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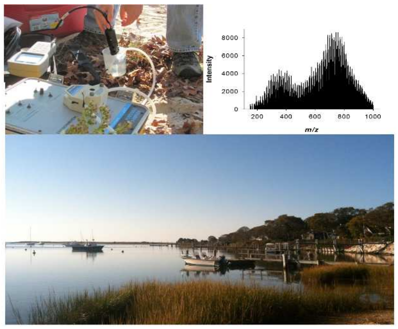

Dissolved organic nitrogen in septic-impacted groundwater is characterized using ultrahigh resolution mass spectrometry.

Environmental Impact Statement

Septic systems are a source of nitrogen to groundwater and coastal systems. Given the known adverse effects of nitrogen pollution, it is important to understand the relative importance of inorganic and organic nitrogen. A limitation is a lack of knowledge of the sources and composition of dissolved organic nitrogen. Using carbamazepine as a marker of septic impact on groundwater, this work compares the molecular-level composition of dissolved organic nitrogen in septic-impacted and non-impacted coastal groundwaters. The results of the work will allow improved assessment and identification of specific sources of dissolved organic nitrogen to aquatic systems.

Molecular signature of organic nitrogen in septic-impacted groundwater

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Abstract

Dissolved inorganic and organic nitrogen levels are elevated in aquatic systems due to anthropogenic activities. Dissolved organic nitrogen (DON) arises from various sources, and its impact could be more clearly constrained if specific sources were identified and if the molecular-level composition of DON were better understood. In this work, the pharmaceutical carbamazepine was used to identify septic-impacted groundwater in a coastal watershed. Using ultrahigh resolution mass spectrometry data, the nitrogen-containing features of the dissolved organic matter in septic-impacted and non-impacted samples were compared. The septic-impacted groundwater samples have a larger abundance of nitrogen-containing formulas. Impacted samples have additional DON features in the regions ascribed as 'protein-like' and 'lipid-like' in van Krevelen space and have more intense nitrogen-containing features in a specific region of a carbon versus mass plot. These features are potential indicators of dissolved organic nitrogen arising from septic effluents, and this work suggests that ultrahigh resolution mass spectrometry is a valuable tool to identify and characterize sources of DON.

Introduction

Groundwater is an important source of freshwater to coastal systems.[1, 2] Thus, groundwater is a source of nitrogen to coastal waters, especially if it has been impacted by human activities. Dissolved nitrogen is comprised of both inorganic (nitrate, nitrite, and ammonia) and organic forms. The quantitative balance between inorganic and organic nitrogen will depend on various factors, including sources and biological activity, but evidence suggests that dissolved organic nitrogen (DON) can be quantitatively as important as inorganic forms of nitrogen in groundwater.[3] For example, Kroeger et al.[3] found that DON comprised 10-93% of total dissolved nitrogen in a set of coastal watersheds. Both the source and environmental conditions influence the bioavailability and processing of DON, with bioavailability ranging from 1-60%.[4, 5] Sources of DON include precipitation, agriculture, natural runoff/infiltration, artificial recharge of wastewater, and septic systems. In populated coastal areas, septic systems may be particularly important sources of both inorganic and organic nitrogen.[3, 6-8] At present, however, it is difficult to link the bulk DON found in impacted aquatic systems to specific sources or processes due to a lack of information about the molecular-level composition of DON.

Information regarding the molecular-level composition of DON is important to improving understanding the reactivity of the pool of nitrogen in aquatic systems. The amount of DON present is changed (both increases and decreases have been observed) by human alteration of the landscape or introduction of runoff/effluents.[9, 10] For example, septic systems are a major source of dissolved nitrogen in low-residential watersheds.[11] Compositional changes in the DON pool in human altered systems have received limited attention, and such changes have potentially important ramifications. For example, DON is more recalcitrant than inorganic nitrogen, leading to longer residence times and a larger zone of impact. Additionally, DON concentration and composition will affect water treatment and human exposure to nitrogenous disinfection byproducts.[12]

