It is the paper published as

Author: S. McDonald, J. Pringle, P. D. Prenzler, A. G. Bishop and K. Robards

Title: Bioavailability of dissolved organic carbon and fulvic acid from an Australian floodplain river and billabong

Journal: Marine and Freshwater Research **ISSN**: 1323-1650

Year: 2007

Volume: 58

Issue: 2

Pages: 222-231

Abstract: Dissolved organic carbon (DOC) is a vital resource for heterotrophic bacteria in aquatic ecosystems. The bioavailability of fulvic acid,which comprises the majority of aquatic DOC, is not well understood.The present study examined the bioavailability of bulk DOC and fulvic acid from two contrasting but inter-relatedwater bodies: the Murrumbidgee River and adjacent Berry Jerry Lagoon. Bacteria utilised fulvic acids; however, bulk DOC was more bioavailable. Bacteria were able to utilise Murrumbidgee River DOC and fulvic acid more readily than Berry Jerry Lagoon DOC and fulvic acid, suggesting that the quality of carbon may be an important factor to consider when evaluating lateral exchange of nutrients between the main channel and floodplain. Chemical characteristics of fulvic acids appeared to explain some of the variation in fulvic acid bioavailability. The higher the molecular weight and complexity of the fulvic acid, the longer it took for bacteria to utilise the substrate (lag phase), but the larger the number of bacteria that grew on the substrate. The present study calls attention to the need for further multidisciplinary studies to address the quality of carbon in riverine-floodplain ecosystems.

Bioavailability of dissolved organic carbon and fulvic acid from an Australian Floodplain River and Billabong

Suzanne McDonald[A]; Jennifer M. Pringle[B]; Paul D. Prenzler[A]; Andrea G. Bishop[A] and Kevin Robards[A, C]

[A]School of Science and Technology, Charles Sturt University,

Wagga Wagga, NSW 2678, Australia.

[B] School of Physics and Materials Engineering, Monash University, Wellington Road, Clayton Vic, 3800, Australia

[C]Corresponding author. email: krobards@csu.edu.au

Abstract

Dissolved organic carbon (DOC) is a vital resource for heterotrophic bacteria in aquatic ecosystems. The bioavailability of fulvic acid, which comprises the majority of aquatic DOC, is not well understood. This study examines the bioavailability of bulk DOC and fulvic acid from two contrasting but inter-related water bodies, the Murrumbidgee River, and adjacent Berry Jerry Lagoon. Bacteria utilized fulvic acids, however, bulk DOC was more bioavailable. Bacteria were able to utilise Murrumbidgee River DOC and fulvic acid more readily than Berry Jerry Lagoon DOC and fulvic acid suggesting that the quality of carbon may be an important factor to consider when evaluating lateral exchange of nutrients between the main channel and floodplain. Chemical characteristics of fulvic acids appeared to explain some of the variation in fulvic acid bioavailability. The higher the molecular weight, and complexity of the fulvic acid, the longer it took for bacteria to utilize the

substrate (lag phase), but the larger the number of bacteria that grew on the substrate. This study points to the need for further multidisciplinary studies addressing the quality of carbon in riverine-floodplain ecosystems.

Introduction

Dissolved organic matter (DOM) is the major form of organic carbon in almost all aquatic ecosystems (Mann and Wetzel 1995; Findlay and Sinsabaugh 1999). It may be considered to be one of the most important factors in determining the biotic functioning of a riverine ecosystem (O'Connell et al. 2000), and plays an important role in energy flow (Fischer et al. 2002). The turnover of dissolved organic carbon (DOC) is almost exclusively the domain of heterotrophic bacteria (Volk et al. 1997; Moran & Hodson 1990; Fischer et al. 2002), due to their abundance, large surface-to-volume ratios, and ability to utilize the substrate at low concentrations (Moran & Hodson 1990).

DOC is operationally defined as the fraction of organic matter that passes through a 0.45 μm filter (McDonald et al. 2004). DOC consists mainly of an extremely complex mixture of organic acids (Sun et al. 1997), and has been further operationally sub-divided into non-humic and humic substances. Non-humic substances include known biomolecular classes of compounds, including lipids, carbohydrates, polysaccharides, amino acids, proteins, waxes and resins; while humic substances may be defined as being a category of naturally occurring, biogenic, heterogeneous, high molecular weight organic substances, that have not yet been chemically defined (McDonald et al. 2004).

Humic substances are further sub-divided into fulvic acids (soluble at all pH conditions), and humic acids (not soluble at pH<2). The division of DOC into humic and non- humic substances is operationally based, and may not be completely distinguishable from each other due to some natural non-humic solutes such as carbohydrates, being an integral part of the structural composition of humic solutes (Peuravuori et al. 2005). Humic substances comprise the majority of DOC (Hertkorn et al. 2002), with fulvic acid being the predominant humic substance in aquatic ecosystems (McKnight and Aiken 1998; Maurice and Namjesnik-Dejanovic 1999).

The role of DOC as an essential component of the microbial food web is well known (Sun et al. 1997). What part of the DOC is consumed by the bacteria is a matter of debate, with two contrasting, although not necessarily mutually exclusive views (Volk et al. 1997). Traditionally, it has been accepted that most of the turnover of DOC was accomplished via the metabolism of a small pool of labile, low molecular weight, and structurally simple compounds within the DOC (Hertkorn et al. 2002; Robertson et al. 1999). Although present in low concentrations, these compounds undergo rapid turnover and satisfy most of the energy for heterotrophic bacteria (Volk et al. 1997).

Secondly, the complex nature of the high molecular weight fraction of DOC has caused dispute as to whether or not this fraction is readily utilized (Hertkorn et al. 2002). Increasing evidence would suggest, however, that the high molecular weight compounds are in fact utilized (Amon and Benner 1996; Wetzel 1995). These high molecular weight compounds, primarily humic substances (Volk et al. 1997), may in part be biologically degradable (Moran and Hodson 1990) and indeed provide a thermodynamic stability to metabolism within lake and river ecosystems that is essential for the maintenance of efficient nutrient recycling (Wetzel 1998). Given the predominance of fulvic acids in aquatic ecosystems, it may be these substances that provide the thermodynamic stability to

metabolism within lake and river ecosystems. While knowledge about the contribution of humic substances to the food web is increasing, further research is needed in this area.

The degree to which DOC is bioavailable is likely a consequence of its source(s), chemical composition, arrangement of the various functional groups, and the ability to complex with other chemicals (McDonald et al. 2004). Two main approaches have been used to investigate the quality of organic matter in terms of its ability to support bacterial growth. The first is to infer bioavailability from direct measurement of selected chemical properties, and the second is to examine the ability of organisms to utilize the organic matter and infer differences among the complex mixtures (Leff and Meyer 1991).

When examining the ability of organisms to utilize the organic matter, DOC has either been studied as a substrate by itself, further fractionated into its operationally defined humic, non-humic substances (Obernosterer and Herndl 2000), or separated into various molecular weight ranges (Engelhaupt and Bianchi 2001; Amon and Benner 1996) using ultrafiltration. The bioavialability of these various DOC components are then compared.

Billabongs, lentic waterbodies common to the floodplain of Australian rivers, differ considerably from the lotic riverine environment in terms of hydrology, physiochemical characteristics, and biological assemblages present (Boon 1991). While bacterial metabolism is a key component of carbon processing in aquatic ecosystems (Rees et al. 2005), there have been few studies on the utilization of carbon from both billabongs, and lotic systems (Rees et al. 2005; Castillo et al. 2003). Billabongs play an important role in the complex relationship that exists between the main channel and floodplain, serving as a nutrient storehouse for exchange with the river upon connection with

the main channel (Gell et al. 2005). This exchange is important, however, few studies have examined the quality of DOC from the two environments. Given the different environmental conditions between a river and an associated billabong, it is possible that the quality of organic matter may differ.

To address these knowledge gaps, we quantified bacterial growth on DOC and fulvic acid from an Australian floodplain river and billabong. Chemical characterisation was conducted on fulvic acid fractions to determine compositional differences between the two environments, and relate these to bioavailability trends. Suwannee River fulvic acid served as a reference.

