Measurement of diffusion coefficients of humic substances in a hydrogel and in water by fluorescence correlation spectroscopy

J.R. Lead¹ and K. J. Wilkinson²
¹Division of Environmental Health and Risk Management, University of Birmingham, U.K.
²CABE, University of Geneva, Switzerland.

Abstract
Diffusion coefficients of three different humic substances (HS) were measured in both water and in agarose hydrogels. The three HS were standard Suwannee River humic and fulvic acid [1] and a peat humic acid [2]. Measurements were made by fluorescence correlation spectroscopy (FCS). In general, values of diffusion coefficients were in the range of 1.9-2.5 x 10⁻¹⁰ m² s⁻¹, for all of the HS under all conditions (except a single peat derived HS at low pH). The small degree of polydispersity of the HS (observed both in the autocorrelation functions of the FCS and by atomic force microscopy, AFM) did not preclude the use of a single diffusion coefficient to explain the data for a single HS at a particular pH. However, at low pH, the peat HS exhibited a high degree of polydispersity with the formation of aggregates of up to 30-50 nm in diameter, as measured by atomic force microscopy and ultrafiltration. In this case, FCS did not give reliable values of diffusion coefficients, most likely due to extensive aggregation. Excluding this extreme case, diffusion coefficients in the gel were approximately 10-20% lower than in the water, because of either interactions with the gel or increased path lengths due to an increased tortuosity of the gel compared to water. Large HS aggregates were excluded from the gel.

1 Introduction
Humic substances (HS) are potentially important in binding trace metals [3] as well as affecting their transport [4,5] and bioavailability [6] in aquatic systems. Diffusion coefficients in water are essential to our understanding of the biogeochemistry of HS and their associated metals. In particular, they are essential to our understanding of the environmental transport in systems where molecular diffusion is the dominant type of movement, e.g. in sediment and soil porewaters.
and in the diffusive layer around particles and surfaces [7] and micro-organisms [8]. In the case of micro-organisms, HS will likely modify the toxic or nutritive effect of metals in complex and poorly understood ways [6, 9]. Diffusion coefficients in gels are essential in order to interpret metal speciation data from several newly developed in-situ analytical techniques, notably voltammetric microelectrodes where gels are used for anti-fouling purposes [10] and in Diffusive Gradients in Thin-films (DGT) [11]. In both techniques, diffusional mass transport of the analyte may be constrained by the gel. It is thus essential to know whether and to what extent diffusion in the gel is hindered. Comparison of diffusion coefficients of HS in both water and gel is therefore required and previously no such study had been undertaken.

There have been a number of studies investigating the diffusion of macromolecules in gels reviewed by Westrin and co-workers [12]. More recently, a fluorescence based study of diffusion coefficients of a variety of standard macromolecules in agarose gels [13] has allowed the calculation of effective pore diameters under similar conditions to those used in this study. For an agarose gel of 2%, they found average diameters of a few hundred nm, indicating that physico-chemical interactions such as sorption between the gel and small macromolecules would be expected to be negligible. Previous work [14, 15] has indicated that under most solution conditions, the HS used in this study are relatively small with diameters < 5 nm. However, at low pH, some HS may form larger aggregates, with Atomic Force Microscopy (AFM)-derived ‘diameters’ of up to 30 nm [16]. Because AFM height measurements are likely to underestimate the true hydrodynamic diameters [16, 17], under some circumstances the HS may be sufficiently large for physical interactions between the gel and humic substances to become significant.

Until recently, diffusion coefficients of HS had not been measured with great certainty in either waters or gels, in large part due to the lack of suitable techniques for analysing these complex, polydisperse molecules which are easily modified by preparative techniques [17]. In an interlaboratory study, we have shown that the diffusion coefficients in water measured by fluorescence correlation spectroscopy (FCS) agree well with two other non-perturbing techniques, flow field-flow fractionation (FIFFF) and pulsed field gradient NMR [14] and we have previously used the techniques to measure effects of solution conditions on one type of HS [15]. In this study, we have investigated the diffusion coefficients of several HS with different characteristics (humic and fulvic acids extracted from different source materials) in both water and gel under identical experimental conditions. This has allowed direct comparison between the two systems and quantification of the retardation of HS by the gel. This should permit a greater understanding of the environmental behaviour of these complex species.

2 Experimental

2.1 Humic Substances

Three different humic substances were selected for this study: the IHSS standard Suwannee River fulvic and humic acids (SRFA, SRHA), and a UK Geological
Survey peat humic acid (GSHA). They have been characterised and the results reported in the literature [1, 2]. Their electrophoretic mobilities and molecular weights are reported in Table 1. Samples were prepared at pH 3-7.5 at 5 mM NaCl and an HS concentration of 10 mg L\(^{-1}\). The pH was adjusted with dilute NaOH or HNO\(_3\), without the use of buffers. The solutions were prepared from the freeze dried solids without further treatment and left for 24 hours for complete rehydration. In the case of the GSHA, solutions were prepared from a concentrated solution (5 g HS L\(^{-1}\)). The dilute HS solutions were stored at 4\(^\circ\)C in the dark and were kept for a maximum of about 14 days before being discarded. The pH was measured with a Metrohm Digital E500 pH meter and a Metrohm AG CH-9101 combination pH electrode, calibrated with standard NBS buffers.