Ultrahigh resolution mass spectrometry techniques, such as Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS), are used to gain insight into the molecular-level composition of dissolved organic matter (DOM).[13-17] Such instruments provide the opportunity to see differences among samples that may not be apparent through bulk analyses (e.g., measurement of dissolved organic carbon (DOC)) or targeted analysis of specific molecules. FT-ICR-MS has been used to analyze incorporation of nitrogen into organic matter and transformations and sources of the DON pool.[18-20] While recent studies have shown compositional differences between effluent organic matter and natural organic matter,[21, 22] a specific focus on the nitrogen-containing component has yet to be undertaken. That said, past work on septic-impacted groundwater on Cape Cod (MA) noted that a large portion of assigned formulas contained nitrogen, potentially due to microbial activity and only partial mineralization of septic contributions to the DON pool.[17]

Groundwater impacted by septic system effluent contains numerous human-use compounds, including antibiotics, over-the-counter pharmaceuticals, steroid estrogens, prescription medications, artificial sweeteners, and personal care products.[23-27] On Cape Cod, studies have found such compounds in impacted groundwater, which also serves as the drinking water source for the population.[27, 28] Some of these compounds, including carbamazepine (an anti-convulsant) and artificial sweeteners, appear to be persistent and sorb to soils or sediments only to a limited extent. Thus, they have been proposed as markers for wastewater/septic impact and for contaminant source tracking.[26, 29-32] In particular, carbamazepine is generally recognized as being persistent in groundwater and an indicator of impact on natural waters by untreated or treated wastewater.[29, 30] Laboratory studies have shown carbamazepine to be stable in on-site treatment scenarios,[33] and recent results demonstrated the utility of carbamazepine in discriminating nutrient sources in coastal groundwater.[8]

On Cape Cod, the vast majority of residents have on-site wastewater treatment,[34] and nationwide, 20% of households rely on septic systems. Even when functioning properly, septic systems are a source of nitrogen to groundwater. Because of the prevalence of septic systems on Cape Cod, past detection of pharmaceuticals in groundwater,[27, 28] known nitrogen pollution issues,[3, 35, 36] and the fact that the aquifer to which septic systems discharge is also the source of drinking water, this is a logical site to investigate the composition of DON in groundwater with and without potential septic system impact. Our goals were to both discern septic impacted sites and begin to characterize the DON arising from septic tanks. In this work, electrospray ionization (ESI) FT-ICR-MS was used to obtain detailed information about the dissolved organic nitrogen component of DOM, with carbamazepine used as a marker to identify impacted vs. non-impacted waters.

Experimental

All solvents were obtained from Fisher and were Optima® grade. Trace metal grade hydrochloric acid was also from Fisher. Ultrapure water (18.2 MΩ·cm) was produced by a Millipore Elix 3 and Gradient system. Carbamazepine (Sigma-Aldrich) standards (0.1-1,000 ng/mL and a d_{10}-carbamazepine (Cambridge Isotopes) spiking solution in methanol were prepared previously.[37] All groundwater samples were collected via a peristaltic pump into prewashed (overnight soak with soap and water, rinsed with ultrapure water, soaked in 10% HCl, rinse with ultrapure water) and autoclaved 2.5 L polycarbonate bottles. The bottles were rinsed three times with ~1 L of collected groundwater and then filled to capacity, with 5 L being collected for each sample. The samples were transported on ice to the laboratory, where they were filtered through 0.2 μm, 47 mm hydrophilic PTFE Omnipore™ membranes (EMD-Millipore) and acidified to pH 3 using concentrated HCl. If not extracted immediately, the filtered and acidified samples were stored at 4 °C.

Sample sites

Four sites in Falmouth (Cape Cod), MA were sampled in September and October of 2013. The shallow groundwater at two of the sites, Head of Waquoit Bay and Sage Lot Pond, with population densities of 190 and 0 people per km^2 respectively, was expected to have minimal, if any, impact from septic systems.[3] The groundwater sample at Head of Waquoit Bay was taken within the Waquoit Bay National Estuarine Research Reserve east of the boathouse at the north point of the transect described by Kroeger and Charette,[36] using a small diameter drive point piezometer.[38] Groundwater at the Sage Lot Pond site was collected in a forested area from previously installed wells. The other two sites, Green Pond and Site D, were near residences in more densely populated areas (1,000-1,500 people per km^2) [3] and were expected to be impacted by septic system effluent. Groundwater at Green Pond was collected on the west shore approximately 0.4 kilometers north of the pond outlet into Vineyard Sound. A small diameter drive point piezometer was used approximately 5 m from shore. At Site D, a densely populated residential area west of Waquoit Bay, samples were collected at three depths (6.30, 9.97, and 16.11 m below ground surface) during ongoing USGS sampling efforts from a multilevel well. These specific depths for characterization of the DOM and quantification of carbamazepine were chosen based on nitrate and ammonia levels observed in previous sampling efforts to capture potentially different redox zones.