Methods

Study Site

The Murrumbidgee River is approximately 1,600 km in length and drains an area of approximately 82,000 km^2. It is one of Australia's largest and most heavily regulated rivers (Page et al. 2005), beginning in the Snowy Mountains, and flowing west across the semi-arid Riverine Plain before joining the Murray River near Balranald.

Berry Jerry Lagoon (billabong) is a 66000 m^2 (117000 m^3, mean depth of 1.8 m) meander cut-off lagoon located approximately 30 km west of Wagga Wagga, south-eastern Australia. It is connected to the Murrumbidgee River by overbank flows, and when the channel flow reaches 193 m^3 s^{-1} (as recorded at the Wagga Wagga gauge). Sampling sites were located at 147°3'54"E, 35°0'60"S (Berry Jerry Lagoon) and 147°3'38"E, 35°1'10"S.

Collection and preparation of water samples. Water samples totaling 1300 L were collected seasonally during 2004 as previously described by McDonald et al. (2006). Briefly, water from Berry Jerry Lagoon and Murrumbidgee River was collected in 10 L and 15 L carboys. Prior to sampling, each carboy was acid washed, rinsed several times with Milli-Q water, and then rinsed twice with sample water before filling.

Water from Berry Jerry Lagoon was sampled in Autumn (14th April 2004) and was used to prepare both DOC and fulvic acid as the concentration of carbon was sufficient for this study. On the other hand, the carbon levels in the Murrumbidgee River were low and large volumes of river water were needed to isolate milligram quantities of fulvic acid. As our previous study (McDonald et al. 2006) showed that seasonal variation in the chemical character of Murrumbidgee River fulvic acid was not statistically significant we were able to combine water samples collected at weekly intervals from 3rd May 2004 to 1st August 2004. This was logistically advantageous to enable sample processing of the large volumes of water. DOC from Murrumbidgee River was collected in Winter (1st August 2004) when levels were sufficiently high to allow this study.

Preparation of DOC

DOC was regarded as the 0.45 μm filtered river or lagoon water. Water samples were pre-filtered first through a 90 mm GF/C filter and then filtered using a SolvCycle Nalgene® filtration apparatus fitted with Advantec MFS 0.45 μm mixed cellulose ester or cellulose acetate membrane filters to obtain DOC. DOC samples (20 L) were frozen until needed for bioavailability experiments. The remaining DOC was used to extract fulvic acid samples.

Isolation of fulvic acids

Fulvic acids were isolated using Supelite DAX-8, as per the method outlined by the International Humic Substance Society (Thurman and Malcolm 1981). Briefly, 50 g of Supelite™ DAX-8 resin was added to the filtered 4 L water sample (adjusted to pH 2.0 with concentrated HCl) and stirred for 30 minutes. The DAX-8 resin was allowed to settle, supernatant decanted, and the slurry collected in a 10 mm diameter glass chromatography column fitted with a frit.

Humic and fulvic acids were eluted from the resin with 0.1 M NaOH, and acidified to pH 2.0 immediately with concentrated HCl to avoid oxidation of the humic substances (Thurman and Malcolm 1981). The humic substances were then re-concentrated onto DAX-8 resin, and again eluted with 0.1 M NaOH. Humic acid was separated from the fulvic acid by lowering the pH to <1.0 (concentrated HCl), and allowing the humic acid to aggregate overnight at 4°C in the dark. The humic and fulvic acid suspension was then centrifuged at 3,000 rpm for 10 min, and filtered through a Whatman GF/C filter to remove the humic acid.

The fulvic acid fraction was adjusted to pH 2.0 with 1.0 M aqueous NaOH and re-adsorbed onto the DAX-8 resin. The DAX-8 resin was then rinsed with one bed volume of Milli-Q water, and fulvic acid

desorbed with 0.1 M NaOH. Fulvic acid was immediately passed through an Amberlite IR-120 (H) resin, frozen, and freeze-dried.

Suwannee River fulvic acid (SRFA 1R101F) was purchased through the International Humic Substance Society to serve as a reference.

Preparation of samples for bioavailability studies

Solutions of Berry Jerry DOC and fulvic acid, Murrumbidgee River DOC and fulvic acid, and Suwannee River fulvic acid were prepared as follows. All fulvic acid solutions were made by re-dissolving fulvic acid powder in Milli-Q water to give a final concentration of 20 mg C L^{-1} and volume of 500 mL.

Berry Jerry DOC and Murrumbidgee River DOC were thawed (refer to collection and preparation of water samples) and made to a volume of 500 mL. The concentrations of DOC in the lagoon and river were 114 mg C L^{-1} and 10.9 mg C L^{-1} respectively.

Inorganic phosphorus (10 μM, analytical grade KH_2PO_4; Ajax Finechem, Sydney, Australia) and nitrogen (20 μM, analytical grade NH_4NO_3; Ajax Finechem, Auburn, Australia) were added to all solutions so that they were not nutrient limited, and the pH adjusted to 7 with NaOH (1.0 M). A solution of Milli-Q water plus inorganic nutrients served as a control.

The solutions were filter-sterilized by passing through a 0.2 μm membrane filter into acid washed, autoclaved 500 mL conical flasks. Sterile stirrer bars were added to the flasks, and the flasks

stoppered with sterile cotton wool plugs. Solutions were stirred using magnetic stirrers and bars, to simulate aerobic conditions in the natural environment. Analyses were carried out in duplicate at ambient.

Preparation of natural microbial inoculum

Water samples were collected from the Murrumbidgee River water column in 10 L carboys. Prior to sampling, each carboy was acid washed, rinsed several times with Milli-Q water. The Milli-Q water was allowed to stand overnight in the containers to ensure the adequate removal of contaminants. Carboys were then rinsed twice with sample water before filling.

Water was collected (1.0 L), and filtered through two GF/F filters (Whatman, 0.7 μm) to remove protozoa and other bacterivores, without removing all bacteria (Wetzel & Likens 2000; Miller & Moran 1997; Goldstone et al. 2002). The water was placed in an acid washed, autoclaved Schott bottles and yeast extract (1 mL of 1000 mg L^{-1} stock solution, giving a final concentration of approximately 1.0 mg L^{-1}) added. These samples were then placed in the dark for 24 hours before collecting bacterial assemblages over a 0.45 μm filter, and re-dissolving them in 5 mL of filter-sterilized Milli-Q water. Immediately, 1.5 mL of the bacterial inoculum was added to each fulvic acid and DOC solutions (containing nitrogen and phosphorus), to give an initial concentration of approximately 3.0 x 10^4 cells per mL.

Bacterial Enumeration

Bacteria were incubated in the DOC and fulvic acid solutions for nine days at ambient temperature, and bacterial numbers counted at 0, 1,

2, 3, 4, 7 and 9 days. Enumeration of the bacteria was achieved using epiflourescence microscopy to identify the stained bacteria (LIVE/DEAD *Bac*Light bacterial viability kit, Molecular Probes, Oregon, USA).

Briefly, using aseptic techniques, ten ml of DOC and fulvic acid solution containing bacteria, were taken at intervals of 0, 1, 2, 3, 4, 7 and 9 days, and added to sterile centrifuge tubes. The live/dead dye (3.0 μL) was added to the centrifuge tubes and mixed. The solutions were stored in the dark for 10 minutes and then fixed with formaldehyde (2% final concentration). Bacteria were collected on 0.22 μm black polycarbonate filters (25mm, Osmonics Inc., USA), the filters transferred onto a glass slide, and the under-side of the slide heated mildly to drive off excess water. A glass cover slip was mounted onto the slide using mounting oil, and the edge of the cover slip was sealed with clear nail polish.

Microscope slides were viewed using a Nikon Eclipse E1000 microscope (Tokyo, Japan), fitted with a Nikon digital still camera DXM 1200, at a magnification of x1000 (oil immersion lens). Images were viewed using ACT-1 version 2.20 software on a Dell, Pentium 4, 37.2 GB, 1.0 GB RAM computer.