Table 1: Weight average molecular weight and electrophoretic mobilities (x \(10^8\) m\(^2\) V\(^{-1}\) s\(^{-1}\)) of selected HS. a-from reference [18], b-from reference [2], c from reference [19]

| Weight-average molecular weight | Electrophoretic mobility (x \(10^8\) m\(^2\) V\(^{-1}\) s\(^{-1}\))
|-------------------------------|------------------------
| SRFA 860\(^a\)                | -5.1                   |
| SRHA 1490\(^a\)               | -4.8                   |
| GSHA 23 000\(^b\)              | -4.8                   |

2.2 Fluorescence correlation spectroscopy

The FCS method has been discussed in detail elsewhere [20, 21]. In brief, laser light (excitation at 488 nm) is focused into the sample of interest using confocal optics. In this manner, a small, illuminated volume element (approximately 0.5-1.0 \(\mu\)m\(^3\)) called the confocal volume is created. In order to optimize the signal-to-noise ratio, the confocal volume should be occupied by a small number of fluorescent molecules at any given point in time. Temporal fluctuations in the measured fluorescence intensity are used to derive an autocorrelation curve. In the absence of any other processes which affect sample fluorescence, such as chemical reactions, the autocorrelation curve will be related to the translational diffusion of the fluorophore across the confocal volume.

Diffusion times of the SRFA molecules are obtained from a best fit of the autocorrelation function following the calibration of the size of the confocal volume using Rhodamine-6G (R6G), which has a diffusion coefficient of 2.8 \(x\) 10\(^{-10}\) m\(^2\) s\(^{-1}\) [21]. The diffusion coefficient, \(D\), of the SRFA was calculated from the following relationship:

\[
D = \frac{\omega_1^2}{4\tau_1}. \tag{1}
\]
where $\omega_1$ is the width of the confocal volume and $\tau_1$ is the characteristic diffusion time of the particle through the confocal volume.

## 3 Results and Discussion

Figure 1a-c shows the variation of diffusion coefficient of several HS samples as a function of pH in water and in an agarose gel. With the exception of the GSHA at low pH, the values in water are in the range $1.9\text{ to }2.8 \times 10^{-10}$ m$^2$ s$^{-1}$ and increase with increasing pH. The lower diffusion coefficient indicates lower mobility, and can most likely be accounted for by the formation of small aggregates as the pH, and therefore the repulsive charge on the HS, is lowered. If we assume that individual macromolecules exist at high pH (about 7), we calculate that at low pH (about 4) small aggregates of 2 or 3 macromolecules are formed. Interestingly, at high pH the GSHA has a significantly higher diffusion coefficient (greater mobility) than the Suwannee River HS despite its greater apparent molecular weight (Table 1).

However, the measured molecular weight is most likely an experimental artifact [2] and is related to ionic strength, concentration of HS and pretreatment. It is likely therefore that the difference in diffusion coefficient between the peat HS and the Suwannee River HS is real and is a consequence of different experimental conditions employed. In addition, analysis of the FCS data and fitting curve indicate that, with the exception of GSHA at low pH, a single diffusion coefficient can adequately model the data, despite a small degree of polydispersity of the HS [15]. Use of a two or three diffusion coefficient model results in a slight improvement of the fits of the autocorrelation curves, but gives parameter values which are often physically unrealistic and not statistically meaningful. Using the Stokes-Einstein approximation [17] the observed change in diffusion coefficient with pH corresponds to diameters of approximately 1.5 nm at high pH and to diameters of 2.3 nm at low pH, in agreement with our previous results [14, 15, 17].

In the case of the GSHA at low pH, adequate fits to the data could not be determined using a model containing one, two or three diffusion coefficients as fitting parameters, most likely because of a significant degree of aggregation of the GSHA and consequent high degree of polydispersity. This was indicated by visual analysis of the raw data, which was generally lower in fluorescence than other samples with large, randomly occurring spikes of fluorescence which corresponded to aggregates moving through the confocal volume. Further data analysis is ongoing in order to interpret the results quantitatively. The aggregation of the GSHA into structures up to a size of 50 nm was confirmed by AFM (Figure 2 and reference 16) and ultrafiltration (data not shown).

Comparison of the data for GSHA at low pH in the gel (where a single diffusion coefficient is sufficient to model the data) and in water (where the model fails due to the aggregation of HS) indicates that the gel acts as an effective barrier to the movement of the large aggregates, either by acting as a complete physical barrier or by slowing diffusion almost completely. These results will have significant implications for the analysis of metal speciation data from the use of in-situ techniques such as DGT and gel-covered voltammetric microelectrodes.
Figure 1. Variation of diffusion coefficient ($x10^{10} \text{ m}^2 \text{ s}^{-1}$) with pH for GSHA (top), SRHA (centre) and SRFA (bottom). Open circles in water, closed circles in gel.
Figure 2. Atomic force microscopy of the U.K. Geological Survey Peat humic acid A. Height histograms of the adsorbed macromolecule. B and C. Tapping AFM images of a peat humic acid (5 mM NaCl) at pH 3.2 (B) and 6.8 (C). Scan size = 2 μm X 2 μm. Taken from [16].

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References