DOC and water quality analysis

For the six samples described above, DOC and total dissolved nitrogen (TDN) were measured with a Shimadzu TOC V_{CSH} with a total nitrogen attachment. Conductivity was measured with a YSI 556MPS handheld meter. Nutrients were measured using an AA3 four-channel segmented flow autoanalyzer (SEAL Analytical). DON was calculated by subtracting the nitrate/nitrite and ammonia concentrations from the TDN. Water quality data from other sampling depths at Site D were provided by the USGS.

Carbamazepine

Duplicate 1-L samples of collected groundwater were spiked with 5 μL of 2 μg/mL d_{10}-carbmazepine for use as a surrogate and extracted following the protocols of ref.[37] using Empore SDB-RPS disks (3M) that had been pre-conditioned with acetone, isopropyl alcohol, methanol, and ultrapure water. The disks were stored at -20 °C until extraction. The disks were warmed to room temperature and eluted with 10 mL of methanol and 10 mL of acetone. The combined extract was blown down with nitrogen to a volume of ~5 mL and then reduced to near-dryness via vacuum centrifugation. The extract was then reconstituted with 60 μL of methanol and 140 μL of ultrapure water. Carbamzepine and d_{10}-carbamazepine were quantified by ultrahigh pressure liquid chromatography tandem mass spectrometry (Thermo TSQ Vantage) with the electrospray ion source in positive mode according to previously described methods.[37, 39]

ESI-FT-ICR-MS

Samples (1-L) were extracted using Bond Elut PPL cartridges (Agilent) following the protocol of Dittmar et al.[40] The resulting methanol extracts were analyzed via direct infusion with the electrospray ionization interface of a 7T LTQ FT-ICR-MS (LTQ FT-Ultra, Thermo Scientific). The D2 sample was analyzed twice to test instrument reproducibility. The methanol extracts were diluted with a solution of 70% methanol/30% water (2-fold dilution by volume for Site D samples, 3-fold for Green, and 10-fold for Head of Waquoit and Sage Lot) to achieve a stable spray and analyzed in positive mode, because nitrogen functional groups are generally more amenable to detection under positive ionization. The resolving power was 400,000 at *m/z* 400, and 200 transients were collected per sample. The transients were co-added and processed using the protocols described in Kido Soule et al.[41]

After correcting the *m/z* values of the features detected via ESI-FT-ICR-MS for the presence of sodium, a previously developed algorithm[42, 43] was used to assign elemental formulas with an allowed error of 1 ppm. The algorithm allows formulas including C, H, O, N, S, and P and allows inclusion of ^{13}C if the error is reduced by doing so. All data analysis and processing were performed in MATLAB R2013b (MathWorks, Inc.).

Results and Discussion

Dissolved organic carbon, dissolved nitrogen and carbamazepine

Sample collection depths, dissolved organic carbon, total dissolved nitrogen, and carbamazepine data are shown in Table 1. The specific conductance values demonstrate that all samples were collected from fresh water zones, rather than salt water intrusion where specific conductance would be > 30,000 μS/cm.

The DOC levels at Head of Waquoit and Sage Lot are higher than those at Green Pond and Site D. The lower DOC concentration for the D1 sample may be due to the deeper vadose zone at Site D, which would allow greater DOC mineralization during transport to the water table. At Site D, the DOC level decreases with depth, consistent with previous observations on Cape Cod,[44] and with a greater extent of degradation for organic matter that has traveled further or is older (see below).