Bacterial numbers were calculated according to the formula in Boulos et al. (1999):

$T = N \times A / a \div V$

Where T is the number of bacteria per ml, N is the average number of bacteria per field, A is the surface of filtration (mm^2), a is the area of the microscopic field, and V is the volume of sample filtered (mL). Bacterial numbers were estimated from counts of 10 microscopic fields using two replicates for each sample (Boulos et al. 1999), achieving at least 300 cells for each sample (Wetzel and Likens 2000).

Enumeration of bacteria in fulvic acid and DOC samples over nine days of incubation resulted in a growth curve with a lag, log and stationary phase. Live cells accounted for greater than 95% of total bacteria growing in all DOC and fulvic acid samples, with only live cells being counted. Bioavailability was determined by comparing the maximum number of bacteria between samples (referred to as bacterial abundance). Further information about the bioavailability of the carbon sources was obtained by measuring the mean growth rate and lag phase of the curve. The mean growth rate was calculated from the slope of the growth curves during the 'log phase' for duplicate samples, and is expressed as cells mgC^{-1} day^{-1}. The lag phase was taken to be the number of days before reaching the log phase.

Size-exclusion chromatography

Many studies have focused on molecular size as being an important factor for determining microbial utilization of humic substances (Hunt et al. 2000). High-performance size exclusion chromatography is the most extensively used (Peuravuori et al. 2005), and well-established (Reemtsma and These 2003) technique for measuring the molecular weight distribution of humic substances. It was therefore used to determine the apparent molecular weight of fulvic acids in this study.

Size-exclusion chromatography was conducted using a Varian Star LC (Melbourne, Australia) solvent delivery system equipped with a 50 μL sample loop and a Star 9050 variable wavelength UV/VIS detector. The HPLC was interfaced with a Pentium 4, 37.2 GB hard drive with 385 MB RAM Dell computer equipped with Varian Star Chromatography Workstation software (version 6.41).

A Waters Ultrahydrogel 250 column (7.8 mm x 300 mm, pore size 250 A°) was used, with an exclusion limit of $8x10^4$ g mol^{-1}. Absorbance of fulvic acids was detected at 254 nm. Isocratic elution was conducted at a flow rate of 1.0 mL min^{-1} with a 10 mM ammonium acetate mobile phase. The Ultrahydrogel 250 column was calibrated with poly (styrenesulfonate) sodium salt standards to give a linear equation of y = -1.0809*x* + 9.8154, where y = log M, and *x* = retention time (min). Number (M_n) and weight-average (M_w) molecular weights were determined using the following equations:

$$M_N = \frac{\sum_i^N h_i}{\sum_i^N \left(\frac{h_i}{M_i} \right)}$$

$$M_W = \frac{\sum_i^N \left(h_i . M_i \right)}{\sum_i^N h_i}$$

Number-average molecular weight is the weight of the average molecule in the mixture (Zhou et al. 2000). The weight-average molecular weight is the weight of the molecule to which the average atom belongs (Zhou et al. 2000). Polydispersity indicates how widely distributed the molecular weights are, and was calculated by dividing weight-average by number-average molecular weight.

Solid-state CP-MAS ^{13}C-NMR spectroscopy

The degree to which organic matter is bioavailable is likely a consequence of its chemical composition. Solid-state CP-MAS ^{13}C-NMR spectroscopy has been widely used for the characterisation of humic substances (Cardoza et al. 2004), with certain peaks corresponding to various functional groups and classes of compounds (Engelhaupt and Bianchi 2001). This technique was used to characterise fulvic acids in this study, and gain a broader understanding of their composition and relation to bioavailability.

Solid-state ^{13}C-NMR spectroscopy was conducted using a Bruker AM300 instrument equipped with a Bruker 4 mm solid-state probe operating at 75.5 MHz. The spectra presented were collected using a magic angle spinning speed of 8 KHz. This was sufficient to eliminate spinning side bands, confirmed by comparison with spectra collected at a MAS rate of 5 KHz. The signal was enhanced using cross polarization techniques, where the ^{1}H polarization is transferred to the ^{13}C nuclei. A contact time of 2,000 μs was used with a 7.5 μs pulse, a recycle delay of 1 s and line broadening of 200 Hz. Chemical shifts were referenced to external samples of glycine.

*0-63 ppm (Al) corresponded to aliphatic carbons, including simple (0-32), complex (32-53) and methoxyl (OCH_3) carbons (53-63). 63-90 ppm (*o*-alkyl) corresponded to C-O resonance arising from* o*-alkyl carbons or carbohydrate type compounds. 90-110 ppm (Anom) resonance was due to anomeric carbons of carbohydrates, while 110-160 ppm (Ar) was due to aromatic carbons. 160-190 ppm (carboxyl) corresponded to carboxyl and aliphatic amide*

carbons, while 190-230 ppm (carbonyl) was due to ketones and aldehydes (Peuravuori et al. 2003; Bianchi et al. 2004).

Total organic carbon analysis

Total organic carbon content of the solutions was measured using a Rosemount Dohrmann DC-190 TOC analyzer according to Zander et al. (2005). Briefly, 8 mL of 0.45 μm filtered fulvic acid and DOC solution was lowered to pH 2 with 20% phosphoric acid (60 μL). The sample was sparged for 3 min with oxygen gas, and injected into the TOC analyzer where it was combusted, and the CO_2 gas analyzed by a non-dispersive infrared (NDIR) detector. The carbon analyzer was calibrated with potassium hydrogen phthalate at a concentration of 10 mg L^{-1}. Analyses were conducted in triplicate. Water quality measurements including temperature, dissolved oxygen, pH and conductivity were measured using a Quanta Hydrolab® (Loveland, CO).

Chlorophyll a

Analysis of chlorophyll *a* (Table 1) was undertaken in quadruplicate using the method of Tett et al. (1975). Briefly, 400-500 mL of water was filtered through a Whatman GF/F filter paper, the filter paper then placed into a 10 mL centrifuge tube with approximately 150 mg of $MgCO_3$ and10 mL of 90% aqueous methanol to stabilize pigments. The centrifuge tubes were then stored for 24 h at 4°C. After 24 h, the tubes were placed in a 70°C water bath, and the aqueous methanol was allowed to boil for 2 min. The tubes were then centrifuged for 3 min at 4500 rpm. Samples were allowed to cool, and the absorbance measured using a Varian Cary 50 Conc UV-vis spectrophotometer at 750

and 666 nm. Correction for pheophytin-*a* was performed by acidification with 1.0 M HCl. 90% methanol solution served as a blank.

Statistics

Data was analysed using S-Plus software (version 7.0). A Kruskal-Wallis rank sum test with multiple comparisons (Pace 1999) was performed to reveal significant differences in the bacterial abundance between carbon sources (Table 2). Bacterial abundance was defined as the maximum number of bacteria per mL. One-way analysis of variance (ANOVA) with Tukey's post hoc multiple comparisons test was used to determine whether there was a significant difference between the number and weight average molecular weight of the fulvic acid samples (Table 3). Significant differences were determined with an α error level of $P<0.05$.

Results

Bacterial growth

Bacterial abundance on Berry Jerry Lagoon DOC was significantly higher than the Murrumbidgee River DOC (31 x 10^6 cells mL^{-1} for Berry Jerry Lagoon DOC and 17 x 10^6 cells mL^{-1} for Murrumbidgee River DOC, Figure 1, C). This may have been due to the higher carbon concentration of the Lagoon DOC (114 mg C L^{-1}) compared with the river DOC (10.9 mg C L^{-1}) (Table 1). After concentration corrections were made, the maximum number of bacteria per mg of carbon was higher for Murrumbidgee River DOC than Berry Jerry DOC (Figure 2, C).

Bacterial abundance was significantly higher for DOC than its fulvic acid counterpart for both the Murrumbidgee River and Berry Jerry Lagoon (Figure 1A and B, and 2 A and B). Further, bacteria grew

almost immediately on the Murrumbidgee River DOC, whereas, there was a two-day lag phase before bacteria were able to utilize the Murrumbidgee River fulvic acid. A one-day lag phase was apparent for both DOC and fulvic acid from Berry Jerry Lagoon.

