks seems to shift up in both excitation and emission, but this is not with higher pH. As seen in "Changes in Fluorescence with pH: Alanine", the linear-looking trend in changing fluorescence with different pH is not, excitation and emission do not increase with increased pH.

Materials not directly associated with peptidoglycan degradation also showed results in the same region of the excitation-emission spectra. As outlined in the "Experimental Peak Locations" (above), both the test materials and the natural water samples aligned along three major $\Delta\lambda$ lines, at 44, 62 and 70.

Discussion: The dramatic changes in a material's fluorescence peak with changing pH suggests that taking pH of natural water samples into account when testing for fluorescence is much more important than previously thought. Future work on pH-dependent fluorescence will yield much more information about how to identify organic materials in natural waters. The impact that pH has on a material's fluorescence also could explain the discrepancies between published fluorescence peaks (Coble et al, 1996; Biers et al, 2007). Without controlling for pH in water samples, the fluorescence peaks are not usable outside the pH at which they were mixed. Further examination of the impact of pH on fluorescence on much finer pH intervals should characterize this fluorescence.

Finally, with many of the material's main fluorescence peaks lying on or very near the $\Delta\lambda$ 44, 62 and 70, scanning for their presence becomes much quicker and easier than running a full excitation-emission scan, which enables us to analyze more unique water samples and better understand karst systems.

REFERENCES

Coble, Paula G. 1996. Characterization of marine and terrestrial DOM in seawater using excitation-emission matrix spectroscopy. Marine Chemistry 51, no. 4: 325-346.