The total dissolved nitrogen and nitrate+nitrite (Table 1) at Green Pond and Site D are substantially higher than the levels at Head of Waquoit and Sage Lot, supporting the expectations regarding the degree of anthropogenic impact at the two sites. The nitrate+nitrite is likely derived from septic discharge and fertilizer application. The DON levels are lowest at the two unimpacted sites (Head of Waquoit and Sage Lot), but at these sites, the DON comprises the largest fraction of TDN (>65%). This indicates decreased processing of the organic nitrogen and less septic impact. At Site D, the DON levels are equal or higher than those at Head of Waquoit or Sage Lot, but DON is only 4-8% of the total TDN. The elevated levels, however, clearly indicate impact from septic systems. While the nitrate levels at Green Pond also indicate septic influence, the calculated DON level is zero. This is in contrast to previous samples from groundwater around Green Pond, which saw levels ~2 mg/L as DON.[3] This does not mean, however, that one would not expect to observe N-containing formulas via ESI-FT-ICR-MS in our sample. The zero value for DON merely indicates that determining this value as the difference between TDN and nutrients does not have the required precision and that additional analyses are needed to evaluate the role of septic systems as a source of organic nitrogen.

For the Head of Waquoit and Sage Lot groundwater samples, average carbamazepine concentrations were below the limit of quantification (1.6 ng/L). Carbamazepine was detected at concentrations ranging from 7.8 to 46.7 ng/L in the samples from Green Pond and Site D. These levels are consistent with those in various wastewater-impacted groundwaters[25, 30, 32] and a recent analysis of drinking waters drawn from the shallow aquifer on Cape Cod.[28]

It is clear that the samples collected from shallow depths (Green and D1) as well as the deeper samples D2 and D3 are impacted by septic effluent based on the presence of carbamazepine. Yet, the trend of values with depth at Site D merits discussion. Groundwater on Cape Cod flows in layers,[45, 46] with deeper waters travelling farther distances and being of greater age. Thus, the water from the shallow depth near the water table (D1) is likely derived from proximal sources and influenced by local septic systems. The water at the greatest depth (D3) is likely 10-20 years old[44, 46, 47] and has travelled from the recharge mound further north. The presence of carbamazepine at this depth suggests that the water is from an area impacted by septic systems or wastewater recharge and supports the notion that carbamazepine is persistent in groundwater systems. The carbamazepine concentration at the intermediate depth is lowest. This may indicate that the region in which this groundwater layer receives recharge is less impacted/populated than the area providing recharge to the deeper groundwater.

Molecular characterization of the organic matter

For the seven samples analyzed via ESI-FT-ICR-MS (including the duplicate runs for sample D2), 12,992 features were detected and 11,434 formulas were assigned. Because of the small number of samples, cluster analysis was performed based on the features found in each sample, and the dendrogram is shown in the Supplementary Information (Figure S1). The two unimpacted sites, Head of Waquoit and Sage Lot, clustered together as did the replicate injections for D2. Overall, the calculated differences were not large (all distances between 0.2 and 0.45), and the clustering was not used to guide further analyses.

The total formulas and the percentage of formulas in each sample containing only CHO and CHON are given in Table 2. Also given are the percentages of all formulas found by the algorithm that contained N in any allowed combination with other elements. In general, Head of Waquoit and Sage Lot (the two unimpacted sites) had a higher percentage (≥56%) of CHO formulas compared to the sites where carbamazepine was detected (≤56%). Using a 1-tail t-test with equal variance (as determined by an F-test) to evaluate if the impacted samples have a lower CHO level, gives a p-value of 0.115 (significant with 88% confidence). Similarly, the CHON percentages (≤26%) and all N-containing formulas (≤40%) at the unimpacted sites are lower than at the impacted sites (28-36% for CHON and 41-56% for all N). In this case, the t-test gives a p-value of 0.063 (significant with 93% confidence). Previous comparisons of effluent organic matter from a wastewater treatment plant with Suwannee River natural organic matter analyzed using negative mode ESI-FT-ICR-MS showed reduced abundance of CHO formulas in the wastewater samples.[21] The molar ratios of O:C and N:C (Table 2) also indicate that the Head of Waquoit and Sage Lot samples are more oxidized (based on O:C ratio) and that the impacted samples have higher nitrogen content. While these differences are subtle and more data would be needed to derive more robust statistical certainty, these results suggest that septic-impacted groundwater has a greater abundance of N-containing formulas.

The van Krevelen plots for all features and for only N-containing features are shown in the Supplementary Information (Figures S2-S7). The four samples collected from shallow depths have a larger number of points with O:C ratios > 0.5, with the Head of Waquoit and Sage Lot samples having more than the Green Pond and D1 samples. This is true of the plots for all of the detected features as well as those containing N, as quantified in Table 2. The exception is the N-containing compounds for the Head of Waquoit sample, but the count is biased by the fact that this sample has the fewest N-containing features. Overall, these results indicate a more reduced character for the septic-impacted samples.