Significant differences in the bacterial abundance for Berry Jerry Lagoon and Murrumbidgee River fulvic acid occurred (Table 2). Bacterial abundance was lower for Berry Jerry Lagoon fulvic acid than for the Murrumbidgee River fulvic acid. Bacterial abundance was not significantly different between Suwannee River and Murrumbidgee River fulvic acid.

Both the time taken to reach maximum abundance, and lag phase differed for each fulvic acid sample. Bacterial abundance reached its maximum in four-days for all samples, except Suwannee River fulvic acid which took seven days. Additionally, the lag phase for the Berry Jerry Lagoon, Murrumbidgee River and Suwannee River fulvic acid samples all differed, being one, two and three days, respectively.

Bacterial growth rate was highest on Murrumbidgee River DOC (Figure 3), followed by Murrumbidgee River fulvic acid, Berry Jerry Lagoon DOC, Suwannee River fulvic acid, and finally Berry Jerry Lagoon fulvic acid.

Chemical characteristics

Fulvic acid samples varied in their chemical characteristics. Suwannee River fulvic acid had a higher apparent molecular weight than Murrumbidgee River fulvic acid, followed by Berry Jerry Lagoon fulvic acid (Table 3). The lower apparent molecular weight and polydispersity of Berry Jerry Lagoon

fulvic acid indicates that this organic matter is not as complex as the Murrumbidgee or Suwannee River fulvic acids. This conclusion was supported by the size-exclusion chromatogram of Berry Jerry fulvic acid having three distinct molecular weight regions, compared to the one broad molecular weight region of the Murrumbidgee and Suwannee River fulvic acid (Figure 4).

Solid-state NMR spectra revealed that Berry Jerry fulvic acid was more aliphatic than either the Murrumbidgee or Suwannee River fulvic acid samples. Murrumbidgee River fulvic acid was slightly more aromatic than Suwannee River fulvic acid. Four main bands were prevalent in the spectra for all fulvic acid samples, corresponding to aliphatic, *o*-alkyl, aromatic, and carboxylic acid functional groups. Most of the variation between the samples occurred in the aliphatic and aromatic regions of the spectra.

Discussion

Fulvic acids isolated from the Murrumbidgee River and Berry Jerry Lagoon were bioavailable. Bacteria grew more readily, however, on the bulk DOC than the fulvic acid fraction from the same source. This would suggest then, that non-humic substances play an important role in the bioavailability of DOC in aquatic ecosystems. The extent to which non-humic substances are bioavailable, or their role in co-metabolism with humic substances is beyond the scope of this study. Other studies have shown that while bacteria do grow on fulvic acid, the ability of bacteria to utilize the non-humic fraction of DOC as a whole is generally better. For example, Rosenstock et al. (2005) found that 74 to 88% of the total growth on oceanic DOC was attributed to non-humic substances. In another study, non-humic substances were shown to support a fourfold increase in the bacterial secondary production compared to the humic substances from lake and blackwater swamp DOC

(Moran and Hodson 1990). 22% and 53% of the total bacterial growth from the lake and blackwater swamp respectively were attributed to the humic substances (Moran and Hodson 1990).

It is also possible that differential growth on the various carbon sources was a consequence of the quality of carbon. Precautions were taken to minimise differences among treatments by using filter sterilisation techniques to remove extraneous microorganisms from DOC and fulvic acid samples, adding a common inoculum, and adding sufficient nitrogen and phosphorus to be non-limiting. Thus, major differences among samples were the carbon concentration (replicating natural conditions) and quality. The data were normalized to carbon concentration (Leff and Meyer 1991) to remove this as a variable, leaving quality as the major difference between treatments. Bacterial utilization differences are evident even when carbon concentration is not normalized, e.g. Figure 1A shows that bacteria grew more readily on the Murrumbidgee river DOC than the fulvic acid fraction, even though the fulvic acid carbon concentration (20 mg C L^{-1}) was almost twice that of the river DOC (10.9 mg C L^{-1}). Upon normalizing carbon concentration, it becomes evident that bacterial abundance per mg of carbon was higher on the Murrumbidgee River DOC than Berry Jerry Lagoon DOC (Figure 2C, c.f. Figure 1C). While it is now recognized that bacterial metabolism is a key component of carbon processing in aquatic ecosystems (Rees et al. 2005), it is unclear as to what component of the carbon is important for metabolism. Given that fulvic acids were less bioavailable in this study than the DOC from their respective environments, it may be possible that the higher humic content of Berry Jerry Lagoon (Table 1) resulted in reduced bacterial abundance on the DOC.

Differences in bioavailability may also be due to intrinsic chemical characteristics of the DOC. Studies have shown a close link between bioavailability and chemical characteristics of organic matter (Waiser and Robarts 2000; Hopkinson et al. 1998; Sun et al.1997). While no chemical

characterisation of River or Lagoon DOC was conducted, the major component, fulvic acid, was characterised. Fulvic acids from the Lagoon and River differed in their chemical characteristics, having implications for their bioavailability. Murrumbidgee River fulvic acid had a higher apparent molecular weight, and was more aromatic than Berry Jerry Lagoon fulvic acid, however, the lagoon had higher bacterial abundance than the river fulvic acid. Furthermore, Suwannee River fulvic acid, which had a higher molecular weight than Murrumbidgee River fulvic acid, also resulted in higher bacterial abundance. This indicates that high molecular weight was not necessarily detrimental to the quality of organic matter.

Another trend relating to the complexity of the fulvic acid was the lag phase, and time taken to reach the maximum number of bacteria. Initial growth on the more complex, higher molecular weight, aromatic fulvic acids was slow (longer lag phase), however, bacteria grew to higher numbers. Bacteria are able to break down and utilize compounds either by direct, ectoenzyme-mediated or sorption-mediated uptake (Findlay & Sinsabaugh 1999). The longer lag phase would suggest that bacteria needed more time to break down the fulvic acids by one or more of these mechanisms. Once the bacteria were able to break down some of the compounds in the fulvic acid fraction, they could then be utilized as a food source.

The more complex the fulvic acid, the more compounds are likely to be present, and available for bacteria to utilize; leading to higher bacterial numbers. For example, Suwannee River fulvic acid had a higher average molecular weight than the Murrumbidgee River fulvic acid, followed by Berry Jerry Lagoon fulvic acid. It took the bacteria three, two and one day respectively (lag phase) to begin to grow on the fulvic acid. After seven days, the maximum number of bacteria growing on Suwannee River fulvic acid was higher than the maximum reached after four days for the Murrumbidgee River

and Berry Jerry Lagoon fulvic acid. These results would suggest that, the less complex carbon sources could be utilized quicker, but the more complex carbon sources would provide substrate for increased bacterial numbers.

Molecular size has received great interest as a factor for indicating bioavailability of humic substances and DOC (Hessen and Tranvik 1998). Apparent molecular weight appeared to explain some of the variation in bioavailability between fulvic acids; however, the results were not consistent with the traditional view that lower molecular weight compounds are more readily utilized than the higher molecular weight (HMW, > 1 KDa) components. Studies conducted by Amon and Benner (1996) on oceanic DOM found that the HMW DOC pool was typically larger than the pool of low molecular weight (LMW, <1 KDa) DOC. They hypothesized that this was due to the origin of the HMW components being more recent than the LMW components. The LMW components have been diagenetically altered, and are less bioavailable than the HMW DOC. Riverine environments are considered transition zones, typically dominated by diagentically younger, and therefore higher molecular weight materials (Amon and Benner 1996). It may be that fulvic acid from Berry Jerry Lagoon had been diagenetically altered to a greater extent than the Murrumbidgee River, rendering it slightly less bioavailable. Indeed, previous studies have shown that the molecular weight of Berry Jerry Lagoon fulvic acid decreased seasonally during 2004, while the molecular weight of Murrumbidgee River fulvic acid did not alter significantly during the same time (McDonald et al. 2006). The molecular weight of the Murrumbidgee River fulvic acid remained higher than Berry Jerry Lagoon (McDonald et al. 2006). As the Lagoon is a lentic environment, and had no connectivity to the Murrumbidgee River during the course of the study due to drought, it may be possible that the DOC in the Lagoon had undergone more biological processing than the Murrumbidgee River.

The relative importance of floodplain carbon inputs and in-stream metabolic processes has not been well quantified in major Australian rivers (Vink et al. 2005). One study has shown that metabolism in the Murrumbidgee River may be fuelled by allochthonous carbon (Vink et al. 2005), although, net heterotrophy was not as strong as other large river systems due to river regulation. It is not clear what type of carbon source fuels the Lagoon, and further studies would have to be conducted to identify the relative importance of allochthonous and autochthonous inputs into the Lagoon.

The relative aromatic/aliphatic nature of the fulvic acids in this study were not consistent with the view that humic matter with a higher aliphatic content was more available as a growth substrate (Hessen and Tranvik 1998). Berry Jerry Lagoon fulvic acid was more aliphatic and less polydisperse than Suwannee River fulvic acid and Murrumbidgee River fulvic acid, but it had the slowest growth rate of bacteria, and lowest maximum number of bacteria. This may also have been a consequence of more processing occurring in the Lagoon.

Billabongs are thought to play an important role in the exchange of resources between the main channel and terrestrial floodplain ecosystems (Gell et al. 2005). These studies indicate that the quality of organic matter from the main channel, in terms of its ability to be utilized by bacteria, may be important for floodplain habitats. In contrast to Berry Jerry Lagoon DOC, bacteria were able to utilize Murrumbidgee River DOC immediately. In terms of the fulvic acid fraction, Berry Jerry Lagoon fulvic acid could be utilized more quickly than Murrumbidgee River fulvic acid, however, Murrumbidgee River fulvic acid resulted in higher bacterial growth. Additional studies on the sources, age, and diagenesis of DOC and fulvic acids across more Australian rivers and associated billabongs would provide further insight into the role of DOC as a food source.

Acknowledgements

The authors wish to thank Prof. Barry Hart for his comments on the manuscript, and Simon McDonald (SPAN, Charles Sturt University) for his help with statistical analysis. The authors would also like to thank the Department of Materials Engineering at Monash University for the use of their NMR instrument, and funding from an ARC discovery grant that made the analyses possible. Charles Sturt University is thanked for financial assistance, and an APA scholarship is gratefully acknowledged. We also thank the reviewers of the manuscript for their helpful comments.

References

Amon, R.M.W., and Benner, R. (1996). Bacterial utilization of different size classes of dissolved organic matter. *Limnology and Oceanography* **41**, 41-51.

Bianchi, T.S., Filley, T., Dria, K. and Hatcher, P.G. (2004). Temporal variability in sources of dissolved organic carbon in the lower Mississippi River. *Geochimica et Cosmochimica Acta* **68**, 959-967.

Boon, P.I. (1991). Bacterial assemblages in rivers and billabongs of southeastern *Australia. Microbial Ecology* **22**, 27-52.

Boulos, L., Prevost, M., Barbeau, B., Coallier, J., and Desjardins, R. (1999). LIVE/DEAD® *Bac*Light™: application of a new rapid staining method for direct enumeration of viable and total bacteria in drinking water. *Journal of Microbiological Methods* **37**, 77-86.

Cardoza, L.A., Korir, A.K., Otto, W.H., Wurrey, C.J., and Larive, C.K. (2004). Applications of NMR spectroscopy in environmental science. *Progress in Nuclear Magnetic Resonance Spectroscopy* **45**, 209-238.

Castillo, M.M., Kling, G.W., and Allan, J.D. (2003). Bottom-up controls on bacterial production in tropical lowland rivers. *Limnology and Oceanography* **48**, 1466-1475.

Engelhaupt, E., and Bianchi, T.S. (2001). Sources and composition of high-molecular-weight dissolved organic carbon in a southern Louisiana tidal stream (Bayou Trepagnier). *Limnology and Oceanography* **46**, 917-926.

Findlay, S., and Sinsabaugh, R.L. (1999). Unravelling the sources and bioavailability of dissolved organic matter in lotic aquatic ecosystems. *Marine and Freshwater Research* **50**, 781-90.

Fischer, H., Sachse, A., Steinberg, C.E.W., Pusch, M., 2002. Differential retention and utilization of dissolved organic carbon by bacteria in river sediments. Limnology and Oceanography 47, 1702-1711.

Gell, P.A., Bulpin, S., Wallbrink, P., Hancock, G., and Bickford, S. (2005). Tareena Billabong – a palaeolimnological history of an ever-changing wetland, Chowilla Floodplain, lower Murray-Darling Basin, Australia. *Marine and Freshwater Research* **56**, 441-456.

Goldstone, J.V., Pullin, M.J., Bertilsson, S., and Voelker, B.M. (2002). Reactions of hydroxyl radical with humic substances: bleaching, mineralization, and production of bioavailable carbon substrates. *Environmental Science and Technology* **36**, 364-372.

Hertkorn, N., Claus, H., Schmitt-Kopplin, P.H., Perdue, E.M., and Filip, Z. (2002). Utilization and transformation of aquatic humic substances by autochthonous microorganisms. *Environmental Science and Technology* **36**, 4334-4345.

Hessen, D.O., and Tranvik, L.J. (1998). Aquatic humic matter: From molecular structure to ecosystem stability. In 'Aquatic humic substances: Ecology and biogeochemistry'. (Eds D.O. Hessen and L.J. Tranvik) pp. 333-342. (Springer: New York, USA).

Hopkinson, C.S., Buffam, I., Hobbie, J., Vallino, J., Perdue, M., Eversmeyer, B., Prahl, F., Covert, J., Hodson, R., Moran, M.A., Smith, E., Baross, J., Crump, B., Findlay, S., and Foreman, K. (1998). Terrestrial inputs of organic matter to coastal ecosystems: and intercomparison of chemical characteristics and bioavailability. *Biogeochemistry* **43**, 211-234.

Hunt, A.P., Parry, J.D., and Hamilton-Taylor, J. (2000). Further evidence of elemental composition as an indicator of the bioavailability of humic substances to bacteria. *Limnology and Oceanography* **45**, 237-241.

Leff, L.G., and Meyer, J.L. (1991). Biological availability of dissolved organic carbon along the Ogeechee River. *Limnology and Oceanography* **36**, 315-323.

Mann, C.J., and Wetzel, R.G. (1995). Dissolved organic carbon and its utilization in a riverine wetland ecosystem. *Biogeochemistry* **31**, 99-120.

Maurice, P.A., and Namjesnik-Dejanovic, K. (1999). Aggregate structures of sorbed humic substances observed in aqueous solution. *Environmental Science and Technology* **33**, 1538-1541.

McDonald, S., Bishop, A.G., Prenzler, P.D., and Robards, K. (2004). Analytical chemistry of freshwater humic substances. *Analytica Chimica Acta* **527**, 105-124. doi: 10.1016/j.aca.2004.10.011.

McDonald, S., Pringle, J.M., Bishop, A.G., Prenzler, P.D., and Robards, K. (2006). Isolation and seasonal effects on characteristics of fulvic acid isolated from an Australian floodplain river and billabong. *Journal of Chromatography A.* In press. Doi: 10.1016/j.chroma.2006.08.086.

McKnight, D.M., and Aiken, G.R. (1998). Sources and age of aquatic humus. In 'Aquatic humic substances: Ecology and biogeochemistry'. (Eds D.O. Hessen and L.J. Tranvik) pp. 9-39. (Springer: New York, USA).

Miller, W.L., and Moran, M.A. (1997). Interaction of photochemical and microbial processes in the degradation of refractory dissolved organic matter from a coastal marine environment. *Limnology and Oceanography* **42**, 1317-1324.

Moran, M.A., and Hodson, R.E. (1990). Bacterial production on humic and nonhumic components of dissolved organic carbon. *Limnology and Oceanography* **35**, 1744-1756.