Differences in the N-containing compounds are more readily assessed by identifying features that are common or unique among the samples. The samples were divided into three pairs for this analysis: Head of Waquoit + Sage Lot, Green + D1, and D2 + D3. In each case, the N-containing features present in both samples of one pair and not in both samples of the other pair (and vice versa) were determined. Also found were the features contained in all four of the compared samples.

In the van Krevelen diagrams shown in Figure 1a and 1b, the septic impacted samples are compared to the unimpacted samples. It is apparent that the impacted samples have N-containing features that are absent from the unimpacted samples. In comparing Green Pond and D1 with Head of Waquoit and Sage Lot, the impacted samples contain features of H:C > 1.5 across the O:C range that are not in the unimpacted samples (green points in Figure 1a). Similarly, D2 and D3 contain features of H:C > 1.5 and O:C < 0.6 that are not in the unimpacted samples (red points in Figure 1b). The features with H:C > 1.5 overlap with the regions of the diagram generally ascribed to be ‘protein-like’ and ‘lipid-like’,[48] and it may be expected that the lipid and protein content of human waste would be different than that of natural systems. Nitrogen-containing compounds may also arise from household products/pharmaceuticals that are washed down the drain.[49] A previous study identified the sulfur-containing linear alkyl benzene sulfonates in wastewater effluent using ESI-FT-ICR-MS, supporting the hypothesis that human-use chemicals alter the composition of the dissolved organic matter.[21]

In Figure 1c, the differences between the shallow and deep samples impacted by septic effluents are shown. The shallow samples have a cluster of features with higher O:C ratios (0.2-0.8, green points), and the deep samples (red points) have additional features with O:C < 0.3. This could be consistent with reduction of the N-containing features in older, deeper samples in which dissolved oxygen has been consumed.[48] Also supporting the greater processing of N-containing compounds in the deep, impacted samples are the number of formulas in the 'protein-like' area of the van Krevelen diagrams for the individual samples (181 and 387 for D2 and D3, respectively), compared to D1 (528). The reduced numbers in the deep samples may indicate degradation of a portion of the septic-derived N-containing features in these samples.

Another means to visualize the differences in nitrogen content are carbon versus mass plots.[50] In such plots, the number of carbon atoms in a molecule is plotted versus the molecular weight of the molecule, and the size of the point is proportional to the intensity of the ESI-FT-ICR-MS signal. Shown in Figure 2 are such plots for each sample. Note that the four impacted samples have abundant (number of dots) and intense (larger size) features with N:C ≥ 0.5 from carbon numbers 10 to 30 with *m/z* values from 400 to 700 (denoted by pink ovals). These features are less intense and less numerous in the Head of Waquoit and Sage Lot samples, indicating this pattern may be indicative of septic impacts. Interestingly, the four samples from shallow depths all have a greater intensity of points in the upper right portion of the plot (carbon number > 30, *m/z* > 600; black ovals) with low nitrogen content. The greatest intensity for these points is in the two impacted samples (D1 and Green). While this is not an indicator of organic nitrogen from the septic system effluent, the greater intensity in this region may be an indicator of recent or less-processed septic input.

Both the van Krevelen diagrams and carbon versus mass plots show that it is possible to identify features/molecular formulas of DON associated with septic-derived organic matter. Elucidating molecular level composition is the first step to a predictive understanding of the reactivity of the DON pool. Such features can be used to identify the relative contributions of different sources of DON to the total nitrogen pool. This has the potential to allow the assessment of the availability of DON in aquatic systems. With additional structural characterization to identify the abundance of functional groups, it would also be possible to evaluate impacts on water treatment (e.g., the formation of nitrogenous disinfection by products).