Obernosterer, I., and Herndl, G.J. (2000). Differences in the optical and biological reactivity of the humic and nonhumic dissolved organic carbon component in two contrasting coastal marine environments. *Limnology and Oceanography* **45**, 1120-1129.

O'Connell, M., Baldwin, D.S., Robertson, A.I., and Rees, G. (2000). Release and bioavailability of dissolved organic matter from floodplain litter: influence of origin and oxygen levels. *Freshwater Biology* **45**, 333-342.

Pace, N.L. (1999). [S] Kruskal-Wallis multiple comparisons, University of Utah, USA, viewed 10 February 2006, http://www.biostat.wustl.edu/archives/html/s-news/1999-03/msg00245.html.

Page, K., Read, A., Frazier, P., and Mount, N. (2005). The effect of altered flow regime on the frequency and duration of bankfull discharge: Murrumbidgee River, Australia. *River Research and Applications* **21**, 567-578.

Peuravuori, J., Ingman, P. and Pihlaja, K. (2003). Critical comments on accuracy of quantitative determination of natural humic matter by solid state ^{13}C NMR spectroscopy. *Talanta* **59**, 177-189.

Peuravuori, J., Monteiro, A., Eglite, L. and Pihlaja, K. (2005). Comparative study for separation of aquatic humic-type organic constituents by DAX-8, PVP and DEAE sorbing solids and tangential ultrafiltration: elemental composition, size-exclusion chromatography, UV-vis and FTIR. *Talanta* **65**, 408-422.

Reemtsma, T., and These, A. (2003). On-line coupling of size exclusion chromatography with electrospray ionization-tandem mass spectrometry for the analysis of aquatic fulvic and humic acids. *Analytical Chemistry* **75**, 1500-1507.

Rees, G.N., Beattie, G., Bowen, P.M., and Hart, B.T. (2005). Heterotrophic bacterial production in the lower Murray River, south-eastern Australia. *Marine and Freshwater Research* **56**, 835-841.

Robertson, A.I., Bunn, S.E., Boon, P.I., and Walker, K.F. (1999). Sources, sinks and transformations of organic carbon in Australian floodplain rivers. *Marine and Freshwater Research* **50**, 813-829.

Rosenstock, B., Zwisler, W., and Simon, M. (2005). Bacterial consumption of humic and non-humic low and high molecular weight DOM and the effect of solar irradiation on the turnover of labile DOM in the Southern Ocean. *Microbial Ecology* **50**, 90-101. doi: 10.1007/s00248-004-0116-5.

Sun, L., Perdue, E.M., Meyer, J.L., and Weis, J. (1997). Use of elemental composition to predict bioavailability of dissolved organic matter in a Georgia river. *Limnology and Oceanography* **42**, 714-721.

Tett, P., Kelly, M.G., and Hornberger, G.M. (1975). A method for the spectrophotometric measurement of chlorophyll *a* and pheophytin *a* in benthic microalgae. *Limnology and Oceanography* **20**, 887-896.

Thurman, E.M., and Malcolm, R.L. (1981). Preparative isolation of aquatic humic substances. *Environmental Science and Technology* **15**, 463-466.

Vink, S., Bormans, M., Ford, P.W., and Grigg, N.J. (2005). Quantifying ecosystem metabolism in the middle reaches of Murrumbidgee River during irrigation flow releases. *Marine and Freshwater Research* **56**, 227-241.

Volk, C.J., Volk, C.B., and Kaplan, L.A. (1997). Chemical composition of biodegradable dissolved organic matter in streamwater. *Limnology and Oceanography* **42**, 39-44.

Waiser, M.J., and Robarts, R.D. (2000). Changes in composition and reactivity of allochthonous DOM in a prairie saline lake. *Limnology and Oceanography* **45**, 763-774.

Wetzel, R. G. (1998). Foreword. In 'Aquatic humic substances: Ecology and biogeochemistry'. (Eds D.O. Hessen and L.J. Tranvik) pp. V-VII. (Springer: New York, USA).

Wetzel, R.G. (1995). Death, detritus, and energy-flow in aquatic ecosystems. *Freshwater Biology* **33**, 83-89.

Wetzel, R.G., and Likens, G.E. (2000). Limnological analysis, 3rd ed. Springer, New York, USA.

Zander, A., Bishop, A., and Prenzler, P.D. (2005). A solid phase microextraction method to fingerprint dissolved organic carbon released from *Eucalyptus camaldulensis* (Dehnh.) (River Red Gum) leaves. *Analytica Chimica Acta* **530**, 325-333.

Zhou, Q., Cabaniss, S.E., and Maurice, P.A. (2000). Considerations in the use of high-pressure size exclusion chromatography (HPSEC) for determining molecular weights of aquatic humic substances. *Water Research* **34**, 3505-3514.

Table 1. Indicative data for the recovery of fulvic acid and water quality for Murrumbidgee River and Berry Jerry Lagoon.

Year: 2004	*Murrumbidgee River*	*Berry Jerry Lagoon*
Isolation of fulvic acid	*1/8/04*	*14/4/04*
Volume of water used to isolate fulvic acid (L)	*56*	*52*
Quantity of fulvic acid recovered (mg)	*17.4*	*662*
Yield (mg L^{-1})	*0.3*	*13*
Water quality data		
Temperature (℃)	*10.1*	*26.1*
Dissolved Oxygen (mg L^{-1})	*11.0*	*13.9*
pH	*7.15*	*8.30*
Conductivity (mS cm^{-1})	*0.17*	*0.47*
DOC (mg L^{-1})	*10.9*	*114*
Chl-a (mg L^{-1})	*0.04*	*2.16*

Table 2. Summary statistics for maximum bacterial numbers in Berry Jerry Lagoon DOC (BJDOC), Berry Jerry Lagoon fulvic acid (BJFA), Murrumbidgee River DOC (MRDOC), Murumbidgee River fulvic acid (MRFA) and Suwannee River fulvic acid (SRFA).

	BJDOC	BJFA	MRDOC	MRFA
BJDOC				
BJFA	**			
MRDOC	**	**		
MRFA	**	**	**	
SRFA	**	**	**	ns

Note: (Kruskal-Wallis test, $\chi^2 = 21.4$, $p < 0.01$, $n = 100$)

**. Significant at the 0.01 level.

ns. Not Significant at the 0.01 level.

Table 3. Number-average (M_n), weight-average (M_w) molecular weight, and polydispersity of Berry Jerry Lagoon fulvic acid, Murrumbidgee River fulvic acid and Suwannee River fulvic acid.

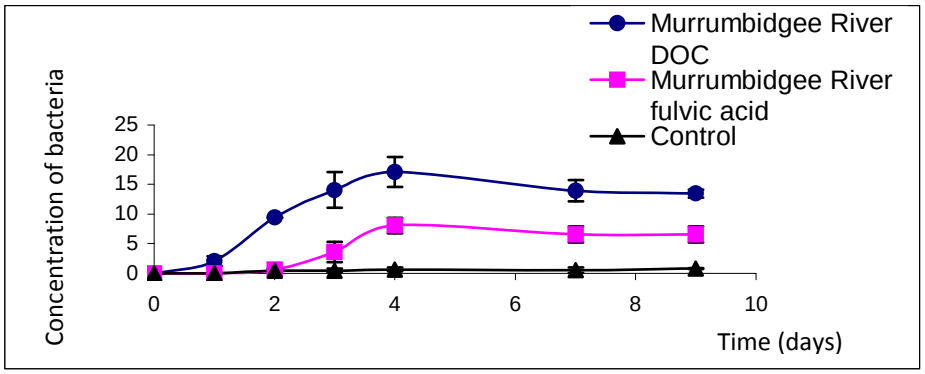
Sample	M_n	M_w	Polydispersity (M_w / M_n)
Berry Jerry Lagoon Fulvic Acid	527 ± 15^a	986 ± 32^a	1.87
Murrumbidgee River Fulvic Acid	588 ± 56^a	1409 ± 9^b	2.40
Suwannee River Fulvic Acid	1118 ± 36^c	2280 ± 128^d	2.04

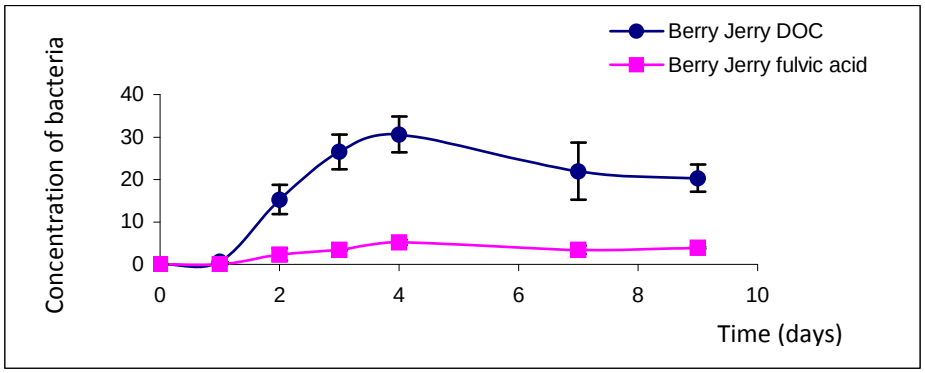
Different superscripts in a column indicate significantly different (p<0.05) molecular weight means for triplicate analyses.

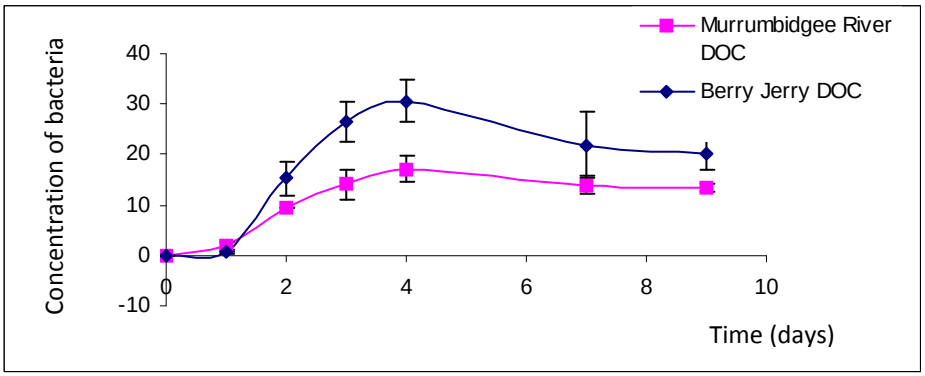
Table 4. ^{13}C NMR integrated intensities of fulvic acids as percentage of total.

Integrated Ranges (ppm)*	Al (0-63)	*o*-alkyl (63-90)	Anom (90-110)	Ar (110-160)	carboxyl (160-190)	carbonyl (190-230)
Berry Jerry Lagoon fulvic acid	56	10	1	12	16	5
Murrumbidgee River fulvic acid	41	10	3	20	18	8
Suwannee River fulvic acid	42	12	4	17	17	8

Al = aliphatic carbons, including simple, complex and methoxyl carbons; o-alkyl = C-O resonance arising from o-alkyl carbons or carbohydrate type compounds; Anom = anomeric carbons of carbohydrates; Ar = aromatic carbons; carboxyl = carboxyl and aliphatic amide carbons; carbonyl = ketones and aldehydes.







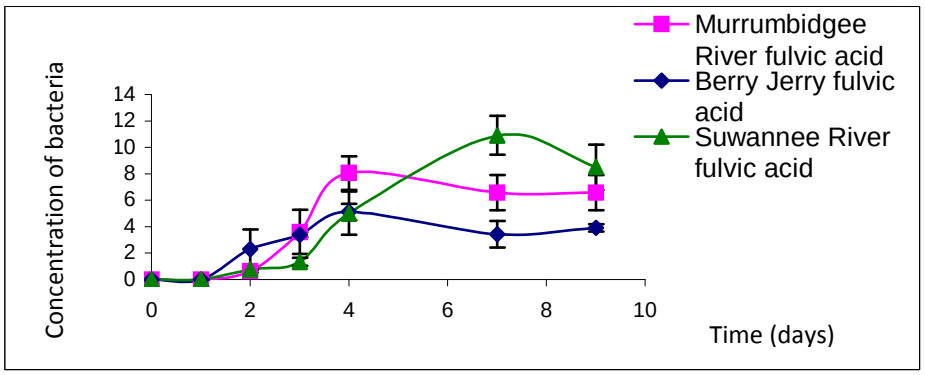
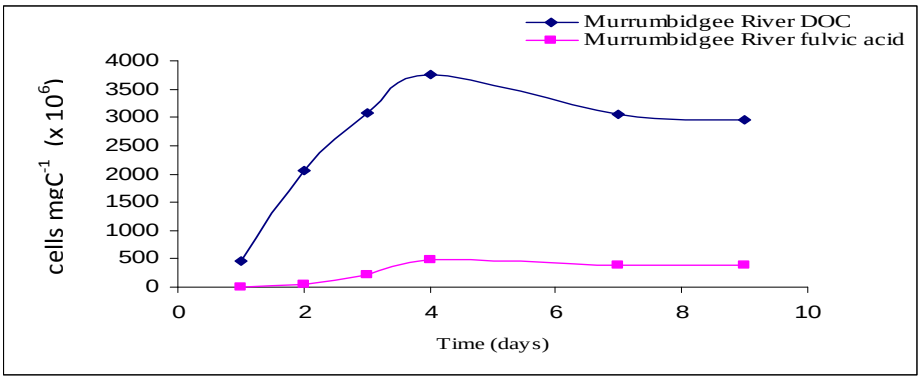
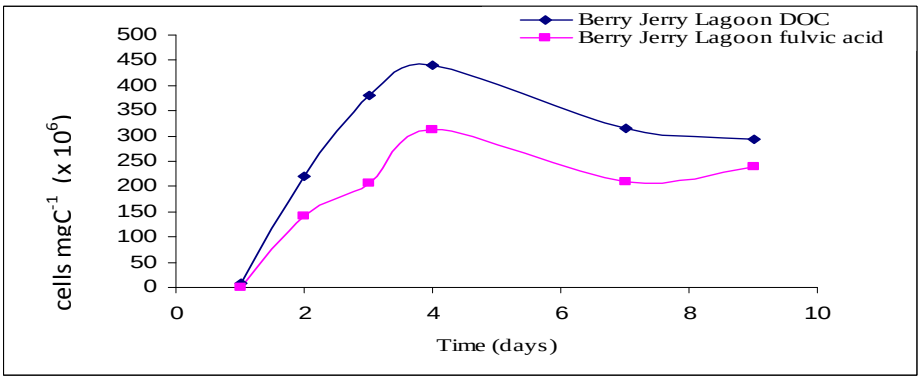
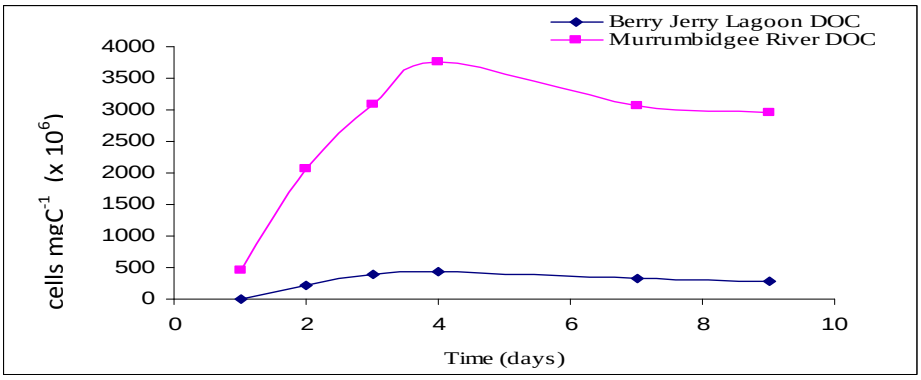


Figure 1. Changes in the concentration of bacteria in Murrumbidgee River DOC, fulvic acid, Berry Jerry Lagoon DOC, fulvic acid, Suwannee River fulvic acid, and control over nine days incubation. Error bars indicate the standard deviation of duplicate analyses.