Conclusion

This work has provided molecular-level information regarding the DON in groundwaters impacted by septic system effluent. Carbamazepine and nitrate/nitrite were found to be valid indicators of septic influence, but depth, age, and recharge location of groundwater layers need to be carefully considered when comparing these measurements. Overall, there appears to be a greater abundance of N-containing features in DOM impacted by septic effluent, even if the water is older and/or deeper. In van Krevelen space, the impacted samples had N-containing features present at $H:C > 1.5$ that plotted in the ‘lipid-like’ and ‘protein-like’ areas of the diagram. An abundance of features in this area of the diagram may be a useful marker of septic impact in groundwater. The carbon versus mass plots also have abundant, intense features in a specific region of the plot for the impacted samples. Thus, these plots may also be useful in identifying the source of DON. Further work will be needed to identify features in van Krevelen space and carbon versus mass plots that are indicators of other sources of DON in groundwater.

This work also demonstrates that unimpacted groundwater samples from the targeted watershed are needed to allow comparisons to be performed to find features indicative of alteration of the organic matter by human activities.

This work focused on abundance and intensity of N-containing features. Quantitative sample-to-sample comparison of intensities is limited using ESI-FT-ICR-MS due to issues associated with variations in ion suppression among samples. Thus, it is important to recognize that while the features detected in this work are a first step in using ESI-FT-ICR-MS to develop a molecular level understanding of DON, the overall impact of DON on aquatic systems will be a combination of abundance, concentration, and bioavailability of specific DON-moieties, and ESI-FT-ICR-MS analysis alone will not allow this assessment.

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Table 1. Specific conductance, nutrient, dissolved organic carbon, and carbamazepine levels in the sampled groundwaters.

Site[a]	Depth below surface (m)	SC (μS/cm)	DOC (mg C/L)	[NO_3^-+ NO_2^-] (mg/L as N)	[NH_4^+] (mg/L as N)	TDN (mg/L as N)	DON (mg/L as N)	Carbamazepine (ng/L; average of n = 2)
Head of Waquoit	1.17	680	4.6	0.05	0.03	0.3	0.3	<LOQ[e]
Sage Lot	2 – 4[b]	-	8.6	0.01	0.03	0.2	0.2	<LOQ
Green	0.86	531	1.7	3.68	0.02	3.7	0	12.1
Site D1	6.30[c]	171	1.8	7.33	0.14	7.8	0.3	46.7
	7.53	181	1.1	1.67	0.57	2.4	0.11	
	8.75	203	1.2	2.34	0.69	3.2	0.17	
Site D2	9.97	236	0.4	12.09	0.51	13.7	1.1	7.8
	11.19	225	0.3	15.7	<0.02	14.9	-[d]	
	12.41	209	0.5	12.9	<0.02	12.3	-[d]	
	13.66	219	0.5	14.5	<0.02	14.9	0.4	
	14.88	255	0.4	19.4	<0.02	18.7	-[d]	
Site D3	16.11	321	0.5	24.39	0.02	26.3	1.9	30.6
	17.33	1081	0.6	23.1	<0.02	16.1	-[d]	

[a] For unlabeled rows at Site D, only water quality data were collected by the USGS (provided courtesy of Denis LeBlanc and John Colman), and no quantification of carbamazepine or analysis by ESI-FT-ICR-MS was performed.

[b] Exact depth between these depths. Water table at ~2 m below ground surface.

[c] The interval at 5.1 m was dry, indicating the water table is between 5.1 and 6.3 m in depth

[d] Subtracting the inorganic components from total nitrogen results in a negative value.

[e] One replicate was above the LOQ of 1.6 ng/L and one below, with the average value below the LOQ.

Table 2. The total number of formulas and percentages (by number) of the types of formulas assigned to the groundwater samples, weighted averages of O:C and N:C molar ratios, and number of formulas with O:C ratio > 0.5.

Site	Total Formulas	%CHO	% CHON	% containing N[b]	$(O:C)_{wa}$	$(N:C)_{wa}$	O:C > 0.5 all formulas	O:C > 0.5 N-containing formulas
Head of Waquoit	5,667	61	25	37	0.34	0.07	1193	278
Sage Lot	6,638	56	26	40	0.35	0.07	1337	522
Green	5,731	54	28	43	0.30	0.09	947	374
Site D1	5,803	56	29	41	0.30	0.07	661	375
Site D2[a]	4,413	41	36	56	0.20	0.17	425	177
Site D3	5,368	53	30	44	0.27	0.09	505	262

[a] Average of duplicate injections.

[b] Includes all possible formulas found by the algorithm that contain N in any allowed combination with C,H,O,S, and/or P.

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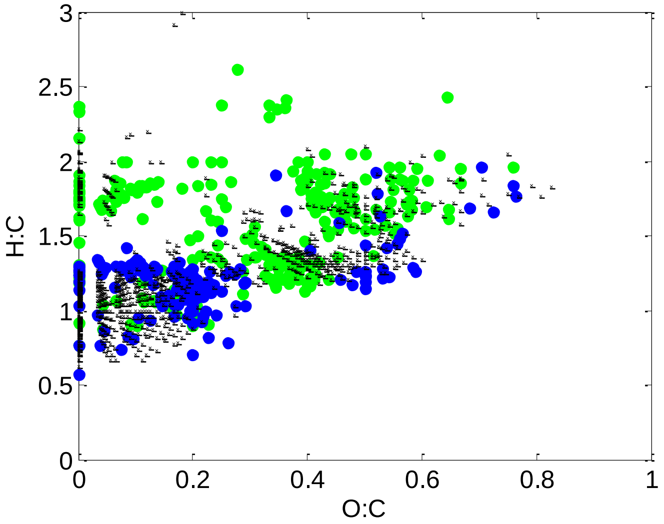

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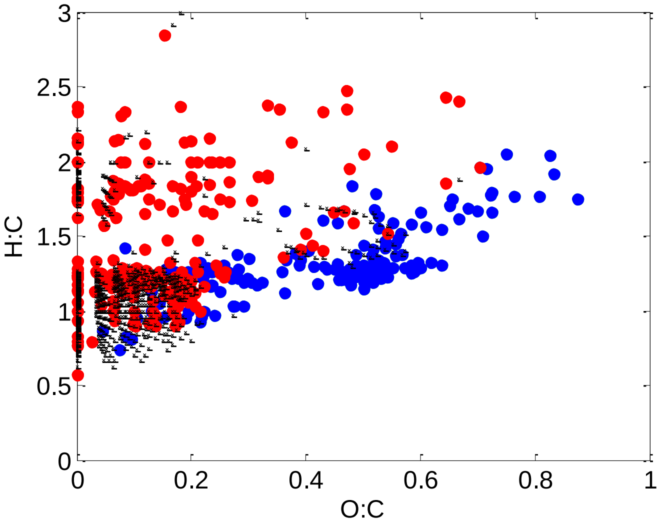

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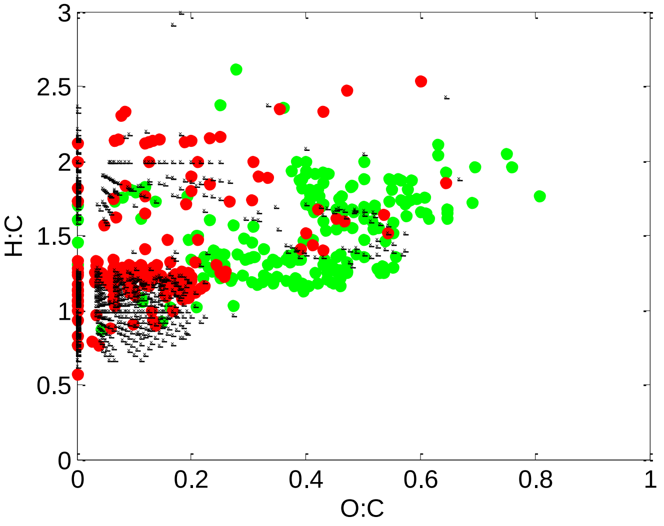