Murrumbidgee River DOC
Murrumbidgee River fulvic acid
cells mgC-1 (x 10^6)
4000
3500
3000
2500
2000
1500
1000
500
0
0
2
4
6
8
10
Time (days)


Berry Jerry Lagoon DOC
Berry Jerry Lagoon fulvic acid
cells mgC-1 (x 10^6)
500
450
400
350
300
250
200
150
100
50
0
0
2
4
6
8
10
Time (days)


Berry Jerry Lagoon DOC
Murrumbidgee River DOC
cells mgC-1 (x 10^6)
4000
3500
3000
2500
2000
1500
1000
500
0
0
2
4
6
8
10
Time (days)

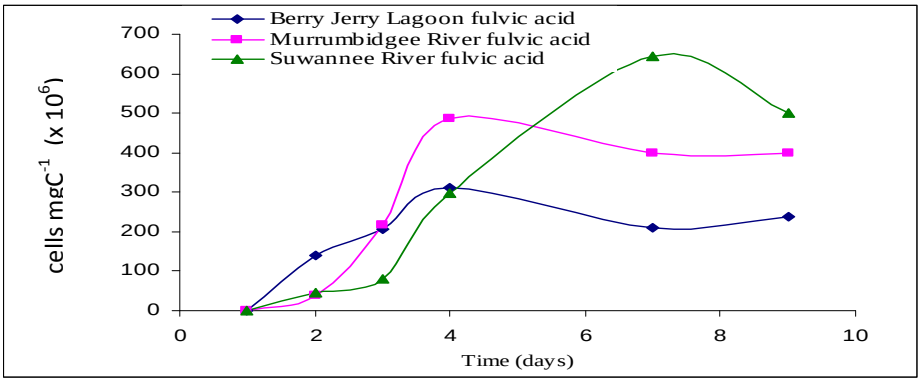


Figure 2. Bacterial growth per mg carbon of Berry Jerry Lagoon DOC, Berry Jerry Lagoon fulvic acid, Murrumbidgee River DOC, Murrumbidgee River fulvic acid and Suwannee River fulvic acid.

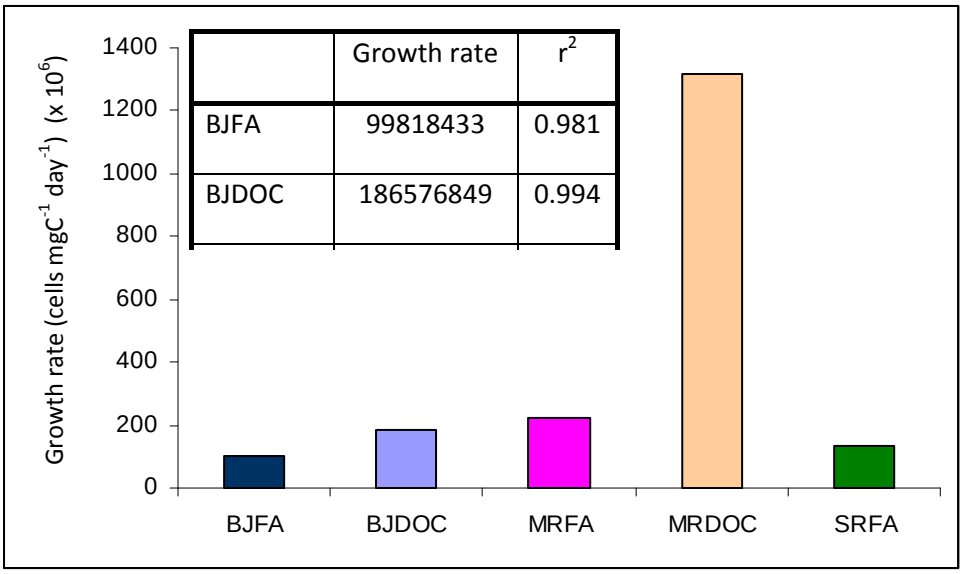


Figure 3. Growth rate of bacteria on DOC and fulvic acids.

BJFA = Berry Jerry fulvic acid, BJDOC = Berry Jerry DOC, MRFA = Murrumbidgee River fulvic acid, MRDOC = Murrumbidgee River DOC, SRFA = Suwannee River fulvic acid.

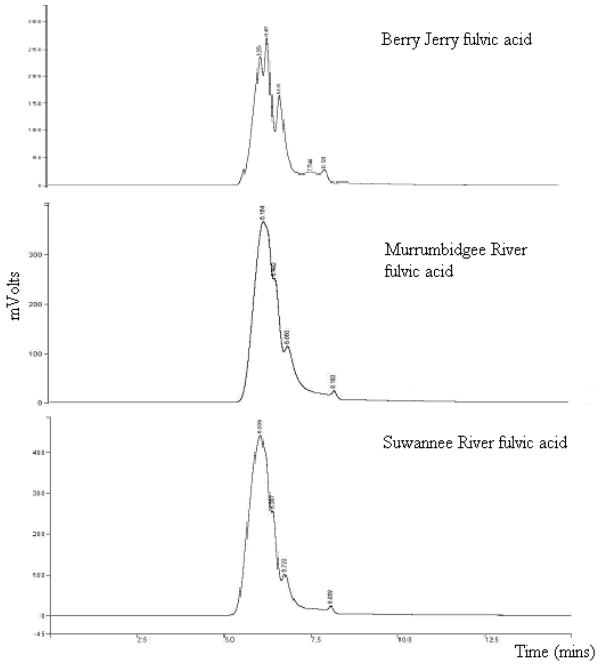


Figure 4. Size-exclusion chromatograms for Berry Jerry fulvic acid, Murrumbidgee River fulvic acid and Suwannee River fulvic acid

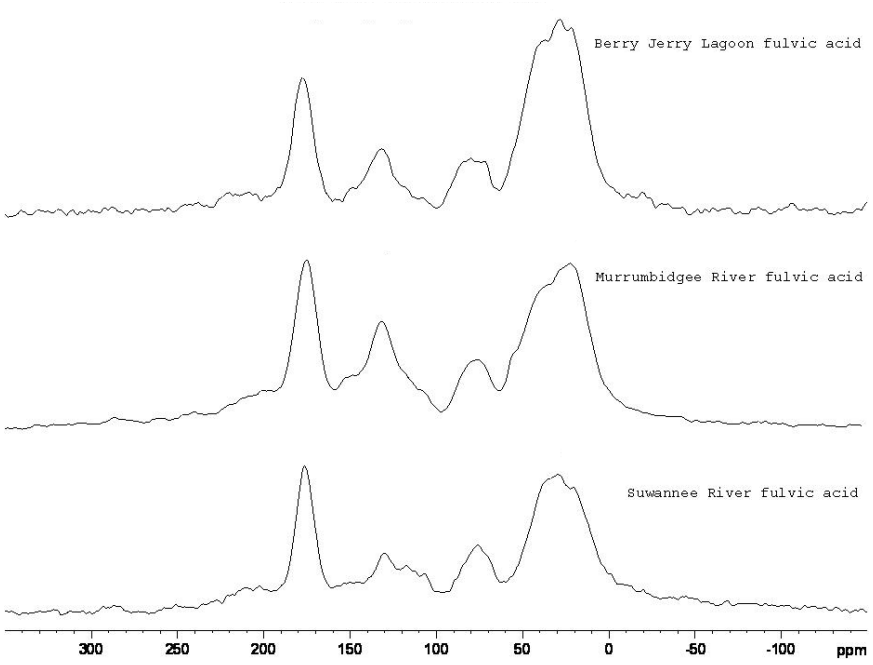


Figure 5. CPMAS ^{13}C-NMR spectra of Berry Jerry Lagoon, Murrumbidgee River and Suwannee River fulvic acid