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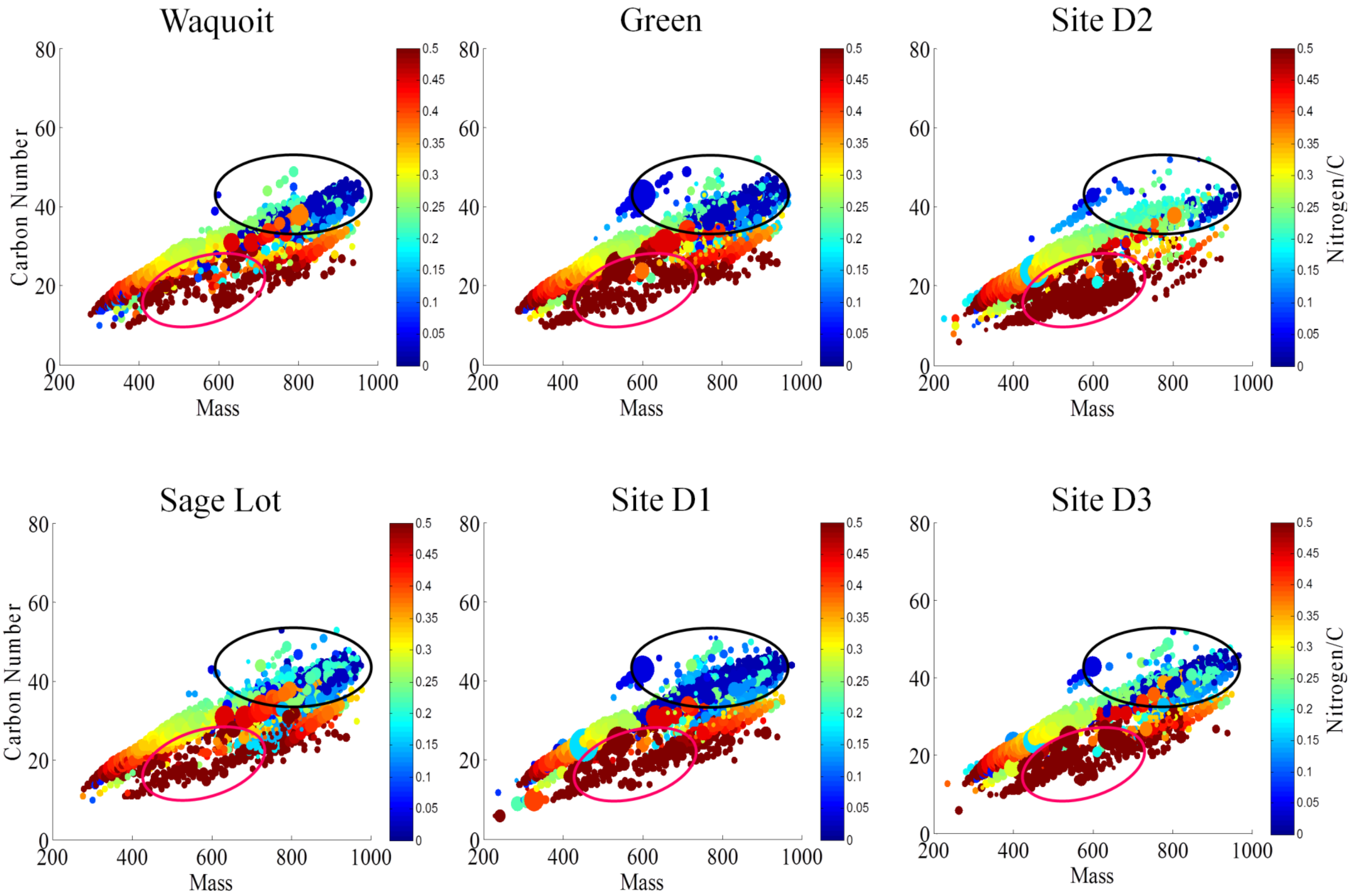
Figure 1. Comparisons of N-containing features in selected samples. (a) The green symbols are features present in the shallow, impacted samples (Green and D1) but not in the unimpacted sites (Head of Waquoit and Sage Lot). The blue are the features in Head of Waquoit and Sage Lot, but not the other two samples. Black symbols are present in all four samples. (b) The blue symbols are features present in the shallow, unimpacted samples (Head of Waquoit and Sage Lot) but not in D2 and D3. The red are the features in D2 and D3, but not the other two samples. Black symbols are present in all four samples. (c) The green symbols are features present in the shallow, impacted samples (Green and D1) but not in D2 and D3. The red are the features in D2 and D3, but not the other two samples. Black symbols are present in all four samples



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440 **Figure 2.** Carbon versus mass plots for the nitrogen containing features detected via ESI-FT-ICR-MS in the groundwater samples.
441 Larger diameter denotes greater intensity and color indicates the N:C ratio. The pink oval marks the region of the plot that is

comprised of high nitrogen ratio features that are more intense in the septic -impacted samples. The black oval indicates low nitrogen features that are more abundant/intense in shallow water samples.